REVIEW ARTICLE

Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling

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The features of three distinct protein phosphorylation cascades in mammalian cells are becoming clear. These signalling pathways link receptor-mediated events at the cell surface or intracellular perturbations such as DNA damage to changes in cytoskeletal structure, vesicle transport and altered transcription factor activity. The best known pathway, the Ras → Raf → MEK → ERK cascade [where ERK is extracellular-signal-regulated kinase and MEK is mitogen-activated protein (MAP) kinase/ERK kinase], is typically stimulated strongly by mitogens and growth factors. The other two pathways, stimulated primarily by assorted cytokines, hormones and various forms of stress, predominantly utilize p21 proteins of the Rho family (Rho, Rac and CDC42), although Ras can also participate. Diagnostic of each pathway is the MAP kinase component, which is phosphorylated by a unique dual-specificity kinase on both tyrosine and threonine in one of three motifs (Thr-Glu-Tyr, Thr-Phe-Tyr or Thr-Gly-Tyr), depending upon the pathway. In addition to activating one or more protein phosphorylation cascades, the initiating stimulus may also mobilize a variety of other signalling molecules (e.g. protein kinase C isoforms, phospholipid kinases, G-protein α and $\beta\gamma$ subunits, phospholipases, intracellular Ca^{2+}). These various signals impact to a greater or lesser extent on multiple downstream effectors. Important concepts are that signal transmission often entails the targeted relocation of specific proteins in the cell, and the reversible formation of protein complexes by means of regulated protein phosphorylation. The signalling circuits may be completed by the phosphorylation of upstream effectors by downstream kinases, resulting in a modulation of the signal. Signalling is terminated and the components returned to the ground state largely by dephosphorylation. There is an indeterminant amount of cross-talk among the pathways, and many of the proteins in the pathways belong to families of closely related proteins. The potential for more than one signal to be conveyed down a pathway simultaneously (multiplex signalling) is discussed. The net effect of a given stimulus on the cell is the result of a complex intracellular integration of the intensity and duration of activation of the individual pathways. The specific outcome depends on the particular signalling molecules expressed by the target cells and on the dynamic balance among the pathways.

INTRODUCTION

The 21 kDa mammalian Ras proteins (p21) consist of 188 or 189 amino acids. They are integral to signal transduction pathways connecting events at many cell surface receptors to intracellular processes. Mammalian cells contain three very similar *ras* genes (H-*ras*, N-*ras* and K-*ras*, which has two splice variants, A and B, that differ in their C-terminal sequences). In this review, Ras is used to refer collectively to the products of these three genes, even though in different cell types one or the other may predominate. At the protein level Ras is present in all cells, with the highest levels in proliferating cells. At the mRNA level H-*ras* is highest in skin and muscle, K-*ras* in gut and thymus, and N-*ras* in thymus and testes. For signalling purposes, there are no known differences among these Ras proteins, though cell-specific differences in the transforming potential of the codon 12 Gly → Asp mutants have been reported [1]. Recent reviews of the Ras-controlled signalling pathways contain references to background material that is assumed or summarized here [2–4].

These three *ras* genes are part of a large superfamily of genes encoding small GTP-binding proteins (G-proteins) [5]. Closely related to Ras are several species of Rap proteins found in granules in the Golgi and endoplasmic reticulum. Rap1A can antagonize the transforming ability of Ras, a function that led to its isolation as a suppressor, called Krev-1, of the K-*ras* oncogene. Also closely related to Ras are the Ral-A and Ral-B proteins, which appear to regulate the activity of exocytic and endocytic vesicles. This Ras/Rap/Ral group is important in growth and development, and certain members are implicated in exocytosis, anabolic processes and/or regulation of the oxidative burst. Less closely related to Ras are several other families in the Ras superfamily [5–10]. The Rho family, which includes Rho-A, -B and -C, Rac-1 and -2, CDC42, Rho-G and TC10, embraces small G-proteins that play dynamic roles in the regulation of the actin cytoskeleton and focal contacts, mediating formation of filopodia and lamellipodia. Rac also controls NADPH oxidase activity in phagocytes. Ran proteins are involved in the transport of RNA and proteins across the nuclear membrane. ARF/SAR proteins are important for vesicle formation and budding. Members of the large and extensively studied Rab/YPT family are involved both in regulating intracellular vesicle trafficking between donor and acceptor membrane-enclosed compartments and in controlling the exocytosis and endocytosis of different types of vesicles.

Abbreviations used: see Table 1 for the names and abbreviations of most of the signalling intermediates mentioned in this review; CREB, cAMP response element-binding protein; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GSK-3, glycogen synthase kinase-3; Hsp90, heat-shock protein of 90 kDa; IL-1, interleukin-1; JAK, Janus kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κB; PH domain, pleckstrin homology domain; PI3-K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC and PLD, phospholipases C and D respectively; PTB domain, phosphotyrosine-binding domain; SH domain, Src homology domain; SRF, serum response factor; STAT, signal transducer and activator of transcription; TNFα, tumour necrosis factor α.

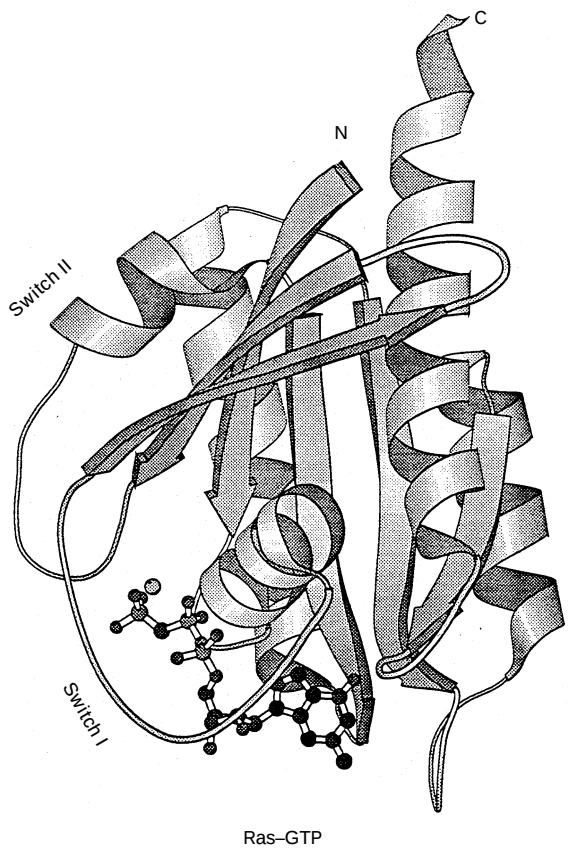

Figure 1 Structure of p21 Ras–GTP

See [11] and the text for details. Figure generously provided by Dr. Sung-Hou Kim.

Figure 1 illustrates the structure of the GTP–Ras protein [11–13]. Presumably all members of the Ras superfamily have a very similar structure. Notable features include the highly conserved catalytic domain made up of amino acids 1–164, the GTP-binding site (constituted from amino acids 12–18, 57–63, 116–119 and 144–147) and the two 'switch' regions (amino acids Asp-30–Asp-38 and Gly-60–Glu-76). The switch regions of the Ras protein are close to the γ-phosphate group of the activating GTP and exhibit different conformations depending upon whether GDP or GTP is bound. This N-terminal proximate region is the effector binding site (involving amino acids Tyr-32–Tyr-40) responsible in part for interactions with the GTPase-activating protein (GAP) and with downstream effectors of Ras action such as Raf, which competes with GAP for binding to Ras. The two Ras-GAPs, p120Ras^{GAP} and neurofibromin, appear to interact somewhat differently with Ras in that substitution of the amino acids Lys-Arg-Val with Leu-Ile-Arg at positions 101–103 had no effect on the intrinsic hydrolytic activity of Ras or its sensitivity to neurofibromin, but did reduce p120Ras^{GAP}-stimulated hydrolysis 2–3-fold [14]. The interaction of the guanine nucleotide exchange factor (GEF) with Ras is, in part, with the switch 2 region [15].

The Ras proteins are bound to the inner surface of the plasma membrane by several lipophilic interactions involving post-translational modifications of the C-terminus [5,12]. A cysteine residue very near to the C-terminus, in a CAAX (where A = aliphatic amino acid) motif, typically has a polyisoprenyl moiety attached to it, either C_{15} (farnesyl) for Ras or C_{20} (geranylgeranyl) for some Rap proteins and members of the Rho superfamily. (These modifications are determined by the specific C-terminal amino acid sequences and they may determine the specific cell membrane targeted by the protein.) Subsequently the three C-terminal amino acids are removed and the newly generated farnesylated Ras C-terminal cysteine is carboxymethylated. H-Ras, N-Ras and K-Ras(A) have in addition a palmitoyl group added to a cysteine residue in the hypervariable region (amino acids 165–185) near the C-terminus; K-Ras(B) does not have a cysteine residue that can be palmitoylated, but does have a polybasic domain that fulfils a similar function, presumably by forming an amphipathic helix.

Activation of Ras induces proliferation in many types of cells, and in its mutant oncogenic form Ras transforms many immortal cell lines, conferring on them a malignant phenotype [16]. Oncogenic Ras can also co-operate with an 'immortalizing oncogene' to transform primary cells. In some cell types, e.g. pheochromocytoma PC12 cells, Ras activation induces differentiation and neurite outgrowth. Oncogenic Ras bearing a mutation that alters the Ras structure so as to prolong the lifetime of the active GTP-bound form (in amino acids 12, 13, 59, 61 and 63 in the phosphate-binding region) generates a constitutive signal that is a major factor in many human cancers. Mutations that increase the guanine nucleotide exchange rate (amino acids 116, 117, 119 and 146) are in the base-binding region and also produce a transforming Ras. Ras is also responsible for transmitting the proliferative signal generated by a number of oncogenes, including the *src*, *fms*, and *fes* oncogenes.

ACTIVATION OF RAS- AND RHO-GTPase CASCADES

Ras and its relatives are activated in response to an extracellular or intracellular signal that generates the GTP-bound form and energizes the signal transducing ability. Typically the Ras–GTP level will increase 2–3-fold and remain high for at least 30 min after receptor activation. Hydrolysis of the bound GTP by an intrinsic GTPase activity relaxes the conformation and terminates the signal. Figure 2 illustrates the cycling of Ras between these two forms. Table 1 lists many of the proteins discussed in this review.

GAPs enhance the GTPase activity of normal Ras (but typically not oncogenic Ras) and thus shorten the lifetime of the signalling form. Presumably Ras–GTP continues to signal as long as the GTP remains intact, although association with GAP may alter the signal. The mammalian GAPs include the well studied p120Ras^{GAP} and the quite distinct and larger ($\sim$ 290 kDa) neurofibromin, NF1, whose expression is largely restricted to neural tissues (including neural-crest-derived tissues such as Schwann cells) and gonadal tissues [17]. There are two members of the GAP1 family, p100GAP1^{m} and GAP1^{IP4BP}, which via a PH (pleckstrin homology) domain bind to and are stimulated by inositol 1,3,4,5-tetrakisphosphate [18,19]. GAPs active on Rho family members include Ral-BP1 (CDC42-GAP), p190Rho^{GAP} and BCR, which are respectively preferentially active on CDC42, Rho and Rac [10,20] (see Table 1 and Figure 3).

Studies with homozygous null mice ('knock-outs') have revealed the importance of some of these proteins. Animals unable to make p120Ras^{GAP} are embryonic lethals that exhibit increased Ras–GTP signalling and possess major defects in endothelial cell organization [21]. Mice unable to produce NF1 also die as embryos. It appears that one function of NF1 is to promote the death of certain neurons when an appropriate signal from certain nerve growth factors (neurotrophins) is not received [22].

GEFs [also known as guanine nucleotide releasing proteins, or guanine nucleotide dissociation stimulators (GDSs)], catalyse

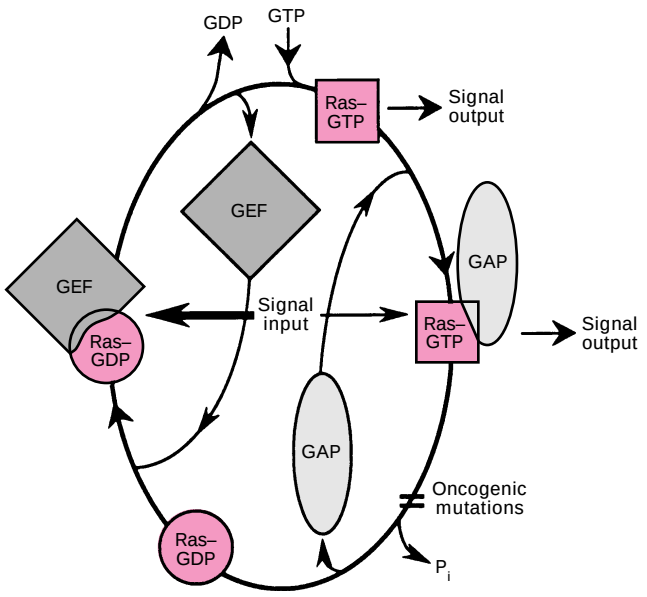

Figure 2 The Ras cycle

Inactive, 'nucleotide-free', Ras (top of cycle), which may exist only as a transition state during which GTP is replacing the GDP, associates with GTP and is activated as a consequence. Ras–GTP continues to interact with and activate effector proteins (as discussed in the text) until GAP enforces hydrolysis of the GTP by the low-level intrinsic Ras-GTPase activity. The inactive Ras–GDP complex associates with a GEF, which when activated catalyses the exchange of GTP for GDP, again activating Ras. The signal from activated Ras is transmitted by protein–protein interactions, which may differ depending upon whether GAP is bound or not. Input signals leading to Ras activation are thought to be transmitted more often via GEF and less often via GAP. See the text for references and further details.

the release of GDP from inactive p21 proteins complexed with GDP; they are major players in signal activation [10,15,22]. Release of GDP allows GTP, present at much higher levels than GDP in the healthy cell, to bind and activate the p21 protein once again. SOS1 and SOS2, CDC25 and C3G are the four known mammalian GEFs with activity towards Ras–GDP; DBL, OST and smg-GDS are known GEFs acting to activate various Rho family members. DBL, isolated from a diffuse B-cell lymphoma, stimulates exchange activity for Rho-A, Rac-1 and CDC42 [15]. OST, isolated from an osteosarcoma, is an effector for Rac-1, acting as a GEF for Rho-A and CDC42, but not for Rho-B or Rho-C [23]. A domain found in DBL, known as the dibble homology domain, along with a PH domain, appears to be characteristic of the Rho-GEFs. To what extent different GEFs act differentially on members of the Ras and Rho (including Rac and CDC42) families *in vivo*, perhaps co-ordinately, remains to be established. Their importance is underscored by the fact that several known or putative Rho-GEFs are proto-oncoproteins (e.g. DBL, VAV, OST) that can be activated by N-terminal truncations. VAV is a 95 kDa protein expressed in haematopoietic cells that is tyrosine phosphorylated in response to the activation of a variety of receptors (e.g. the T-cell antigen receptor, the IgM receptor or c-Kit). It has a dibble domain, suggesting it may be a GEF, and it is capable of binding both to tubulin and to the nuclear protein Ku-70, the DNA-binding element of the DNA-dependent protein kinase [24,25].

Rho-GDI (guanine nucleotide dissociation inhibitor) binds equally well to both the GDP- and GTP-bound forms of CDC42, inhibiting both GDP dissociation and GTP hydrolysis [25a]. It inhibits the interaction of the p21Rac, Rho and CDC42 proteins with GAP and stimulates their release from cell membranes, perhaps to allow them to relocate to another membrane site [10].

The interactions among Ras, Ral, CDC42, Rac and Rho and both the downstream effectors of their actions and their diverse upstream activators, particularly physical and chemical stress, are poorly understood [9,10,15]. Via their respective and sometimes shared GEFs and GAPs, they have the potential to socialize as suggested in Figure 3, although which interactions are important is likely to depend on the abundance and location of relevant proteins in the cell in question. An important consequence of these shared interactions may be to place a particular GAP or GEF in a location where it can act more efficiently on its target p21 protein. $p120Ras^{GAP}$ interacts with $p190Rho^{GAP}$, which can deactivate GTP-bound Rho, Rac and CDC42. The precise consequences of the interaction between these two GAP proteins is not known. BCR, which contains a serine/threonine kinase domain, also contains both a dibble domain that may function as a GEF for certain Rho family members (CDC42 > Rho-A > Rac in terms of relative activity) and a GAP domain targeted to CDC42 and Rac, but not to Rho-A [25b]. N-chimerin and B-chimerin are Rho-GAPs specific for Rac. Ral is a downstream effector of Ras, as shown by the ability of oncogenic Ha-Ras to activate the guanine nucleotide dissociation stimulator Ral-GDS, which (as a GEF) activates Ral-A and Ral-B; this appears to enhance the transforming activity of Ras (and Raf). Ral interacts with Ral-binding protein 1 [Ral-BP1; also known as Ral interacting protein 1 (RIP1)], which is a GAP protein for CDC42 [26–28]. Elegant microinjection experiments by Nobes and Hall [29] and Kozma et al. [30] have revealed that CDC42 can activate Rac, which in turn activates Rho. Each of these members of the Rho family can generate specific changes in cytoskeletal elements in response to specific extracellular factors (bradykinin for CDC42, PDGF or insulin for Rac and lysophosphatidic acid for Rho) [29,30]. In this so-called 'GTPase cascade', activation of CDC42 promotes the formation of shaft-like filopodia and microspikes, activated Rac promotes membrane ruffling and the formation of curtain-like lamellipodia, and activated Rho promotes the formation of stress fibres and focal adhesions. The formation of these different cytoskeletal structures presumably reflects the specific cytoskeletal components recruited in each case. The different ways in which these signalling elements can be arranged, determined by which proteins are actually present, generate specific cell surface structures and choreograph complex activities such as cell migration and neurite outgrowth [9].

Khosravi-Far et al. [31] have presented compelling evidence that oncogenic Ras, possibly acting via CDC42, requires both Rac-1 and Rho-A in order to establish a fully transformed state. They showed that dominant negative mutants of Rac-1 and Rho-A blocked Ras transformation, and that constitutively activated but weakly transforming Rac-1 and Rho-A could synergize with a weakly transforming Raf-1 to transform cells efficiently. Rho-A appeared to be downstream of Rac-1 on a pathway that had its major impact on the actin cytoskeleton, as contrasted with the action of the Raf-1 → MEK → ERK pathway on gene expression.

Signalling initiated by receptor tyrosine kinases

In one-pass receptor-initiated signal transduction pathways, activation of the cell surface receptor tyrosine kinase by its ligand generally stimulates receptor homo- or hetero-dimerization and/or a conformational change that typically results in autophosphorylation of multiple tyrosine residues in the cytoplasmic portion of the receptor [3,32]. Receptor dimerization can be accomplished

Table 1 Names, abbreviations and terminology

Receptors and growth factors
- TrkA: 'tropomyosin receptor kinase'; the receptor for NGF (nerve growth factor)
- TrkB and TrkC bind NGF-related neurotrophins
- EGF: epidermal growth factor
- PDGF: platelet-derived growth factor
- FGF: fibroblast growth factor
- IGF: insulin-like growth factor
- CSF: colony-stimulating factor

Small-molecular-mass p21 GTPase proteins
- Ras: rat sarcoma virus
- Rac: Ras-related C_3-botulinum toxin substrate
- Rho: Ras homologous
- Ral: Ras-related protein
- cdc42: homologous to yeast cell division cycle gene 42
- ARF: ADP-ribosylation factor
- SAR: secretion-associated and ras-superfamily-related gene
- YPT: yeast protein two

GAPs (GTPase-activating proteins)
- $p120Ras^{GAP}$: 120 kDa Ras-GAP
- NF1: neurofibromin; also a Ras-GAP
- $p100GAP1^{m}$: a rat Ras-GAP1
- $GAP1^{IP4BP}$: a human Ras-GAP1
- CDC42-GAP (Rho-GAP)
- $p190Rho^{GAP}$: 190 kDa protein; binds $p120Ras^{GAP}$
- BCR: product of breakpoint cluster region gene
- ABR: active BCR-related

GDS (guanine nucleotide dissociation stimulator)

GEFs (guanine nucleotide exchange factors)
- SOS: son of sevenless (a *Drosophila* gene product); SOS1 and 2 are mammalian homologues
- CDC25: mammalian homologue of yeast cdc25
- C3G: Crk SH3-binding GEF
- DBL: diffuse B cell lymphoma
- VAV: named from the sixth letter of the Hebrew alphabet
- OST: oncogene from rat osteosarcoma cells
- smgGDS: small G-protein GDP dissociation stimulator; possible GEF for K-Ras
- Ral-GDS: GDS protein active on Ral

Adaptor proteins
- GRB2: growth-factor-receptor-bound protein 2
- SHC: SH2-domain-containing α2-collagen-related
- NCK: a novel cytoplasmic protein

MAPKKKs (mitogen*-activated protein kinase kinase kinases)
- Raf-1, A-Raf, B-Raf
- PAK: p21-activated kinase
- MEKK: MEK kinase
- TAK: transforming-growth-factor-β-activated kinase

MAPKKs (mitogen*-activated protein kinase kinases)
- MEK1 (MKK1) and MEK2 (MKK2): MEK = MAPK/ERK kinase; MKK = MAP kinase kinase
- JNKK: JNK kinase
- SEK1: SAPK/ERK kinase 1; vastly prefers SAPK as substrate
- MKK3 and MKK 4: MAP kinase kinases 3 and 4
- RKK: RK kinase

MAPKs (mitogen*-activated protein kinases)
- ERK: extracellular-signal-regulated kinase; ERK1 = p44; ERK2 = p42
- FRK: Fos-related kinase
- JNK: Jun N-terminal kinase, also known as:
- SAPK: stress-activated protein kinase; several splice variants are known, producing proteins of about 46 and 54 kDa; thus there are p46 and p54 versions of SAPKα1, SAPKα2, SAPKβ and SAPKγ (p46SAPKα1 = JNK2)
- p38: RK (reactivating kinase)
- CSBP: cytokine-suppressive anti-inflammatory drug binding protein

TCFs (ternary-complex factors)
- Elk-1: Ets-like transcription factor
- SAP-1: SRF accessory protein 1 or stress-activated protein 1

MAPKAPK (mitogen-activated protein kinase activated protein kinase)
- MAPKAPK-1: RSK, the ribosomal S6 kinase $p90^{rsk}$
- MAPKAPK-2: 50 kDa protein kinase that phosphorylates Hsp25/Hsp27

Phosphatases
- SHP-1: also known as SHP, PTP1C, SHPTP-1 and HCP
- SHP-2: also known as SYP, PTP1D, SHPTP-2, SHPTP-3 and PTP2C
- SH-PTP: SH-domain-containing protein tyrosine phosphatase
- MKP-1: MAP kinase phosphatase-1 (also known as 3CH134 and CL100)
- PTP: protein tyrosine phosphatase
- PAC: phosphatase of activated cells
- PP1G: protein phosphatase-1 associated with hepatic glycogen

* Only some of these kinases are strongly activated by mitogens.

either by dimeric ligands such as PDGF or by monomeric ligands such as EGF, presumably because there are two ligand-binding sites on the monomer. One of the first tyrosines to be autophosphorylated in the PDGF receptor is in the kinase activation domain of the receptor; this enhances the activity of the kinase. Different growth factor receptors (e.g. the PDGF, EGF, FGF and NGF receptors) present phosphorylated tyrosines in different constellations of amino acids. Thus each receptor interacts with its own characteristic set of proteins, endowing each receptor type with a unique composite signal. Cytokine receptors present a variation on this theme in that the receptor is not a tyrosine kinase itself, but rather upon activation stimulates an associated protein, a Janus kinase (JAK), to phosphorylate tyrosine residues on both itself and the receptor, thereby providing docking sites for various proteins including STATs (signal transducer and activator of transcription) and other signalling molecules such as SHC, SHP-2, phospholipase Cγ (PLCγ) and p85 [33].

Some of the proteins known to associate with particular tyrosine phosphates in the cytoplasmic domain of the dimerized and activated PDGF receptor are depicted in Figure 4 [34]. The proteins interact with the tyrosine phosphates via the SH2 (Src homology 2) domain or the more recently described PTB (phosphotyrosine-binding) sequence [35–37]. Structural studies have revealed a similarity between the PTB and PH domains that suggests a possible role for the PTB domain in membrane localization, a conclusion that is supported by its ability to bind acidic phospholipids [38]. Only a subset of those proteins potentially able to associate with the fully phosphorylated receptor are actually able to bind with any one particular activated receptor molecule. In some cases (e.g. binding of p85 to the PDGF receptor) more than one interaction may be involved (e.g. two SH2–phosphotyrosine interactions, or one SH2 and one PTB interaction), presumably reinforcing what is otherwise a fairly weak single interaction (K_d = 0.3–4.0 μM) [39]. In other cases two different proteins may compete for the same phosphotyrosine. For example, NCK can bind to pTyr-751; SHC, which has more relaxed binding site characteristics, competes for pTyr-579, -740, -751 and -771 [34]. Src, with only one SH2 site, appears able to bind to either Tyr-579 or, less strongly, Tyr-581. All these proteins contribute to the consequences of PDGF activation. Some of them have the potential to interact with each other, and other proteins, via SH3 domains, which recognize a left-handed polyproline type II helix domain in their partner [40]. Via such an interaction, both NCK and SHC (non-enzymic adaptor proteins) form a complex with SOS (SHC can also associate with SOS via GRB2) [41,42]. van der Geer et al. [3] have reviewed in detail the

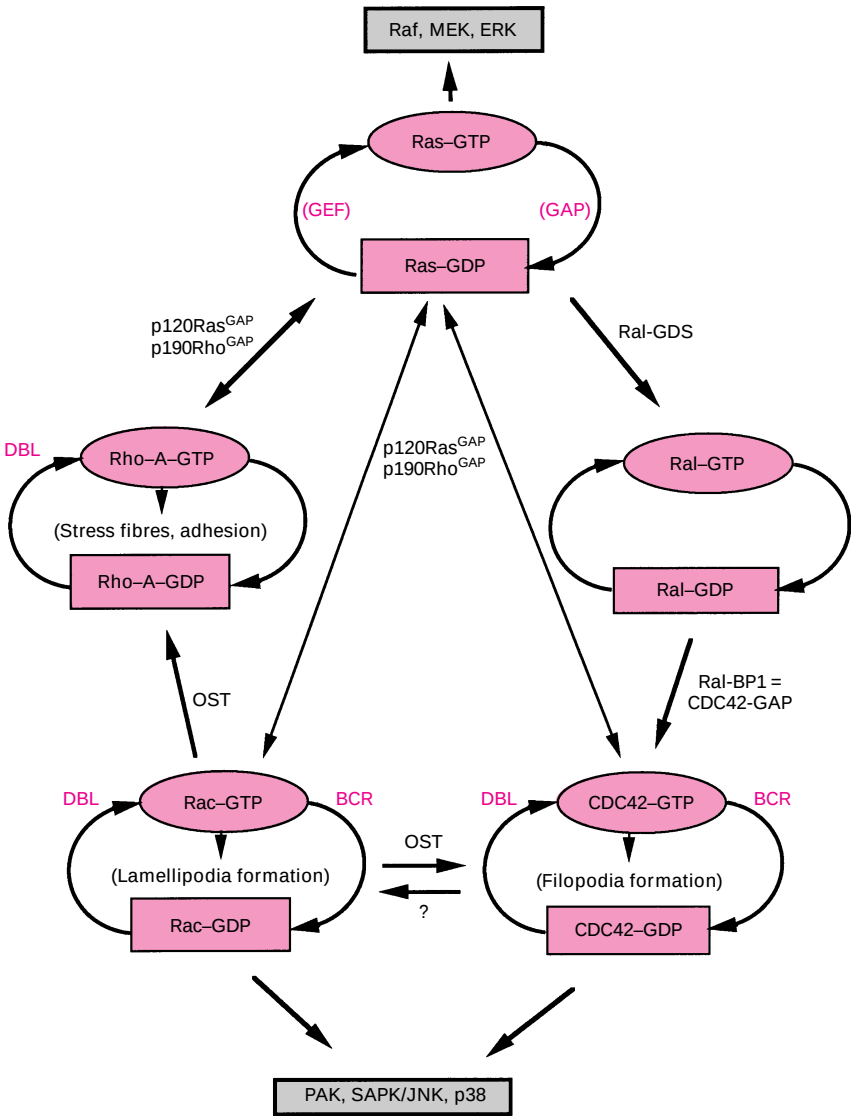

Figure 3 Potential interactions, or 'GTPase cascades', among the p21 GTP-binding proteins of the Ras/Rho/Ral family

Which interactions, indicated by the arrows, actually occur will depend on which proteins are present. Depending upon the circumstances, the GTP-bound and activated form of one p21 protein may promote the activation or deactivation of a second p21 protein via an interaction with the appropriate GEF or GAP. Known or suspected interactions involve the indicated GAP and GEF proteins, which interact respectively with the GTP-bound and GDP-bound forms of the enzymes. p190Rho^{GAP} acts more efficiently on Rho than on CDC42 [10]. BCR (and ABR) have GAP activity towards Rac and CDC42, while their DBL homology domains are the GEFs CDC42, Rho-A and Rac [25b]. DBL is a GEF for CDC42 and Rho [10]. OST interacts specifically with the GTP-bound form of Rac-1 and is a GEF for Rho-A and CDC42 [23]. Output signals from Ras–GTP and CDC42/Rac–GTP are described in Figure 6.

properties of the various proteins that can bind to tyrosine-phosphorylated receptors, and they suggest that activation of several distinct species of receptor-binding proteins is essential for a full response to any given stimulus.

The EGF receptor possesses five tyrosines located in a cluster spanning residues 992–1173 near the C-terminus that are strongly autophosphorylated after receptor activation and are thus assumed to be important in signal transmission. However, a mutant EGF receptor in which these tyrosines have been deleted or mutated to phenylalanine can still deliver mitogenic and transforming signals, albeit not as vigorously [43,44]. Although protein tyrosine phosphorylation in general was substantially reduced, SHC appeared to be fully phosphorylated after EGF stimulation of the mutant receptor, probably accounting for the activation of Ras and the ERKs. The process leading to SHC phosphorylation remains obscure, but could be due to interaction of the mutant EGF receptor, which still contained an active kinase domain, with susceptible target proteins (e.g. cErbB2). Alternatively, Tyr-845 may become phosphorylated by Src in an EGF-dependent manner, thus enabling the mutant protein to transmit a signal [45]. When Ras action is blocked by a dominant negative Ras mutant (p21$^{rasAsn-17}$), EGF-induced phosphorylation of ERK2 can still occur, possibly as a result either of PKC activation or of an elevation of the free intracellular Ca^{2+}concentration, depending upon the cell type [46]. In contrast, ERK2 phosphorylation induced by insulin or PDGF was abrogated by expression of the dominant negative Ras mutant. The ERK proteins, reviewed below, are mitogen-activated protein kinases (MAPKs) that are responsible for the phosphorylation of many target proteins in response to mitogen stimulation.

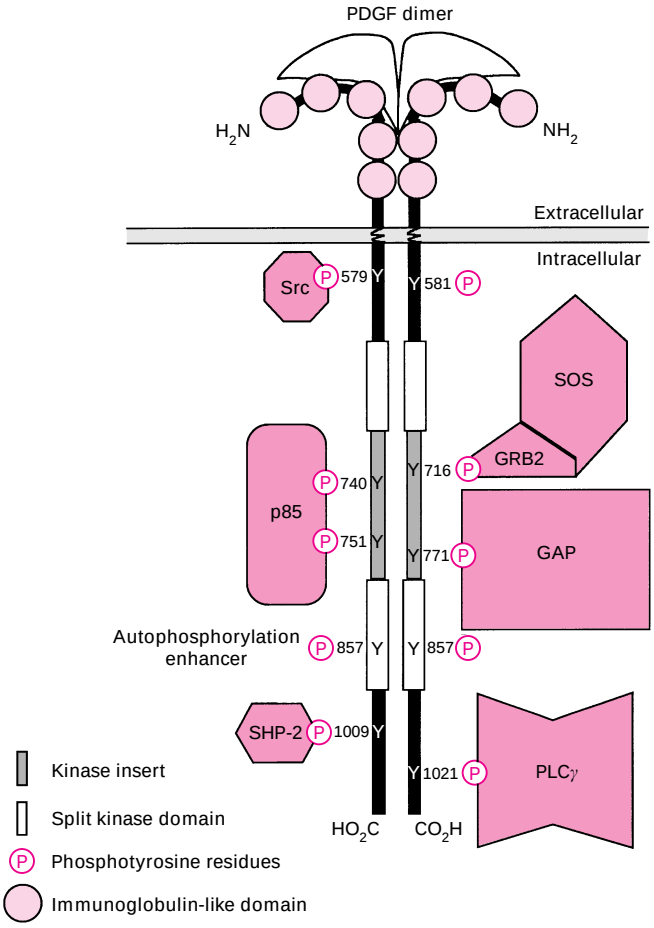

Figure 4 Cartoon of the activated human PDGFβ receptor dimer

A PDGF dimer brings two PDGF receptor monomers into close proximity, allowing cross-phosphorylation to occur at multiple tyrosine residues. Various proteins are able to bind to the phosphotyrosines with a specificity determined by the neighbouring amino acids. As discussed in the text, the proteins binding to the activated receptor may also be subject to tyrosine phosphorylation by the receptor. They also have the potential to interact with each other. Since the stoichiometry of receptor autophosphorylation is not high, it is likely that on average any one receptor molecule will only be phosphorylated at a few sites and be able to bind only a subset of all the proteins with the potential to bind.

Proteins that interact with protein tyrosine phosphates

Proteins with SH2-binding sites, such as Src, p85, SHP-2 (= syp), PLCγ, GAP, GRB2, SHC and NCK, bind via these domains only to those tyrosine phosphates that are found in the appropriate amino acid sequence context. This context is usually determined by the 3–6 amino acids on the C-terminal side of the phosphotyrosine. In contrast, the ability to bind the PTB sequence, which has been identified in nine proteins [37], appears to be determined largely by the 3–5 amino acids on the N-terminal side of the phosphotyrosine. Certain of the amino acids in the SH2 domains that are important in determining which phosphotyrosines will be most strongly bound have been identified and used to classify SH2 domains into groups which distinguish the various contexts in which the phosphotyrosine in found [47].

Like the phosphotyrosine-binding proteins, the protein tyrosine kinases (both receptor and non-receptor kinases) target different tyrosines depending upon the local amino acid context. Cytosolic protein tyrosine kinases prefer to phosphorylate tyrosines in binding motifs recognized by group I SH2 domains (e.g. Src, GRB2 and GAP), whereas receptor protein tyrosine kinases phosphorylate tyrosines in motifs recognized by group III SH2 domains (e.g. SHC, p85 and PLCγ) [48]. (These groups are distinguished on the basis of the βD5 residue in the SH2 domain.) Proteins containing phosphotyrosine-binding sites (i.e. SH2 or PTB domains) compete with protein phosphotyrosine phosphatases for the tyrosine phosphate, and by doing so prolong the lifetime of those phosphotyrosine residues that are particularly strongly bound. Some of these SH2-containing proteins are enzymes [e.g. p120Ras^{GAP}, SHP-2, phosphoinositide 3-kinase (PI3-K), Src and PLCγ] whose enzymic activity is enhanced, whereas others are adaptor proteins (GRB2, SHC and NCK) that bind a specific target protein and deliver it to the activated receptor.

Enzymes regulated by phosphotyrosine interactions

Association of p120Ras^{GAP} with tyrosine-phosphorylated receptors is mediated by its SH2 domains. By virtue of its ability to activate the Ras GTPase it is a negative regulator of Ras function [49]. p120Ras^{GAP} can also acquire a signalling function, mediated by the SH2 or SH3 domains in its N-terminal region, as a consequence of the interaction of its C-terminal region with Ras. This signalling may involve two phosphorylated proteins: p62, a membrane-associated RNA-binding protein, and p190Rho^{GAP}, a protein that possesses GTPase-activating activity towards proteins in the Rho/Rac family. As discussed above (see Figure 3), p120Ras^{GAP} may serve to co-ordinate the activity of the Ras and Rho signalling pathways [9]. The N-terminal domain of p120Ras^{GAP} can modify cellular cytoskeletal structure (disruption of the actin cytoskeleton and focal contacts), possibly as a result of its interaction with p190Rho^{GAP} [50]. NF1 may also have a signalling function separate from its GTPase-activating ability, since it can inhibit transformation by v-*ras*, even though the GTPase activity of v-Ras cannot be stimulated by NF1 [51]. Deficits in NF1-GAP activity have been detected in a number of human tumours, including von Recklinghausen's neurofibromatosis, consistent with an important signalling role.

SHP-2 (the recently proposed name for the independently isolated but identical SYP, PTP1D, SH-PTP2, and PTP2C protein tyrosine phosphatases [52]) is a protein tyrosine phosphatase that contains two N-terminal SH2 domains through which it can associate with tyrosine-phosphorylated receptors, e.g. the PDGF receptor, and become activated. Phosphorylation of a C-terminal tyrosine in SHP-2 produces a docking site for GRB2, thus facilitating activation of SOS [53–55]. SHP-2 can activate Src by dephosphorylating Tyr-527 in inactive Src. When phosphorylated, Tyr-527 inhibits Src activity by forming an intramolecular complex with the Src SH2 domain [55]; freed, the Src SH2 domain can, for example, interact with an appropriately phosphorylated PDGF receptor (see Figure 4). (Alternatively, interaction of the Src SH2 domain with the phosphorylated receptor, should it be able to compete, would expose the Tyr-527 phosphate to the action of SHP-2 [3].) GRB2 has been reported to bind tyrosine-phosphorylated focal adhesion kinase, thus providing a potential link between integrin-mediated signalling and Ras-mediated signalling. This may explain the activation of the p42 and p44 ERKs when integrins are engaged by their ligands [56,57].

PI3-K is a dimeric enzyme (subunits of 110 and 85 kDa) capable of phosphorylating suitable phosphoinositides in the 3-position; it is also a serine/threonine protein kinase able to phosphorylate various proteins, including its own regulatory

85 kDa subunit and the insulin receptor substrate-1 [58,59]. The relationship between the lipid- and peptide-binding sites is not known. p85 possesses one SH3 group, two SH2 groups and a domain that may have GAP activity towards an unidentified target. It probably activates the kinase holoenzyme by an allosteric effect and by fostering relocation of the p110 catalytic subunit to the membrane. Tyrosine phosphorylation of the p85 subunit down-regulates PI3-K activity. p110 can also interact directly with activated, membrane-bound, Ras and thereby itself be activated. One function of the 3-phosphorylated phosphatidylinositol phosphates is to participate with Rac in inducing membrane ruffling. PtdIns(3,4,5)P_3 interacts with the SH2 domains of some proteins, including p85 and pp60$^{c\text{-}src}$, competing directly with receptor protein tyrosine phosphates for binding to these proteins [60]. PtdIns(3,4,5)P_3 also directly activates protein kinase (PKC) ζ, thereby stimulating phosphorylation of serine/threonine residues in proteins targeted by this kinase in a calcium- and diacylglycerol-independent process [61,62].

Src is a protein tyrosine kinase that is attached to the plasma membrane by its N-terminal myristate modification and is essential for the mitogenic action of certain receptors, e.g. that for PDGF. When activated, it phosphorylates a number of cytoskeleton-associated proteins (focal adhesion protein tyrosine kinase, paxillin) and probably contributes to the activation of both Raf-1 and PI3-K [63]. v-Src has been found to activate phospholipase D (PLD) by inducing Ras, possibly by phosphorylation of SHC with subsequent activation of GRB2/SOS, to activate a Ral-GDS activity, the exchange factor responsible for the activation of Ral [64] (see Figure 3). PLD is found in a complex with Ral-A, and when it is activated as a consequence of the Ras–Ral interaction it generates several lipid second messengers, e.g. phosphatidic acid derived from the action of PLD on phosphatidylcholine. v-Src augments both the Ras/MEKK1/JNK and Ras/Raf-1/ERK pathways, which among other actions contribute to the activation of a cAMP response element in the mouse prostaglandin synthase-2 promoter to enhance transcription of the gene [65].

Adaptor proteins can mediate phosphotyrosine interactions

SOS is a cytoplasmic Ras-GEF that is typically constitutively associated with the adaptor protein GRB2 and can be stimulated to activate Ras in various ways [66]. When GRB2 interacts with a tyrosine-phosphorylated membrane receptor, it positions SOS at the plasma membrane where it can promote activation of Ras. Emphasizing the importance of the cellular location of an enzyme is the observation that SOS derivatives engineered to localize at the membrane are able to activate Ras in a receptor-independent manner and to transform cells. Cell transformation by SOS (achieved by targeting SOS to the cell membrane by providing it with a farnesylation or myristoylation signal) is accentuated by deletion of the C-terminal region, suggesting that this region can fold to inhibit the interaction of SOS with Ras [67]. Alternatively, instead of GRB2 binding directly to a tyrosine-phosphorylated receptor such as the activated EGF receptor (Figure 5a), the SOS–GRB2 complex may interact with a phosphotyrosine on SHC, which in turn can associate with a receptor phosphotyrosine, as illustrated for the NGF receptor TrkA (Figure 5b). The importance of the SHC–GRB2–SOS pathway in *ErbB2* (p185, or *HER-2/neu*) signalling was demonstrated by the finding that GRB2 in which the N-terminal SH3 domain was deleted was able to block substantially the oncogenic signal from activated *ErbB2*; although still able to bind SHC, the ΔN-GRB2 could no longer bind SOS [68].

The SHC gene encodes several variant proteins that become tyrosine phosphorylated when various receptors are activated. SHC can itself transform fibroblasts and promote PC12 differentiation in a Ras-dependent fashion. It appears that SHC first binds to a tyrosine-phosphorylated receptor, which phosphorylates a tyrosine in SHC that can then serve as bait for GRB2 docking and consequent SOS activation. The FGF receptor-1 stimulates the GRB2–SOS complex not only via their mutual interactions with SHC but also via p89, another adaptor protein that is in addition membrane-associated [69]. Although the SH2 domain of SHC has been considered as its primary receptor interaction domain, a recently identified PTB domain that recognizes NPXY(P) may mediate this interaction instead [70–72]. By computer analysis, Bork and Margolis [36] detected this domain in a number of other proteins and designated it PID (for phosphotyrosine interaction domain).

Signalling via the heterotrimeric G-proteins

Activation of heterotrimeric G-proteins (consisting of α, β and γ subunits), typically by stimulated serpentine (7-pass) membrane receptors [74], is effected by the replacement of GDP with GTP on the Gα component and the consequent dissociation of Gα from the membrane-bound G$\beta\gamma$ heterodimer. Like Ras, Gα is active only when bound to GTP; it is inactivated by its intrinsic GTPase. Research during the past few years has revealed that signals from the heterotrimeric G-proteins, particularly those stimulated by pertussis toxin-inhibitible receptors (G_i), can impact on the Ras/MAPK signal transduction cascade [75–77]. The G$\beta\gamma$ subunit, by virtue of its ability to interact with certain PH domains, may influence the activity of either SOS or Ras-GAP, both of which have PH domains, perhaps by attracting them to the plasma membrane. In COS-7 cells, agonists acting on G_s-, G_q- or G_i-coupled receptors stimulated p44^{MAPK} (ERK1) expressed as an epitope-tagged molecule in transiently transfected cells; both the Gα and G$\beta\gamma$ subunits were implicated in the signalling process [78].

Several groups have transfected COS-7 cells with various expression vectors in order to elucidate the mechanisms of G-protein-coupled receptor action. Crespo et al. [79] found that G$\beta\gamma$ generated by stimulation of a muscarinic m2 receptor activated ERK2 via a Ras-dependent pathway, possibly involving SHC. Touhara et al. [80] used anti-SHC antibodies to demonstrate directly that the pertussis toxin-sensitive G_i-coupled receptor (the α2-C10 adrenergic receptor) stimulated tyrosine phosphorylation of the $\sim$ 50 kDa SHC protein via an action of G$\beta\gamma$. These and other results established that the G$\beta\gamma$ heterodimer, or at least certain species thereof [77], can stimulate phosphorylation of SHC, facilitating the association of the latter with GRB2–SOS, activating Ras and resulting in Raf-dependent ERK activation [81]. This same group also reported that, in Rat 1 fibroblasts, activation of the IGF receptor by IGF-1 also required input from G$\beta\gamma$ subunits (e.g. derived from the G_i-coupled receptor for lysophosphatidic acid) in order for full ERK activation to occur [82]. Signalling from the β-adrenergic receptor, assessed by ERK activation, involves both a positive signal conveyed by G$\beta\gamma$ acting on a Ras-dependent pathway and a negative signal resulting from activation of adenylate cyclase by Gα_s. The increase in cAMP activates protein kinase A (PKA), which suppresses activation of the Ras pathway by an inhibitory phosphorylation of Raf-1 [77,83].

The PH domain (named for its presence in the abundant platelet protein pleckstrin) is an approx. 120-amino-acid module with a characteristic structure; it is found in many signalling proteins and mediates protein–protein interactions and membrane attachment [84]. The membrane-bound G$\beta\gamma$ complex is

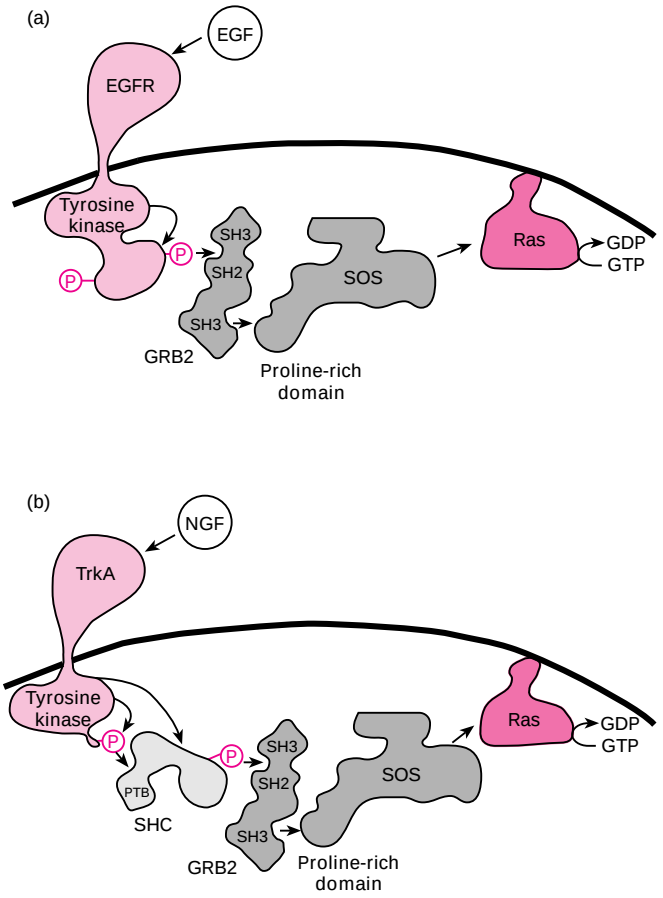

Figure 5 Model for the regulation of Ras by activated receptors

(**a**) The EGF receptor (EGFR) transmits its signal directly through GRB2 to SOS and on to Ras. (**b**) The TrkA receptor, unable to bind GRB2 directly, interacts with SHC via a PTB domain. SHC then provides a mechanism for GRB2 and SOS to be activated. In this representation the locations of tyrosine phosphates (P), PTB, SH2, SH3 and the proline-rich SH3-binding domain are for illustrative purposes only. (Modified from FEBS Lett., **338** Downward, J., 113–117, ©1994, with kind permission of Elsevier Science – NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.)

believed to interact with PH domains in target proteins, causing them to relocate to the cell membrane [76]. The PH domain has also been implicated in the binding of both PtdIns(4,5)P_2 [85–87] and PKC (both calcium-dependent and calcium-independent isoforms) [88]. The PH domain in PLCδ_1, but not in certain other proteins, can bind to Ins(1,4,5)P_3 with stereospecific high affinity [89]. Lee and Rhee [90] have reviewed the significance of PtdIns(4,5)P_2 hydrolysis by PtdIns-specific PLC isoenzymes with regard to the role of PtdIns(4,5)P_2 in regulating PLC isoenzymes, activating PLD and controlling actin polymerization. There are four PLCβ, two PLCγ and four PLCδ enzymes, all possessing a PH domain near the N-terminus. The PLCγ isoenzymes are activated by receptor (and non-receptor) tyrosine kinases, which phosphorylate three tyrosines, one of which (Tyr-783) is essential for activating the phospholipase activity and also for association with the cytoskeleton. The PLCβ isoenzymes are activated by GTP-bound α_q and $\beta\gamma$ subunits of receptor-activated G$\alpha\beta\gamma$. Activated PLC not only generates diacylglycerol and Ins(1,4,5)P_3 but also reduces the level of PtdIns(4,5)P_2 and consequently the number of membrane attachment sites for proteins with PH domains.

THE p21 SIGNALLING PATHWAYS

Ras is normally found attached to the inner face of the plasma membrane by the lipid interactions discussed above. Mobilized Ras–GTP is capable of interacting with and activating a number of proteins; known direct targets include p120Ras^{GAP}, the p110 subunit of PI3-K, Ral-GDS, PKCζ, Raf-1, A-Raf, B-Raf, and possibly certain MEKKs [91,92]. Each of these proteins is likely to be activated by Ras–GTP via a unique interaction with a particular site on the p21 protein, and each contributes to the overall consequence of Ras activation. With regard to interaction sites, Joneson et al. [93] reported recently that the mutation Tyr-40 → Cys abrogated ERK activation but not membrane ruffling, whereas the Thr-35 → Ser mutation impaired membrane ruffling but not ERK activation when quiescent fibroblasts were transfected with the indicated mutant of Ha-Ras. This is consistent with the idea that distinct downstream consequences of Ras activation may be caused by different Ras effectors generating different signals.

Figure 6 shows a generic schematic of the Ras → Raf → MEK → ERK pathway, along with what is known about two related pathways that for the most part operate independently of Ras. These pathways are not equally activated by any single stimulus. Growth factors and mitogens preferentially activate ERK1 and ERK2, whereas inflammatory cytokines and various forms of stress preferentially activate SAPK/JNK and/or p38/RK (see Table 1 for details of nomenclature). Different laboratories using different cells and different activating strategies have identified various different components in these cascades and have often given them different names. The 'take-home message' is that there are several parallel tracks down which a signal can be transmitted. These three phosphorylation cascades are activated to different extents depending upon the particular stimulus and the various other signalling components that are activated (e.g. PI3-K, PKC isotypes, non-receptor Src-family tyrosine kinases and phospholipases). The degree of activation of a particular signalling pathway depends upon the number of activated receptors and the specific upstream effectors that are activated. Phosphorylation of specific residues in some proteins may be inhibitory, thus allowing modulation of the signal intensity. As discussed later (see Figure 9), if different phosphorylated sites in a protein convey separate signals, for example to phosphorylate different kinases, then one has the potential for a pathway to transmit more than one signal simultaneously (multiplex signalling).

The categorizing of various 'levels' in the different pathways in Figure 6 more likely reflects a human desire than nature's intent to arrange things this systematically. A comparison of these pathways with the five or so known pathways in yeast suggests that additional signalling cascades remain to be defined in mammalian cells [94,95]. Consistent with this thought is the existence of several enzymes that might be parts of as-yet-undefined mammalian pathways (e.g. ERK3 [96] and MEK5 [97]). Although 'mitogen-activated protein kinase' has been used as a generic term to describe collectively all of the MAP kinases that are activated by dual phosphorylation in Figure 6, only a subset of them (the ERKs) are typically strongly activated by a proliferative stimulus.

The determination of which proteins are true *in vivo* substrates for the various kinases is not trivial. Conclusions drawn from experiments with purified enzymes and substrates can be extrapolated to the intracellular situation only with certain caveats, particularly concerning protein concentrations and macromolecular associations. Since the concentrations of a particular kinase and its substrate, and their mutual affinity, determine

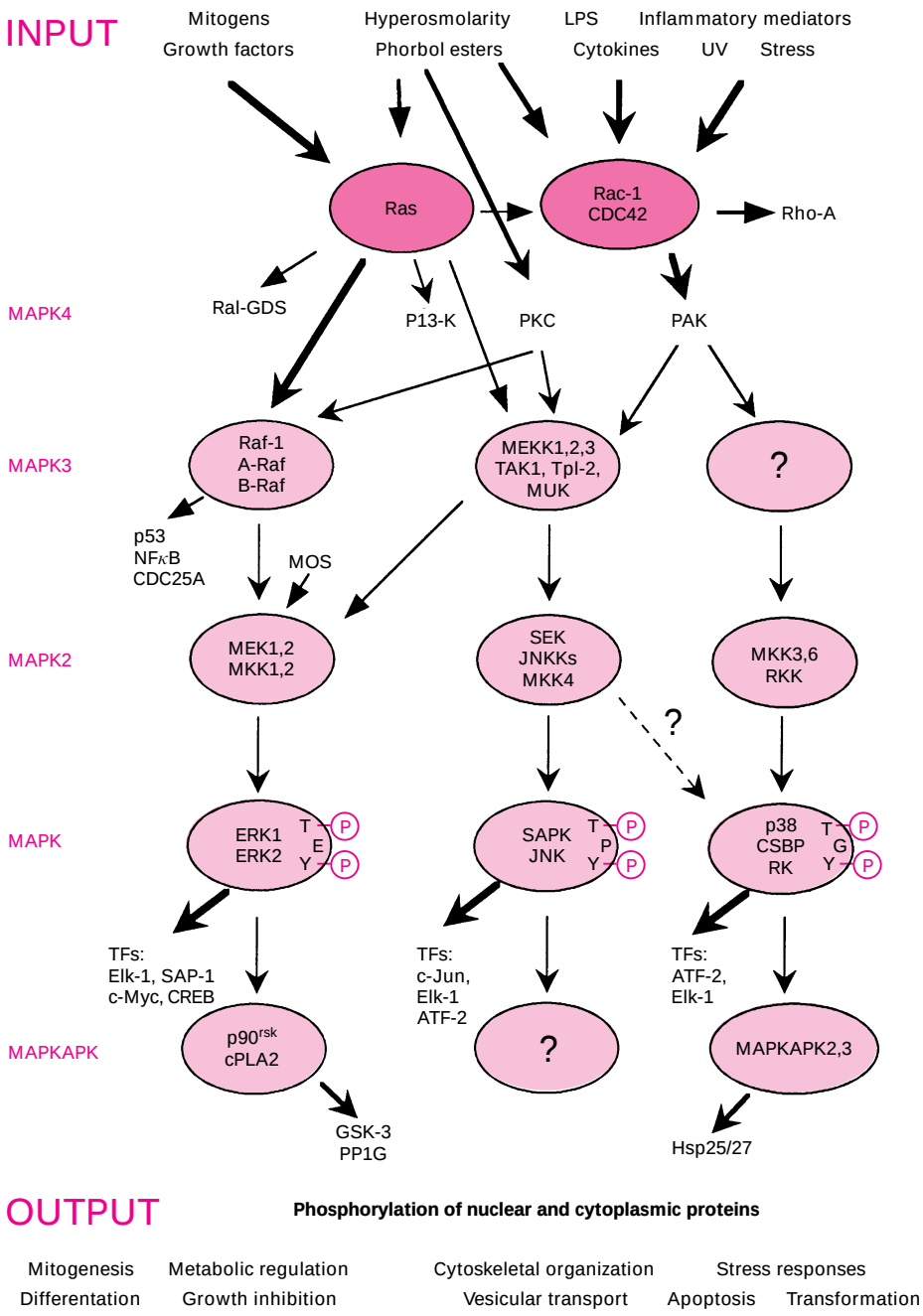

Figure 6 Multiple signal transduction pathways initiated by various stimuli

Considerable evidence suggests the existence of three reasonably discrete protein phosphorylation cascades in mammalian cells, although with an unknown amount of cross-talk and variable activation relative to each other depending upon the stimulus. Arrows indicate some of the major routes of signal transmission; many interactions are not shown. As discussed in the text, localization of the components in the pathway and their recruitment into macromolecular complexes by factors that nucleate specific interactions [116] may endow the pathways with a specificity that is not apparent when efforts are made to dissect the pathways *in vitro*. Phosphorylation of upstream proteins by downstream effector kinases (not illustrated) completes the signalling circuit. Abbreviations: TF, transcription factor; MOS, cellular homologue of the oncogene of Moloney murine sarcoma virus; cPLA2, cytosolic phospholipase A2.

whether that substrate is phosphorylated, *in vitro* experiments should in principle be performed using *in vivo* concentrations, which of course cannot easily be known if the proteins are not homogeneously distributed in the cell. There is also the potential that consecutive elements in a phosphorylation cascade exist in a specific complex, thus providing exquisite specificity with regard to transmission of the signal. Furthermore, if the signalling molecules are normally organized in a complex (a 'signalsome') with a particular scaffold protein present in limiting quantities, then results obtained using transient transfection protocols may not mimic the real situation *in vivo*, thus further clouding the interpretation of various experiments [93]. Signals transmitted within a complex of proteins will not be amplified. Amplification does occur at the level of the downstream effectors, particularly

the MAPKs, and also as the result of positive synergistic interactions of individual signalling pathways.

The 'MAPK4' level

This 'level' encompasses everything between the receptor and MAPK3, i.e. mitogen-activated protein kinase kinase kinase. In this group, which is not as well defined a level as those that follow, are the GAPs and GEFs, the PKC isoforms, PI3-K and $p65^{pak}$. One could argue that PKA and assorted phospholipases should be included also.

The serine/threonine PKC family is divided into three subgroups depending on whether both Ca^{2+} and diacylglycerol are required, whether only diacylglycerol is required, or whether some other lipid (e.g. ceramide) is required [59,62,98]. Second messengers generated from the breakdown of various membrane lipids, especially phospholipids, enhance PKC activity, for example by recruiting the cytosolic protein to the plasma membrane or stimulating proteolysis of the inhibitory pseudosubstrate. The tumour-promoting phorbol esters, analogues of the natural activator diacylglycerol, are potent activators of most PKC isoforms. PKC is regulated by phosphorylation, which is necessary for activity, and by lipids such as phosphoserine and diacylglycerol, the product along with $Ins(1,4,5)P_3$ of the action of PtdIns-specific PLC on $PtdIns(4,5)P_2$. This PLCγ is activated by many receptor tyrosine kinases via its SH2-mediated association with the appropriate phosphotyrosine (Figure 4), and it may make a definite contribution to the mitogenic response. *In vitro* the PKC isoforms are rather promiscuous with regard to which substrates, typically Arg-rich proteins, they will phosphorylate. Although the critical targets of the various PKC isoforms *in vivo* remain for the most part to be discovered, it is clear that PKC is an important constituent of many signalling pathways.

PAK ($p65^{pak}$; p21-activated kinase) was discovered as a brain serine/threonine kinase that is a potential downstream target for Rac-1 and CDC42 signalling. PAK binds to the activated GTP-bound p21 proteins CDC42 and Rac-1, inhibiting their intrinsic GTPase activity and promoting its own phosphorylation [99]. Because PAK-I is active in *Xenopus* oocytes and in quiescent and serum-starved cells, and because (when microinjected) it can inhibit cleavage in blastomers, Jakobi et al. [100] have suggested that PAK-I may be involved in the regulation of cytostasis and the response of the cell to stress. PAK is activated in phagocytes as a result of its interaction with Rac–GTP, and it can phosphorylate the $p47^{phox}$ NADPH oxidase in a Rac–GTP-dependent manner. Rac–GTP is itself generated via an interaction with a Gα protein activated, for example, by a chemoattractant receptor [101]. Coso et al. [102] have suggested that PAK might be an intermediate linking Rac-1 and CDC42 to MEKK1 on the basis of their observation that mutationally activated CDC42 or Rac-1 stimulated JNK/SAPK, but not ERK, activity. *In vivo* experiments with constitutively activated CDC42 and PAK3 revealed that PAK3 was activated by CDC42, and that JNK1 and p38 were in turn activated more efficiently than ERK2 [103]. Studies by Minden et al. [104] and Qiu et al. [105] using constitutively active or dominant interfering alleles of the various signalling intermediates placed Rac-1, possibly followed by PAK, downstream of Ras and upstream of MEKK1 in a signalling cascade resulting in JNKK and p38 activation; attempts to locate CDC42 in the pathway were inconclusive. Studies of interleukin-1 (IL-1) signalling suggested a pathway (Rac/CDC42) → PAK → (unidentified intermediates) leading to p38 and JNK activation [106]. The observation that a dominant negative CDC42 mutant inhibited IL-1 signalling was consistent with such a pathway [103]. Mutationally activated Rac-1 and CDC42 GTPases, and also the GEFs for these Rho family proteins, activate JNK/SAPK without affecting the ERKs [102] (see Figure 3). In addition to PAK, CDC42 may activate activated CDC42-associated kinase (ACK), a non-receptor tyrosine kinase that inhibits the GTPase activity of CDC42, and PI3-K [9,10].

The 'MAPK3' level

Although Ras can interact with a number of effector proteins in a GTP-dependent manner, it is likely that there will be competition among effectors, with certain interactions being preferred over other interactions. Each of these Ras effectors also has its own differing target protein affinities. Thus A-Raf can activate MEK1 but not MEK2 in EGF-stimulated HeLa cells [107]. Ras and Raf-1 form a signalling complex with MEK1 but not MEK2 [108], whereas the Ras–MEKK1 and –MEKK2 interactions preferentially activate JNKK [109]. MEKK1, 2 and 3 are all able to stimulate the ERK and SAPK/JNK pathways, although MEKK1 and 2 preferentially stimulate SAPK/JNK, whereas MEKK3 preferentially activates MEK/ERK [92]. MEKK1 is activated in resting PC12 cells by EGF, and to a lesser extent by NGF, phorbol 12-myristate 13-acetate and oncogenic Ras [110]. EGF-induced activation of MEKK1 and B-Raf can be inhibited by a dominant negative Ras and also by PKA activation (which inhibits Raf). Both the Raf-1 and MEKK1 pathways are stimulated in rat 3Y1 fibroblasts by hyperosmolar (0.5 M NaCl) shock, with consequent activation of ERK1 and ERK2 ($p43/44^{MAPK}$ and $p41/42^{MAPK}$) and the SAPKs (p54 and p46) [111]. How osmotic shock activates these pathways, including that leading to p38 phosphorylation, and the extent to which Ras is involved, is not known.

Downstream elements in Ras-controlled signalling pathways may be activated by various forms of stress-initiated signals that do not function by activating Ras itself. However, Ras does appear to be an important sensor of oxidative stress. Lander et al. [112] observed in PC12 and Jurkat cells that inhibitors of Ras function (a dominant negative mutant p21 and an inhibitor of farnesyltransferase) blocked nuclear factor κB (NFκB) activation by various oxidative agents that modulate cellular redox stress (peroxide, haemin, Hg^{2+}). The authors concluded from these and other experiments that free radicals can activate $p21^{ras}$ and generate a nuclear signal. Other studies have found that the MEK/ERK pathway is activated in neutrophils exposed to an oxidant (diamide or peroxide with a catalase inhibitor) [113] and in HeLa cells exposed to lysophosphatidic acid, which is believed to elevate reactive oxygen species via lysophosphatidic acid-induced arachidonic acid release [114]. However, in these latter two studies the mechanism of MEK activation was not determined.

The interaction of MEKK1 with Ras, at least in the test tube, involves the kinase domain of MEKK1 and the effector domain of Ras. When MEKK1 is complexed with activated Ras it is capable of phosphorylating MEK1 [115]. Xu et al. [116] have shown that a recombinant fragment of MEKK1 containing the catalytic domain could phosphorylate MEK1 and MEK2, and that MEK1 and MEK2 could be activated when co-transfected with MEKK1. However, ERK2 was not activated to the full extent expected, possibly, the authors suggest, because of an inhibitory effect of the up-regulated MEKK1 activity upon ERK2 activity. The relevance of these *in vitro* observations to the *in vivo* situation remains unclear, since it is possible that the highly specific interactions of MEK1/2 with Raf-1 and with

ERK1/2 are the consequence of the formation of macromolecular complexes. It remains unclear to what extent Ras can activate MEKK1 directly, or whether it normally proceeds indirectly via Rac and PAK, or even by a paracrine process involving a secreted extracellular mediator.

In proliferating cells, transforming growth factor-β is usually a negative regulator of proliferation. However, this factor can deliver a mitogenic signal to quiescent (confluent, serum-starved) 3T3 cells in the absence, surprisingly, of detectable tyrosine phosphorylation of the 41 or 43 kDa ERKs (ERK activation typically is associated with a proliferative stimulus) [117]. A kinase called TAK1 has recently been identified as a possible mediator of transforming growth factor-β and bone morphogenetic protein signalling; consistent with this possibility was its inability to activate the MEK $\rightarrow$ ERK pathway and its ability (*in vitro* at least) to activate SEK1 [118]. Another recently identified MEKK, a proto-oncogene called Tpl-2, is of interest in that it has the potential to activate, independently of Ras and Raf, both MEK1 and SEK1 (but not p38/RK); how Tpl-2 is activated is not known [119]. The so-called mixed-lineage kinases have sequence motifs that are associated with both serine/threonine kinases and tyrosine kinases, and at least one of them (called MUK) has the properties of a MEKK that is a selective activator of the SAPK/JNK pathway [120,121].

Reuter et al. [122] have recently characterized several MEK activators, including the $\sim$95 kDa B-Raf and an unidentified 40–50 kDa MEK activator, and shown that they, rather than Raf-1, are the major serum-stimulated MEK activators in fibroblasts. (Lysophosphatidic acid, which stimulates G-protein-coupled receptors, may be the active serum component [122a].) Raf-1 does respond well to both PDGF and phorbol esters. In PC12 cells B-Raf was responsible for the NGF-stimulated, $p21^{ras}$-mediated, activation of MEK [123]. Pritchard et al. [124] expressed the protein kinase domains of Raf-1, A-Raf and B-Raf coupled to the oestrogen receptor and found that after oestradiol activation the B-Raf construct was more effective than the Raf-1 or A-Raf constructs in activating MEK and the ERKs. All three constructs could (after oestradiol activation) morphologically transform 3T3 cells, but only the A-Raf construct could cause quiescent (G_0) 3T3 cells to enter the cell cycle.

The interaction of Ras with Raf-1, illustrated in Figure 7, has been extensively studied [125]. Raf-1 is normally located in the cytosol in association with Hsp90 (heat-shock protein of 90 kDa) and Hsp50, which appear to stabilize the protein and facilitate a proper interaction with Ras [126]. Activation of the 74 kDa Raf-1 protein by Ras involves relocation to the cell membrane via direct association of two regions in the N-terminal conserved region 1 of Raf-1, a previously recognized Ras-binding domain and a more recently recognized cysteine-rich region, with the switch 1 and switch 2 domains respectively of Ras [127,128]. Full activation of Raf-1 requires both an interaction with the plasma membrane and phosphorylation of key amino acids [129–131]. Phosporylation events include tyrosine phosphorylation by membrane-bound Src [132] and serine/threonine phosphorylation by a PKC isoform [133] or by KSR (kinase suppressor of Ras) [134]. The latter is a protein kinase with some similarity to Raf-1 that has been implicated in Ras signalling either upstream of or in parallel to Raf-1. Unlike Raf-1, which requires Ras-induced membrane localization and tyrosine phosphorylation, B-Raf contains aspartic acids in place of the phosphorylatable tyrosines (at 340 and 341) and is not localized to the plasma membrane upon Ras activation [135].

Sites of serine phosphorylation on Raf-1 include Ser-43, Ser-259 and Ser-621; the latter two occur in a consensus sequence RSXSXP, which is conserved among all Raf family members

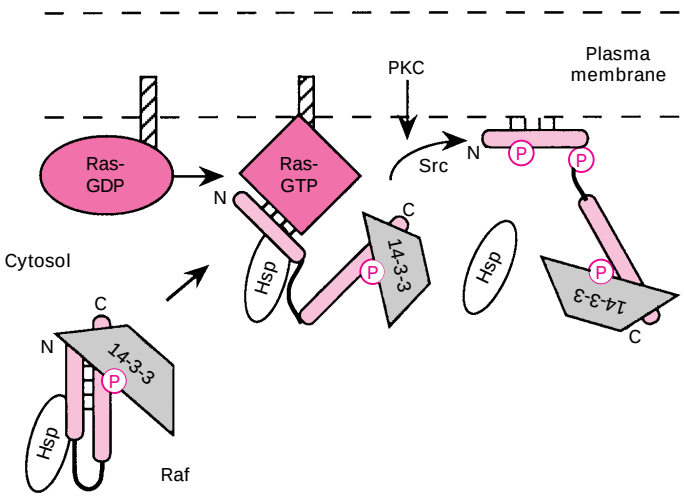

Figure 7 Proposed mechanism for the activation of Raf by Ras and protein phosphoylation

Activated Ras binds to the conserved region near the N-terminus of Raf-1 and attracts it to the plasma membrane, where it undergoes further conformational changes and phosphorylation by PKC and a protein tyrosine kinase, enhancing its ability to phosphorylate MEK. The involvement of 14-3-3 is discussed in the text. The location of phosphate groups, known to include both Ser and Tyr residues, is for illustrative purposes only.

[136]. Phosphorylation of at least some of the serine/threonine residues [those just mentioned and also Ser-499 and Thr-268 (the site of *in vitro* autophosphorylation)], and Tyr-340 and Tyr-341 is essential for complete Raf-1 activation [137]. Ser-43 and Ser-621 are both constitutively phosphorylated, whereas Ser-259 is phosphorylated in response to PDGF. The conformational changes induced in Raf-1 activate its serine/threonine kinase activity, which is specific for Ser-218 and Ser-222 in human MEK1 and for comparable conserved residues in other MEK family kinases [138]. The specificity appears to be provided by a proline-rich sequence in MEK1 and MEK2 that is missing from the other MAPKKs (JNKK/SEK1/MKK4 and RKK/MKK3, described below) and appears essential for the interaction of the MEKs with Raf-1 and downstream signalling proteins [139]. Other possible *in vivo* Raf-1 substrates include the NFκB inhibitor IκB [140], p53 [141] and the dual-specificity phosphatase CDC25A [142]. Thus Raf-1 activation can directly stimulate the expression of genes under the control of NFκB and p53, and can initiate cell cycle progression by dephosphorylation and activation of the cyclin kinases. Two of the three CDC25 isoforms associate with Raf-1 and are phosphorylated when Ras is activated, perhaps accounting for the synergism between CDC25A or CDC25B and Ras in transforming primary cells [143]. CDC25 is a dual-specificity phosphatase that regulates CDC2 kinase, activating CDC2 by dephosphorylating adjacent pThr-14 and pTyr-15 residues.

Hafner et al. [144] showed in studies with purified proteins that phosphorylation of Raf-1 by PKA was inhibitory both to Ras binding and to the functioning of the kinase domain. However, *in vivo* the situation becomes more complex. For example, Raf-1 and the downstream signalling pathway are inhibited by cAMP in fibroblasts, whereas in PC12 cells cAMP synergizes, in the presence of serum, with NGF to enhance neurite outgrowth induced by B-Raf. This difference in sensitivity is the result of the presence only in Raf-1 of Ser-43 in a consensus site for phosphorylation by PKA [145]. Because Raf-1 is rapidly and negatively regulated by PKA phosphorylation *in vivo*, Burgering and

Bos [77] have suggested that Raf-1 activation is a reiterative process and that Ras action is repeatedly required to maintain Raf-1 activity. Raf-1 is also subject to an activating phosphorylation on Thr-269 by a membrane-bound and proline-directed kinase that is activated by ceramide, the second messenger of the sphingomyelin pathway [146]. Both tumour necrosis factor α (TNFα) and IL-1β signal through this pathway, enhancing the activity of Raf-1 towards MEK; presumably Ras–GTP is required to localize Raf-1 to the cell membrane, although this was not investigated. If the different phosphorylations on Raf-1 send different signals downstream, differentially activating MEK1 or MEK2 for example, then Raf-1 would be an example of a multiplex signalling element.

Proteins known as 14-3-3 proteins are typically acidic, dimeric proteins of about 60 kDa that are found in a broad range of organisms. Several isoforms are known, some of which are simply phosphorylation variants. They are found to associate with, and often to modify the activity of, a number of other proteins (e.g. tyrosine hydroxylase, PKC), not unlike chaperones [147]. The catalytic p110 subunit of PI3-K binds to and is inhibited by 14-3-3 [148]. Various isoforms of 14-3-3 have different effects on the different species of PKC; both activation and inhibition of PKC isoforms by preparations of 14-3-3 have been reported [149]. Binding of 14-3-3 to target proteins is via a phosphoserine residue in the motif RSXSXP [150], identical to the conserved consensus sequence for serine phosphorylation in Raf-1 noted above [136]. 14-3-3 associates efficiently with Raf-1 (via the constitutively phosphorylated Ser-621), but does not activate its kinase activity in immunoprecipitates [151]. The association of 14-3-3 (β and ζ isoforms) with inactive Raf-1 may stabilize that conformation, and upon Raf-1 activation it may facilitate activation, perhaps via a specific interaction with the kinase domain [152,153]. As indicated in Figure 7, there are two sites of interaction of 14-3-3ζ with Raf-1, one of which can be competed for by activated Ras [154]. Dent et al. [137] showed that both 14-3-3ζ and Hsp90 could inhibit the inactivation of purified Raf-1 by either serine/threonine or tyrosine protein phosphatases. Because of the dimer structure of 14-3-3, the protein has the potential to foster interactions between proteins to which it can bind, and Braselmann and McCormick [154a] have demonstrated that 14-3-3 can indeed perform such an adaptor function, generating a complex with BCR and Raf, particularly the membrane-bound form. This may be important in facilitating the action of BCR on target proteins (see Figure 3).

Buscher et al. [155] investigated in the macrophage-like line BAC-1.2F5 the signal transduction pathways used by CSF-1 and lipopolysaccharide (LPS) to induce phosphorylation of the two ternary-complex factors Elk and SAP. Although CSF-1, a mitogen, stimulated Ras–GTP formation, Raf-1 activation curiously did not seem to depend on Ras activation, as judged by its insensitivity to a dominant negative Ras. LPS, a stimulator of macrophage differentiation, clearly did not utilize Ras at all, and instead activated Raf-1, MEK and ERK via a PKC and phosphatidylcholine-specific PLC pathway. Elk, but not SAP, activation was dependent upon ERK activation. The CSF-1 receptor appeared to activate Src, which in turn activated Raf-1, probably by tyrosine phosphorylation; full mobilization of the downstream ERKs and Elk was accomplished synergistically with other signals generated simultaneously by the CSF receptor. The LPS receptor CD14 was also able to induce activation of Src family kinases.

The 'MAPK2' level

The MAPKKs are typically activated by serine/threonine phosphorylation catalysed by one of the MAPK3s (Raf, MEKK). The MAPKKs (MEKs, MKKs, JNKKs, SEKs, RKK; see Figure 6 and Table 1) are a subset of protein kinases that possess unique dual (or mixed-lineage?) kinase specificities. When activated (e.g. by phosphorylation of Ser-222 in MKK1a [156]) the MAPKKs phosphorylate only very specific native MAPKs on both Thr and Tyr residues in a Thr-Xaa-Tyr motif, where Xaa is Glu, Pro or Gly.

One subgroup of the MAPKKs includes MEK1a, MEK1b and MEK2, which phosphorylate different ERKs [157] but nevertheless appear to elicit similar transcriptional and morphological responses [158]. Using dominant negative and constitutively active forms of MEK1a (MKK1a), Seger et al. [156] demonstrated that this signalling element controlled NIH-3T3 cell proliferation and morphology. Mansour et al. [159] have shown that NIH-3T3 cells expressing constitutively active forms of MEK were transformed, as judged from the formation of both foci in culture and tumours in nude mice. Cowley et al. [160] have shown that, in the appropriate cell context, activation of MEK is necessary and sufficient for growth-factor-induced proliferation (NIH-3T3 cells) or differentiation (PC12 cells).

A second subgroup of these dual-specificity kinases includes SEK/JNKK/MKK4. These are preferentially activated by MEKK1 and 2, but not by the Raf proteins, and their target proteins are the SAPKs/JNKs [161,162]. JNK was identified on the basis of its ability to phosphorylate the N-terminal region of c-Jun on Ser-63 and Ser-73; its activity is stimulated by inflammatory mediators (e.g. LPS, interferon, TNFα, IL-1) and UV light, and can be further augmented by oncogenic Ras [163]. The SAPKs were identified independently as protein kinases that are activated by stress (e.g. heat shock, inflammatory cytokines, inhibitors of protein synthesis, and DNA-damaging agents such as UV light, ionizing radiation, arabinosylcytosine, alkylating agents and topoisomerase inhibitors). For some of these the activating signal is conveyed by the c-Abl non-receptor tyrosine kinase, a nuclear and cytoplasmic protein that shares structural features with Src and activates SAPK via SEK1 [164]. The presence of both SAPK and SEK1 in the nucleus suggests that this cascade may be activated independently of strictly cytosolic or plasma-membrane-bound kinases [164]. Activated (e.g. by AraC) SAPK binds to the SH3 domain of GRB2, which in turn can bind to the (tyrosine phosphorylated) p85 subunit of PI3-K via its SH2 group, forming a SAPK–GRB2–PI3-K complex that effectively inhibits the lipid kinase and protein serine kinase activities of PI3-K [165]. The significance of this may be to favour the SEK $\rightarrow$ SAPK pathway over the MEK $\rightarrow$ ERK pathway.

The JNKs and SAPKs are the same set of proteins, and they are activated by SEK1, the same enzyme as JNKK [166]. Because of the greater efficiency with which MEKK1 activates JNKK/SEK/MKK4 as compared with MEK, it appears that (despite its name) these may be the normal substrates for MEKK1 in the cell, while the 'true' activators of MEK are the Rafs and possibly MEKK3. Since MEKK1 can at high concentrations activate MEK, it is not excluded that there are circumstances whereby MEK is a substrate for MEKK1 *in vivo*. Ras and the Ras-coupled agonist Raf-1 are inefficient activators of SEK and SAPK. Thus there appear to be two pathways under the influence of Ras (see Figure 6): (1) the Raf $\rightarrow$ MEK/MKK $\rightarrow$ ERK pathway activated by growth factors, mitogens and tumour promoters that is highly dependent upon Ras activation; and (2) the MEKK $\rightarrow$ SEK/JNKK/MKK4 $\rightarrow$ SAPK/JNK pathway that can be stimulated through Ras but is more strongly activated by various stressors and cytokines in a largely Ras-, Raf- and MEK-independent manner.

MKK3, along with MKK4, was first cloned from human brain tissue [167]. It, and the closely related MKK6, appear to be related to a yeast gene involved in responding to hyperosmotic conditions (pBS2), and are responsible for phosphorylating p38, which was cloned on the basis of its enhanced phosphorylation in a mouse pre-B cell line by LPS [168,169]. The sequence of p38 most closely resembles that of the yeast kinase HOG-1, which is involved in adaptation to osmotic stress and is the substrate for pBS2. IL-1 and hyperosmolar conditions also induce p38 phosphorylation. MKK3/6 preferentially phosphorylate p38, whereas MKK4 preferentially phosphorylates JNK [166,170]. p38, which is not significantly activated via Ras-controlled pathways, is efficiently activated by MKK3 and MKK6, and can in turn activate both ATF-2 and Elk-1 [171].

The MAPK level

The activated serine/threonine protein kinases collectively referred to here as 'MAPKs' constitute a superfamily of proteins that includes the ERKs, JNKs/SAPKs and p38/RK MAP kinases. These are uniquely identified by the Thr-Xaa-Tyr dual-phosphorylation motif, where Xaa is respectively Glu, Pro and Gly for the ERKs, JNKs/SAPKs and p38/RK/CSBP. Phosphorylation of both tyrosine and threonine residues, which are found in the activation segment of the kinase domain, is essential for full kinase activity of the MAPKs [172]. (In the literature, 'MAPK' may refer either specifically to the ERKs or more generally to this superfamily.) Upon activation, the activated MAPK may be translocated to the nucleus (if it is not already there), where it can phosphorylate targeted transcription factors [e.g. c-Jun, SAP-1, Elk-1, c-Myc, activating transcription factor (ATF)-2 and the cAMP response element-binding protein (CREB)]. [SAP-1 was cloned as the serum response factor (SRF) accessory protein [173]; it is not a 'stress-activated protein' and is not activated by JNK.] The MAPKs are proline-directed in the sense that they target only serine and threonine residues that are closely followed by one or more prolines in a motif recognized preferentially by a particular MAPK. Whereas the ERKs are strongly activated by one-pass receptor tyrosine kinases (e.g. the PDGF, EGF, CSF, NGF and FGF receptors), the JNK/SAPKs are potently activated by heterotrimeric-G$\alpha\beta\gamma$-protein-coupled receptors, such as the muscarinic acetylcholine receptor, in a Ras- and PKC-independent manner that also does not involve the ERKs [174].

ERK1 (p43/44^{MAPK}) and ERK2 (p41/42^{MAPK}) have been extensively studied; they are discriminating with regard to the target proteins they phosphorylate, recognizing a proline domain that identifies the substrate and producing (usually) multiple phosphorylations on a Ser/Thr-Pro motif. ERK1 targets Elk-1, a TCF that associates with the SRF on the serum response element, whereas ERK2 exhibits a preference for c-Myc [175]. Potential cytoplasmic targets of ERK1/2 phosphorylation include cytoskeletal elements (microtubule-associated proteins, tau), various kinases [ribosomal protein S6 kinase (both p70^{s6k} and p90^{rsk}), cytoplasmic phospholipase A2, SHP-2, glycogen synthase kinase-3 (GSK-3) and protamine kinase], and upstream signalling elements (e.g. the EGF receptor, SOS, Raf-1 and MEK) [157]. The extent to which phosphorylation of upstream signalling elements reflects significant negative-feedback regulation remains to be determined. Elk-1 and SAP-1 bind to an Ets domain in the serum response element in the c-*fos* promoter along with the SRF. Serum induction of c-*fos* transcription, stimulated by lysophosphatidic acid acting through a serpentine receptor and a heterotrimeric G-protein, is the result of ERK-induced phosphorylation of these serum-response-element-bound proteins (Elk-1 or SAP-1; SRF) [176]. Elk-1 is also phosphorylated by the JNKs, which are activated by MEKK1 in response to UV radiation, heat shock, protein synthesis inhibition and other forms of stress [177].

Mouse p38 (RK) is a kinase at the MAPK level with the unique Thr-Gly-Tyr motif as the site of Thr/Tyr phosphorylation, which is accomplished by the MKK3/RKK dual-specificity kinase [178,179]. Raingeaud et al. [170], working with HeLa and COS cells, reported that UV light, osmotic shock, LPS, TNFα and IL-1 activated JNK and p38, but not the ERKs, whereas EGF and phorbol 12-myristate 13-acetate gave maximal activation of the ERKs but only a modest activation of JNK or p38. p38 did not phosphorylate cytosolic phospholipase A2, c-Myc or c-Jun, whereas it did phosphorylate ATF-2 and small heat-shock proteins, and stimulated expression of inflammatory cytokines. There are two versions of the human p38 protein, CSBP1 and CSBP2, that are splice variants differing in an internal 25-amino-acid sequence; when expressed in yeast, the protein kinase activity of CSBP1, but not CSBP2, was increased under hyperosmolar conditions [180].

The outline of several parallel pathways leading to activation of different subsets of MAPKs is taking shape (Figure 6), although the extent to which there is cross-talk among these pathways is unknown [181–183]. In some cases apparent cross-talk can instead result from the induced secretion of a protein or hormone that acts back on the cell in a paracrine manner [184]. In U937 human leukaemic cells, transient expression of a constitutively active MEK1 stimulated SAPK as well as ERK activity by an apparent intracellular signalling process [185]. Whether or not this happens under normal signalling conditions remains to be determined. It is important to note that, usually, multiple elements are activated by a given stimulus or receptor, generating parallel signalling tracks that may or may not be self-reinforcing. One consequence of this is that loss of an individual component may have a relatively small effect. The extent to which the signal is amplified at each step is unclear. The signal is unlikely to be amplified as it passes through a multiprotein complex, for example a MAPKK–MAPK complex. Upstream (e.g. Ras, Rac) and downstream (e.g. ERKs, JNKs) elements almost certainly do amplify the signal.

The 'MAPKAPK' level

This group of MAPK-activated protein kinases consists of various kinases phosphorylated by the MAPKs. Included are upstream signalling elements whose phosphorylation may modulate the signal.

Ribosomal protein S6 kinase, p90^{rsk} (which apparently is capable of phosphorylating the ribosomal protein S6 only *in vitro*; the related p70^{s6k} does the job *in vivo*), can enter the nucleus, phosphorylate c-Fos and thus contribute to the control of transcription of genes under the control of AP-1 [157,186]. It, along with c-Myc and cytoplasmic phospholipase A2, are among the proteins phosphorylated (but not necessarily activated) by the Ras → Raf → MEK → ERK pathway in response to the activation of the T-cell receptor [187]. Phospholipase A2, activated by a single serine phosphorylation, preferentially releases arachidonic acid, a precursor of prostaglandins and leukotrienes, from the 2-position of various phospholipids. Bohm et al. [188] reported that, in normal human melanocytes stimulated with various growth factors, p90^{rsk} was likely to be responsible for the activation of the transcription factor CREB by phosphorylating it. Ginty et al. [189] made somewhat similar observations in PC12 cells and primary rat cortical neurons stimulated with NGF, although in this case the Ras-dependent protein kinase

responsible for activating CREB did not appear to be $p90^{rsk}$. The $p90^{rsk}$ kinase has also been called MAPKAPK-1.

The authentic S6 kinase, $p70^{s6k}$, is strongly but indirectly activated by PI3-K and by PLCγ1 [190]. It is considered to regulate a range of signalling pathways by phosphorylation of important target proteins in addition to the ribosomal protein S6. Stimulation of $p70^{s6k}$ via PI3-K may be mediated by certain PKC isoforms or by $p65^{PAK}$; stimulation by PLC is also likely to be via PKC, a known activator of $p70^{s6k}$, via formation of diacylglycerol. Interestingly, there is an 85 kDa splice variant of $p70^{s6k}$ that possesses an N-terminal addition with a nuclear localization signal that places this isoform in the nucleus. Activation of $p70^{s6k}$ is blocked by the immunosuppressive drug rapamycin, which has a number of effects on the cell, including the suppression of translation of specific mRNAs and inhibition of NFκB activation. CDC42/Rac contributes to $p70^{s6k}$ activation also, possibly via $p65^{PAK}$ [190].

MAPKAPK-2 is a 50 kDa protein kinase that can be activated in various cells (KB, HeLa, PC12, monocytes and macrophages) by chemical or physical stress (sodium arsenite, heat shock, osmotic stress) and by certain cytokines and inflammatory mediators (IL-1 and LPS). It is responsible for phosphorylation of the small heat-shock proteins Hsp25/Hsp27. Phosphorylation of Hsp27 facilitates actin polymerization, probably contributing to the repair of stress-damaged actin microfilaments [191]. Activation, by phosphorylation, of any two of the three residues Thr-222, Ser-272 and Thr-334 is accomplished by the 38–40 kDa protein p38/RK, which is itself activated by an RKK [169,178,179,192]. Although *in vitro* the ERKs can phosphorylate MAPKAPK-2, this does not typically happen *in vivo*, in that activators of the Ras → Raf → MEK → ERK pathway do not stimulate phosphorylation of MAPKAPK-2 and, conversely, strong activators of p38/RK do not typically cause significant phosphorylation of the ERKs. Exceptions to this include both LPS and hyperosmolality, each of which can activate both ERK and p38 [169]. MAPKAPK-3 has also been identified as a substrate for CSBP p38 that is activated by stress, LPS, IL-1 or TNFα [193].

GSK-3 has a larger role than simply that of regulating intermediary metabolism [194]. Its control of early *Drosophila* and *Xenopus* development suggests strongly a comparable role in mammalian development. Interestingly, it also has properties of a dual-specificity protein kinase in the MAPK group [195] and it functions to complete the activation of CREB previously phosphorylated by PKA on a nearby serine residue (hierarchical phosphorylation) [196]. It can also phosphorylate multiple metabolic enzymes, cytoskeletal proteins, and nuclear factors including c-Jun, CREB and $p90^{rsk}$ [197]. GSK-3 itself is subject to multiple levels of control, including both serine/threonine and tyrosine phosphorylation, both activating and deactivating, by PKC and MAPKAPK-1 ($p90^{rsk}$) [198,199]. Thus GSK-3 is a candidate for a multiplex signalling element. The insulin-stimulated inhibition of GSK-3 is mediated by protein kinase B, which is also known as AKT/Rac, a serine/threonine protein kinase distantly related to the PKC family and regulated by *D*-3-phosphorylated phosphoinositides, products of PI3-K [200,201].

Some extracellular cytokines that do not typically activate Ras nevertheless activate downstream elements of Ras-mediated signal transduction pathways. Such cytokines include IL-1 [178], interferons [202] and TNFα [109]. For example, IL-1 activates SAPK/JNK, but not ERK1 or ERK2, by a pathway that does not require Ras, Raf-1 or MEK activation [203]. David et al. [204] discovered an interaction between ERK2 and the α subunit of the interferon α/β receptor, and suggested that ERK2 may regulate the interferon → JAK → STAT signalling cascade. TNFα stimulates a sphingomyelinase activity that generates the second messenger ceramide, which appears to act via MEK1 to activate the SAPKs/JNKs, and possibly NFκB [205,206]. IL-3 appears to require Ras to deliver certain signals (e.g. for apoptosis) but not certain other signals (e.g. for growth stimulation), at least in a mouse pro-B-cell line [207]. Interestingly, growth hormone appears to stimulate a signalling pathway mediated by JAK2 activation of ERK2 in a Ras- and Raf-dependent process [208]. Clearly, there is the potential for an immense variety of different signalling pathways.

SIGNAL DOWN-REGULATION AND TRANSCRIPTION UP-REGULATION

Receptor signalling can be terminated in several ways. Phosphorylation by serine/threonine kinases activated as a consequence of receptor activation is one (negative feedback, e.g. by PKA, PKC or one of the MAPKs), and dephosphorylation of key phosphotyrosines by phosphotyrosine phosphatases (e.g. SHP-1) is another. In contrast to this easily reversed down-regulation is the more permanent shut-off resulting from ligand-induced internalization of the receptor and its degradation in lysosomes. An interesting example of signal desensitization is provided by the EGF receptor vIII transforming mutant, which is deleted for much of the extracellular domain of the receptor and undergoes spontaneous homodimerization, autophosphorylation and constitutive activation. It binds much of the cell's GRB2 and strongly activates MEK; nevertheless, ERK1 and ERK2 are activated to a lesser extent and seem to resist further activation by various agonists, apparently because of the activation of a tyrosine phosphatase that negatively regulates their activity [209].

Phosphorylation of upstream signalling elements by activated downstream kinases is one mechanism for shutting down a signalling pathway. For example, phosphorylation of SOS (on serine/threonine residues in proline-rich motifs in the C-terminal domain) by ERK (p44/p42 MAPK) inhibits its ability to activate Ras and results in separation of an *intact* SOS–GRB2 complex from the activated receptor (e.g. SHC or the EGF receptor) [210,211]. Somewhat at odds with this are the reports that ERK activation, either by an insulin-stimulated Ras/MEK pathway or by osmotic shock acting via a MEK-dependent but Ras-independent pathway, led to phosphorylation of SOS and *dissociation* of the GRB2–SOS complex, terminating the ability of SOS to promote disengagement of GDP from Ras–GDP [212,212a]. These differences could, of course, reflect differences in the input signals and the regulatory circuits activated in the different cell types in these studies. Specific activation of JNK but not ERK by anisomycin, which inhibits protein synthesis and activates the stress-activated signalling pathways, did not cause GRB2–SOS dissociation, suggesting that SOS is not a substrate for JNK [213]. In cells with a wild-type Ras requiring GEF activity, this negative-feedback regulatory circuit attenuates the Ras-activated signal transduction pathway. The signal would be more persistent in cells containing a mutant oncogenic Ras that did not require GEF activity.

The length of time a signalling pathway remains active varies with the cell type and the receptor involved. For example, PC12 cells stimulated with FGF or NGF exhibited a persistent elevation of Ras–GTP levels and a sustained activation of the ERKs, which were translocated to the nucleus, resulting ultimately in differentiation as shown by neurite outgrowth. In the same cells stimulated with EGF, Ras–GTP levels increased only transiently and ERK activation was short-lived. Besides differences in the details of just which intracellular signalling molecules are con-

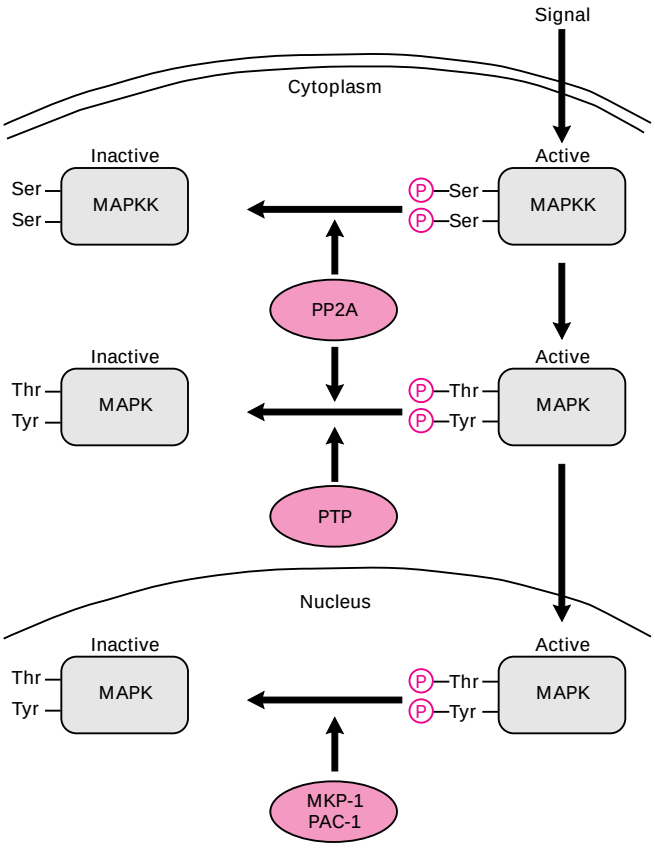

Figure 8 Dephosphorylation of active signalling intermediates by protein phosphatases acting in the cytoplasm or the nucleus with varying specificities

PP2A and other serine/threonine phosphatases dephosphorylate phosphoserine and phosphothreonine residues as indicated. Several protein tyrosine phosphatases have the potential to dephosphorylate the pTyr moieties. PTP represents a generic protein tyrosine phosphatase. Dual-specificity protein phosphatases with a predominantly nuclear location include MKP-1 (previously known as 3CH134 and CL100) and PAC-1. (Redrawn from Biochim. Biophys. Acta, **1265** Keyse, S. M., 152–160, ©1995, with kind permission of Elsevier Science – NL, Sara Burgerhartstraat 25, 1055 KV Amsterdam, The Netherlands.)

trolled by each receptor, there is also the fact that the EGF receptor is more rapidly down-regulated than the NGF receptor through phosphorylation and internalization. Appropriate manipulation of EGF or NGF receptor levels can lead to either response, suggesting that the critical element is the intensity of ERK activation [181].

Many of the signalling elements are returned to their ground state by cytoplasmic and nuclear phosphatases (Figure 8) that remove the activating phosphates from the various intermediates [54]. A corollary of this is that inhibitors of phosphatase activity can mimic the effect of a kinase by prolonging the state of activation of a signalling intermediate. Protein serine/threonine phosphatases (e.g. PP1, PP4, PP5, PP2A, PP2B, PP2C) remove phosphates from protein phosphoserine and phosphothreonine residues [214]. Many of them are sensitive to okadaic acid. Barford [215] has reviewed structural studies of the protein phosphatases, noting their classification into four structurally distinct families and the fact that the depth of the catalytic site cleft determines whether the phosphatase will target a tyrosine phosphate or serine/threonine phosphate.

Various protein tyrosine phosphatase activities have been identified (e.g. CD45, SHP-1, SHP-2), and much current research is directed at discovering their specificities [216]. Because of the number of names given to the same protein tyrosine phosphatases, a uniform nomenclature has recently been proposed [52]. Thus SHP-1 (also known as SHP, PTP1C, SHPTP-1 and HCP) dephosphorylates many tyrosine-phosphorylated receptor proteins and has been implicated in attentuating the mitogenic response [216]. SHP-2 (also known as SYP, SHPTP-2, SHPTP-3, PTP2C and PTP1D) is a protein tyrosine phosphatase that appears to act immediately downstream of Ras as a positive mediator of growth factor signalling by a number of receptors (those for insulin, IGF-1 and EGF). Phosphatases (e.g. SHP-2) can also provide a positive signal by removing inhibitory phosphates, for example the phosphate in (unactivated) Src that obstructs an SH2 domain necessary for activity. It is not known whether SHP-2 phosphatase action itself is necessary for transmission of the signal from activated receptors such as the PDGF receptor, or whether its effect is because of its potential to associate with GRB2–SOS [217–219].

Dual-specificity protein phosphatases (e.g. MKP-1, PAC-1) are predominately nuclear proteins that dephosphorylate both phosphothreonine and phosphotyrosine residues. They exhibit considerable specificity for individual MAPKs [220]. Growth factor stimulation and many forms of physical or chemical trauma enhance the mRNA levels of these enzymes, suggesting an important role for PAC-1 and MKP-1 in modulating the signal transduction pathways initiated by mitogens and stress respectively.

In cells with wild-type Ras, activation of Ras in response to the binding of a ligand to an appropriate receptor is transient, and consequently the transmitted signal has the nature of a pulse. Importantly, the Ras-transmitted signal is also accompanied by parallel signals generated by other signalling elements mobilized by the receptor; some of these signals may serve in a negative-feedback capacity. As shown in Figure 6, the signal often results in the modification of one or more transcription factors, giving rise to changes in gene expression. These changes in gene expression are the consequence of a cell-specific integration of all the input signals, including both their intensity (i.e. number of activated receptors) and duration (which varies with the receptor activated), and although the complexity of the pathways and their cross-talk is bewildering, there do seem to be some consistent patterns emerging [221].

Oncogenic forms of Ras have been shown to activate all three of the protein phosphorylation cascades illustrated in Figure 6 [94]. In contrast to wild-type Ras, the signal from a mutant oncogenic Ras (a transformation signal) is continuous, is not accompanied by independently generated upstream signals and results in the perpetual stimulation of certain downstream signalling elements, probably giving rise to an altered relationship among the signalling intermediates and a permanent state of modification of particular downstream transcription factors [16]. As a consequence of the particular Ras mutation or the permanent stimulation of certain pathways, there may also be changes in the way Ras interacts with proteins such as Ral-GDS, PI3-K, and the GAPs, GEFs, Rafs and MEKKs. Many of the identified Ras response elements in promoters and enhancers involve either AP-1 (Fos/Jun) or Ets transcription factors, often in situations where the two binding sites overlap. The resulting changes include the increased expression of genes that are important in making cells tumorigenic and metastatic, e.g. proteinases and adhesion molecules [222,223]. It is interesting to note that in at least some cell types both Rac and Rho make important contributions to Ras transformation [224]. Finally, the fact that most of the studies that have led to our current picture of all these signalling pathways have been done with

immortal cell lines must not be ignored. It remains to be determined how the immortalization process has impacted on these signalling pathways.

PERSPECTIVE: IMPLICATIONS OF MULTIPLE SIGNALLING CASCADES AND MULTIPLEX SIGNAL TRANSMISSION

When a receptor is activated, a number of signalling pathways are typically stimulated to various degrees. Receptor, non-receptor and receptor-associated protein tyrosine kinases phosphorylate themselves and specific target proteins. Other proteins are then activated by virtue of their association with tyrosine phosphate groups on these proteins, directly or via an adaptor. Heterotrimeric G-proteins coupled to serpentine receptors trigger signalling pathways through both their Gα and G$\beta\gamma$ subunits. The resulting cascades of interactions provide a self-reinforcing stability to the signal; the contribution of any one element is important to, but may not be critical to, the successful delivery of the signal. The branching of the pathways permits a degree of signal diversification, while at the same time cross-talk among pathways permits one pathway to reinforce or attenuate the signal transmitted by a second pathway. Signals initiated by different receptors are distinguished by the different mix of pathways stimulated and the intensity of their stimulation. Thus NGF stimulation of 3T3 cells expressing the human TrkA receptor inhibits proliferation, possibly via the induction of the cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1}$; this occurs despite the fact that NGF is more effective at activating the p42 and p44 ERKs than is EGF, which stimulates proliferation [225]. It may be instructive that EGF binds GRB2–SOS directly, whereas TrkA acts via SHC phosphorylation and the formation of SHC–GRB2–SOS complexes [181].

A very important principle to appreciate is that most of these pathways in the cell are likely to be functioning at a modest level in a healthy cell in normal (unperturbed) conditions. The balance among the pathways results in a homoeostatic state. Perturbations of different sorts upset the balance in different ways, leading for example to cell differentiation or proliferation on the one hand, or to growth inhibition or apoptosis on the other. For example, removal of NGF from PC12 cells reduces the stimulation of the Ras → Raf → MEK → ERK1/2 pathway and tilts the balance towards net activation of the MEKK → SEK → JNK and MKK3 → p38 pathways, resulting in apoptosis [226]. Similarly, activation of the B-cell surface IgM receptor activates the Ras → Raf → MEK → ERK (but not the JNK) pathway, leading to cell activation, differentiation or apoptosis (depending upon other variables), whereas activation of the CD40 receptor (a member of the TNF receptor family that recognizes a ligand expressed on activated T-cells) activates JNK/SAPK, but not ERK, and inhibits the apoptotic response induced by anti-IgM [227]. Insulin promotes the survival of neurons in culture, perhaps by virtue of the fact that it negatively regulates p38 kinase activity [228].

The fact that some of the proteins in a pathway become multiply phosphorylated (e.g. Raf-1, MEK, GSK-3) raises the possibility that more than one signal may be transmitted simultaneously via the same pathway (Figure 9). This is known as multiplex signalling. For example, MEK is preferentially phosphorylated on Ser-218 by MEKK1, but on both Ser-218 and Ser-

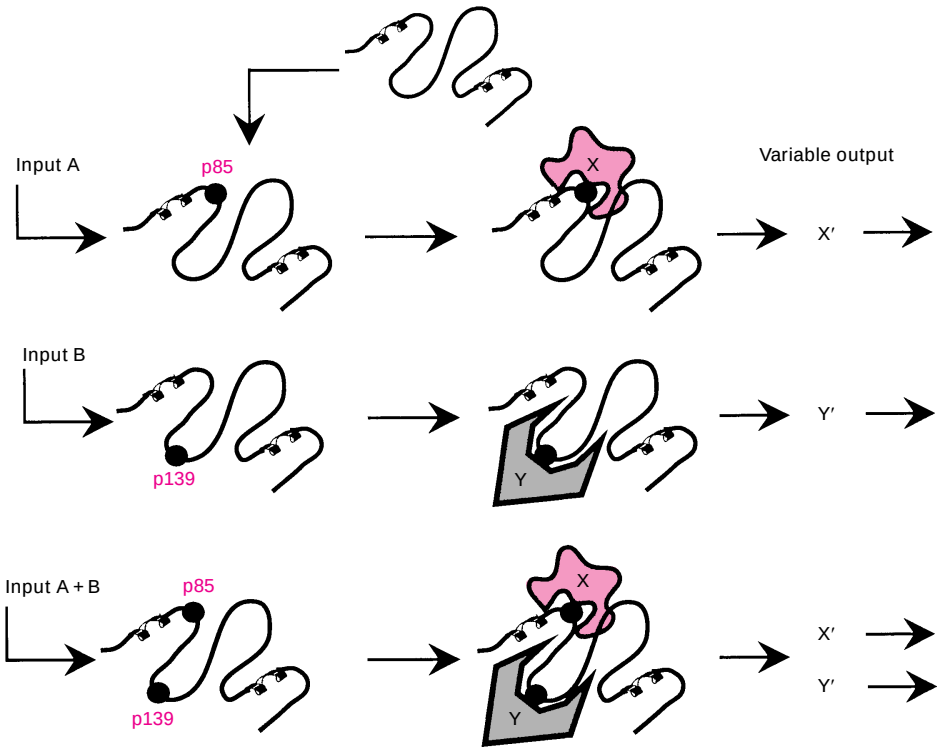

Figure 9 Model for multiplex signalling via a multiply phosphorylated protein

In this hypothetical example, one input signal leads to phosphorylation of amino acid 85, whereas input of a second signal phosphorylates residue 139. Each of the phosphorylated amino acids must interact independently with a different downstream signalling element, providing a distinct output. When both amino acids are phosphorylated, then the protein conveys both signals simultaneously.

222 by Raf-1 [4]. Is it possible that the type of signal MEK delivers is different in these two cases? As illustrated by the hypothetical example in Figure 9, when amino acid 85 is phosphorylated it interacts with and passes the signal on to protein X, whereas when residue 139 is phosphorylated it interacts with and passes a signal on to protein Y; phosphorylation of both amino acids leads to both signals being transmitted. Selective mutation of one or other of the relevant amino acids followed by determination of the type of signal transmitted would give some indication of whether different signals are being sent or not. Activating and inhibiting phosphorylations of a particular element, e.g. a transcription factor, are a form of multiplex signalling only in the sense that the second signal is a Null signal. If multiplex signalling is occurring, it adds yet another layer of complexity to the signalling process, one that will be as fascinating as it will be difficult to sort out in future research.

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