Leaders

The mammalian mitogen activated protein kinase network

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The families of MAP kinases cascades (mitogen activated protein kinases) form major signalling systems by which cells transduce extracellular signals into cellular responses. Studying the regulation of these MAP kinase signalling pathways is of utmost importance in the understanding of the triggering of cell proliferation versus cell differentiation and the responses of the cell to stress or osmotic factors.

This review will describe in detail the archetypal MAP kinase cascade (Raf-MEK-ERK); it will present the more recently discovered MAP kinase cascades (SAPK and p38) and provide some indications on the regulation of this network of signalling pathways. The scope of the review is not to provide an exhaustive listing of all aspects of research in this field; it aims rather to provide a basis for readers unfamiliar with the subject to scrutinise specific points of interest. Special attention will be given to introduce clearly the nomenclature of mammalian MAP kinase cascades, since it is often confusing with up to five names being used for the same mammalian enzyme. Readers requiring more detailed information on the MAP kinase cascades are referred to other recent reviews.¹⁻⁵

Importance of protein phosphorylation in cell signalling

Phosphorylation is a reversible means of covalently modifying proteins. The phosphate group being added is highly hydroxylated and negatively charged; thus it is sterically large and able to alter markedly the tridimensional conformation of proteins. Phosphorylation may be permanent and necessary for correct folding of many proteins. Reversible phosphorylation consists of the addition of a phosphate from ATP by a specific protein kinase, and subsequent removal of this phosphate through a protein phosphatase. Phosphorylation of many enzymes affects their catalytic properties. The importance of protein phosphorylation in regulating cellular activities is illustrated by the large number of protein kinase genes that are present in eukaryotic genomes. The latest estimate is that humans have as many as 2000 conventional protein kinase genes.³ It is becoming increasingly obvious that protein phosphatases play a specific role in regulating cellular activities and some aspects of these regulating activities will be dealt with in the final section of this review.

Protein kinases have broadly been classified into two main categories according to the amino acid that they phosphorylate: the tyrosine kinases and the serine/threonine kinases. Recently a new class has been discovered, the dual specificity protein kinases that phosphorylate both tyrosine and serine/threonine residues. Additionally, in bacteria⁶ and yeasts,⁷ histidine kinases have been identified which function as osmosensors. In yeast the histidine kinase osmosensor triggers activation of one of the three yeast MAP kinase cascades.⁷ Each newly cloned kinase can be classified in one of these categories by sequence homology, and thus its specificity rapidly revealed.

The MAP kinase cascade module

MAP kinases (MAPK) are serine/threonine kinases which are activated by a dual phosphorylation on threonine and tyrosine residues⁸ in a sequence of three amino acids: TXY (T = threonine, Y = tyrosine).⁹ The central amino acid (X) of this triad defines each family of MAP kinase as is indicated in fig 1. Their specific upstream activators, called MAP kinase kinases (MAPKK),¹⁰ constitute a family of dual specific threonine/tyrosine kinases, which in turn are activated by MAP kinase kinase kinases (MAPKKK) which are serine/threonine kinases.¹¹ A schematic diagram at the left of fig 1 illustrates the MAP kinase cascade module. There are several families of MAP kinase cascades, and members of each family are conserved among various species such as mammals, frogs, flies, worms, or yeasts (reviewed in ¹). This high degree of conservation during evolution points towards a fundamental role for these enzymes in cell physiology.

Theoretical enzymological analyses indicate that an amplification factor of 10 can readily be achieved at each stage of these kinase cascades.³ A three protein kinase cascade, such as the MAP kinase cascade, could generate a theoretical amplification of 1000-fold. This provides an easy means to augment a specific signal received at the cell surface by a relatively small number of receptors. The actual amplification achieved by a MAPK pathway *in vivo* is not yet known, but experimental evidence shows that binding of less than 5% of Raf-1 molecules to Ras is sufficient to induce maximum activation of the archetypal MAP kinase ERK (cascade illustrated in fig 1).¹² As

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Figure 1 Diagram indicating the nomenclature of the mammalian MAP kinase network. On the left are represented the three levels of the MAP kinase cascade module. Each box represents a cascade and provides alternative names for each step. At the bottom is indicated the amino acid sequence of the phosphorylation sites of the MAPKs (T=threonine; Y=tyrosine; E=glutamic acid; G=glycine; P=proline).

will be described later, activation of the ERK cascade is an absolute requirement for growth factor mediated cell division. Therefore it may be expected that any deregulation of the signalling component leading to ERK activation would have a profound effect on cell proliferation because of signal amplification by the cascade. For analytical purposes, amplification of the signal at the end of the cascade may prove to be a useful tool for detecting aberrant cellular pathologies initiated by undetectable abnormal regulation of an upstream component.

Nomenclature

While searching for cytoplasmic substrates of tyrosine kinase receptors, Ray and Sturgill were the first to purify two proteins of 42 kDa and 44 kDa phosphorylated on tyrosine and threonine residues when 3T3-L1 adipocytes were stimulated by insulin.^{8,13} The preferential substrate of these proteins was the brain Microtubule Associated Protein 2, and thus they were named MAP kinase (mapk).^{8,13} At the same time, other laboratories working with these p42^{mapk} and p44^{mapk} gave the following names to the same enzymes: MBP kinase (Myelin Basic Protein) kinase^{14,15} and RSK kinase (90 kDa Ribosomal S6 Kinase-kinase).¹⁶

As numerous agonists were able to stimulate MAP kinase activity, Sturgill and Weber proposed that the same acronym MAP kinase could stand for Mitogen Activated Protein kinase. Cobb and colleagues^{17,18} then identified the cDNAs corresponding to several isoforms

of the MAP kinase proteins and named them ERK, for Extracellularly Regulated Kinases (ERK1 for the p44^{mapk}, ERK2 for the p42^{mapk}).

Accordingly, the same protein may be called MAP kinase, ERK1, p44^{mapk}, MBP kinase, and RSK kinase. Similar confusion occurred later with the direct upstream activator of ERK which was named MAP kinase kinase (MAPKK),¹⁰ or MEK for "MAP or ERK Kinase" in an attempt to reconcile the two acronyms MAP and ERK.¹⁹

Recently, two other families of enzymes presenting some homologies to ERKs have been cloned and termed SAPK for Stress Activated Protein Kinases^{20,21} and p38.²² Their activation is also achieved by dual specificity threonine/tyrosine kinases presenting homologies to the ERK activator.^{23,24} Thus a reasonable consensus exists at the time of writing for calling "MAP kinase cascade" a protein kinase cascade of three enzymes activated sequentially as follows: a serine/threonine kinase (MAPKKK) which activates a dual specificity tyrosine-serine/threonine kinase (MAPKK), which activates a serine/threonine kinase (MAPK) (fig 1).

MAPK (interchangeably MAP kinase) is thus the generic name of the third member of each cascade, whereas the name ERK is reserved for the third enzyme of one of the MAP cascades, the Raf-MEK-ERK cascade. Figure 2 gives a synopsis of the actual nomenclature used for the three mammalian MAP kinase cascades and provides alternative names that can be encountered in published reports. The names may correspond to isoforms or enzymes

Figure 2 Schematic representation of the identified and putative signalling pathways leading to activation of the three MAP kinase cascades. The black stars represent cloned members of the MAP kinase cascades. Known phosphorylation sites are indicated on the left of the stars. Arrows represent direct activation or protein-protein interactions; see text for details.

* Indication of the cellular responses resulting from the activation of these cascades should be interpreted with care, as crosstalk and multiple activation occur, and some responses may be cell specific.

from different species, and thus a precise equivalence between the names of each isoenzyme is still lacking.

Activation of the Raf-MEK-ERK cascade, the archetypal MAP kinase cascade

ACTIVATION BY TYROSINE KINASE RECEPTORS (TKR)

Following ligand binding, TKRs dimerise and transphosphorylation occurs on several tyrosine residues of the receptor. Src homology 2 (SH2) domain containing proteins are recruited to these phosphorylated tyrosine residues (reviewed in ²⁵). Binding specificity for each SH2 domain is provided by the four amino acids upstream of the tyrosine.²⁶ Among the numerous SH2 proteins recruited, the adaptor protein Grb2 leads to activation of the Raf-MEK-ERK pathway, at least in the case of the EGF (epidermal growth factor) and PDGF (platelet derived growth factor) pathways.²⁵ Grb2 has no intrinsic catalytic activity; it is composed of one SH2 and two SH3 domains which promote protein-protein interactions. In this case, the role of Grb2 is solely to recruit proteins bound to its SH3 domain (Src homology 3), such as the Ras guanine-nucleotide exchange factor, Sos, to the phosphorylated receptor bound to its SH2 domain. The complex Grb2-Sos is

brought to the membrane where Sos interaction with Ras-GDP catalyses the dissociation of GDP from Ras allowing GTP loading and activation of Ras. Then by direct interaction with Ras-GTP, Raf-1 is brought to the membrane^{12,27-30} where it is likely to be activated by another mechanism which may involve phosphorylation. At present this specific issue is not resolved and two groups have shown recently that the targeting of Raf to the membrane was sufficient to induce Raf activation fully.^{31,32} Activated Raf-1 phosphorylates MEK³³ on two serine residues S222 and S228.³⁴ In turn MEK phosphorylates ERK on a tyrosine (Y183) and a threonine residue (T185) in the sequence TEY (E = glutamic acid).¹³

An alternative pathway to transmit Ras activation to MEK is thought to involve MEK kinase (MEKK),³⁵ which phosphorylates MEK on the same sites as Raf. Furthermore, it appears that Ras may have a third effector, PI3 kinase,³⁶ and thus activated Ras could exert a multiplicity of effects. Understanding all the cellular signalling pathways induced by Ras is a great challenge for future research; matters are complicated by the existence of three isoforms of Ras in each cell: H-Ras-1, K-Ras-2, and N-Ras (reviewed in ³⁷). What is the rationale for having three isoforms in one cell? How many direct partners interact with Ras? The contribution of multiple Ras functions to

mammalian cell transformation has recently been demonstrated.³⁸

ERK1 and ERK2 are widely expressed in most cell types, and in virtually all cells these two isoforms are coordinately regulated and display the same substrate specificity. Hence it is not understood why cells need both isoforms.³⁹ ERK1 and ERK2 have been shown to phosphorylate numerous substrates on the consensus site PXS/TP (reviewed in ¹), but in vitro many kinases show an apparently broader substrate specificity; it is thus difficult to identify unambiguously substrates of ERK in vivo. Putative in vivo substrates of ERK include surface receptors, cytosolic protein, and nuclear proteins.¹

One of the best candidates for nuclear targets of ERK is the transcription factor Elk-1 (also named p62^{TCF}).⁴⁰⁻⁴² ERK phosphorylates Elk-1 on identical sites in vitro and in vivo. Elk-1 plays an essential role in increasing the rate of transcription of many immediate early genes such as c-Fos (reviewed in ⁴³). Elk-1 interacts with SRF (serum response factor) and forms a ternary complex with an SRF dimer.⁴⁴ The phosphorylation of ELK on the sites phosphorylated by ERK has been shown to increase the transcription in vitro of genes containing an SRE (Serum Response Element, binding site for SRF).⁴⁵

In quiescent cells, ERK is cytosolic^{46,47} and thus unable to be in contact with nuclear proteins such as Elk-1. Upon mitogenic stimulation, ERK translocates to the nucleus,^{46,47} and can thus phosphorylate several nuclear targets. It is noteworthy that MEK⁴⁷ and Raf (⁴⁸ and Lenormand P, personal communication) are exclusively located in the cytoplasm and Ras is membrane bound following specific post-translational modifications at the C terminus (reviewed in ⁴⁹); thus ERK is the first member of this MAP kinase cascade able to translocate and transmit extracellular signals to the nucleus.

Another means of transmitting signals from the Ras-MEK-ERK cascade to the nucleus is performed by ERK activation of the cytosolic kinase p90^{RSK} (p90 ribosomal S6 kinase), which in turn can translocate to the nucleus upon stimulation.⁴⁶ The mechanism of nuclear translocation of ERK is not understood as ERK does not possess nuclear localisation sequences (NLS), and we have shown in this laboratory that the nuclear translocation of ERK occurs even when the two phosphorylation sites are mutated and the kinase is no longer activatable.⁴⁷ As will be discussed later, nuclear translocation of ERK is thought to be a critical step in triggering cell proliferation or differentiation; thus understanding the mechanism of this translocation will prove to be very important.

ACTIVATION BY NON-TYROSINE-KINASE RECEPTORS

Many hormones and neurotransmitters acting through G protein coupled receptors also activate the Raf-MEK-ERK pathway by a mechanism that involves Ras activation. It has been

demonstrated that the thrombin, muscarinic acetylcholine M2, and α_2 adrenergic receptors activate Ras (conversion from Ras-GDP to Ras-GTP).⁵⁰ The link by which the G protein coupled receptors activate Ras is not at present well established, and may involve proteins of the shc, src family, and (or) PTP1D (Syp). It was shown recently that coexpression of the β and γ subunits of heterotrimeric G proteins was able to activate Ras.⁵¹

Many G protein coupled receptors activate protein kinase C (PKC) and ERK, but it is not clear yet how PKC activation is transmitted to the Raf-MEK-ERK pathway, nor which isoforms of PKC are implicated.¹ One report indicates that PKC can activate Raf-1 directly by phosphorylation⁵²; alternatively PKC is known readily to phosphorylate members of the 14-3-3 protein family (reviewed in ⁵³) which have been shown to be bound to Raf. There is some controversy as to whether or not the 14-3-3 protein is indeed able to activate Raf.⁵⁴⁻⁵⁷ This shows that deciphering the molecular events leading to Ras and (or) Raf activation will be an area of intense research.

Roles of the Raf-MEK-ERK cascade

Cells express many types of tyrosine kinase receptors at their surface. Interestingly only some of them can induce cell proliferation (or cell differentiation in a different cell context). We shall see that the duration of the Raf-MEK-ERK cascade activation provides clues to understanding the final outcome of the cellular response elicited by various tyrosine kinase receptors.

CELL PROLIFERATION

The importance of the Raf-MEK-ERK cascade in conducting the mitogenic signal is deduced from an array of data. (1) Transfection of oncogenic forms of Ras or Raf induce the proliferation of many cell lines.³⁸ (2) Injection of oncogenic Ras into quiescent fibroblasts is sufficient by itself to stimulate DNA synthesis.^{58,59} (3) Expression of constitutively activated forms of MEK, generated by site directed mutagenesis, is sufficient to induce mitogenesis and transformation in fibroblasts.⁶⁰⁻⁶² Constitutively active MEK1 mutants were generated by replacing the two serines that are the sites of activation by Raf by negatively active amino acids that mimic the negative charge brought by the phosphorylation (S222D and S228D, or S222E and S228E; D=Aspartic acid, E=glutamic acid). (4) The dominant negative form of ERK can block mitogenic signalling.⁶³ These dominant negative forms are generated by mutation of the phosphorylation sites (T183 or Y185) or mutation of the ATP binding site. The dominant negative forms of ERK are only effective when highly expressed in a cell, and the mechanism of their action is not understood; they may act by making stable complexes with MEK.⁶³

In most cell types, many agonists stimulate ERK (reviewed in ¹), but there are several

noticeable differences in the pattern of ERK activation according to the mitogenic potential of these agonists. For example in CCL39 fibroblasts it has been shown that non-mitogenic stimuli activate ERK in a transient manner (maximum of activation between 10 and 30 minutes), whereas mitogenic stimuli induce a second phase of activation that persists for hours.⁶⁴ In addition, only mitogenic stimuli are able to induce nuclear translocation of ERK.⁴⁷ Thus mitogenic stimuli activate ERK for a long duration and induce its nuclear translocation, which may then induce phosphorylation of transcription factors. In this way several stimuli can activate ERK, but the quantitative difference in ERK activation that they induce (durable versus transitory) is translated into a qualitative difference (phosphorylation of transcription factors or not). Establishing the full cascade of events linking the prolonged ERK activation by mitogenic stimuli to cell proliferation is an immense challenge for future research in this field.

DIFFERENTIATION

The response of PC12 cells to receptor tyrosine kinase activation has been extensively used as an experimental system to study transduction and how activation of some receptors triggers differentiation, while the activation of others leads to proliferation. Treatment of PC12 cells with nerve growth factor (NGF) triggers neurite outgrowth and stops cell division,^{65,66} whereas treatment with EGF leads to cell proliferation.⁶⁷ ERK activation is sustained for several hours following NGF stimulation, but it is transitory with EGF stimulation.⁶⁸⁻⁷⁰ The association of prolonged ERK activation with NGF stimulation of PC12 cells has led to the idea that it is sustained activation of this pathway that leads to differentiation. Confirmation of this theory came from three sets of experiments. (1) In PC12 cells, stimulation of the endogenous EGF receptor induces transient activation of ERK and does not lead to differentiation, but overexpression of the EGF receptor leads to EGF dependent differentiation and sustained activation of ERKs.⁷¹ (2) Expression of constitutively active MEK mutants in PC12 cells induces neurite outgrowth in the absence of differentiating factors.⁶⁰ (3) Dominant negative mutants of ERK block ligand activation of endogenous ERK and stop differentiation of PC12 cells.⁶⁰ These experiments show that ERK activation is necessary and prolonged activation is sufficient to trigger PC12 cell differentiation.⁷¹ There is a correlation between sustained activation of ERK induced by NGF and differentiation. Thus, as for ERK involvement in fibroblast proliferation, these experiments lead us to propose in PC12 that the sustained ERK activation induces marked changes in gene expression which induce cell differentiation. In conclusion, one can propose that prolonged ERK activation triggers a cell fate that was preprogrammed in the cell, leading to differentiation in PC12 cells and cell proliferation in fibroblasts.

The newly discovered MAP kinase families

STRESS ACTIVATED MAP KINASES: SAPK

In 1991, Kyriakis *et al*⁷² discovered a 54 kDa protein kinase that was phosphorylated on serine, threonine, and tyrosine residues and activated in liver extracts from cycloheximide treated rats. Subsequent protein purification, peptide sequencing, and cDNA cloning revealed a family of closely related stress activated protein kinases (SAPKs) activated by stresses such as heat shock and by inflammatory cytokines such as tumour necrosis factor α .²⁰ Independently, other laboratories identified two kinases that phosphorylate the amino terminus of *c-Jun* (on serines 63 and 73) and potentiate *c-Jun* transcriptional transactivating activity.^{73,74} These enzymes were thus named *jun* N terminal kinase: JNK1 and JNK2.²¹ JNK1 is closely related to SAPK γ , and JNK2 to SAPK α . These enzymes constitute a new family of MAP kinases since their activation requires dual phosphorylation on a tyrosine and a threonine residue at the sequence TPY (P = proline), instead of the sequence TEY required for ERK.

The race to identify the SAPK activator led to the nearly simultaneous discovery of SEK1^{23,24} and MKK4⁷⁵ which are highly homologous. Two initiation codons can potentially be used to start translation of the SAPK activator, and it appears that the proteins engineered for in vitro studies by these two groups differ: SEK1 starts at the first potential initiation codon and is 34 amino acids larger than MKK4. The SAPK activation cascade was further defined by discovering that MEKK is able to activate in vivo and in vitro both SEK²⁴ and MKK4.⁷⁵ Initially MEKK was thought to contribute in parallel with Raf-1 to the activation of MEK, and thus was named MEK kinase (MEKK).³⁵ However, MEKK is able to activate both MEK and SEK when highly expressed in a cell line, but upon mild (normal?) activation it solely phosphorylates and activates SEK.^{23,75} In fact, it is more likely that MEKK activates the stress pathway of SAPKs (thus SEK) than the mitogenic pathway of ERKs (thus MEK) as expression of MEKK in fibroblasts arrests cell growth (²⁴, and Brunet A, personal communication).

Matters are complicated by the knowledge that both Raf and MEKK can be activated by Ras^{35,76}; the action of cofactors in Ras activation must intervene to lead specifically to the activation of one of these two pathways. In addition to Raf and MEKK, it has been shown recently that Ras was able to activate PI3 kinase.³⁶ Understanding precisely the events leading to Ras and Raf activation and all the pathways initiated following Ras activation is a great challenge for the future, and for cancer research in particular, as the presence of oncogenic forms of Ras has been found in at least 10% of all human cancers.³⁷

OSMOREGULATED MAP KINASES: p38

Three closely related proteins, p38, RK, and CSBP kinase, were cloned independently in 1994 and belong to a new MAP kinase family,

as they are activated upon dual phosphorylation of both a tyrosine and a threonine residue in the sequence TGY (G = glycine).

p38

p38 is a mammalian protein kinase phosphorylated on tyrosine in response to lipopolysaccharide (LPS) that was cloned from peptide sequencing after its purification²² and was shown to be closely related to the yeast protein HOG1 (*S cerevisiae*). HOG1 lies in a signalling pathway that restores the osmotic gradient across the yeast cell membrane in response to increased external osmolarity.⁷⁷

RK

RK was cloned after purification of the enzyme that phosphorylated MAPKAP 2 (MAP kinase activated protein 2) upon heat shock or arsenite stimulation, in the absence of ERK activation.⁷⁸ This protein was named RK after its capability to reactivate MAPKAP 2 which is then able to phosphorylate HSP27 (heat shock protein of 27 kDa). HSP27 is induced in response to stress⁷⁹ to protect cells against thermal injuries.⁸⁰

CSBP KINASE

CSBP kinase was identified as the target of a new drug, CSAID (cytokine suppressive anti-inflammatory drug), that inhibits inflammation caused by the action of cytokines such as interleukin-1 and tumour necrosis factor (TNF).⁸¹ CSBP kinase was purified according to its ability to bind to radiolabelled and radio-photoaffinity labelled CSAID. It was thus called CSAID binding protein kinase, CSBP kinase. CSAID blocks the biosynthesis of cytokines through inhibition of the CSBP kinase; it has the extraordinary interest of being the first drug able to block specifically one of the three known MAP kinase cascades.

The three kinases p38, RK, and CSBP kinase represent the same protein and will be referred to in this review as p38. One putative nuclear substrate of p38 is the transcription factor ATF-2⁷⁵, and Brunet A, personal communication). One activator of p38 has been partially purified: RKK (RK kinase)⁷⁸; two others, MKK3 and MKK4, were cloned by polymerase chain reaction (PCR) using degenerated oligonucleotides corresponding to PBS2, the known activator of HOG1 which is the yeast equivalent of p38.⁷⁵ MKK3 has been shown to activate p38 specifically, whereas MKK4 (= SEK) can also phosphorylate SAPK, as was described in the previous section.^{24,75} No upstream activator of MKK3 has yet been cloned but it seems that Ras and Raf are not implicated in the activation of this cascade.⁷⁸ Interestingly if one makes a parallel with the yeast cascade (PBS2 and HOG1⁷⁷ which are highly homologous to MKK3 and p38 respectively), one can envision that an osmosensor capable of triggering the activation of this kinase cascade could be a histidine kinase, a type of kinase unknown in mammalian cells at present. The yeast osmosensor is called SLN1⁷; its mutational deletion

leads to lethality, which can be suppressed by four complementation groups. One suppressor of the SLN1 mutation is HOG1 and another is its activator PBS2. These data clearly indicate that the yeast osmosensor SLN1 activates a kinase cascade similar to the mammalian MKK3-p38 cascade. The two other complementation groups led to the discovery of a cytoplasmic protein homologous to the regulatory domain of the osmosensor SLN1, and of phosphatases.⁷ It remains to be seen whether mammalian proteins homologous to each yeast protein of this osmoregulatory pathway will be found.

Understanding the regulation of the mammalian MAP kinase network

Studying the regulation of such a complex kinase network is fraught with difficulties. This section will provide some reflections about limitations in interpreting current results, and indicate research trends in this field.

THE DIFFICULTIES IN IDENTIFICATION OF IN VIVO SUBSTRATE

In vitro the catalytic properties of a kinase are often modified, usually with a reduction in substrate specificity. To identify substrates unambiguously in vivo, one can only correlate in vitro and in vivo phosphorylation at the same sites, but these sites may be phosphorylated in vivo by several kinases. Phosphorylation may also occur on sites that do not affect the function of a particular protein as we can assess it. For example, *c-fun* was thought to be an in vivo substrate of ERK because ERK was shown to phosphorylate the full *c-fun* protein readily in vitro.⁸² The significance of this ERK dependent phosphorylation of *c-fun* is not understood because C terminal phosphorylation does not affect *c-fun* transcriptional transactivation. It is now acknowledged that SAPKs (JNKs) are the cellular activators of *c-fun* as they specifically phosphorylate both the serines 63 and 73 implicated in activation of *c-fun* transcriptional transactivation.⁸³

The discovery of the p38 specific inhibitor, CSAID,⁸¹ will allow the specific blocking of this kinase cascade and should permit the identification of specific roles of the cascade in cell physiology. Specific inhibitors of the other MAP kinases may be found, and this would allow us to assess the precise contribution of each cascade in cellular responses to mitogenic and non-mitogenic stimuli or to stress factors. One incentive for finding specific inhibitors of ERK is the possibility of blocking many upstream pathological disorders of this pathway, disorders that often lead to aberrant cell proliferation. For example, it is known that mutations of Ras are implicated in about 70% of all pancreatic cancers³⁷; as blocking ERK activation was shown to stop mitogenicity in fibroblasts,⁶³ it would be of interest to test whether this is also true for some types of cancer cells, such as those with constitutively active Ras.

OVEREXPRESSION OF THE KINASE STUDIED MAY CREATE ARTEFACTUAL EFFECTS

MEKK was named from its ability to activate MEK both in vitro and in vivo when overexpressed in fibroblasts.³⁵ Recent work indicates that transfection of MEKK induces both MEK and SEK activation, whereas low expression of MEKK upon conditional induction activates SEK specifically, and not MEK.^{23 24 75} The use of inducible promoters to drive expression of the kinase of interest should be more widely applied. Unfortunately, all available inducible expression vectors present drawbacks such as leaky expression without induction or weak induction, or even secondary effects by the inducer. An alternative strategy is to make fusion proteins with the catalytic domain of the protein of interest and the oestradiol receptor binding domain (ER). In some cases, such as with Raf, the oestradiol binding domain is able to mask the catalytic domain of the kinase, and upon addition of oestradiol the catalytic domain is revealed.^{84 85} Cell lines expressing this Raf-ER have been established, and it has been shown that addition of oestradiol leads to MEK activation within two minutes and ERK activation within five minutes, the maximum of activation being reached by 30 minutes^{(85 and Lenormand P, unpublished data).}

CELL TYPE SPECIFIC DIFFERENCES

One stimulus may provoke opposite effects in two cell types, which may be the result of marked differences in the differentiation programme engaged. I shall illustrate this complexity by two examples, the response of MAP kinase pathways to osmotic shock, and the role of PKA activation on the Raf-MEK-ERK pathway.

Variability in osmotic shock responses

In some fibroblasts, osmotic shock has no significant effects on ERK activation.²² On the contrary, cells of renal origin such as MDCK cells (Madin-Darby canine kidney epithelial cells) respond to hyperosmolarity by activation of Raf, MEK, and ERK.⁸⁶ Clearly fibroblasts are not exposed to the same variations of osmotic pressure as are renal cells; none the less no molecular mechanism can yet explain such differences.

In the cell lines tested so far (mostly fibroblasts), hyperosmolarity modulates both SAPK and p38.^{20 22 87} This can be explained by the fact that p38 and SAPK can be activated by a SEK/MKK4⁷⁵; in addition they could be activated by distinct MAPKKs that are both osmoregulated.

Variability in response to PKA activation

ERK activation is required for fibroblast cell proliferation,⁶³ and it has been appreciated for some time that a rise in cAMP, which activates PKA, inhibits cell proliferation.⁸⁸ In contrast, in dog thyrocytes (cells of epithelial origin) PKA stimulates growth in the absence of ERK

stimulation.⁸⁹ The molecular mechanisms explaining these differences are lacking; however progress was obtained by showing in NIH 3T3 fibroblasts that PKA activation inhibits the ERK pathway at the level of Raf1.⁹⁰⁻⁹⁴ This effect may result from direct inhibitory phosphorylation of Raf1 by PKA.⁹⁵ However, it has recently been shown in CCL39 fibroblasts that PKA inhibition of ERK, although rapid, is very transient (McKenzie F, personal communication). Finally, in PC12 cells, PKA stimulates ERK activation by NGF instead of blocking it.^{96 97}

Interestingly, in PC12 cells PKA inhibits Raf activated by NGF.^{98 99} Thus cAMP activation of ERK would have to be accomplished by Raf independent activation of a distinct MAPKKK such as MEKK, which is activated by a Ras dependent pathway and has been shown to activate MEK in vitro or when overexpressed.³⁵

The different effects of PKA activation on the ERK pathway in fibroblasts, PC12 cells, and thyrocytes provide another illustration of fundamental cell type specific differences. Understanding these differences at the molecular level is a great challenge.

SPECIFICITY IN MAP KINASE ACTIVATION

In some cell lines the activation of MAP kinase pathways can be kept separated; for example in PC12 cells, NGF activates ERK but not p38, and arsenite activates p38 but not ERKs.⁷⁸

This isolation of one MAP kinase pathway from the others may be due to the specificities of the kinases involved; it may also be helped by arranging the cascades of kinases in complexes. The only example of such complex in MAP kinase signalling is provided by the mating-type pathway in the yeast *S cerevisiae*. The kinases of this pathway (homologous to MEK and ERK) seem to be held together by a scaffolding protein, STE5.^{100 101} Kinases from the two other yeast MAP kinase pathways (one for osmosensing and one dedicated to cell wall biosynthesis) cannot bind to STE5.¹⁰⁰ STE5 may bring kinases into close proximity to ensure specificity and speed of activation. Several laboratories are searching for mammalian homologues of STE5, but there is no report of success at present.

CROSS TALK BETWEEN MAP KINASE PATHWAYS

Negative feedback is a widely used mechanism for signal attenuation and desensitisation, though it is not clear yet whether negative feedback operates in MAP kinase signalling pathways. Activated ERK was shown to phosphorylate SOS,¹⁰² Raf,¹⁰³ and MEK.¹⁰⁴⁻¹⁰⁶ It seems that these feedback phosphorylations have little or no effect on MEK and Raf activities, but this lack of effect could be due to our incapacity to measure fine regulation with in vitro studies. Negative feedback by ERK was thought to occur by phosphorylation of the EGF receptor at threonine 669, which does not seem to affect the kinase activity but could increase receptor internalisation.^{107 108}

Two groups reported that ERK phosphorylates SEK,²⁴⁷⁵ but the significance of this crosstalk among MAP kinase pathways is still not understood.

ROLE OF THE PHOSPHATASES

It is increasingly obvious that protein phosphatases are involved in the regulation of signal transduction (reviewed in ³¹⁰⁹¹¹⁰). ERK activation is biphasic, and some phosphatases are likely to be responsible for both phases of inactivation. The phosphatase responsible for inactivating the first peak of activation is unknown, but for the second phase of activation MKP-1 (MAP kinase phosphatase-1, also called CL100) is a good candidate.¹¹¹ It belongs to a new family of dual specificity phosphatases able to dephosphorylate ERK on both the threonine and the tyrosine residue. MKP-1 is an early gene which is not expressed in arrested cells: upon stimulation MKP-1 accumulates after 30 minutes and is nuclear.¹¹² It could be responsible for inactivating ERK in the nucleus; none the less its precise role still needs to be well defined since it is now apparent that a family of MKP-1-like proteins exists which may be specific inactivators of each MAP kinase. However, ERK kinase inactivation is often too rapid to be explained by the MKP-1 family of proteins.¹¹³ Recent data suggest that rapid inactivation of the MAP cascade at the level of ERK is mediated by PP2A (protein phosphatase 2A) which removes phosphothreonine and an as yet unidentified tyrosine phosphatase distinct from MKP-1.¹¹⁴

Concluding remarks

In recent years, signalling by MAP kinase has blossomed into three distinct kinase cascades. The crossover points between individual members of these cascades, and hence the specificity of agonist activation of each cascade, remain to be defined. One of the greatest challenges is to identify the specific *in vivo* substrates of each of the three end members of the ERK, SAPK, and p38 cascades. For pathologists, fundamental research on this pathway will provide tools that may permit the correlation of the aberrant status of an MAP kinase pathway with pathophysiological conditions. For example it is at present impossible to evaluate Ras activation in one cell, and it may remain impossible because of the small percentage of GTP-Ras sufficient to induce full activation of ERK. None the less signal magnification may render the indirect assessment of Ras activation possible from the development of new tools such as monoclonal antibodies that specifically recognise phosphorylated ERK (Nishida E, personal communication). With these antibodies, it is possible to evaluate the subcellular localisation of activated ERK. Will it be possible to distinguish between aberrantly proliferating cells and normal ones by detecting nuclear accumulation of activated ERK?

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