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Comment

Dimerization in protein kinase signaling

Steven Pelech

Address: The Brain Research Centre, Division of Neurology, 2211 Wesbrook Mall, University of British Columbia, Vancouver, BC V6T 2B5, Canada. Email: spelech@kinexus.ca

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Abstract

The closely related mitogen-activated protein kinases ERK1 and ERK2 have now been shown to have opposing roles in Ras-mediated cell proliferation. I propose that dimerization of these highly related protein kinases could underlie these surprising observations and that this could be a common paradigm for widespread regulation of protein phosphorylation by kinase-substrate interactions.

Two closely related mitogen-activated protein (MAP) kinases, extracellular signal-regulated protein kinase (ERK)1 and ERK2, are known to be involved in the regulation of cell proliferation. These ubiquitous protein-serine/threonine kinases are well known as key players in signaling pathways downstream of growth-factor receptor-tyrosine kinases, cytokine receptors and G-protein-coupled receptors [1]; they often indirectly mediate the actions of members of the Ras family of small GTPases. Gain-of-function mutations have been implicated in more than 30% of human tumors, but chronic activation of Ras by mutated mitogen receptors occurs in even higher frequency than this [2]. Most previously published work has inferred that ERK1 and ERK2 are commonly regulated and that they target the same substrates. In this issue of the *Journal of Biology*, however, Riccardo Brambilla and colleagues [3] provide compelling evidence that the two ERK proteins in fact counteract each other in the regulation of the cell-proliferation effects of Ras in mouse fibroblasts.

Vantaggiato and Formentini *et al.* [3] have demonstrated that induced reduction of ERK1 expression using antisense constructs leads to enhanced ERK2 function and increased Ras-dependent cell proliferation, whereas knockdown of ERK2 expression has the opposite effect on cell growth. Furthermore, they found that catalytically inactive (knockdown or KD) and active (wild-type or WT) forms of ERK1 were equally capable of inhibiting oncogenic Ras-mediated cell proliferation, cell colony growth in soft agar, and tumor formation in nude mice. These findings run counter to the popular notion that the ERK1 and ERK2 MAP kinases, which share 83% amino-acid identity, have similar if not the same functions [1].

At first glance, it is extraordinary that ERK1 can inhibit oncogenic Ras-mediated cell proliferation, given that it was thought that ERK1 and ERK2 have the same targets and functions. Ras mediates the recruitment of the protein-serine/threonine kinases Raf1 and RafB to the plasma membrane, where they become phosphorylated and activated by several other protein kinases. In turn, the Rafs phosphorylate and activate the MAP kinase kinases MEK1 and MEK2, which then phosphorylate and stimulate ERK1 and ERK2. Hyperactivation of Ras and other oncoproteins that stimulate this canonical MAP kinase pathway can induce apoptosis; Vantaggiato and Formentini *et al.* [3] have shown, however, that the antagonistic effects of ERK1 on Ras action are not simply due to an overall gain of MAP kinase activity that elicits a feedback inhibition response.

To explain their surprising observations, Vantaggiato and Formentini *et al.* [3] have proposed a simple competition model for the interaction of ERK1 and ERK2 with their immediate upstream activating kinases MEK1 and MEK2. They argue that ERK1 might act by displacing ERK2 from MEK1 and MEK2. If this were the case, it might be possible to compensate for the effect of WT-ERK1 or KD-ERK1 on reduction of phosphorylation of ERK2 by increasing the levels of MEK1 or MEK2, thus reducing the amount of competition. The authors [3] also found, however, that the suppressive effects of WT-ERK1 or KD-ERK1 on Ras-induced cell proliferation were even greater when a version of ERK2 was used that was defective in its kinase activity. This indicates that simple competition for MEK1 or MEK2 is insufficient to account for the results entirely; there is in fact no evidence that ERK1 and ERK2 do not compete equally for binding to MEK1 and MEK2.

The KiNET proteomics database [4] holds expression and phosphorylation data for MAP kinases and hundreds of other signaling proteins that have been quantified by western blotting of thousands of cell and tissue extracts. Using KiNET, it is possible to perform meta-analyses and correlate these proteins, in order to uncover their interrelationships. As shown in Figure 1, this analysis reveals a broad range of differential expression levels of ERK1, ERK2, MEK1 and MEK2 in organs, tissues and cultured cell lines. The protein levels of ERK1 were more than double the ERK2 levels in two-thirds of 30 different mouse and human tumor cell lines examined; only one cell line showed a modest 30% increase in levels of ERK2 relative to ERK1 (data not shown). Remarkably, MEK2 levels were also typically double those of MEK1 in these cell lines. These same trends were found when 33 different mouse and human tissues and organs were also tested for expression of these kinases (Figure 1). In view of these findings, it is somewhat ironic that MEK1 tends to dominate the discussion within the scientific literature, as revealed by a simple PubMed search (1,772 MEK1 citations; 156 MEK2 citations).

Although measurement of the expression levels of target proteins can provide some clues about their potential roles in biological processes, specific quantification of the functionally active forms of the proteins can give far more insights. Queries of the KiNET database [4] enabled me to assess the phosphorylation status of ERK1, ERK2, and MEK1/MEK2 at their activation sites in 116 human and rodent cell lines. Only aggregate data was available for MEK1 and MEK2, because MEK1 phosphorylation-site-specific antibodies recognize both kinases identically, and the two MEKs also co-migrate closely on western blots. Figure 2 shows the results from the specific analysis of 69 human cell lines. It is evident that there is huge variability in the phosphorylation status of these kinases across the cell lines examined, and several lines lacked detectable phosphorylation of one or more kinases. These findings show no apparent correlation between the levels of either active ERK1 or active ERK2 and cell proliferation. Of the 116 cell lines, however, 40% had twofold or higher levels of phospho-ERK2 than of phospho-ERK1 (59% had 25% or more phospho-ERK2 than phospho-ERK1). By contrast, only 8.6% of the cell lines showed twofold or higher levels of phospho-ERK1 relative to phospho-ERK2 (18% of the cell lines showed 25% or more phospho-ERK1 than phospho-ERK1).

Elevated phosphoprotein levels detected by western blotting with phosphorylation-site-specific antibodies can reflect a rise in the number of protein molecules (if the stoichiometry of phosphorylation is unchanged), increases in the rates of phosphorylation, or reductions in the rates of dephosphorylation of these proteins. In most cell lines the phosphorylation signals were higher for ERK2 than for ERK1, whereas the total protein levels of ERK2 were generally much lower than those of ERK1. This indicates that, in general, ERK2 was preferentially activated over ERK1 in the proliferating cells. If phospho-ERK2 is more susceptible to proteolysis when it is activated, that could also account for the lower protein levels of ERK2 relative to ERK1 in proliferating cells.

In their study, Vantaggiato and Formentini *et al.* [3] have speculated that the rates of translocation and sequestration of ERK1 and ERK2 to the nucleus or their dephosphorylation may differ. They also point out that there could be subtle differences in the substrate specificity of ERK1 and ERK2. Even though these two kinases are both directed to their phosphorylation site by proline-rich motifs and appear to have identical preferences for the consensus phosphorylation site sequence in their substrates (Pro-X-Ser/Thr-Pro) [5,6], there are additional specialized docking sites on MAP kinase substrates, such as D-domains (a cluster of basic amino-acid residues surrounded by hydrophobic amino-acid residues) and DEF domains (Phe/Tyr-X-Phe/Tyr-Pro) that might confer additional specificity [7,8].

The ability of MAP kinases to dimerize contributes yet another level of complexity to their regulation and substrate specificity. Over the past few years, there has been mounting evidence that ERK1 and ERK2 are retained in inactive states in the cytoplasm of cells, bound in dimeric complexes with MEK1 and MEK2 [9,10]. Direct phosphorylation of these MEK isoforms (human MEK1 at Ser217 and Ser221; MEK2 at Ser222 and Ser226) by upstream kinases (such as

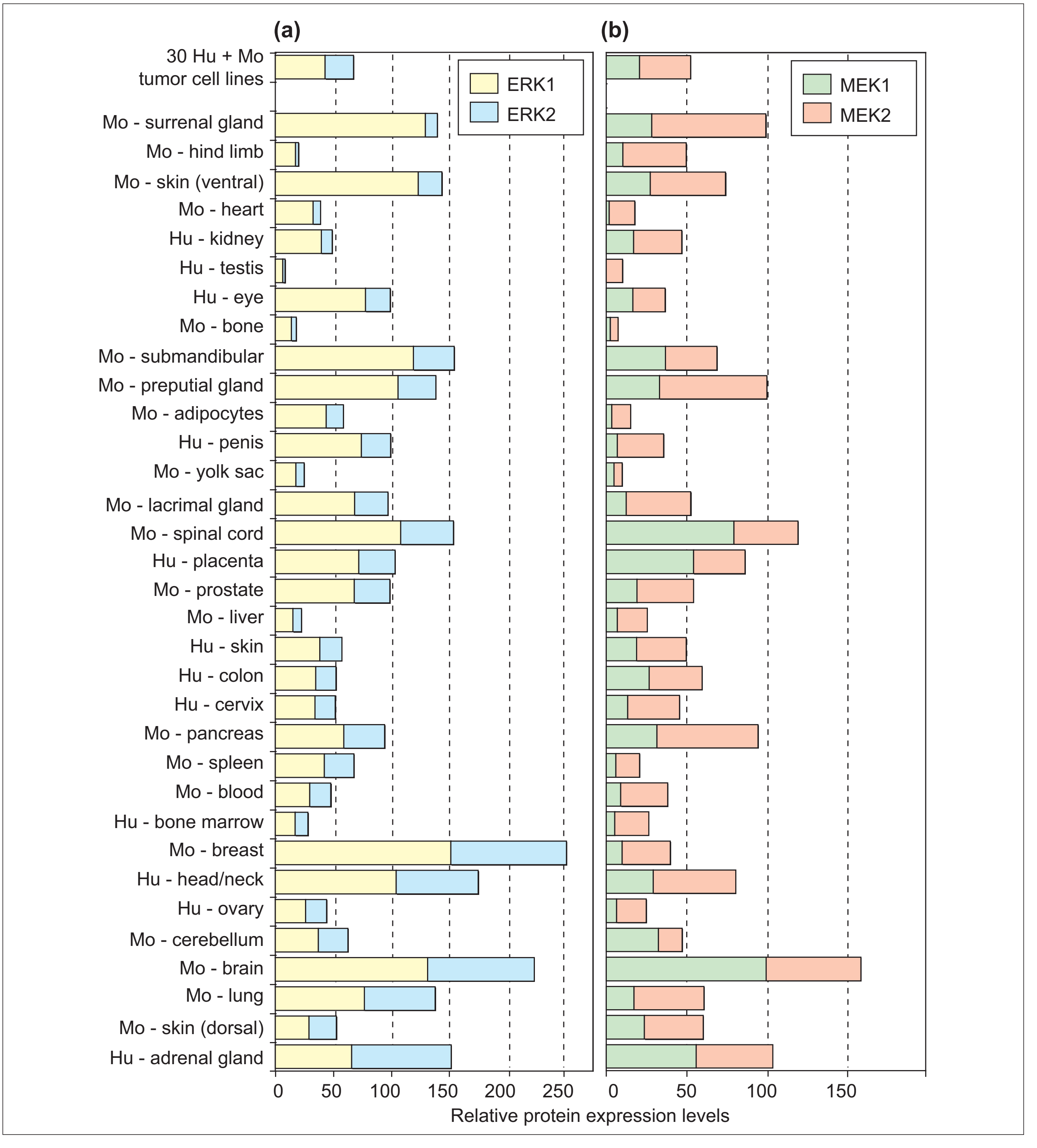

Figure 1

Relative expression levels of MAP kinases and MAP kinase kinases in diverse tissues and organs. Western blotting was used to quantify the relative protein levels of **(a)** ERK1 and ERK2 and **(b)** MEK1 and MEK2 in 306 human (Hu) and mouse (Mo) tissue and organ specimens. Values are the mean of at least triplicate (range 3 to 38) determinations from measurements for each kinase in 33 diverse tissues and organs analyzed by Kinetworks™ Protein Kinase Screen (KPKS) immunoblotting [4]. The mean values for kinase expression from the pooled average values from 30 different cultured tumor cell lines evaluated with another 111 Kinetworks™ KPKS immunoblots are also shown at the top of each panel. Equivalent total amounts of proteins from tissue or cell lysates were assayed on each immunoblot, and the relative affinities for the antibodies for their target proteins were comparable.

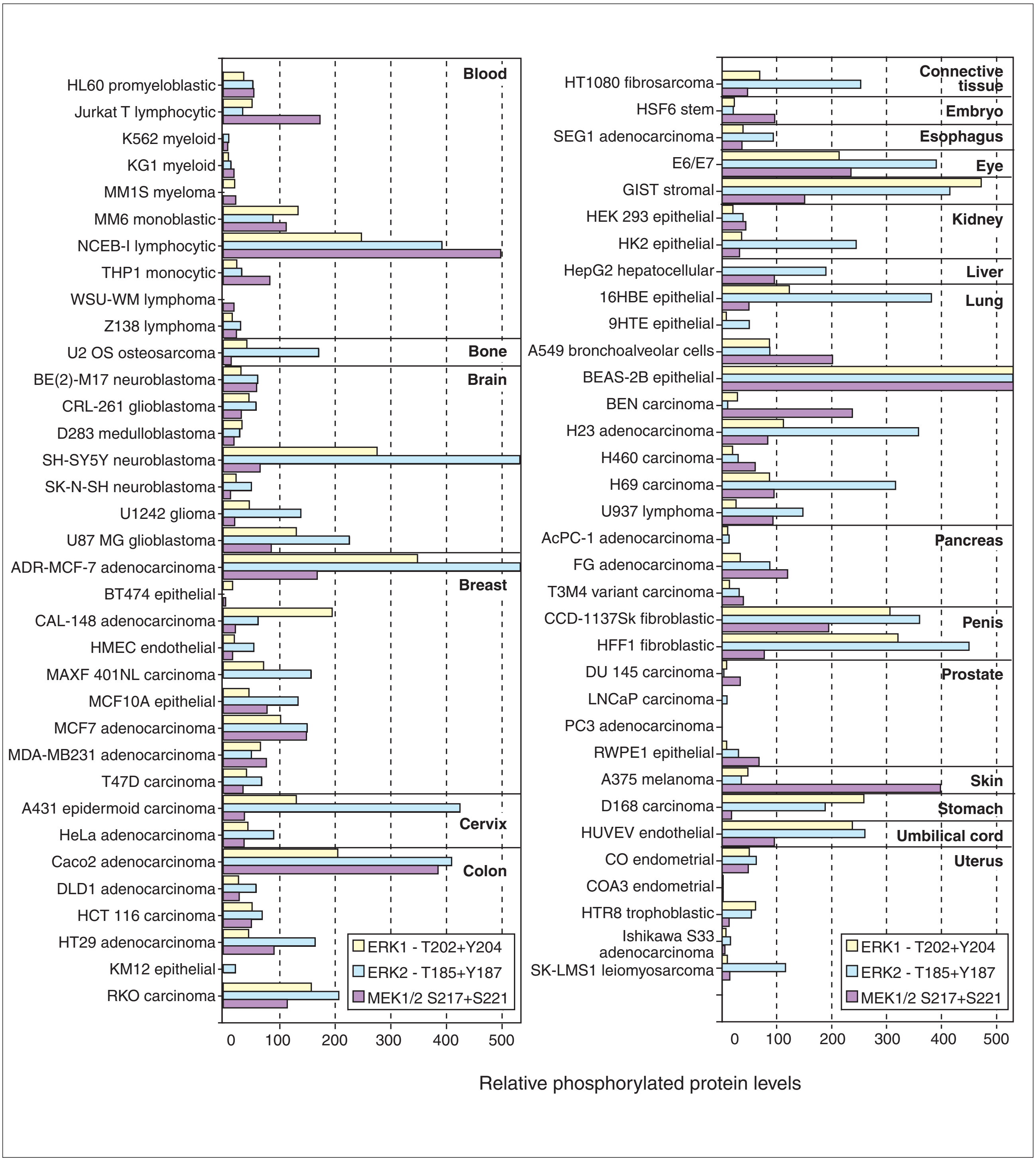

Figure 2
Relative phosphorylation levels of MAP kinases and MAP kinase kinases in human cell lines. Western blotting was performed to quantify the relative phosphorylation of ERK1 (yellow), ERK2 (blue) and MEK1 or MEK2 (purple) at their activation sites in subconfluent cultures of proliferating cells. MEK1 and MEK2 cannot be distinguished with the antibody used. Values are the means of at least triplicate (range 3 to 54) determinations for measurements of the phosphorylated forms of the kinases in 69 diverse human cell lines analyzed with 588 lysates by Kinetworks™ Phospho-Site Screen (KPSS) multi-immunoblotting [4]. Cell lines have been divided into groups on the basis of their organ of origin.

Raf1, RafB, RafA and Mos) stimulates their ability to phosphorylate and activate the associated ERK isoform (human ERK1 at Thr202 and Tyr204; human ERK2 at Thr185 and Tyr187) [1]. This also triggers the release of the ERK isoform from its MEK partner and its subsequent reassociation into active ERK homodimers [11-14]. MEK1 and MEK2 have nuclear exclusion sequences that normally prevent MEK-ERK heterodimers from accumulating in the nucleus [15]. Following their phosphorylation and release, however, activated ERK1 and ERK2 can enter the cell nucleus both by passive diffusion and by active transport [9-11,13,16]. Once in the nucleus, the MAP kinases can phosphorylate transcription factors that are important for cell-cycle progression. Careful studies have revealed that ERK1 and ERK2 homodimers are more catalytically active than their monomeric counterparts [14,17].

The ERK and MEK expression data presented in Figure 1 supports this model. There is a strong correlation between the total combined levels of expression of ERK1 and ERK2 and the total combined expression levels of MEK1 and MEK2 across the many organs examined (a notable exception appears to be the mouse breast). This indicates that most of the inactive ERK1 and ERK2 in cells is bound to MEK1 and MEK2, although there is no obvious preferential binding of either ERK to either MEK.

Melanie Cobb and Elizabeth Goldsmith [18], starting from their solution of the dimeric X-ray crystallographic structure of ERK2, proposed that the formation of an ERK2 homodimer could be important for the recognition of dimeric substrates. Many transcription factors that are targeted by MAP kinases, such as the AP1 Fos-Jun complex, also occur as dimers. They predicted that the occurrence of heterodimeric complexes of WT-ERK2 and KD-ERK2 would result in incomplete phosphorylation of a dimeric substrate [11]. They also noted that ERK1 and ERK2 can form heterodimeric complexes, but that these are unstable. It would seem that this model would account nicely for the findings of Vantaggiato and Formentini *et al*. [3], as KD-ERK2 should be a more potent inhibitor of active ERK2 dimer formation than WT-ERK1 or KD-ERK1. Apart from reduced stability, however, why would the WT-ERK1-WT-ERK2 heterodimer not be as functional as a WT-ERK2-WT-ERK2 homodimer? One possibility is the WT-ERK1-WT-ERK2 heterodimer will not dock transcription-factor substrates as efficiently, as the amino-terminal regions of ERK1 and ERK2, which are located near the active sites of these enzymes in the dimeric complex, are quite distinct, with ERK1 featuring an additional 17 amino acids that are not present in ERK2. Interestingly, in a study of protein kinases that interact with AP1 transcription-factor complexes, ERK2 but not ERK1 was detected [19].

The related stress-activated MAP kinase p38 would not be expected to interact with MEK1 or MEK2, but rather with its own upstream activating kinases, MEK3 and MEK6 [2]. As a control, the Vantaggiato and Formentini *et al.* study [3] also transfected mouse fibroblasts with p38-α, which appeared to have relatively little effect on ERK1 and ERK2 phosphorylation or Ras-induced cell proliferation. In these experiments, however, p38 was not stimulated. Activation of p38 by diverse cellular insults is known to inhibit ERK1 and ERK2 activation [20,21]. Furthermore, high ratios of either phospho-ERK1 or phospho-ERK2 relative to phospho-p38, or ERK1/2 activity relative to p38 activity, were observed to be strong predictors of tumorigenicity of breast, prostate, melanoma, and fibrosarcoma cell lines *in vivo* [22]. One explanation for these findings is that phosphorylated and active p38-α and p38-δ isoforms appear to form inhibitory complexes with ERK1 and ERK2 [20,21]. But there is also one report of a splice variant of p38 called Mxi2 that seems to bind and stabilize both ERK1 and ERK2 in the nucleus to prolong their signaling [23]. There have been no reports of p38 homodimers, although ERK5 [24] and the c-Jun N-terminal kinase (JNK) family of MAP kinases appear to form homodimers [11]. Heterodimerization of c-Jun with other transcription proteins seems to be important for their recognition for phosphorylation by JNK MAP kinases [25].

Dimerization is not only widespread among the MAP kinases, but is also rampant in many of their upstream-acting kinases. Although homodimerization of MEK isoforms has yet to be described in cells, MEK2 has been crystallized as a homodimer [26]. Furthermore, there are several reports of interactions of Raf1 and RafB isoforms and the related kinase 'kinase suppressor of Ras' (KSR) in homodimeric and heterodimeric complexes [27-30]. Dimerization of Ras in the plasma membrane may be essential for Raf1 homodimerization [27]. Dimers of members of the multifunctional 14-3-3 protein family can also promote complex formation of KSR with Raf1 [31]. There are also numerous reports of homodimerization for many of the other upstream kinases in the p38 and JNK MAP kinase signaling pathways. These include: the Ste20-like kinases MST1 [32,33], MST2 [34], SLK [35], and TAO1 [36]; the Ste11-like kinases ASK1 [37], MEKK2 [38], and MEKK4 [39]; and the mixed lineage kinases DLK [40,41], MLK3 [42], and LZK [43] (see 'Kinases' box for more information).

For a substantial proportion of the 515 known human protein kinases, the appearance of two or more kinase catalytic domains in the holoenzyme forms has been directly reported or can be inferred from the high levels of homology among related kinase subfamily members. All of the 58 receptor-tyrosine kinases probably dimerize when activated, and this may also be true for the 20 receptor-serine/threonine

Kinases

Receptor tyrosine kinases

EGFR - Epidermal growth factor receptor
ErbB2 or HER2 - Human epidermal growth factor receptor 2
IGFR1 - Insulin-like growth factor receptor 1
PDGFR - Platelet-derived growth factor receptor

Non-receptor tyrosine kinases

- **Doubled catalytic domain kinases**

JAK1, JAK2, JAK3 - Janus kinases 1, 2 and 3
TYK2 - Tyrosine kinase 2

- **Others**

Abl - Ableson kinase
Arg - Able-related gene kinase
BMX - Bone marrow kinase in chromosome X
BTK - Bruton's tyrosine kinase
FAK - Focal adhesion kinase
ITK - T cell specific kinase
Pyk2 - Protein-tyrosine kinase 2
Syk - Spleen tyrosine kinase
TEC - Tec protein-tyrosine kinase
ZAP70 - 70 kDa zeta T chain-associated kinase

Non-receptor protein-serine/threonine kinases

- **Doubled catalytic domain kinases**

GCN2 - Kinase related to yeast GCN2
MSK1, MSK2 - Mitogen- and stress-activated kinases 1 and 2
RSK1, RSK2, RSK3, RSK4 - Ribosomal S6 protein kinases 1, 2, 3 and 4
SgK069 - Bsk146-related protein kinase

- **Ste20-like kinases**

MST1, MST2 - Mammalian sterile 20-like kinases 1 and 2
SLK - Sterile 20-like kinase
TAO1 - Thousand and one amino acid protein 1

- **Ste11-like kinases**

ASK1 - Apoptosis signal-regulationg kinase 1
MEKK2, MEKK4 - Mitogen- and extracellular-signal kinase kinase 2 and 4

- **Mixed lineage kinases**

DLK - Dual leucine zipper bearing kinase
LZK - Leucine zipper bearing kinase
MLK3 - Mixed lineage kinase 3

kinases. Furthermore, the existence of heterodimeric complexes between diverse receptor-tyrosine kinases (such as between IGF1 receptor and ErbB2 [44], and between the receptors for PDGF and EGF [45]) has been described. At least eight non-receptor-tyrosine kinases have multiple kinase catalytic domains, either within the same polypeptide chain (JAK1-3, TYK2) or in holoenzymes (Abl, FAK, BMX, BTK) and, on the basis of the levels of homology, Arg, Pyk2, ITK, and TEC are strong candidates as well. By contrast, there is no evidence for dimerization of Syk, ZAP70 or any of the Src kinase family members, despite exhaustive studies of these enzymes.

When it comes to the non-receptor protein-serine/threonine kinases, eight have tandem catalytic domains (SgK069, GCN2, MSK1, MSK2 and RSK1-4), whereas at least 59 others have been reported to dimerize or oligomerize. Again, on the basis of strong homology, at least another 36 protein kinases are likely to also undergo complex formation. Some notable exceptions for dimerization include all of the protein kinase C isoforms and the cyclin-dependent kinases. In view of the very limited enzymological characterization of most protein-serine/threonine kinases, however, it may well be that more than half of them are subject to homo- and heterodimeric catalytic kinase domain interactions. Like the MAP kinases, dimerization may have a profound impact on their regulation and their substrate selectivity.

In conclusion, dimerization has a crucial role in the regulation of many kinases, and this might help to explain the seemingly paradoxical results of Vantaggiato and Formentini *et al.* [3]. Another important ramification of the study [3] is that chemotherapy drugs that inhibit ERK2 more than ERK1 could be more optimal for inhibition of oncogenic cell proliferation, but that selective inhibition of ERK1 might actually enhance cell growth and division and tumorigenesis. Overall, it is clear that detailed studies of the differences in regulation between related members of kinase families can yield considerable insights into their specialized functions.

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