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Invited review

ERK1/2 MAP kinases: Structure, function, and regulation

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ABSTRACT

ERK1 and ERK2 are related protein-serine/threonine kinases that participate in the Ras-Raf-MEK-ERK signal transduction cascade. This cascade participates in the regulation of a large variety of processes including cell adhesion, cell cycle progression, cell migration, cell survival, differentiation, metabolism, proliferation, and transcription. MEK1/2 catalyze the phosphorylation of human ERK1/2 at Tyr204/187 and then Thr202/185. The phosphorylation of both tyrosine and threonine is required for enzyme activation. Whereas the Raf kinase and MEK families have narrow substrate specificity, ERK1/2 catalyze the phosphorylation of hundreds of cytoplasmic and nuclear substrates including regulatory molecules and transcription factors. ERK1/2 are proline-directed kinases that preferentially catalyze the phosphorylation of substrates containing a Pro-Xxx-Ser/Thr-Pro sequence. Besides this primary structure requirement, many ERK1/2 substrates possess a D-docking site, an F-docking site, or both. A variety of scaffold proteins including KSR1/2, IQGAP1, MP1, β-Arrestin1/2 participate in the regulation of the ERK1/2 MAP kinase cascade. The regulatory dephosphorylation of ERK1/2 is mediated by protein-tyrosine specific phosphatases, protein-serine/threonine phosphatases, and dual specificity phosphatases. The combination of kinases and phosphatases make the overall process reversible. The ERK1/2 catalyzed phosphorylation of nuclear transcription factors including those of Ets, Elk, and c-Fos represents an important function and requires the translocation of ERK1/2 into the nucleus by active and passive processes involving the nuclear pore. These transcription factors participate in the immediate early gene response. The activity of the Ras-Raf-MEK-ERK cascade is increased in about one-third of all human cancers, and inhibition of components of this cascade by targeted inhibitors represents an important anti-tumor strategy. Thus far, however, only inhibition of mutant B-Raf (Val600Glu) has been found to be therapeutically efficacious.

Contents

Abbreviations: C-spine, catalytic spine; CK, casein kinase; $cPLA_2$, cytosolic phospholipase A_2; CRM1, chromosome region maintenance 1; CTKD, C-terminal kinase domain; DRS, D-site recruitment site; DUSP, dual-specificity phosphatase; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated protein kinase; FAK, focal adhesion kinase; FLAG, DYKDDDDK epitope; FRET, fluorescent resonance energy transfer; FRS, F-site recruitment site; FTase, farnesyltransferase; GFP, green fluorescent protein; GGTase, geranylgeranyltransferase; GST, glutathione *S*-transferase; HA, hemagglutinin; HGF, hepatocyte growth factor; JNK, c-Jun N-terminal kinase; KD, kinase domain; KIM, kinase interaction motif; KSR, kinase suppressor of Ras; MAPK, mitogen-activated protein kinase; MAPKAP kinase, mitogen-activated protein kinase activated protein kinase; MBP, myelin basic protein; MEK, MAP/ERK kinase; MKP, MAP kinase phosphatase; MLK, mixed lineage kinase; MNK, MAP kinase interacting kinase; MORG1, mitogen-activated protein kinase organizer 1; MST, mammalian Ste-20 like kinase; MP1, MEK partner 1; MSK, mitogen- and stress-activated kinase; NES, nuclear export signal; NLS, nuclear localization signal; NTKD, N-terminal kinase domain; NTS, nuclear transport signal; P–PI, protein–protein interaction; PKA, protein kinase A; PKC, protein kinase C; ppERK, bisphosphorylated ERK; pT, phosphothreonine; pY, phosphotyrosine; PTP, protein-tyrosine phosphatase; R-spine, regulatory spine; Raf, regulator of α-fetoprotein; RSK, ribosomal protein 6 kinase; siRNA, small inhibitory RNA; TBB, 4,5,6,7-tetrabromo-2-azabenzimidazole; TGF, transforming growth factor; TPA, tetradecanoyl phorbol acetate; VEGFR, vascular endothelial growth factor receptor.

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1. Introduction

1.1. MAP kinases

Protein kinases play a predominant regulatory role in nearly every aspect of cell biology [1]. The human protein kinase family consists of 518 genes, which correspond to about 1.7% of the genome, thereby making it one of the largest gene families [2]. Protein kinases catalyze the reaction illustrated in Fig. 1. Note that the phosphoryl group (PO_3^{2-}) and not the phosphate group (PO_4^{2-}) is transferred to the protein substrate. Based upon the nature of the phosphorylated —OH group, these proteins are classified as protein-serine/threonine kinases (385 members), protein-tyrosine kinases (90 members), and tyrosine-kinase like proteins (43 members) [2]. Moreover, there are 106 protein kinase pseudogenes. A small group of dual-specificity kinases including MEK1/2 catalyze the phosphorylation of both tyrosine and threonine in target proteins such as ERK1/2. Dual-specificity kinases belong to the protein-serine/threonine kinase family. Protein phosphorylation is the most widespread class of post-translational modification used in signal transduction. Families of protein phosphatases catalyze the dephosphorylation of proteins [3,4] thus making phosphorylation–dephosphorylation an overall reversible process.

The mammalian MAP kinases consist of cytoplasmic protein-serine/threonine kinases that participate in the transduction of signals from the surface to the interior of the cell. This group includes the extracellular signal-regulated kinase (ERK) family, the p38 kinase family, and the c-Jun N-terminal kinase family (JNK, also known as stress-activated protein kinase or SAPK). The following enzyme forms have been described: ERK1–ERK8, p38α/β/γ/δ, and JNK1–3 [5–7]. Each MAPK signaling cascade consists of at least three components, or tiers: a MAPK kinase kinase (MAP3K),

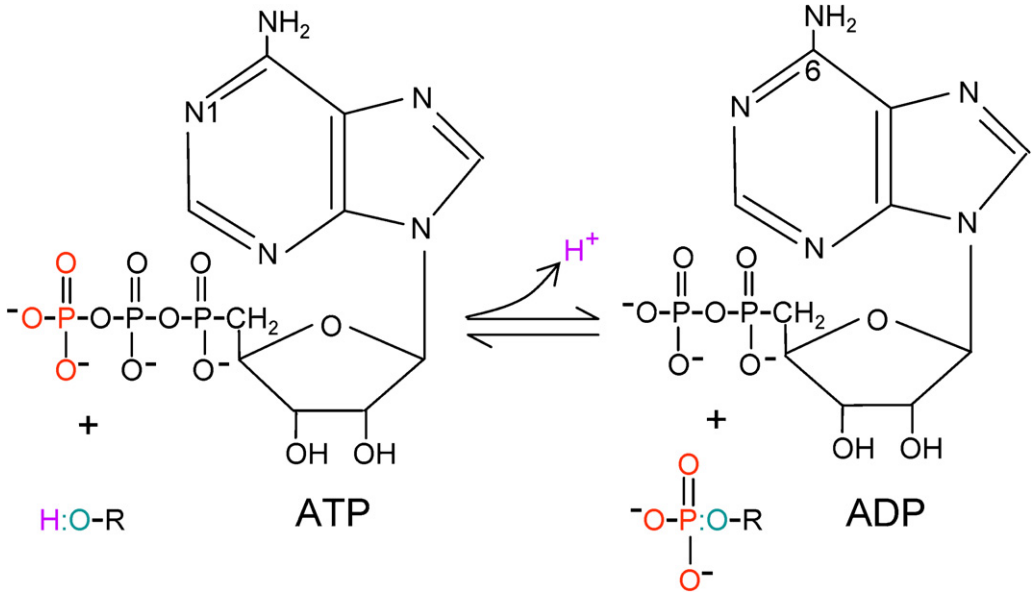

Fig. 1. The protein kinase reaction involves the transfer of the γ-phosphoryl group from ATP to the hydroxyl group of the protein substrate (HO-R). Positions 1 and 6 of adenine are indicated.

a MAPK kinase (MAP2K), and a MAPK (Fig. 2). The activated MAP kinases catalyze the phosphorylation of numerous substrate proteins including transcription factors, protein kinases and phosphatases, and other functional proteins.

Scaffold proteins, which interact with more than one component in a given cascade, mediate the activation of the MAP kinase signaling pathways. For example, the kinase suppressor of Ras (KSR) and MEK partner 1 (MP1) function as scaffolds for the ERK1/2

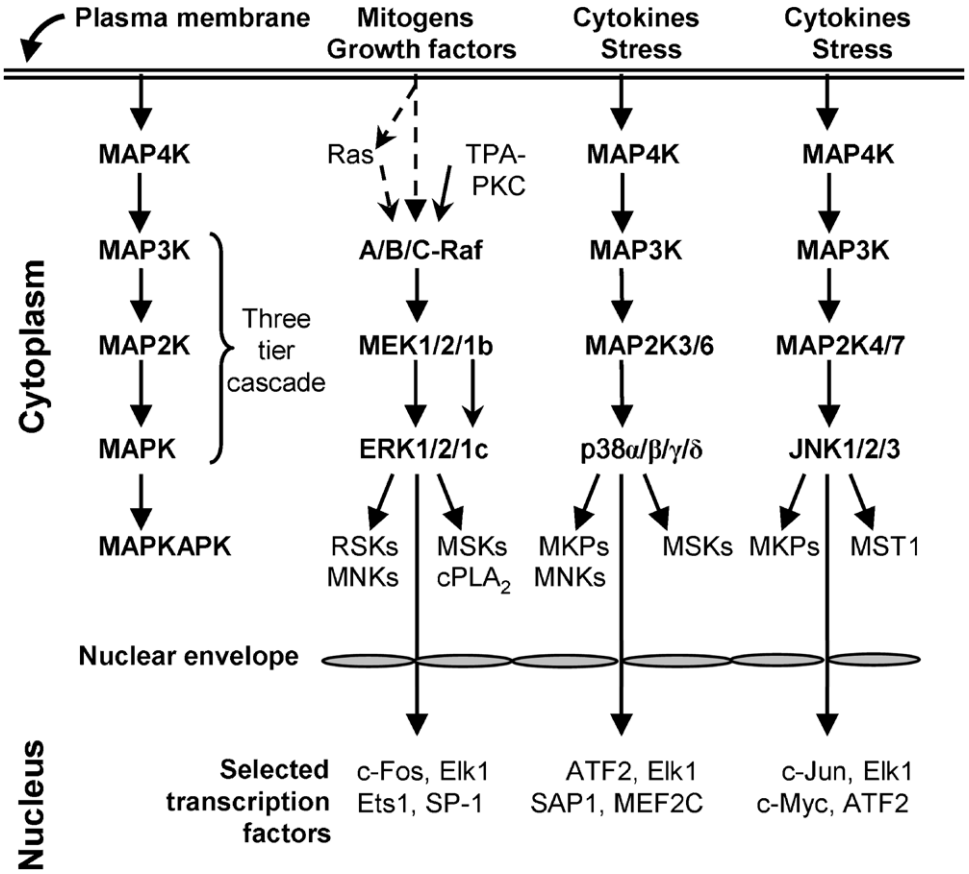

Fig. 2. The ERK, p38, and JNK MAP kinase cascades. The MAP kinases, which occur in the cytoplasm and can be translocated into the nucleus, catalyze the phosphorylation of dozens of cytosolic proteins and numerous nuclear transcription factors.
Adapted from Ref. [8].

signaling pathway, and the JNK-interacting protein group (JIP) serves as scaffolds for the JNK pathway [9,10]. KSR1/2 interact with B-Raf, C-Raf, MEK1/2 and ERK1/2, and MP1 interacts with MEK1/2 and ERK1/2. JIP interacts with MLK3, MKK7, and JNK. β-arrestin2 is a scaffold protein for both the ERK1/2 and JNK1–3 signaling pathways. β-Arrestin2 interacts with the Raf and ERK families and with apoptosis signal-regulating kinase (ASK1, or MAP3K5) and JNK3.

The p38 and JNK signaling pathways are activated by proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β or in response to cellular stresses such as genotoxic, osmotic, hypoxic, or oxidative stress [8]. The p38 signaling pathway consists of p38α/β/γ/δ, a MAP2K such as MKK3 or MKK6, and a MAP3K such as ASK1 or TAK1 (transforming growth factor-β-activated kinase 1) [11]. The p38 family plays a role in angiogenesis, cell proliferation, inflammation, and the production of cytokines, which are immunomodulating agents. The JNK pathway consists of JNK1–3, a MAP2K such as MAP2K4 (also known as SEK1) or MAP2K7, and a MAP3K such as ASK1 or TAK1 (both of which also function in the p38 pathway), MEKK1, or mixed-lineage kinase (MLK) [12]. The JNK family participates in apoptosis and the development of multiple cell types of the immune system.

1.2. The ERK1/2 MAP kinases

1.2.1. Essential versus nonessential nature of ERK1 and ERK2

Human ERK1 and ERK2 are 84% identical in sequence and share many if not all functions [13]. For this reason they will be referred to as ERK1/2. ERK1/2, like nearly all protein kinases, contain unique N- and C-terminal extensions that provide signaling specificity. ERK1 contains a 17-amino-acid-residue insertion in its N-terminal extension (Fig. 3). The ERK1/2 family contains a 31-amino-acid-residue insertion within the kinase domain (kinase insert domain) that provides additional functional specificity. The cyclin-dependent kinase family also contains a comparable kinase insert domain [14]. In this review we will focus on the biochemistry and molecular biology of ERK1/2 MAP kinases.

The ERK2 enzyme has been more widely studied than the ERK1 enzyme. Human ERK2 consists of 360 amino acid residues while the rat and mouse enzyme consist of 358 residues. Although care has been taken in this review to document the enzyme species under investigation, the difference of two residues is most likely inconsequential. Human ERK1 consists of 379 amino acid residues while rat and mouse ERK1 consist of 380 residues. ERK1 and ERK2 differ more from one another in a given species more than either ERK1 or ERK2 differs among the three species.

All known cellular stimulants of the ERK1/2 pathway lead to the parallel activation of ERK1 and ERK2 [15]. Furthermore, Robbins et al. demonstrated that bacterially expressed ERK1 and ERK2 possess identical substrate specific activity in vitro [16]. Of course not all ERK1/2 substrates were known then, or now. Lefloch et al. observed that the activation ratio of ERK1/ERK2 in cells corresponds with their expression ratio indicating that the isoforms are activated in parallel [17]. Despite numerous efforts to establish differences, the functions of the two isoforms are similar. Gene ablation studies have provided provisional evidence for differential functions of ERK1 and ERK2, as described next.

Yao et al. reported that ERK1 and ERK2 are not entirely functionally redundant [18]. The *erk1* gene is dispensable for the development of mice, but ablation of the *erk2* gene is embryonic lethal. Pagès et al. [19] and Nekrasova et al. [20] found that *erk1*-deficient mice were viable, fertile, and of normal size. However, thymocyte maturation beyond the $CD4^+CD8^+$ stage was reduced by half in $erk1^{-/-}$ mice, with a similar diminution in the thymocyte subpopulation expressing high levels of T cell receptor (CD^{high}) [19]. Thus, ERK1 appears to play an important role in thymocyte development.

Yao et al. found that ablation of *erk2* in mice is embryonic lethal [18]. They reported that these mice fail to form mesoderm. Although *erk2*-null embryonic stem (ES) cells exhibit augmented ERK1 phosphorylation following cellular stimulation, they exhibit reduced total ERK activity and decreased downstream RSK phosphorylation; yet embryonic stem cell proliferation is unaffected. Under these conditions in vitro, ERK1 is apparently able to compensate for ERK2 loss. Hatano et al. found that the development of placental vasculature is severely impaired in *erk2*-deficient mice, which leads to embryonic lethality [21]. Moreover, Saba-El-Leil et al. observed that mouse embryonic trophoblast development is impaired in *erk2*-deficient mice [22]. They found that ERK1 is widely expressed in wild-type and *erk2*-null mouse embryos. These studies taken together suggested that ERK1 is unable to compensate for ERK2 deficiency in vivo. However, does the embryonic lethality of $erk2^{-/-}$ mice, but not $erk1^{-/-}$ mice, represent a difference in function or a difference in isoform expression?

Lefloch et al. examined the role of ERK1 and ERK2 expression on their effects in cells and in whole animals [17]. Although $erk1^{-/-}$, but not $erk2^{-/-}$, mice survive, they observed that an unfavorable embryonic outcome results when an *erk1* allele is not expressed in mice expressing a single *erk2* allele. Based upon a series of gene ablation experiments, they conclude that *erk* gene dosage is critical for mouse survival. They observed that no animal can survive with only one *erk* allele. However, mice with two *erk2* alleles or with

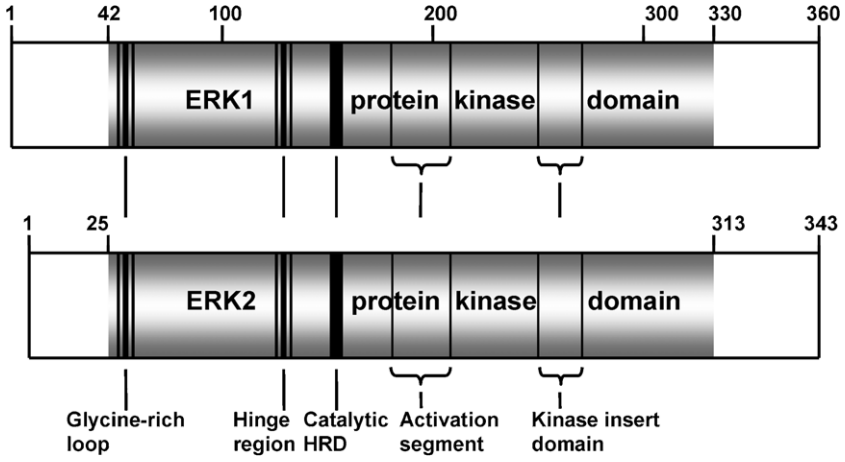

Fig. 3. Architecture of human ERK1 and ERK2. The numbers are those of amino acid residues.

one *erk1* allele and one *erk2* allele are able to survive. Since ERK2 expression exceeds that of ERK1 in most cells, they ascribe the findings of the severe effect of knocking out ERK2 expression more to the role of diminishing total ERK content as opposed to decreasing the expression of a protein with unique biological functions [17]. Thus, whether functions exist that are unique or preferred to one or the other ERK1/2 isoform is still an open question. At one time or another during the lifetime of an animal, ERK1 or ERK2 may perform functions unique to that isoform. However, the detection of such distinctive functions will be difficult to pinpoint.

Table 1
Concentrations of Ras, Raf, MEK, and ERK in cells.[a]

	HeLa cells nM	COS-7 cells
Ras	400	530
Raf	13	5.4
MEK	1400	1800
ERK	960	810

[a] Data from Ref. [38].

1.2.2. The ERK1/2 signaling cascade

ERK1/2 are ubiquitously expressed hydrophilic non-receptor proteins that participate in the Ras-Raf-MEK-ERK signal transduction cascade, which is sometimes denoted as the mitogen-activated protein kinase (MAPK) cascade [23]. This cascade participates in the regulation of a large variety of processes including cell adhesion, cell cycle progression, cell migration, cell survival, differentiation, metabolism, proliferation, and transcription. Moreover, oncogenic mutations in human *KRAS* occur in about 58% of pancreatic, 33% of colorectal, and 31% of biliary cancers, and *NRAS* mutations occur in about 18% of melanomas [24]. Overall, the *RAS* genes are activated in about 30% of all human cancers [25]. Activating mutations in *RAF* occur in perhaps 7% of all human cancers [26,27]. For example, *BRAF* mutations occur in about 40–60% of melanomas, 40% of thyroid cancers, 30% of ovarian cancers, and 20% of colorectal cancers [26,27]. The Ras-Raf-MEK-ERK pathway is upregulated in a variety of cancers even in the absence of oncogenic mutations.

H-Ras, K-Ras, and N-Ras, three gene products, have a molecular weight of about 21 kDa. These molecules function as molecular switches as an inactive Ras-GDP is converted into an active Ras-GTP in a process that is mediated by a guanine nucleotide exchange factor (GEF), which is also known as Sos1/2 (from *Drosophila* son of sevenless) [28]. This conversion of Ras-GDP to Ras-GTP is promoted by the action of several receptor protein-tyrosine kinases including those of the EGFR family, the insulin-like growth factor receptor, the VEGFR family, and many others [29]. Ligand-induced receptor dimerization promotes receptor autophosphorylation in *trans* thus resulting in receptor activation [30]. Such phosphorylated residues serve as binding sites for proteins that contain a Src homology 2 (SH2) domain, a phosphotyrosine binding domain (PTB), or both domains, which are expressed in Shc [31]. Shc in turn recruits Grb2 (growth factor receptor-bound protein 2) and Sos1/2 leading to Ras activation. The best characterized route of Ras activation occurs at the plasma membrane and is mediated by Sos1/2, as noted above. Activated G-protein coupled receptors and integrins, which are integral membrane proteins, can also lead to the formation of active Ras-GTP. Ras-GTP has about a dozen downstream effector pathways including the Raf-MEK-ERK signaling cascade [28]. Active Ras-GTP is converted to the inactive Ras-GDP as the intrinsic Ras-GTPase activity is stimulated by GTPase activating protein (GAP) [32].

Ras-GTP leads to the activation of the Raf kinase family (A-, B-, and C-Raf) by an intricate multistage process that involves homodimer and heterodimer formation [33]. The Raf kinases have restricted substrate specificity and catalyze the phosphorylation and activation of MEK1 and MEK2. MEK1/2 are dual-specificity protein kinases that mediate the phosphorylation of tyrosine and threonine in ERK1 and ERK2, their only known physiological substrates [34,35]. This phosphorylation activates ERK1/2, which are protein-serine/threonine kinases. Unlike the Raf kinases and MEK1/2, which have narrow substrate specificity, ERK1 and ERK2 have more than 175 documented cytoplasmic and nuclear substrates [36] and surely more will be found. While the Raf isoforms are the primary MAP3Ks in the ERK1/2 module, MEKK1, Mos, and COT (MAP3K8, or TPL2) are additional ERK1/2 MAP3Ks utilized in more restricted cell type- and stimulation-specific situations (reviewed in Refs. [11,37]).

One potential of a signaling cascade is that of amplification. One protein kinase can catalyze the phosphorylation of many substrate molecules. If the substrate is a protein kinase, it too can catalyze the phosphorylation of many substrate molecules, etc. Fujioka et al. measured the concentrations of Ras, Raf, MEK, and ERK in human HeLa and African green monkey COS-7 cells and obtained the results shown in Table 1 [38]. The concentration of Ras in HeLa cells is about 30 times that of Raf. The concentration of MEK is about 100 times that of Raf, but the concentration of ERK is only 2/3rds that of MEK. Thus, in the ERK signaling module, the possibility of a 100-fold amplification from Raf to MEK exists. In contrast, such amplification from MEK to ERK is unlikely. The likelihood that one kinase can activate 1000 kinase substrates and the second kinase can activate 1000 substrates for a total amplification of 1×10^6 is remote for the Raf-MEK-ERK module. Thus, amplification of a signaling cascade is not obligatory. Moreover, the original definition of a cascade is a series of waterfalls. The amount of water that goes over the last waterfall is the same as that going over the first, and amplification in a waterfall cascade is impossible. Under conditions whereby EGF stimulation leads to the phosphorylation (activation) of 5% of endogenous MEK in HeLa cells, Fujioka et al. observed that about 60% of endogenous ERK is phosphorylated [38]. This observation is consistent with the notion that an approximate 10-fold amplification occurs in response to stimulation. This finding indicates that significant amplification took place, but not a hypothetical increase amounting to several orders of magnitude.

ERK1/2 MAP kinases are activated in a wide variety of cell types by mitogenic and other stimuli [39,40]. 1n 1987, Ray and Sturgill investigated the insulin-stimulated activation of a microtubule-associated protein 2 kinase (MAP2 kinase) from mouse 3T3-L1 adipocytes [41]. In 1989, Silliman and Sturgill renamed this enzyme "mitogen-activated protein" kinase maintaining the MAP kinase acronym [42]. Boulton et al. cloned the cDNA of rat ERK1 [43], purified the enzyme from a rat fibroblast cell line that over expresses human insulin receptors (Rat 1 HIRc B cells) [44], and cloned the cDNA of two additional family members (ERK2 and ERK3) [45]. Boulton et al. coined the acronym ERK for extracellular signal-regulated protein kinase and applied it to MAP2 kinase because of the wide variety of extracellular signals that lead to its activation [45]. Early studies indicated that these enzymes are activated following cellular stimulation by bradykinin, epidermal growth factor, fibroblast growth factor, insulin, insulin-like growth factor-1, nerve growth factor, and platelet-derived growth factor [39]. ERK1/2 are also activated by cytokines, osmotic stress, and activated seven transmembrane G-protein coupled receptors [11].

In addition to the classical MEK1/2-ERK1/2 signaling component, MEK1b and ERK1c constitute a distinct signaling pathway [46]. MEK1b and ERK1c are alternatively spliced forms of MEK1 and ERK1, respectively. Zheng and Guan reported that human MEK1b, which possesses a 23-amino-acid deletion in its kinase domain, is unable to activate recombinant human ERK1 or ERK2 [47]. Aebersold et al. showed that human ERK1c results from the insertion of intron 7 into the coding region of ERK1 [48]. The

insertion contains a stop codon leading to the expression of a 41-kDa protein that contains 18 different amino acids that replace the C-terminus of ERK1. ERK1c influences mitotic Golgi fragmentation and mitosis progression. Shaul et al. demonstrated that MEK1b, which is unable to activate ERK1 or ERK2, activates ERK1c [46]. This provides a signaling component consisting of MEK1b-ERK1c (Fig. 2). However, the mechanism of activation of MEK1b is unclear [46]. Yung et al. reported that a different alternatively spliced form of ERK1 (ERK1b) occurs in rat [49].

The Ras-Raf-MEK-ERK signaling cascade is dysregulated in a variety of diseases including brain injury, cancer, cardiac hypertrophy, diabetes, and inflammation [50–55]. Moreover, oncogenic mutations of *RAS* or *BRAF* are responsible for a large proportion of human cancers as noted earlier in this section [25,26]. Owing to the importance of protein kinases in general and in the ERK1/2 signaling cascade in particular, protein kinases represent bona fide drug targets that are receiving considerable attention from a large cadre of biomedical scientists [56]. Anecdotal evidence indicates that perhaps one-quarter to one-third of drug discovery research performed by commercial pharmaceutical firms is directed toward protein kinases. Moreover, a significant fraction of academic research is directed toward understanding the molecular biology and physiology of protein kinase signaling pathways.

2. ERK1/2 structures

2.1. Catalytic residues in the N- and C-lobes

ERK1/2, like all protein kinases, have a small amino-terminal lobe and large carboxyterminal lobe that contain several conserved α-helices and β-strands, first described by Knighton et al. for PKA [57]. The small lobe is dominated by a five-stranded antiparallel β-sheet (β1–β5) [58]. It also contains an important and conserved αC-helix that occurs in active or inactive orientations. The small lobe contains a conserved glycine-rich (GxGxxG) ATP-phosphate-binding loop, sometimes called the P-loop, which occurs between the β1- and β2-strands (Fig. 4). The glycine-rich loop, which is the most flexible part of the N-lobe, helps position the β- and γ-phosphates of ATP for catalysis. The β1- and β2-strands harbor the adenine component of ATP. The glycine-rich loop is followed by a conserved valine (V56/V39 in ERK1/2) that makes a hydrophobic contact with the adenine of ATP (unless otherwise specified, all residue numbers correspond to the human isoforms even when experiments were performed with enzymes from other species). The β3-strand typically contains an AXK sequence, the lysine of which (K71/54 of ERK1/2) couples the α- and β-phosphates of ATP to the αC-helix. A conserved glutamate occurs near the center of the αC-helix (E88/71 in ERK1/2) in protein kinases. The presence of a salt-bridge between the β3-lysine and the αC-glutamate is a prerequisite for the formation of the activated state and corresponds to the "αC-in" conformation. The αC-in conformation is necessary but not sufficient for the expression of full kinase activity. However, the absence of this salt bridge indicates that the kinase is inactive.

The large C-terminal lobe is mainly α-helical (Fig. 4) with six conserved segments (αD-αI) [58]. It also contains four short conserved β-strands (β6-β9) that contain most of the catalytic residues associated with the phosphoryl transfer from ATP to the ERK1/2 substrates. The primary structure of the β-strands occurs between those of the αE- and αF-helices.

Hanks et al. identified 12 subdomains (I–VIa, VIb–XI) with conserved amino acid residue signatures that constitute the catalytic core of protein kinases (Fig. 5) [59]. Of these, the following three amino acids, which define a K/D/D (Lys/Asp/Asp) motif, illustrate the catalytic properties of ERK1/2. An invariant β3-strand lysine (K88/71 in ERK1/2) forms salt bridges with the α- and β-phosphates of ATP (Fig. 6). Asp166/149, which is a base occurring within the catalytic loop, plays an important role in catalysis. Gibbs and Zoller observed that replacement of the equivalent residue in yeast PKA resulted in a k_{cat} ($0.05\ s^{-1}$) that was about 0.3% that of the wild-type enzyme ($16.9\ s^{-1}$) with a 3.5-fold increase in the K_m value of the peptide substrate and a 50% increase in the K_m value of ATP [60]. Madhusudan et al. suggested that this aspartate abstracts the proton from the —OH group of the protein substrate thereby facilitating the nucleophilic attack of oxygen on the γ-phosphorus atom

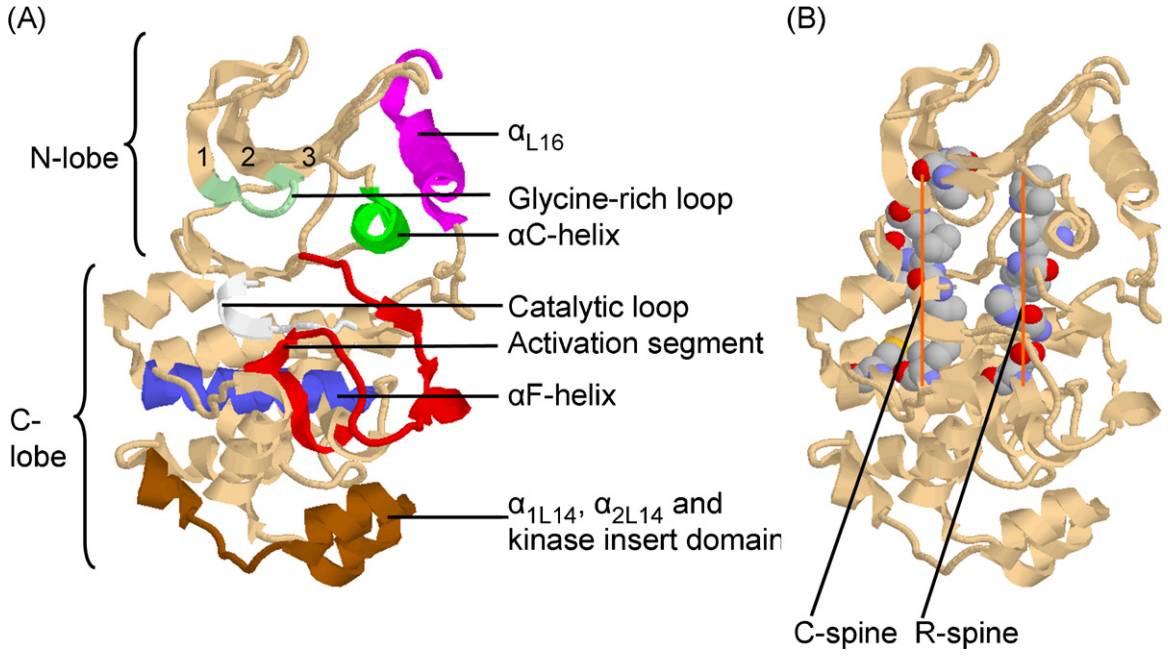

Fig. 4. (A) Ribbon diagram of human ERK2. The numbers in the N-lobe label β-strands 1–3; β-strands 4–5 are hidden. This structure corresponds to an inactive enzyme with the DFG-aspartate out (not shown), but with the αC-helix in. The αC-helix is viewed from its N-terminus. Note that helix α_{L16}, which is adjacent to the N-lobe, occurs at the C-terminus of ERK2. (B) The orange lines denote the residues (space-filling models) that constitute the catalytic and regulatory spines. The view is the same as that of (A). Prepared from the following protein data bank file of human ERK2; PDB ID: 2OJJ.

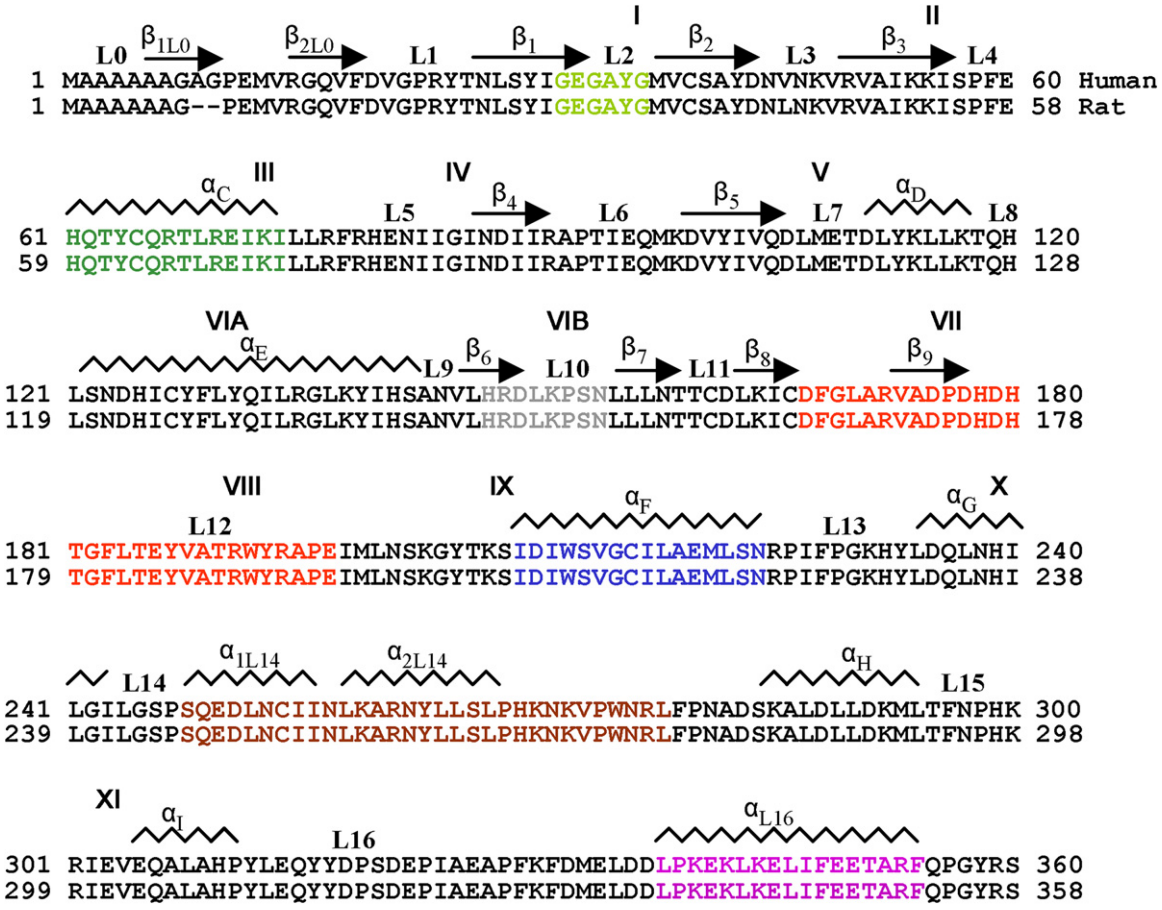

Fig. 5. The primary structure of human and rat ERK2. The color code is the same as that of Fig. 4. The roman numerals correspond to the Hanks et al. subdivisions of protein kinases [59]. The location of α-helices (zigzag lines) and β-strands (arrows) are noted.

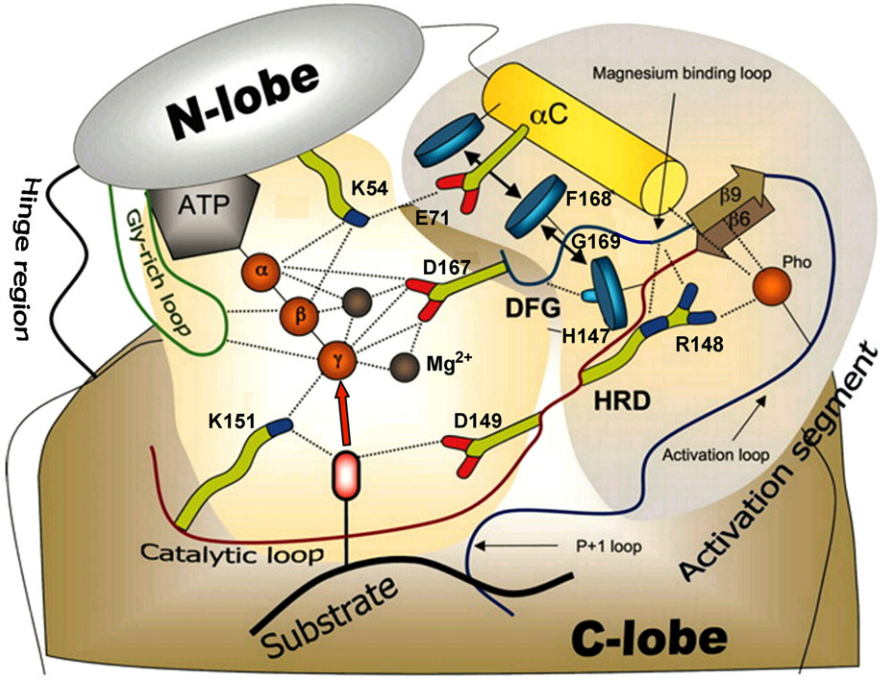

Fig. 6. Diagram of the inferred interactions between the human ERK2 kinase catalytic core residues, ATP, and the protein substrates. Catalytically important residues that are in contact with ATP and the protein substrate occur within the light khaki background. Secondary structures and residues that are involved in the regulation of catalytic activity occur within the gray background. Hydrophobic interactions between the HRD motif (the first D of K/D/D), the DFG motif (the second D of K/D/D), and the αC-helix are shown by the double arrows while polar contacts are depicted as dashed lines. Pho is the phosphate attached to Thr185.
This figure is adapted from Ref. [64] copyright *Proceedings of the National Academy of Sciences USA*.

Table 2
Important residues in human and rat ERK1/2.

	Human ERK1	Human ERK2	Rat ERK1	Rat ERK2
Protein kinase domain	42–330	25–313	43–331	23–311
Glycine-rich loop	49–54	32–37	50–55	30–35
The K of K/D/D, or the β3-lysine	71	54	72	52
αC-glutamate	88	71	89	69
Hinge residues	123–126	106–109	124–127	104–107
Gatekeeper residue	Gln122	Gln105	Gln123	Gln103
Catalytic HRD	164–166	147–149	165–167	145–147
Catalytic loop lysine	168	151	169	149
DFG of the activation segment	184–186	167–169	185–187	165–167
Activation lip phosphorylation sites	T202, Y204	T185, Y187	T203, Y205	T183, Y185
APE end of the activation segment	212–214	195–197	213–215	193–195
No. of residues	379	360	380	358
Molecular weight (kDa)	43.1	41.4	40.0	41.3
UniProtKB ID	P27361	P28482	Q63844	P63086

of MgATP [61]. Zhou and Adams suggested that this aspartate positions the substrate hydroxyl for an in-line nucleophilic attack [62]. See Ref. [63] for a general discussion of the enzymology of protein kinases.

The second aspartate of the K/D/D signature, Asp186/169, is the first residue of the activation segment. The activation segments of nearly all protein kinases including ERK1/2 begins with DFG and ends with APE. Asp186/169 binds Mg^{2+}ions, which in turn coordinate the α-, β- and γ-phosphates of ATP. The primary structure of the catalytic loop of ERK2, which occurs near the β6- and β7-strands, contains His147, Arg148, Asp149, and Lys151 (Fig. 6). The primary structure of the activation segment occurs after that of the catalytic loop and before that of the αF-helix. Functionally important ERK1/2 residues are listed in Table 2. The large lobe characteristically binds the peptide/protein substrates.

The activation segment is the most important regulatory element in protein kinases [65]. This segment influences both substrate binding and catalytic efficiency. The five-residue magnesium positioning loop begins with the DFG of the activation segment. The middle of the activation segment, which is the most diverse part of the segment in terms of length and sequence, is known as the activation loop in protein kinases in general, but as the activation lip in ERK1/2. This lip in ERK1/2 contains a phosphorylatable tyrosine that is two residues downstream from a phosphorylatable threonine. The lip is located close to the magnesium-binding loop, the amino-terminus of the αC-helix, and the conserved HRD component of the catalytic loop. The negatively charged phosphates serve as an organizer for the active site and for the proline-substrate P + 1 binding site as noted in Section 4.1.

Zhang et al. reported that ERK2 has N- and C-terminal extensions that lie on the surface of the molecule [66]. Rat ERK2 has a four-amino acid insertion in the L_6 linker between the β-4 and β-5 strands in the small amino-terminal lobe, and it has a 31-residue insertion after the G-helix in the large carboxyterminal lobe. The latter insertion consists of two helices ($\alpha 1L_{14}$ and $\alpha 2\ L_{14}$) that contact the L_{12} phosphorylation lip, which contains the rat Thr183 and Tyr185 phosphorylation sites [66]. The carboxyterminal extension (residues 315–358) forms part of the domain interface and residues 338–353 form an α-helix (αL_{16}) juxtaposed with the body of the small amino-terminal lobe (Fig. 4).

2.2. The ERK1/2 protein kinase hydrophobic skeletons

Taylor and Kornev [58] and Kornev et al. [64] analyzed the structures of active and inactive conformations of some two dozen protein kinases and determined functionally important residues by a local spatial alignment (LSP) algorithm. This analysis reveals a skeleton of four non-consecutive hydrophobic residues that constitute a regulatory or R-spine and eight hydrophobic residues that constitute a catalytic or C-spine. Each spine consists of residues derived from both the small and large lobes. The regulatory spine contains residues from the activation lip and the αC-helix, whose conformations are important in defining active and inactive states. The catalytic spine governs catalysis by directing ATP binding. The two spines dictate the positioning of ATP (C-spine) and the protein substrate (R-spine) so that catalysis results. The proper alignment of the spines is necessary but not sufficient for the assembly of an active kinase.

The ERK1/2 regulatory spines consist of a residue from the beginning of the β4-strand (I103/86), from the C-terminal end of the αC-helix (L92/75), after a hydrophobic residue following the activation loop DFG (L187/170), along with the HRD-histidine (H164/147) of the catalytic loop. L92/75 and comparable residues from other protein kinases are four residues C-terminal to the conserved αC-glutamate. The backbone of H164/147 is anchored to the F-helix by a hydrogen bond to a conserved aspartate residue (D227/210). The R-spine binds to the F-helix by hydrophobic bonds as do the catalytic loop, the P + 1 loop, the activation lip, and the αH-αI loop [64]. Table 3 lists the residues of the spines in human and rat ERK1and ERK2 and the catalytic subunit of murine PKA, and Fig. 4(B) shows the location of the catalytic and regulatory spines of ERK2.

The catalytic spine of protein kinases consists of residues from the amino-terminal and carboxyterminal lobes that is completed by the adenine moiety of ATP [58]. This spine mediates catalysis by directing ATP localization, and thereby accounting for the term catalytic. The two residues of the N-terminal lobe of ERK1/2 that bind to the adenine component of the nucleotide substrate

Table 3
Human and rat ERK1/2 and murine PKA residues that form the R-spine and C-spine.

	Human ERK1/2	Rat ERK1/2	Murine PKA[a]
Regulatory spine			
β4-Strand (N-lobe)	I103/86	I104/84	L106
C-Helix (N-lobe)	L92/75	L93/73	L95
Activation loop (C-lobe)	L187/170	F186/166	F185
Catalytic loop H or Y (C-lobe)[b]	H164/147	H165/145	Y164
F-helix (C-lobe)	D227/210	D228/208	D220
Catalytic spine			
β2-Strand (N-lobe)	V56/39	V57/37	V57
β3-AxK motif (N-lobe)	A69/52	A70/50	A70
β7-Strand (C-lobe)	L173/156	L174/154	L173
β7-Strand (C-lobe)	L172/155	L173/153	L172
β7-Strand (C-lobe)	I174/L157	I175/L155	I174
D-Helix (C-lobe)	M125/108	M126/106	M128
F-Helix (C-lobe)	I234/217	I235/215	L227
F-Helix (C-lobe)	M238/221	M239/219	M231

[a] From Ref. [64].
[b] Part of the HRD or YRD sequence.

include V56/39 from the beginning of the β2-strand and A69/52 from the conserved AXK of the β3-strand. Moreover, L173/156 from the middle of the β7-strand binds to the adenine base in the active enzyme. L172/155 and I174/L157, hydrophobic residues that flank L173/156, bind to M125/108 at the beginning of the D-helix. The D-helix M125/108 residues bind to I234/217 and M239/222 in the F-helix. Besides the hydrophobic interactions with the adenine moiety, the exocyclic 6-amino nitrogen of ATP characteristically forms a hydrogen bond with a backbone residue in the hinge region that connects the N- and C-lobes. Most small-molecule inhibitors of protein kinases that compete with ATP binding also make hydrogen bonds with the backbone residues of the hinge region [67].

2.3. Structures of active and inactive ERK1/2

The protein kinase catalytic site lies in the cleft between the small and large lobes. In the open and catalytically inactive form of the enzyme, the lobes are modestly tilted away from each other. In the closed and catalytically active form of the enzyme, the lobes are closer together, but the two lobes of protein kinases can still move relative to each other during the catalytic cycle, which allows for the binding of ATP and the release ADP [68]. In the case of rat ERK2, the lobes rotate 5.4° closer when going from the unphosphorylated inactive to the phosphorylated active conformation [70]. After the MgATP and protein substrate bind, additional movement of the closed enzyme form brings residues into the catalytically active state during which time the phosphoryl transfer from ATP to the protein substrate occurs.

The αC-helix of the small lobe has active (αC-helix in) and inactive (αC-helix out) conformations [69]. The αC-helix rotates and translates with respect to the rest of the lobe, making or breaking part of the active site. In the active state, the conserved lysine from the β3-strand (ERK1/2 K71/54) forms a salt bridge with the conserved glutamate from the αC-helix (ERK1/2 E88/71) (Fig. 6). In the dormant activation segment conformation, the aspartate side chain (ERK1/2 D184/167) of the conserved DFG sequence faces away from the active site. This is called the "DFG-aspartate out" conformation. In the active state, the aspartate side chain faces into the ATP-binding pocket and coordinates Mg^{2+}. This is called the "DFG-aspartate in" conformation. This terminology is better than "DFG-in" and "DFG-out" because, in the inactive state, the DFG-phenylalanine may move into the active site while the DFG-aspartate moves out [69]; it is the ability of aspartate to bind (aspartate in) or not bind (aspartate out) to Mg^{2+} in the active site that is the key. The X-ray crystallographic structure of bisphosphorylated active rat ERK2 was determined in the absence of Mg^{2+}, but the DFG-aspartate still assumes its "aspartate in" active conformation [70]. The active enzyme is of the form DFG-aspartate in/αC-helix in. The DFG-aspartate in/αC-helix out, DFG-aspartate out/αC-helix in, and DFG-aspartate out/αC-helix out represent inactive enzyme forms.

The conversion of inactive ERK1/2 to the active form requires phosphorylation of two residues within the activation lip as catalyzed by MEK1/2. These two residues occur in the sequence Thr-Glu-Tyr. All MAP kinases contain a Thr-Xxx-Tyr sequence in their activation segment; the JNK isoforms contain Thr-Pro-Tyr, and the p38 isoforms contain Thr-Gly-Tyr. MEK1/2 first mediate the phosphorylation of the tyrosine residue in the ERK1/2 activation lips [71,72]. Tyrosine-phosphorylated ERK1/2 dissociates from MEK1/2 and then reassociates with the same or another active MEK1/2 that then catalyzes the phosphorylation of the activation-lip threonine, which is two residues upstream from the ERK1/2 phosphotyrosine [73]. Anderson et al. [74] reported that ERK2 can be deactivated completely by treatment with either phosphatase 2A, a protein phosphatase specific for phosphoserine/threonine [75], or CD45, a protein phosphatase specific for phosphotyrosine [76]. They demonstrated that MAP kinase is active only when both the tyrosyl and threonyl residues are phosphorylated.

2.4. Steady-state enzyme kinetics of active and inactive ERK2

Prowse and Lew compared the steady-state kinetic parameters for the phosphorylation of myelin basic protein (MBP) by bisphosphorylated (active) and unphosphorylated (less active) rat ERK2 [77] They reported that the k_{cat} for active ERK2 is 50,000-fold greater than that of the unphosphorylated form, and the specificity constants ($k_{cat}/K_{m(MBP)}$, $k_{cat}/K_{m(ATP)}$) are 600,000–700,000-fold greater than those of the less active form (Table 4). However, the K_m value of MBP for the unphosphorylated enzyme is 12-fold greater than that of the bisphosphorylated enzyme, and the K_m value of ATP for the less active enzyme is 15-fold greater than that of the bisphosphorylated enzyme.

Table 4
Kinetic parameters of bisphosphorylated (ppERK2) and unphosphorylated rat ERK2.

	(pp)ERK2	ERK2	Fold alteration (pp)ERK2/ERK2
MBP Kinase[a]			
k_{cat}	$600 \pm 120\ min^{-1}$	$0.012 \pm 0.009\ min^{-1}$	50,000
$k_{cat}/K_{m(MBP)}$	$2.4 \pm 1 \times 10^6\ M^{-1}\ s^{-1}$	$4.0 \pm 1\ M^{-1}\ s^{-1}$	600,000
$k_{cat}/K_{m(ATP)}$	$0.2 \pm 0.1 \times 10^6\ M^{-1}\ s^{-1}$	$0.3 \pm 0.1\ M^{-1}\ s^{-1}$	700,000
k_3	$720 \pm 240\ min^{-1}$	$0.012 \pm 0.009\ min^{-1}$	60,000
$K_{m(MBP)}$	$4.2 \pm 0.8\ \mu M$	$50 \pm 0\ \mu M$	0.1
$K_{m(ATP)}$	$47 \pm 8\ \mu M$	$700 \pm 150\ \mu M$	0.07
$K_{d(MBP)}$	<0.5 μM	$50 \pm 10\ \mu M$	≤0.01
$K_{d(ATP)}$	57 8 μM	$700 \pm 150\ \mu M$	0.07
ATPase[a]			
k_{cat}	$4.8 \pm 3\ min^{-1}$	$0.0026 \pm 0.0003\ min^{-1}$	2000
k_3	$4.8 \pm 3\ min^{-1}$	$0.0026 \pm 0.0003\ min^{-1}$	2000
$K_{m(ATP)}$	$180 \pm 20\ \mu M$	$1600 \pm 500\ \mu M$	0.1
$K_{d(ATP)}$	$180 \pm 20\ \mu M$	$1600 \pm 500\ \mu M$	0.1
ERKtide kinase[b]			
k_{cat}	$\geq 336\ min^{-1}$		
$k_{cat}/K_{m(ERKtide)}$	$4 \pm 2 \times 10^3\ M^{-1}\ s^{-1}$		
k_3	$\geq 336\ min^{-1}$		
$K_{m(ERKtide)}$	≥1500 μM		
$K_{d(ERKtide)}$	≥1500 μM		

[a] From Ref. [77]. k_3 is the rate of phosphoryl transfer from ATP to myelin basic protein (kinase), water (ATPase), or ERKtide (kinase).
[b] From Ref. [78].

ERK2, PKA, and other protein kinases exhibit ATPase activity where the phosphoryl group is transferred to water and not to a protein or peptide substrate as indicated in the following chemical equation:

$$MgATP^{-1} + HO:H \rightarrow HO:PO_3^{2-} + MgADP + H^+$$

The ATPase k_{cat} for the bisphosphorylated and active enzyme is 2000-fold that of the unphosphorylated enzyme, and the K_m of ATP is 1/9th that of the inactive enzyme (Table 4) [77]. The relative ratios of k_{cat} for MBP kinase reaction (600,000) and the ATPase reaction (2000) between bisphosphorylated and unphosphorylated ERK2 suggests that net stabilization of the transition-state complex for phosphoryl group transfer to MBP occurs only in part by stabilization of ATP and that significant stabilization of the protein phosphoacceptor substrate occurs [77].

Prowse et al. performed steady-state kinetic analysis with ERKtide as substrate [78]. This peptide, which has the sequence ATGPL-S-PGPFGRR, represents an optimal substrate for ERK1 and ERK2 as determined from a random peptide combinatorial library screen [79]. The k_{cat} for ERKtide is about half that for MBP (Table 4). However, the apparent K_m for ERKtide is about 360-fold that of MBP. The ratio of the specificity constants (k_{cat}/K_m) for MBP/ERKtide is 600. The dramatically lower catalytic efficiency of ERKtide compared with MBP is largely attributable to the greater rate of unphosphorylated ERKtide dissociation from ERK2 (ERKtide, $\geq 56\,s^{-1}$; MBP, $\leq 1.2\,s^{-1}$) [78]. A consequence of the slow off-rate for MBP is that its K_D value is only 1/10th that of its K_m value (Table 4). Based upon steady-state enzyme kinetic analysis, Prowse et al. reported that the kinetic mechanism is either (i) random or (ii) ordered with ATP binding first [78]. Similar findings were reported for the steady-state kinetic mechanism of PKA [80,81].

3. Selected ERK1/2 substrates

3.1. Nuclear substrates

Activated ERK1/2 catalyzes the phosphorylation of transcription factors and some of their regulators [36,82]. ERK1/2 nuclear targets include the ternary complex factor (TCF) family of transcription factors. These proteins play a major role in inducing the expression of the immediate early genes (IEGs). The immediate early gene products such as c-Fos and c-Myc induce late-response genes that promote cell survival, cell division, and cell motility [6,83,84]. The ERK1/2 cascade also regulates transcriptional repression and chromatin remodeling [8].

Elk1 is one of the most thoroughly studied targets of the ERK1/2 MAP kinase cascade [36]. Elk1 is a member of the ternary complex factor subfamily of Ets (E-twenty six)-domain transcription factors. The Ets family is one of the largest classes of transcription factors and is unique to metazoans [84]. There are 28 genes in humans, 28 in mice, 10 in *Caenorhabditis elegans* and 9 in *Drosophila*. The founding member of this family was identified as a gene transduced by the leukemia virus, E26. Cesari et al. found that *elk1*-deficient mice are viable, fertile, and display no abnormalities [85]. Surprisingly, cells from these mice display a normal pattern for the expression of immediate early genes. These investigators hypothesize that the closely related Elk3 (Sap2), Elk4 (Sap1), or both may compensate for Elk1 deficiency. Elk1 contains a carboxyterminal transcriptional activation domain with multiple ERK1/2 core consensus phosphorylation sites [84,86]. These include Ser324, Thr336, Ser383, Ser389, and Ser422 (human residue numbers). Phosphorylation of Elk1 leads to increased transcriptional activity. Elk1, Elk3, and Elk4 contain both D- and F-docking sites, described in Section 4, which interact with ERK1/2.

c-Fos is a transcription factor that together with c-Jun make up one form of the AP1 (activating protein 1) transcription factors that regulate early transcriptional processes following the extracellular stimulation of cells [87]. Without c-Fos phosphorylation, both c-Fos mRNA and protein undergo rapid degradation. c-Fos phosphorylation catalyzed by ERK1/2 at Ser374 and that catalyzed by their downstream MAPKAP kinase RSK at Ser362 stabilizes c-Fos for several hours [88,89]. This double phosphorylation occurs within the nucleus. Morton et al. reported that murine ERK1/2 and JNK1/2 catalyze the phosphorylation of the mouse transcription factor c-Jun at Ser63 and at Ser73 in wild-type mouse fibroblasts, which results in increased transcriptional activity [90]. They also reported that ERK1/2 catalyzes the phosphorylation of these two residues in fibroblasts prepared from JNK-deficient mice. c-Fos and c-Jun contain an F-docking site that interacts with ERK1/2, as described in Section 4.

3.2. Cytoplasmic substrates

3.2.1. The RSK family

The ERK1/2 family of protein kinases participates in a wide variety of cellular processes as attested to by the nature of their substrates. More than 50 cytoplasmic substrates have been identified thus far including phosphoprotein phosphatases, RSK family protein kinases, cAMP phosphodiesterase (PDE4), cytosolic phospholipase A_2, cytoskeletal proteins, apoptotic proteins, and regulatory and signaling molecules [36]. The 90 kDa ribosomal S6 kinase (RSK) family of proteins is a group of protein-serine/threonine kinases that regulate cell growth, motility, proliferation, and survival [91]. This family of six enzymes consists of four human isoforms (RSK1–4), MSK1 (mitogen- and stress-activated kinase 1), and MSK2. These enzymes are directly activated by ERK1/2 in response to growth factors, polypeptide hormones, neurotransmitters, and chemokines. RSK1–4 are key components downstream from the Raf-MEK-ERK signaling cascade. They catalyze the phosphorylation of 35 proteins [91] and surely more will be identified. The up regulation of RSK1 and RSK2 expression in different types of cancer suggests that they may be involved in oncogenesis and could potentially be targeted in anticancer therapies [92].

The RSK family enzymes surprisingly contain two protein kinase domains (an N-terminal kinase domain, or NTKD, and a C-terminal kinase domain, or CTKD) within a single polypeptide chain [91]. In addition to N- and C-terminal tails, these proteins contain a linker of about 100 amino acid residues between the two kinase domains (Fig. 7). The NTKD is homologous to the AGC family (PKA, PKG, PKC), and the CTKD is homologous to the calcium/calmodulin-dependent protein kinase family. The CTKD is involved in the autophosphorylation of RSK, and the NTKD is responsible for substrate phosphorylation. That NTKD and CTKD are in different kinase families indicates that the two RSK kinases did not result from the duplication of a single gene. An ERK-docking motif known as the D domain, which is described in Section 4.1, occurs within the C-terminal tail.

Six different phosphorylation sites have been identified in RSK1/2 (and are conserved in RSK3/4) of which four have been shown to be important for enzyme activity (Ser221, Ser363, Ser380, and Thr573 of human RSK1) (Fig. 7) [91,93]. Following activation, ERK1/2 catalyzes the phosphorylation of the CTKD activation loop Thr573 of RSK, which results in the activation of the CTKD. This phosphorylation requires the proline-directed ERK1/2 consensus sequence in the RSK activation loop (Thr573 is followed by a proline residue) and ERK1/2 binding to the D-docking site at the RSK carboxyterminus. ERK1/2 might also catalyze the phosphorylation of Ser363 and Thr359 in the linker region. The activated CTKD of RSK1/2 catalyzes the phosphorylation of

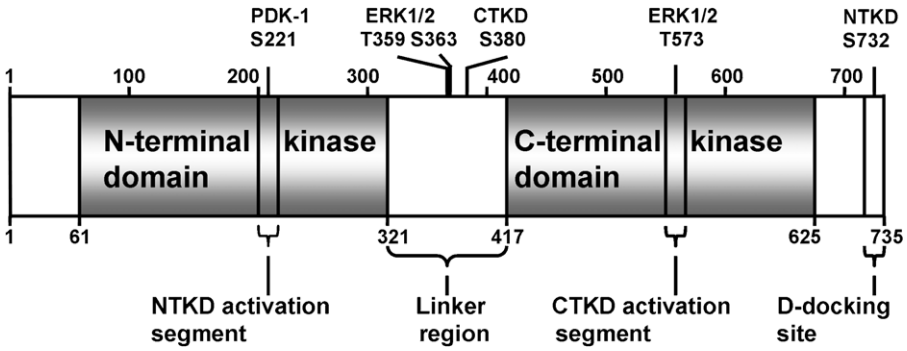

Fig. 7. Architecture of human RSK1. The identity of the protein kinases that catalyze the phosphorylation of the serine (S) and threonine (T) residues are noted above each phosphorylation site. The numbers are those of amino acid residues. CTKD, C-terminal kinase domain; NTKD, N-terminal kinase domain; PDK-1, phosphoinositide-dependent protein kinase 1.

Ser380 in a hydrophobic motif, thereby creating a docking site for phosphoinositide-dependent kinase 1 (PDK-1), a constitutively active protein-serine/threonine kinase. PDK-1 then catalyzes the phosphorylation of Ser221 in the activation segment of the NTKD, which results in the complete activation of RSK. Ser363 is a proline-directed site; whether this residue is solely phosphorylated by ERK1/2 or whether it undergoes autophosphorylation, and/or phosphorylation by a heterologous protein kinase is unclear [93].

The RSK family regulates transcription by mediating the phosphorylation of a number of transcription factors including CREB, serum response factor (SRF), estrogen receptor-α, nuclear factor-κB (NF-κB), nuclear factor of activated T cells 3 (NFAT3), and the transcription initiation factor TIF1A [91]. Activated RSK enzymes catalyze the phosphorylation of several ribosome-associated proteins that enhance protein synthesis. These include ribosomal protein S6 and eukaryotic initiation factor 4B (eIF4B). RSK-mediated cell-survival signaling is due to inactivation of pro-apoptotic proteins including Bcl-2-associated death promoter (Bad) and death-associated protein kinase (DAPK). RSK1/2 promote G1-phase progression of the cell cycle by catalyzing the phosphorylation and inhibition of the cyclin-dependent kinase inhibitor $p27^{KIP}$ [91].

3.2.2. Cytoskeletal proteins

Phosphorylation of actin-binding proteins plays a pivotal role in the remodeling of the actin cytoskeleton, which influences mitogenic, morphological, and migratory cell behavior. Asano et al. reported that human palladin is an ERK1/2 substrate in vivo and in vitro [94]. Palladin is an actin-binding protein that is phosphorylated following cellular growth factor stimulation. They identified Ser77 and Ser197 as phosphorylation sites, and they showed that ERK-dependent palladin phosphorylation has an anti-migratory function. Furthermore, ERK1/2 mediate the phosphorylation of mouse paxillin, which regulates focal adhesion kinase, which in turn, promotes cell morphogenesis [36]. ERK1/2 also mediate the phosphorylation of myosin light chain kinase (MLCK), which enhances its activity and facilitates African green monkey COS-7 cell and human pancreatic FG carcinoma cell motility [95].

3.3. Proteins of the nuclear pore complex

The nuclear pore complex consists of about 30 different proteins collectively called nucleoporins or Nups [96–98]. Kosako et al. demonstrated that mouse Nup50, Nup153, and Nup214 are ERK1/2 substrates [99]. They showed that ERK1/2 pathway activation leads to Nup50 phosphorylation and to repressed nuclear accumulation of proteins that can be translocated. The number associated with each nucleoporin (e.g., 50, 153, 214) corresponds to its molecular weight in kilodaltons.

The nuclear pore complex is made up of three concentric layers [100]. The first and innermost is the FG repeat layer that coats the nuclear pore channel and interacts with the cargo undergoing transport. This layer is composed of phenylalanine/glycine (FG)-rich repeat domains, and these repeats account for perhaps 12–20% of the mass of the nuclear pore complex. About one-third of the nucleoporins contain FG repeat domains. Many ERK1/2 substrates contain a Phe-Xxx-Phe docking motif, which promotes high-affinity binding to the kinase and enhances the substrate phosphorylation rate as noted in Section 4. The second concentric layer is the scaffold, which provides structure to the nuclear pore complex. The third component, which is called the membrane layer, anchors the nuclear pore complex to the nuclear envelope. The role of the nuclear pore complex in the translocation of ERK1/2 is described in Section 6.

4. ERK1/2 substrate docking and enzyme recruitment sites

4.1. ERK1/2 enzyme–substrate and enzyme–protein interactions

ERK1/2 catalyze the phosphorylation of serine/threonine residues that occur in the sequence Ser/Thr-Pro [101]. Proline at the P + 1 position is the most reliable primary sequence determinant of ERK1/2 and other MAP kinase substrates. The phosphorylation site is numbered 0 (zero), the residue immediately after the phosphorylation site is +1, and the residue immediately before the phosphorylation site is −1. The requirement for proline arises from the nature of the ERK1/2 binding site. Many protein kinases such as PKA contain a pocket for a large hydrophobic residue immediately after the phosphorylatable serine/threonine [68,102]. However, the three-dimensional structure of activated bisphosphorylated rat ERK2 reveals a surface depression where the ERK2 phosphotyrosine occupies the would-be pocket observed in other kinases [40,70]. Proline is preferred because its favored backbone conformation places the side chain away from the kinase surface. A detailed analysis of substrate specificity using synthetic peptides indicates that Pro-Xxx-Ser/Thr-Pro represents the optimal primary sequence for ERK1/2 MAP kinase phosphorylation with proline at the −2 and +1 positions [101].

ERK1/2 interact with two independent docking domains (D-site and F-site) that occur within their substrate proteins, their upstream activating enzymes, and their inactivating phosphatases. ERK1/2 possess a D-site recruitment site (DRS) that interacts with the D-docking domain of substrates, and ERK1/2 possess an F-site recruitment site (FRS) that interacts with the F-docking domain of substrates. Some substrates possess a D-docking site, some possess an F-docking site, others possess both [103], and some possess neither. The design of small-molecule inhibitors that block the

Table 5
Substrate docking site and kinase recruitment site properties and nomenclature.

D-site of substrate	D-site recruitment site (DRS) of human ERK2 kinase	F-site of substrate	F-site recruitment site (FRS) of human ERK2 kinase
$(R/K)_{2-3}$-X_{2-6}-Φ_A-X-Φ_B is a canonical sequence[a]	Amino acid residues[b] that make up the DRS and its three subdomains: ED domain (T159, T160), CD domain (D318, D321), hydrophobic groove (L115, L121, L157, H125, Y128)	FXFP or FXF are canonical sequences	Amino acid residues[b] that make up the FRS: Y233, L234, L237, Y263, M199, L200
Binds to ERK and JNK	Found diametrically opposite to the catalytic cleft, on the "Derriere" of the enzyme	Binds to ERK	Found near the activation segment of the catalytic cleft, in the "Front" of the enzyme
Motif may occur N- or C-terminal to the phosphorylation site	More that 10 Å from the active site	Motif occurs between 6 and 20 amino acids C-terminal to the phosphorylation site	Close to the active site
DEJL (Docking site for ERK, and JNK, LXL) = D-site δ domain = D box = KIM (kinase interaction motif) = CRS (cytoplasmic retention sequence) = D-site	CD/ED (common docking) domain/ED (glutamate-aspartate) domain[c]	DEF site (Docking site for ERK, FXFP) = F-site C box = F-site	

[a] Φ represents a hydrophobic residue, usually Val, Leu, or Ile.
[b] Residue numbers correspond to human ERK2; subtract two for the corresponding rat ERK2 residue.
[c] The ED domain occurs in p38 MAP kinase at E160 and D161; this functionality in ERK2 is replaced by T159 and T160 [104].

interaction of the ERK1/2 recruitment sites and the corresponding substrate docking sites represents a potential strategy for inhibiting the Ras-Raf-MEK-ERK signaling cascade (Section 8.2.3). See Table 5 for a summary of the properties of the substrate docking sites and the kinase recruitment sites.

The D-site of substrates contains both hydrophobic (Φ) and positively charged basic residues with the following canonical sequence: $(R/K)_{2-3}$-X_{2-6}-Φ_A-X-Φ_B. This sequence occurs in several transcription factors (Elk1, Lin1, TFII) and other substrates and molecules that interact with ERK1/2 (STEP, MKP3, PDE4E, MEK1, MNK1, MSK1, and RSK1–3) [103,105]. The ERK1/2 D-site recruitment site occurs on the side of the protein that is opposite to the catalytic cleft and consists of a negatively charged component (Φ_{chg}) and a hydrophobic component (Φ_{hyd}). The positively charged basic residues of the substrate D-site bind to the negatively charged ERK1/2 Φ_{chg} (Fig. 8) that contains two aspartate residues (D318/321) previously identified as the common-docking (CD) domain [106]. The Φ_A-X-Φ_B sequence of the ERK1/2 substrates binds to a nearby hydrophobic patch (Φ_{hyd}) that contains L115, L121, L157, H125, and Y128 (human ERK2 residues).

The ERK1/2 F-site recruitment site, which binds to a substrate F-site, is found near the activation segment of ERK1/2 (Fig. 9). The F-site was first characterized as an FXFP sequence occurring in substrates [103]. This site is conserved in multiple ERK1/2 interacting proteins including the transcription factors Elk1, c-Fos, Lin1, Sap1, the kinase suppressor of Ras (KSR), C-Raf protein kinase, and dual specificity protein phosphatases such as MKP1 (DUSP1) and MKP4 (DUSP4) [103,107].

4.2. Interaction of the substrate docking sites with the ERK2 recruitment sites

Lee et al. studied the interaction of peptides containing the D-site or F-site with rat ERK2 using hydrogen exchange mass spectrometry (HX-MS) and site-directed mutagenesis [104]. In HX-MS, ERK2 is incubated with and without effector peptides and with D_2O to allow for the exchange of the ERK2 N—H backbone protons with the deuterium solvent. After various times, the exchange reaction is quenched by lowering the temperature and pH. Following pepsin-catalyzed proteolytic digestion, the resulting ERK2-derived peptides are separated and analyzed by mass spectrometry. This strategy takes advantage of the pepsin pH optimum (pH ≈ 1). Differences in the rate of exchange of the peptidic backbone N-H hydrogens in the presence or absence of D-site or F-site peptides were used to measure steric protection of various segments of ERK2 from the bulk solvent. Decreased deuterium incorporation and decreased peptide masses correspond to protection afforded by the D-site or F-site peptides. These experiments indicate that the D-recruitment site of ERK2 corresponds to human residues T159, T160, D318, D321, L115, L121, L157, H125, and Y128 (subtract 2 for

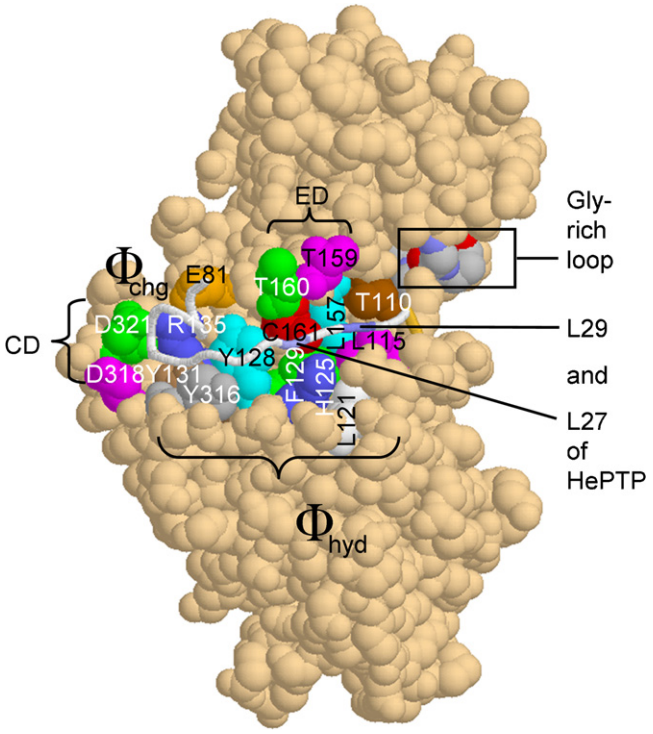

Fig. 8. Model of the human unphosphorylated inactive ERK2 D-site recruitment site, which occurs on the "rear" of protein kinases as usually shown, is indicated by the colored space-filling residues that interact with a peptide (depicted as the gray trace) corresponding to the D-docking site of HePTP. Only a small portion of the glycine-rich loop is visible because it is located in the "front" of protein kinases. ED corresponds to the glutamate/aspartate region of p38 MAP kinase, which is replaced by two threonine residues in ERK1/2. CD is the common docking domain. Φ_{chg} corresponds to human ERK2 charged residues, and Φ_{hyd} corresponds to human ERK2 hydrophobic residues. Adapted from the protein data bank file of rat ERK2, PDB ID: 2GPH. The residue numbers correspond to those of human ERK2.

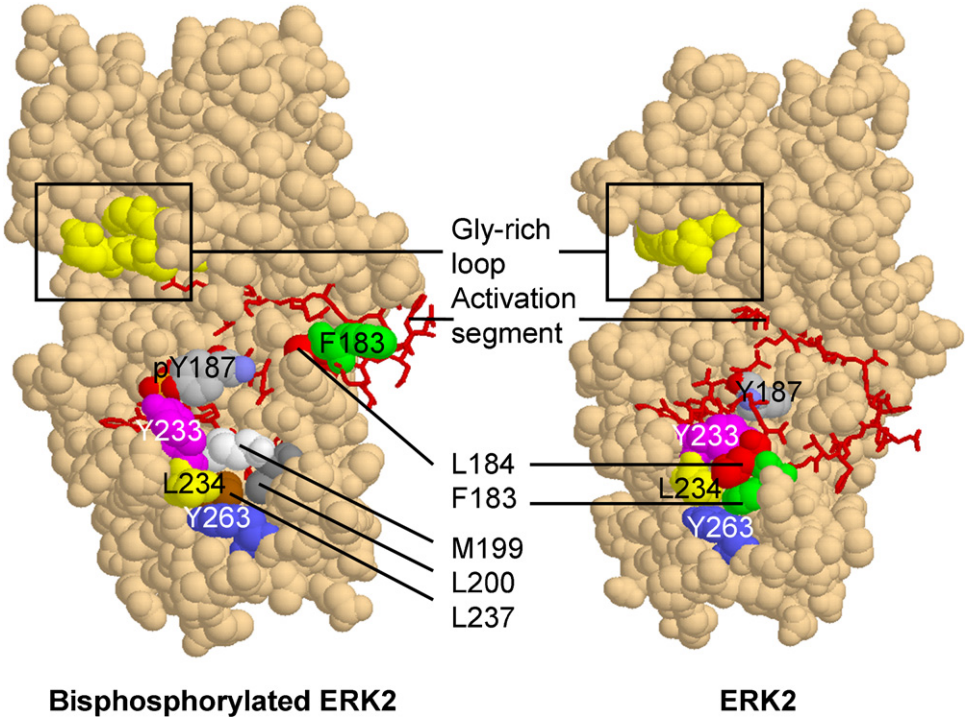

Fig. 9. Models of the ERK2 F-site recruitment sites in bisphosphorylated ERK2 (active) and unphosphorylated ERK2 (inactive) are depicted as the colored residues (space-filling models). Note that M199, L200, and L237, which can be observed in bisphosphorylated ERK2, are buried in ERK2. The glycine-rich loop overlays the ATP binding site. The activation segment is shown in the red stick format. Note the large change in position of L184 and F183 when going from one state to the other. Residue numbers correspond to human ERK2. Adapted from the following protein data bank files of active and inactive rat ERK2; PDB IDs: 2ERK (active) and 1ERK (inactive).

the corresponding rat ERK2 residue) (Fig. 8) [104]. The DRS residues are situated on the side of the protein that is opposite the catalytic cleft.

These investigators demonstrated that the F-site recruitment site of rat ERK2 corresponds to Y233, L234, and L237 on one side of a hydrophobic groove together with M199, L200, and Y263 (equivalent human ERK2 residues) (Fig. 9) [104]. The FRS residues occur near the activation lip and catalytic cleft. To independently test the identity of the F-site recruitment residues of rat ERK2, Lee et al. performed site-directed mutagenesis studies and pull-down assays in vitro to assess the binding of Elk1 to ERK2 [104]. The Y233A, L234A, L237A, and Y263A mutations decrease Elk1 binding by 85–90%, and the M199A and L200A mutations decrease binding by 70–75%. Double mutations combining L200A with Y233A, L234A, L237A, or Y263A decrease Elk1 binding to the ERK2 mutant by more than 90%. The enzyme specific catalytic phosphorylation activity of bisphosphorylated and activated ERK2 mutants toward Elk1 decreases in parallel with Elk1 binding. Based upon rat ERK2 mutagenesis studies, Sheridan et al. reported that the first phenylalanine in the FXFP sequence of the F-site binds to M199 and L200 and the second phenylalanine in the FXFP sequence makes contact with L237 (human residues) [108]. Note that these three residues are buried in unphosphorylated ERK2 (Fig. 9(B)) and are therefore not expected to make contact with the F-docking site of substrates.

4.3. Interaction of the ERK2 D-site recruitment site and the substrate D-docking site

Using X-ray crystallography, Zhou et al. investigated the binding of unphosphorylated (inactive) rat ERK2 to D-motif peptides based upon the sequences of (i) rat hematopoietic protein-tyrosine phosphatase (pepHePTP) and (ii) rat MEK2 (pepMEK2) [109]. Recall that MEK2 catalyzes the phosphorylation and activation of ERK1/2 (Fig. 2). They also studied the interaction of a peptide derivative (pepHePTPm) that contains a cysteinyl residue that forms a disulfide bond with a rat ERK2 mutant (rat T116C). The DRS consists of the CD site (rat/human Asp316/318 and Asp 319/321) and a hydrophobic docking groove that was observed in mouse p38α [110] and human JNK1 [111]. Both Arg20 and Arg21 of pepHePTP bind intimately to the rat/human ERK2 Asp319/321. The rat/human ERK2 Asp316/318, which occurs in the DRS, appears to stabilize the CD site of the kinase rather than forming salt bridges with the paired arginine residues.

Based upon X-ray crystallographic studies, the Φ_A-X-Φ_B sequences within D-sites bind to mouse p38α [110] and human JNK1 [111] in a hydrophobic groove flanked by helices αD and αE and the β7-β8 reverse turn. This motif in HePTP consists of ^{27}LML^{29}. X-ray crystallographic studies of rat ERK2 reveal that Leu27 (Φ_A of pepHePTP) binds to a hydrophobic patch on the surface of helix αE formed by H125 and Y128, and C161 (human residues) in the β7–β8 hairpin turn (Fig. 8) [109]. Leu29 (Φ_B of pepHePTP) makes hydrophobic contacts with L115 from αD, F129 from αE, L155 from β7, and C161 from the β7–β8 hairpin turn. Three hydrogen bonds connect ERK2 to the backbone of the Φ_A-X-Φ_B substrate motif. Two hydrogen bonds link ERK2 Q119 to the main chain carbonyl of M28 (the X in Φ_A-X-Φ_B of HePTP), and one hydrogen bond links ERK2 H125 to the main chain carbonyl of M28.

In addition to binding to residues in the DRS, Zhou observed that pepHePTP induces conformational changes in ERK2 that shed light on the mechanism of the docking activation of kinase activity [109]. The CD site is located at the amino-terminus of L_{16}, which is a locus of major change between inactive, unphosphorylated ERK2 and active, bisphosphorylated ERK2 [66,70]. The peptide-induced conformation of L_{16} involves new activation lip contacts that stabilize the lip. The activation lip of ERK2–pepHePTP differs from both active bisphosphorylated and inactive ERK2. In inactive ERK2, Y187 and T185 are buried. After binding to pepHePTP, these residues face outward where they are in a better position to be phosphorylated in reactions catalyzed by MEK1/2. Moreover, Y187 moves more than 15 Å in response to pepHePTP binding. pepHePTP also induces

conformational changes in the amino-terminus, in the glycine-rich loop, and in the MAP kinase insert. These authors reported that the D-motif interactions involving rat ERK2, mouse p38α, and human JNK1 are distinct. For example, Leu29 (Φ_B) of pepHePTP binds to the portion of the hydrophobic groove of rat ERK2 as described above, but it occupies the position corresponding to Φ_A in mouse p38α and human JNK1. Note that these studies involve changes in unphosphorylated inactive ERK2; any changes resulting from D-site containing substrates interacting with bisphosphorylated active ERK2 remain to be established.

4.4. Substrate D- and F-site docking interactions with the ERK2 D- and F-site recruitment sites

Lee et al. prepared D-site specific (Sub-D) and F-site specific (Sub-F) ERK2 substrates and non-substrate ligands that contain the D-site and F-site specific amino acid residue signatures (Lig-D, Lig-F), and they examined their interactions with rat ERK2 [112]. Using steady-state kinetic analysis, they reported that both Sub-D (k_{cat}/K_m, $6 \times 10^6\ M^{-1}\ s^{-1}$) and Sub-F ($k_{cat}/K_m$, $2 \times 10^6\ M^{-1}\ s^{-1}$) are phosphorylated through a random-ordered sequential mechanism with k_{cat}/K_m values comparable to that of Ets1 ($6 \times 10^6\ M^{-1}\ s^{-1}$), a physiological ERK2 substrate. Occupancy of FRS with a peptide containing a modular docking sequence has no effect on the ability of ERK2 to catalyze the phosphorylation of Sub-D, but it does inhibit the phosphorylation of Sub-F. Although occupancy of DRS with a peptide containing a modular docking sequence inhibits the phosphorylation of Sub-D, it slightly increases the k_{cat} of the ERK2-catalyzed phosphorylation of Sub-F. DRS or FRS specific ERK2 inhibitors fail to decrease ERK2-catalyzed phosphorylation of substrates that interact with the other docking site. Therefore, it appears that it would be possible to develop effective small-molecule inhibitors that are specific for the ERK1/2 DRS or FRS (Section 8.2.3) [112].

Burkhard et al. studied the interaction of rat ERK2 with Elk1, RSK1, and c-Fos [113] using surface plasmon resonance [114]. Elk1 contains both a D-site and F-site, RSK1 contains only a D-site, and c-Fos contains only an F-site. Each of the three substrates binds to ERK2 with submicromolar affinity. A rat T157A mutation in the ED subdomain of the ERK2 DRS increases the K_D for Elk1 by 20-fold and that for RSK1 by 4.5-fold [113]. A D319N mutation in the CD subdomain of the rat ERK2 DRS increases the K_D for Elk1 by 14-fold and that for RSK1 by 7-fold. Surprisingly, the double mutation (T157A/D319N) increases the K_D of Elk1 by less than that of either single mutation. The double mutation increases the K_D for RSK1 about the same as that of the D319N single mutation. An L198A/L235A double mutation of the rat ERK2 FRS domain increases the K_D for Elk1 by 20-fold. However, this L198A/L235A double mutation actually decreases the K_D for c-Fos to one-third of the wild-type value. A triple ERK2 mutation encompassing the ED subdomain of DRS (T157A) and the FRS (L198A/L235A) abolishes the binding of Elk1 and c-Fos to ERK2, and it increases the K_D for RSK1 by 6-fold when compared with wild-type ERK2.

These data are consistent with the notion that RSK1 interacts with ERK2 through the DRS but not the FRS of ERK2. These experiments also support the idea that Elk1 interacts with ERK2 through the FRS but not the DRS. Mutations in both the DRS and FRS completely abolish interactions of ERK2 with Elk1 supporting the idea that both docking sites contribute to the binding of Elk1.

In addition to substrate binding, Burkhard et al. investigated the role that docking interactions play in the ability of ERK2 to catalyze the phosphorylation of Elk1, RSK1, and c-Fos [113]. Mutations in the ERK2 DRS domain inhibit RSK1 phosphorylation but have little effect on Elk1 phosphorylation. This result supports the notion that the DRS is the major determinant of RSK1 interactions with and phosphorylation by ERK2. These experiments indicate that the DRS fails to play a role in mediating Elk1 phosphorylation despite the presence of an Elk1 D-docking site. The ERK2 FRS mutant lacks detectable activity toward RSK1 and Elk1. Although the FRS mutant binds to RSK1 as well as wild-type ERK2, this interaction is non-productive and fails to support catalysis. Moreover, the double mutant (L200A/L237A) lacks catalytic activity toward myelin basic protein, a useful but non-physiological substrate, suggesting that these residues are necessary for the formation of an active enzyme. These residues (L200A/L237A) are far from the regulatory and catalytic spines suggesting that disruption of the hydrophobic skeleton is not directly involved in producing a catalytically impaired kinase.

Piserchio et al. studied the interaction of inactive, unphosphorylated rat ERK2 with peptides modeled after the (i) yeast Ste-2 and (ii) human Elk1 D-sites, the (iii) N-terminal 138 amino acids of mouse Ets1, and (iv) full length Chinese hamster PEA-15 using NMR [115]. These four substrates lack canonical D- and F-sites. PEA-15 is a 15-kDa cytoplasmic ERK1/2 anchor that was initially described as a phosphoprotein enriched in astrocytes (PEA) [116]. Studies with the Ste-2 and Elk1 D-site peptides confirm their interaction with the DRS residues of ERK2 (D318, D321, L115, L157, H125, and Y128; equivalent human ERK2 residues). These peptides fail to interact with the FRS residues. The Ste-2 peptide displayed a long-range interaction with Q103 (the rat ERK2 gatekeeper residue), indicating that the interaction of a small molecule with an enzyme can induce global structural changes.

Although Ets1 lacks canonical D- or F-sites, Piserchio et al. reported that the D-site peptide modeled after Elk1 displaces Ets1 from inactive rat ERK2 suggesting that Ets1 uses the DRS to interact with ERK2 [115]. Using a construct consisting of the 138 amino-terminal residues of mouse Ets1 (Ets1Δ138), they identified L115, L157, and Y128 of the DRS as sites of interaction. However, there was no interaction with D318 or D321. In contrast with the D-site peptides (Ste-2 and Elk1), they found that Ets1 interacts with Y263 of the FRS and its flanking residues. In summary, they observed that Ets1Δ138 binding to inactive ERK2 involves both the DRS and at least part of the FRS. Moreover, Piserchio and collaborators reported that PEA-15, which lacks canonical D- or F-sites, interacts with D318, H125, and Y128 of the ERK2 DRS [115]. They found that this protein produces substantial perturbations in the FRS residues. These NMR studies with D-site ligands indicate that the DRS of ERK2 is rigid with discrete chemical shift perturbations in the DRS residues following ligand binding. The hydrophobic region of the DRS is able to accommodate ligands that are D-site specific and also non-canonical ligands (Ets1 and PEA-15). They suggest that this hydrophobic site represents a likely target of universal inhibitors of the docking interactions involving the DRS. Moreover, these workers observed that D-site ligand binding fails to alter the backbone configuration of ERK2.

4.5. Role of peptide substrate docking sites on steady-state enzyme kinetics

Fernandes and Allbritton prepared ERKSub (TGPL-S-PGPF) and examined the role of D- and F-site docking peptides on its ability to serve as substrate [117] using the D- and F-site consensus motifs identified in ERK1/2 protein substrates [79,101,103]. Fernandes and Allbritton attached a D-site docking peptide corresponding to MEK1 using an 8-amino-3,6-dioxaoctanoyl trimer on the amino-terminus of ERKsub, an F-site docking peptide corresponding to Elk1 using an 8-amino-3,6-dioxaoctanoyl dimer on the carboxyterminus of ERKsub, and they attached both docking-site peptides to ERKsub using the corresponding linkers [117]. All of the docking-site-containing peptides possessed higher catalytic efficiency than the parent substrate peptide alone (Table 6). Surprisingly, the addition of only the F-docking site yielded the most efficient ERK substrate with a k_{cat}/K_m value 70-fold greater than the unmodified

Table 6
Kinetic parameters of rat ERK2 and peptides with D- and F-docking sites[a]

Peptide	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
ERKsub	250 ± 20	123 ± 13	3.9×10^4
ERKsub(−)$_2$F-docking site	310 ± 13	2.2 ± 0.3	2.3×10^6
D-docking site(−)$_3$ERKsub(−)$_2$F-docking site	210 ± 17	4.0 ± 0.1	8.6×10^5
D-docking site(−)$_3$ERKsub	120 ± 40	3.7 ± 3.3	5.4×10^5

[a] Data from Ref. [117]. ERKSub, TGPL-S-PGPF; D-docking site, MP-KKK-PTP-IQL-NP; F-docking site, FQFP; (−), the (8-amino-3,6-dioxaoctanoyl) linker.

substrate peptide. This k_{cat}/K_m value ($2.3 \times 10^6\,M^{-1}\,s^{-1}$) is the same as that obtained with myelin basic protein as substrate (Table 4) and may represent an upper limit. Linking ERKsub to the D-docking site peptide increases the k_{cat}/K_m value by 14-fold. Addition of both docking-site peptides actually decreases the relative efficiency compared with the F-site docking peptide alone, but the addition of both docking-site peptides enhances the k_{cat}/K_m value 22-fold when compared with ERKsub without any docking sites. Studies using different docking site peptides and varying the length of linker between the docking sites and the phosphorylation site peptide component promise to provide additional information on the role of combinations of docking sites in enhancing substrate efficiency.

5. ERK1/2 scaffolds

5.1. Scaffolds and anchors

Scaffolds play a pivotal role in the spatial and temporal regulation of the ERK1/2 signaling cascade. Scaffolds are proteins that bind to more than one component of a signaling module, and they regulate and integrate overall signal transduction [118,119]. Binding multiple components of a signaling cascade brings them into close proximity and facilitates the efficient propagation of the signal while also segregating the module from related pathways. However, scaffolds have the potential to mediate crosstalk with other pathways. Some scaffolds play an essential role in targeting signals to specific subcellular locations.

In resting cells, ERK1/2 bind to a variety of cytoplasmic scaffold and anchor proteins [120]. The scaffold proteins of the ERK1/2 signaling cascade include KSR1/2, IQGAP1, MP1, MORG1, β-arrestin1/2, Sef, MEKK1, and paxillin. ERK1/2 binding to these scaffolds is regulated by growth factor stimulation. Anchor proteins bind to only one element of a signaling module. MEK1/2, the protein kinases that activate ERK1/2, also anchor ERK1/2 in the cytoplasm [121]. PTP-SL (STEP-like protein-tyrosine phosphatase), which inactivates bisphosphorylated ERK1/2, also functions to anchor ERK1/2 in the cytoplasm [122]. Cytoskeletal ERK1/2 anchors include tubulin in microtubules [123], actin and calponin in actin filaments [124], and vimentin in intermediate filaments [125]. PEA-15 is a cytoplasmic anchor protein that binds to and sequesters ERK1/2. ERK1/2 binding to cytoskeletal elements [120] or PEA-15 [118] is not readily reversed following growth factor stimulation.

5.2. KSR1/2

KSR1/2 (kinase suppressor of Ras) binds to C-Raf, MEK1/2, and ERK1/2 [126]. MEK1/2 are constitutively associated with KSR1/2 while ERK1/2 binds to KSR1/2 only after cellular stimulation. It was first thought that KSR1/2 were catalytically inactive owing to the absence of critical conserved amino acid residues. Whereas most active kinases possess His-Arg-Asp at the beginning of the catalytic loop, KSR1/2 have His-Lys-Asp in its place in humans. Of greater significance, the essential lysine residue in the β3-strand is replaced by an arginine in mammalian KSR1 and KSR2 (human KSR1/2 with R637/R692). Prior to 2011, most investigators and authors thought that KRS1/2 were kinase dead. Recent work, however, indicates that KSR1 [127] and KSR2 [128] possess catalytic activity, which is necessary for their function.

Brennan et al. reported that the human KSR2 scaffold participates in the assembly of a MEK1/KSR2/B-Raf ternary complex that is responsible for promoting rabbit MEK1 phosphorylation by mouse B-Raf. KSR2(KD)/MEK1 heterodimers form tetramers through a KSR2(KD) homodimer interface (where KD refers to the kinase domain) [128]. A KSR2 (Arg718His) mutation abolishes the activation of MEK1 by B-Raf indicating the importance of the KSR2 dimer interface in the overall process of MEK1 activation. Brennan et al. showed that the addition of a kinase-impaired mouse B-Raf (K483S) mutant to KSR2(KD)/MEK1 increases MEK1 phosphorylation 15-fold [128]. They concluded that KSR2 is the major protein kinase responsible for increased MEK1 phosphorylation, and this phosphorylation results from a B-Raf (K483S)-induced increase in KSR2 catalytic activity. These experiments indicate that KSR2 possesses catalytic activity despite the substitution for canonical kinase residues as noted previously in this Section. B-Raf forms active side-to-side homodimers [33], and B-Raf presumably allosterically stimulates KSR2 activity by formation of similar heterodimers. Interaction with KSR2 leads to the exposure of the MEK1 activation segment for phosphorylation. Brennan et al. hypothesize that regulatory B-Raf interacts with KSR2 in *cis* to induce a conformational change in KSR2 thereby facilitating phosphorylation of MEK1 by an independent catalytic B-Raf molecule in *trans* [128].

Hu et al. generated a mouse KSR1 (A587F) gatekeeper mutant that cannot bind ATP (Section 8.1) but stabilizes the C- and R-spines critical for the formation of its closed active conformation, and they used this mutant to assess its scaffold versus kinase functions with other mouse proteins [127]. This catalytically inactive KSR1 mutant binds to C-Raf and to MEK1 but fails to activate MEK1, unlike wild-type KSR1. They reported that wild-type KSR1 alone lacks kinase activity. However, co-expression and binding of KSR1 with C-Raf results in KSR1 kinase activity. These experiments suggest that KSR1 is a bona fide kinase whose activity, in cooperation with C-Raf, is required for MEK1 activation. Thus, both the scaffolding function and catalytic activity of KSR1/2 are required for MEK1 phosphorylation and activation by catalytic C-Raf [127]. The KSR1/2 scaffolds perform their functions preferentially from plasma-membrane cholesterol-rich domains [129,130].

5.3. IQGAP1

IQGAP1 is a large (189 kDa) ubiquitously expressed protein that regulates many signaling pathways and cellular functions including cell-cell adhesion, cellular proliferation and differentiation, cytoskeletal architecture, and transcription [131,132]. This protein contains four tandem IQ motifs, which contain isoleucine (I) and glutamine (Q) along with arginine (R) and glycine (G) with a consensus sequence of IQ-XXX-RG-XXX-R [133]. This protein also contains a sequence that resembles the GAP, but it lacks this activity [131]. IQGAP1 binds directly to and modulates the functions of B-Raf [134], MEK1/2 [135], and ERK2 [136]. Analogous to the

situation with the KSR1/2 scaffolds, Roy et al. demonstrated that either an increase (too much) or decrease (too little) in IQGAP1 expression attenuates the EGF-dependent activation of MEK1/2 and ERK2 indicating that the correct ratio of IQGAP1 to MAPK components is required for efficient signaling propagation [135]. Roy et al. postulated that when the concentration of IQGAP1 is too low, few functional IQGAP1 complexes containing B-Raf, MEK1/2, and ERK2 form [135]. At the optimal IQGAP1 concentration, the bound kinases are in an appropriate stoichiometric ratio, and functional ERK2 signaling complexes are formed. When the concentration of IQGAP1 is excessive, many different IQGAP1 molecules form incomplete complexes, which cannot function optimally, or at all.

Roy et al. reported that purified recombinant human ERK2 associates with human IQGAP1 [136]. Moreover, endogenous ERK2 co-immunoprecipitates with IQGAP1 from untreated human breast epithelial cell lysates [136]. Roy et al. demonstrated that purified human IQGAP1 forms complexes with purified human MEK1 or MEK2 in vitro [135]. They also showed that EGF enhances the binding of human MEK1 to human IQGAP1 six-fold in transiently transfected human breast adenocarcinoma MCF7 cells. In contrast, under identical assay conditions, EGF reduces the amount of human MEK2 that co-immunoprecipitates with IQGAP1 to about one-fourth that of the control value [135]. This finding implies that IQGAP1 preferentially activates the MEK1 signaling pathway.

Ussar and Voss demonstrated that MEK1 and MEK2 activities have different effects on the intensity and duration of ERK1/2 signaling and, thus, on the biological response [137]. They showed that the balance of MEK1 and MEK2 activities regulates the G1/S transition by modulating the kinetics and intensity of ERK1/2 activation in human colon cancer HCT 116 cells. Increased MEK2 activity leads to growth arrest. Increased MEK1 activity, in contrast, leads to proliferative signals. Preferential activation of MEK1 signaling may play an important role in stimulating cell proliferation, suggesting that targeting MEK1 as an anti-tumor agent may be advantageous. Additional studies to substantiate this notion of differential MEK1 and MEK2 sequelae are warranted owing to the importance of identifying optimal therapeutic targets.

Ren et al. observed that human B-Raf binds directly to human IQGAP1 in vitro and co-immunoprecipitates with IQGAP1 from cell lysates [134]. Moreover, they reported that IQGAP1 modulates B-Raf function. They further demonstrated that EGF is unable to stimulate B-Raf activity in *iqgap1*-null cells or in cells expressing an IQGAP1 mutant that is unable to bind to B-Raf. This implies that IQGAP1 is necessary for the activation of B-Raf by growth factors. This observation warrants confirmation and follow up to determine its generality. Furthermore, they showed that B-Raf binding to IQGAP1 significantly enhances its kinase activity in vitro.

5.4. MP1 and MORG1

Schaeffer et al. identified MEK partner 1 (MP1), which is a small protein of 13.5 kDa molecular weight that interacts with MEK1 and ERK1 but not with (i) the Raf kinase family, (ii) MEK2, or (iii) ERK2 [138]. They demonstrated that MP1 enhances the ERK1-dependent activation of the Elk1 transcription factor. Teis et al. found that MP1 requires an interacting protein called p14 to augment ERK1 signaling in cells [139]. MP1 and p14 interact constitutively, in contrast to the growth-factor-regulated association of MP1 with MEK1 and ERK1. Although MEK1 binds directly to MP1 but not to p14, the latter protein is required for MEK1 localization at endosomes and for efficient signaling in response to EGF. Kurzbauer et al. reported that MP1 forms a tight complex with p14 with a dissociation constant of about 13 nM, and they determined the X-ray crystal structure of the complex [140]. Nada et al. reported that a novel lipid raft adaptor protein called p18 interacts with the MP1/p14 complex [141]. By immunoprecipitation of recombinant tagged proteins from mouse embryonic fibroblasts and by pull-down assays in vitro, they demonstrated that p18 binds to MP1, to p14, and to the MP1/p14 complex. Furthermore, Magee and Cygler observed that p18 binds to the MP1/p14 complex with a dissociation constant of about 0.5 μM [142]. The MP1/p14/p18 complex probably plays a role in controlling late endosomal traffic [120].

Based upon the yeast two-hybrid methodology, Vomastek et al. identified MORG1 (mitogen-activated protein kinase organizer 1) as an MP1 binding partner [143]. MORG1 is a member of the WD-40 protein family with a molecular weight of 34.5 kDa, which is larger that p14 and thus is able to form higher aggregate complexes. The WD-40 repeat is a short structural motif of approximately 40 amino acids often terminating with tryptophan/aspartate (WD). MORG1 contains seven of these repeats to form a WD domain. MORG1 associates with several components of the ERK1/2 MAP kinase signaling module including MP1, B-Raf, C-Raf, MEK1, MEK2, ERK1, and ERK2 as determined by immunoprecipitation. MORG1 stabilizes the assembly of these components into an oligomeric complex. Ectopic expression of MORG1 and ERK1 in African green monkey kidney COS-1 cells facilitates ERK1 activation following serum stimulation. Similarly, TPA treatment, but not EGF stimulation, of these cells leads to enhanced ERK1 activity. Suppression of MORG1 in HeLa cells by siRNA leads to a marked reduction in ERK activity following serum stimulation when compared with serum stimulation of non-siRNA treated cells. In agreement with the theoretical model of MAPK scaffolds, MORG1 biphasically modulates the activation of the ERK1/2 MAP kinase cascade. Low optimal concentrations of MORG1 enhance ERK activation, and excessively high concentrations of MORG1 lead to the inhibition of ERK activation owing to the binding of suboptimal ratios of the ERK modular components [143]. Note that lower molecular weight MP1 interacts only with MEK1 and ERK1 while the higher molecular weight MORG1 interacts with all of the components of the three-tier cascade. Based on the association of MP1 and MORG1, it appears likely that these proteins cooperate in the regulation of the ERK pathway, but this has not been established experimentally.

5.5. β-Arrestin1/2

β-Arrestin1/2 are small proteins (46 kDa) that were first characterized as participating in the desensitization of seven-transmembrane G-protein coupled receptors following agonist stimulation [144]. G-protein-coupled receptor protein-serine/threonine kinases catalyze the phosphorylation of agonist-occupied receptors to create high-affinity binding sites for β-arrestin1/2. After binding, the receptor is inactivated because it cannot bind to G-proteins, and the complex undergoes β-arrestin-dependent endocytosis. β-Arrestins can function as molecular mediators of G-protein-independent signaling by acting as scaffolds for signaling modules including the Raf-MEK-ERK and ASK1-MAP2K4-JNK3 cascades [144].

Following stimulation of the protease-activated receptor 2 (PAR2) in Kirsten murine sarcoma virus-transformed rat kidney epithelial cells (KNRK) and in the hBRIE380 rat enterocyte cell line, DeFea et al. demonstrated that agonist stimulation induces the formation of a complex containing the receptor, β-arrestin1, C-Raf, and phosphorylated ERK1/2 [145]. Following stimulation of the angiotensin AT_{1A} receptor in the human embryonic kidney HEK 293 cell line, Luttrell et al. described an agonist-induced signaling complex that contained β-arrestin2, C-Raf, MEK1, and ERK1/2 [146]. They showed that β-arrestin2 directly binds C-Raf and ERK1/2 and indirectly binds MEK1. MEK1 presumably interacts with C-Raf and ERK1/2, which are bound to β-arrestin2. Importantly, this complex is not translocated to the nucleus, thereby restricting activated ERK1/2 to non-nuclear substrates [145].

5.6. Sef

Fürthauer et al. identified Sef (similar expression to *fgf* genes) originally as an inhibitor of fibroblast growth factor (FGF)-dependent MEK/ERK signaling in zebrafish [147]. Sef-a is a transmembrane protein that occurs within the plasma membrane, endosomes, and Golgi [148,149]. Sef-b is an alternatively spliced form that is not an integral membrane protein and occurs in the cytoplasm [150]. Torii et al. reported that human Sef acts as a molecular switch for ERK1/2 signaling by blocking their nuclear translocation in human HEK 293 cells without inhibiting their cytoplasmic functions [148]. They demonstrated that Sef binds to activated forms of MEK, inhibits the dissociation of the MEK–ERK1/2 complex, and blocks nuclear translocation of activated ERK1/2. Consequently, Sef inhibits phosphorylation and activation of the nuclear ERK1/2 substrate Elk1, while it does not affect phosphorylation of cytoplasmic RSK2. Torri et al. reported that down regulation of endogenous Sef by siRNA enhances the stimulus-induced ERK1/2 nuclear translocation and the activity of Elk1 [148]. These experiments thus demonstrate that Sef acts as a spatial regulator for ERK1/2 signaling and retains ERK1/2 in the cytoplasm.

5.7. MEK kinase 1

MAP/ERK kinase kinase 1 (MEKK1), a MAP3 kinase, is both a protein kinase and a scaffold for the ERK and the JNK MAP kinase pathways (see Ref. [119] for a review). MEKK1, which is a large (164 kDa) protein, catalyzes the phosphorylation of MEK1 and MEK2 of the ERK pathway [151]. Xu et al. [151] and Karandikar et al. [152] demonstrated that MEKK1 binds to C-Raf, MEK1, and ERK2 of the ERK1/2 MAP kinase signaling module; MEKK1 also binds to MEK7 and JNK of the JNK MAP kinase signaling module (Fig. 2) [153]. Because MEKK1 is a cytoplasmic protein, its actions are most likely confined to this cellular compartment. MEKK1 may also participate in the crosstalk between different signaling pathways. For example, Li et al. reported that RhoA, a small G-protein, enhances the duration and magnitude of ERK1/2 activation via the MEKK1-mediated crosstalk between RhoA and the canonical ERK1/2 signaling module [154]. The activation of ERK1/2 by this mechanism is Ras-independent.

5.8. Paxillin

Paxillin is a phosphoserine- and phosphotyrosine-containing protein that plays a role in several signaling pathways [155]. It is localized in focal adhesions, which are sites of contact of cells with the underlying extracellular matrix. Paxillin binds to protein-tyrosine kinases such as Src and focal adhesion kinase (FAK) and structural proteins such as vinculin and actopaxin. FAK is a non-receptor protein-tyrosine kinase that plays an essential role in regulating cell migration, adhesion, spreading, and reorganization of the actin cytoskeleton, formation and disassembly of focal adhesions, cell cycle progression, cell proliferation, and apoptosis [156].

Liu et al. reported that hepatocyte growth factor (HGF) stimulation of mouse kidney inner medullary collecting duct mIMCD-3 cells induces the ERK-dependent association of paxillin and FAK [157]. Moreover, they demonstrated that the ERK-catalyzed phosphorylation of paxillin in vitro also increases the association of FAK with paxillin. In follow up experiments, Ishibe et al. found that HGF or EGF stimulation of mIMCD-3 cells results in the association of inactive ERK with paxillin at focal adhesions [158]. These investigators demonstrated that bacterially expressed GST-paxillin binds readily with purified inactive ERK but not with activated ERK. This association is enhanced following Src catalyzed protein-tyrosine phosphorylation of paxillin. Ishibe et al. demonstrated that MEK binds to paxillin in unstimulated cells [158]. They also observed that HGF stimulation of mIMCD-3 cells leads to the association of activated Raf with paxillin. The binding of activated Raf, MEK, and ERK, which are components of the ERK signaling cascade, to paxillin demonstrates its scaffolding function. Following their binding to the paxillin scaffold, activated Raf catalyzes the phosphorylation and activation of MEK, which in turn, catalyzes the phosphorylation and activation of ERK. Ishibe and co-workers demonstrated that this scaffolding function plays a role in the initiation of HGF-induced cell spreading. Thus, paxillin is an ERK substrate and ERK cascade scaffold that participates in the regulation of focal adhesion kinase and in cell spreading [36].

Takino et al. demonstrated that membrane type 1 matrix metalloproteinase (MT1-MPP) induces ERK activation in human oral squamous cell carcinoma HSC-4 cells cultured in 3-D collagen [159]. They demonstrated that MT1-MPP and integrin $\alpha_v\beta_3$ participate in Src activation, which in turn leads to FAK and ERK activation. Src catalyzes the phosphorylation of FAK Tyr576 and Tyr577, which leads to FAK activation. Src activation also leads to the stimulation of the Raf-MEK-ERK signaling cascade. Knock-down of paxillin, but not FAK, reduces ERK activation and cell growth. Following activation of Src and FAK, these investigators hypothesize that paxillin functions as a scaffold for activated Raf, MEK and ERK, and thereby leading to cell growth [159]. They propose that expression of MT1-MPP in cancer cells contributes to tumor invasion and to proliferation by activating the ERK signaling cascade by a process that involves the scaffolding function of paxillin.

6. Subcellular localization of ERK1/2

6.1. Nuclear and cytoplasmic localization of proteins

The nucleus is the defining feature of eukaryotic cells. It segregates the chromosomes and transcriptional machinery from the translational and metabolic machinery in the cytoplasm. The nucleus is separated from the cytoplasm by a double membrane envelope that allows for the selective entrance of proteins of molecular weight greater than 40 kDa through specialized nuclear pore complexes (NPC) [96,98]. Smaller proteins, ions, and metabolites pass through the nuclear pore by diffusion. The nuclear pore complex is made up of approximately 30 different proteins collectively known as nucleoporins [96–98]. Intricate pathways exist for both the nuclear import and export of cellular macromolecules.

The classical pathway for the nuclear uptake of proteins involves a nuclear localization signal (NLS) containing either one (monopartite classical-NLS) or two (bipartite classical-NLS) clusters of positively charged amino acids that bind to the importin-α armadillo (ARM) domain [160]. The armadillo repeat, which is a sequence of about 42 amino acids long that is tandemly repeated, was first identified in *Drosophila* [161]. The armadillo gene product mediates cell adhesion. Highly conserved importin-α armadillo repeats 2–4 define the major NLS binding site while a similar arrangement in armadillo repeats 7–8 make up a minor binding site. The monopartite-NLS conforms to the consensus sequence Lys-Lys/Arg-Xxx-Lys/Arg (where Xxx is any amino acid). Polar and acidic side chains of importin-α bind to the basic NLS side chains. The bipartite nuclear localization signal contains a small amino-terminal cluster of basic residues that is connected to the larger carboxyterminal cluster by a linker of 9–29 amino acid residues [162]. The bipartite consensus corresponds to (Lys/Arg) (Lys/Arg) Xxx_{9-29} $(Lys/Arg)_{3/5}$ where $(Lys/Arg)_{3-5}$ represents a total of three lysine and arginine residues out of five consecutive amino acids. The carboxyterminal cluster binds to the major NLS binding site of importin-α while the amino-terminal cluster binds to the minor site [160]. The binary importin-α/cargo complex binds to

importin-β, and a ternary complex (importin-β/importin-α/cargo) is translocated into the nucleus. Transport involves the interaction of importin-β with the nucleoporins that constitute the nuclear pore complex. The ternary complex is disassembled on binding Ran-GTP in the nucleus [163]. The cargo is retained in the nucleus and the importins shuttle back into the cytoplasm.

Active Ran-GTP is converted to inactive Ran-GDP by the intrinsic GTPase activity of Ran. This hydrolysis is aided by a Ran-GAP (Ran GTPase activating protein) that is localized in the nucleus. Ran-GDP moves to the cytoplasm where a Ran-GEF (Ran guanine nucleotide exchange factor) mediates the exchange of GTP for GDP. Energy (ATP) is required to convert GDP to GTP and reactivate Ran as Ran-GTP [96]. Ran is localized in its active GTP-bound form in the nucleus and inactive GDP-bound form in the cytoplasm. The resulting active/inactive Ran gradient participates in the regulation of the import and export of macromolecules through the nucleopore complex. Importins mediate the nuclear import of NF-κB, NFAT, and a multitude of other signaling molecules [96]. The nuclear localization signal pathway has been characterized in digitonin-permeabilized cells using cytoplasmic extracts as a source of importin factors along with added creatine phosphate and creatine phosphokinase to regenerate ATP in situ. Examination of the primary structures of ERK1/2 shows that they lack classical nuclear localization signals. ERK2, β-catenin, and Smad1–4 are examples of proteins that are translocated into the nucleus by importin-independent pathways [164,165].

Using immunofluorescence and confocal microscopy, Chen et al. demonstrated that ERK1/2 and RSK1/2 are localized evenly in the cytoplasm and nucleus of quiescent (G0) human HeLa cells [166]. Within 5 min of serum stimulation, ERK1/2 and RSK1/2 are concentrated in the nucleus and remain there after 2 h. Nevertheless, considerable ERK1/2 and RSK1/2 remain in the cytoplasm. Both ERK1/2 and RSK1/2 remain concentrated in the nucleus well into G1, after their respective kinase activities have begun to return to basal levels. Chen et al. examined the localization of these proteins in subcellular fractions [166]. However, these studies were plagued by the leakage of nuclear proteins into the cytosol during the fractionation procedure. Nevertheless, they were able to demonstrate that serum stimulation of HeLa cells increases the nuclear localization of ERK1/2 and RSK1/2 within 10 min. Biosynthetic labeling of cells with $^{32}P_i$ showed that there is an increase in phosphorylation of cytoplasmic and nuclear ERK1/2 following serum stimulation and immunoprecipitation. Chen et al. also observed that nuclear ERK1/2 have more phosphoserine relative to phosphothreonine and phosphotyrosine [166].

Phosphorylated proteins typically migrate slower than unphosphorylated proteins during denaturing polyacrylamide gel electrophoresis. Based upon this methodology, Chen et al. demonstrated that serum stimulation leads to increased ERK1/2 and RSK1/2 phosphorylation and activation in both the cytoplasmic and nuclear fractions [166]. Chen et al. reported that maximal ERK1/2 stimulation occurs between 2 and 5 min and begins to decline after 10–20 min. The rate of RSK1/2 activation was somewhat slower than that of ERK1/2, which is consistent with the placement of RSK1/2 downstream from ERK1/2. They also demonstrated that ERK1 and ERK2 catalyze the phosphorylation in vitro of c-Fos and c-Jun. In another study, Brunet et al. demonstrated that ERK1/2 nuclear translocation is required for Elk1-dependent gene transcription in Chinese hamster fibroblast cell lines in response to fetal calf serum treatment [167]. This finding suggests that the nuclear translocation of ERK1/2 is a prerequisite for many physiological responses following cellular stimulation.

In resting cells, ERK1/2 bind to a variety of cytoplasmic scaffold and anchor proteins that hold them in the cytoplasm [120]. The scaffold proteins, which bind to more than one component of the ERK1/2 signaling module, include KSR1/2, β-arrestin1/2, IQGAP1, MP1, Sef, MEKK1, and paxillin as described in Section 5. Binding to these proteins is regulated and is reduced following growth factor stimulation. Anchor proteins, which include some cytoskeletal proteins, bind to only one component of a signaling cascade.

6.2. Nuclear uptake by active and passive processes

The mechanism for the nuclear translocation of ERK1/2 is intricate and involves more than a single process [8]. The existence of more than one mechanism adds to the difficulty in deciphering and understanding the physiology of ERK1/2 cellular localization. This is because the blockade or stimulation of one of a few translocation processes makes it difficult to pinpoint and identify the relevant mechanisms. Adachi et al. studied the nuclear translocation of *Xenopus* ERK2 and hemagglutinin-tagged *Xenopus* MEK1 expressed in African green monkey fibroblast COS-7 cells [168]. They found that MEK1 and ERK2 co-immunoprecipitated in unstimulated cells indicating the constitutive formation of MEK1/ERK2 complexes. Co-expression of an activated form of Ras (Ras-V12), which stimulates the ERK1/2 MAP kinase cascade, results in the dissociation of ERK2 from MEK1. This dissociation requires the phosphorylation of ERK2, particularly on the activation lip tyrosine, which is a requirement for its stimulated nuclear translocation [169]. RanQ69L, a dominant negative Ran, fails to alter the nuclear uptake of wild-type ERK2 [168]. In contrast, RanQ69L inhibits the nuclear import of a β-galactosidase–ERK2 fusion protein. These experiments suggest that ERK2 is transported into the nucleus by both passive and active nuclear translocation mechanisms. Note that this comparison involves two forms of ERK2: a β-galactosidase-ERK2 fusion protein and ERK2 per se. These results are consistent with the notion that monomeric bisphosphorylated ERK2, which has not dimerized, is small enough (41.2 kDa) to pass through a nuclear pore in energy- and importin-independent processes, whereas the fusion protein is too large (157 kDa) to diffuse through a nuclear pore.

Matsubayashi et al. examined the nuclear uptake of purified green fluorescent protein (GFP) fused to His_6-tagged *Xenopus* ERK2 in digitonin-permeabilized HeLa cells [165]. They showed that this ERK2 construct translocates into the nucleus in both its phosphorylated and unphosphorylated states. However, when GFP-ERK2 was co-expressed with *Xenopus* MEK1, the ERK2 construct is retained in the cytoplasm in its unphosphorylated state (bound to MEK1), but following phosphorylation it dissociates from MEK1 and is translocated into the nucleus. Wheat germ agglutinin (WGA), which binds to *N*-acetylglucosamine residues linked to the nucleoporins, blocks the nucleopore complex-mediated transport of GFP-ERK2. They observed that wheat germ agglutinin inhibits the translocation of the phosphorylated and unphosphorylated ERK2 constructs. Although the nucleopore complex is required for this translocation, they reported that ATP, cytoplasmic components, or importins are unnecessary. Matsubayashi et al. also demonstrated that their ERK2 construct interacts with human nucleoporin 214.

Whitehurst et al. studied the nuclear uptake of GFP-tagged rat His_6-ERK2 into digitonin-permeabilized rat embryo fibroblast REF52 cells and reported that the construct enters the nucleus through the nuclear pore complex without a carrier and without a need for energy [164]. They demonstrated that the ERK2 construct interacts with nucleoporin 153. These studies show that ERK2 translocation occurs by both passive and active mechanisms [168] and by energy-independent processes involving the nuclear pore complex and nucleoporins 153 and 214 [165]. As noted previously, Kosako et al. reported that nucleoporin 50 is an ERK1/2 substrate [99]. Nucleoporins 50 and 153 are part of the nuclear filament that resides on the inner aspect of the nuclear pore complex, and nucleoporin 214 is part of the cytoplasmic filament that resides on the outer aspect of the nuclear pore complex [100].

Yazicioglu et al. investigated the effect of mutations of rat GFP-ERK2 on nuclear entry in digitonin-permeabilized HeLa, human embryonic kidney HEK 293, and BJ (human foreskin fibroblast) cells [170]. Two regions of ERK2 have been identified as protein–protein interaction sites that bind substrates and regulators as noted in Section 4. One region includes acidic residues and a hydrophobic groove on the D-docking site recruitment site (DRS); this region binds to basic/hydrophobic docking motifs of interacting proteins. The second region includes residues in the αG-helix and the MAP kinase insert domain and binds proteins with FXF motifs (F-docking sites). They observed that mutations in the kinase insert domain (part of the F-docking site) decrease the nuclear uptake of bisphosphorylated and unphosphorylated ERK2. Deletion of the entire insert (rat residues 241–272) has the greatest effect, and the rat Y261N point mutation within the kinase insert domain has a lesser effect.

Yazicioglu et al. reported that mutations of the DRS of ERK2 fail to alter nuclear translocation [170]. This laboratory reported that the kinase insert domain participates in ERK2 binding to PEA-15, a cytosolic anchor protein, and to nucleoporin 153 [164]. Together these studies indicate that the kinase insert domain participates in the nuclear uptake of ERK2 (and probably ERK1) by a process that involves the nuclear pore complex and particularly nucleoporins that contain the FXF motifs that interact with the F-site recruitment site. These investigators reported that the primary mechanism for the nuclear uptake of unphosphorylated ERK2 occurs by an energy-independent process that involves nucleoporins. This mechanism is also responsible for a significant fraction of the nuclear translocation of bisphosphorylated ERK2. However, a major component of nuclear uptake of phosphorylated ERK2 is energy-dependent. This implies a role for Ran-GTP in the import mechanism.

6.3. Mxi2 promotes ERK1/2 nuclear translocation

Mxi2 is an alternatively spliced p38α isoform that is identical with p38α from amino acids 1–280 while containing a unique 17-amino-acid carboxyterminus. Zervos et al. identified this protein in a HeLa cell cDNA library using the yeast two-hybrid methodology [171]. Sanz et al. found that Mxi2 activity with p38α substrates including c-Jun, Elk1, Sap1, and ATF2 is low when compared with the parent isoform [172]. Casar et al. observed that exogenously expressed Mxi2 is predominantly located in the nucleus of COS-7 cells under basal conditions; these cells ordinarily lack Mxi2 [173]. They reported that the nuclear content of exogenously expressed Mxi2 increases further following EGF stimulation. Transfection of these cells with wild-type Mxi2 leads to an increased nuclear accumulation of ERK1/2, whereas transfection with a Δ17 mutant form of Mxi2 that is unable to bind ERK1/2 fails to alter the ERK1/2 distribution. The wild-type MDCK (Madin–Darby canine kidney) epithelial cell line physiologically expresses Mxi2, which occurs predominantly in the nucleus. Casar et al. found that ERK1/2 are predominantly localized in the nucleus under starved, proliferating, or EGF-stimulated conditions in MDCK cells [173]. However, down regulation of Mxi2 with siRNA in these cells leads to a decrease in nuclear ERK1/2 content.

Casar et al. studied the role of the nuclear pore in mediating the translocation of ERK1/2 into the nucleus of COS-7 cells transfected with HA-Mxi2 [173]. These investigators found that Mxi2 increases the association of ERK1/2 with nucleoporin 153 [173]. They found that wheat germ agglutinin blocks the nuclear entrance of both Mxi2 and ERK1/2 into the nucleus of COS-7 cells expressing the HA-Mxi2 construct, thus implicating a role for the nuclear pore in this translocation process. Moreover, the dominant negative RanQ69L inhibits the Mxi2-mediated nuclear translocation of ERK1/2. They observed that neither wheat germ agglutinin nor RanQ69L decreases the binding of ERK1/2 to Mxi2. These investigators demonstrated that Mxi2 promotes the translocation of an ERK2 mutant that lacks activation segment phosphorylation sites, indicating that Mxi2 potentially mediates the translocation of both phosphorylated and unphosphorylated ERK2. They observed that Mxi2 binds to a dimerization-deficient ERK2 mutant (H176E L_4), described in Section 6.5, and promotes its nuclear translocation. Although ERK1/2 can be imported into the nucleus by energy- and Ran-independent processes as documented in Section 6.2, the import of Mxi2 requires both energy and Ran function as Casar et al. demonstrated in digitonin-permeabilized BJ cells [173]. These workers reported that the binding of Mxi2 to ERK1/2 involves the unique carboxyterminal region of Mxi2 and the kinase insert domain of ERK1/2.

Casar et al. observed that COS-7 cells transfected with HA-Mxi2 sustain EGF-stimulated ERK1/2 phosphorylation in the nucleus for more than 10 h, when compared with control-vector transfected EGF-stimulated cells in which nuclear ERK1/2 phosphorylation begins to decrease 10 min after stimulation [174]. They studied the role of phosphatases in this process by transfecting COS-7 cells with either of two nuclear phosphatases (MKP1 or DUSP1, and hVH3 or DUSP5) or either of two cytoplasmic phosphatases (MKP3 or DUSP6, and PTP-SL), each of which can catalyze the dephosphorylation and inactivation of bisphosphorylated ERK1/2. Each of these enzymes decreases EGF-induced ERK1/2 phosphorylation. However, in the presence of ectopically expressed Mxi2, ERK1/2 dephosphorylation catalyzed by nuclear phosphatases was abolished, whereas that by cytoplasmic enzymes was not. When cells expressed an Mxi2 (E293A) mutant that fails to bind to ERK1/2, ERK1/2 nuclear dephosphorylation occurred readily. This last result indicates that Mxi2 binds to ERK1/2 and blocks their dephosphorylation as catalyzed by nuclear phosphatases.

Casar et al. demonstrated that ERK1/2 binds to MKP1 and hVH3 as demonstrated in Myc-MKP1 and Myc-hVH3 immunoprecipitates prepared from COS-7 cells [174]. Upon co-transfection with Mxi2, the amount of ERK1/2 that associates with the phosphatases decreases markedly thereby demonstrating that Mxi2 blocks the association with nuclear phosphatases. Although ERK1/2 binds to substrates and phosphatases by the same docking-site recruitment sites, Mxi2 fails to alter the binding of ERK1/2 with Elk1, one of their nuclear substrates. These experiments demonstrate that Mxi2 sustains nuclear ERK1/2 phosphorylation by preventing the association of ERK1/2 and their nuclear phosphatases without preventing their binding to nuclear substrates. These investigators suggest that a greater affinity of ERK1/2 for their substrates when compared with the phosphatases accounts for the differential effects toward substrates and phosphatases that bind to the same ERK1/2 sites.

6.4. Role of a nuclear translocation signal (NTS) in nuclear import

Chuderland et al. studied the role of mutations of the kinase insert domain of rat GFP-ERK2 expressed in Chinese hamster ovary cells on cellular localization [175]. Transfection of MEK1 (an ERK1/2 activator and anchoring protein) with the ERK2 constructs results in the cytoplasmic localization of the co-transfected ERK2 constructs. Treatment with tetradecanoyl phorbol acetate, or TPA, results in the translocation of the GFP-ERK2 construct into the nucleus. TPA is a protein kinase C agonist that leads to the activation of the Raf-MEK-ERK pathway (Fig. 2). However, deletion of the kinase insert domain Ser244, Pro245 and Ser246 (rat ERK2 residues), or mutation of each of these three residues to alanine, inhibits nuclear translocation of the ERK2 constructs. TPA stimulation of Chinese hamster ovary cells leads to the phosphorylation of Ser244 and Ser246. Replacement of both of these two serines with the phosphomimetic glutamate leads to nuclear localization in unstimulated cells with Glu246 being more effective. They found similar results in digitonin-permeabilized cells. Note that the

phosphorylation of a particular serine or threonine can be functionally mimicked by mutating the corresponding site to an acidic residue such as aspartate or glutamate, which introduces a negative charge.

Chuderland et al. demonstrated that phosphorylation of the activation loop tyrosine and threonine following TPA stimulation is important for the detachment from cytoplasmic anchors but is not required for nuclear translocation [175]. In contrast, phosphorylation of Ser244 and Ser246 promotes translocation into the nucleus. They reported that deletion of the Ser-Pro-Ser residues or their mutation to alanines prevents serum-induced cell proliferation. Chuderland et al. found that importin subunit α-7 is an essential participant in the nuclear uptake of ERK2 [175]. Activated ERK2 binds to importin α-7 as determined by co-immunoprecipitation, and the nuclear accumulation of rat GFP-ERK2 is partially prevented by importin α-7 siRNA treatment. Further experiments demonstrated that a phosphorylated Ser-Pro-Ser sequence in Smad3 and a Thr-Pro-Thr sequence in MEK1 play a role in their nuclear translocation, thereby suggesting that phosphorylation of these residues in target proteins is part of a general nuclear translocation process.

The nuclear translocation Ser-Pro-Ser site in the ERK2 kinase insert domain contains downstream acidic residues (Ser-Pro-Ser-Gln-Glu-Asp) that correspond to casein kinase 2 (CK2) phosphorylation sites: Ser-Xxx-Xxx-Glu/Asp. CK2 is a tetramer consisting of two catalytic (α and α′) and two regulatory (β) subunits with the following compositions: αββα, α′ββα, and α′ββ α′ [176]. This enzyme plays a role in development, cell proliferation, and cell survival [177]. Inhibition of CK2 leads to apoptosis, and CK2 thus represents an attractive target for anti-cancer drug design. Plotnikov et al. treated Chinese hamster ovary cells with TPA and found that this stimulus leads to the translocation of endogenous ERK2 into the nucleus as determined with an anti-ERK2 antibody [178]. They found that TBB (4,5,6,7-tetrabromo-2-azabenzimidazole), a CK2 inhibitor, blocks TPA-stimulated ERK2 nuclear translocation. Similar effects were seen in COS-7 and HeLa cell lines. TBB does not decrease the TPA-stimulated phosphorylation of the threonine and tyrosine residues in the activation loop of ERK2. They reported that CK2 catalyzes the phosphorylation of wild-type ERK2 in vitro but does not catalyze the phosphorylation of the ERK2 (S244A/P245A/S246A) triple mutant. This experimental result indicates that CK2 catalyzes the phosphorylation of only these ERK2 residues. Pretreatment of HeLa cells with TBB or knockdown of CK2 in Chinese hamster ovary cells decreases the TPA-stimulated phosphorylation and activation of Elk1, a downstream target of ERK1/2. These investigators found that mutation of the glutamate and aspartate downstream from the Ser-Pro-Ser site to alanine abolishes the ability of TPA to induce the translocation of the ERK2 mutant into the nucleus of Chinese hamster ovary cells . The Ser-Pro-Ser sequence in the mutant lacking the downstream glutamate and aspartate is not a phosphorylation substrate of CK2, thereby confirming the requirement for CK2-dependent phosphorylation for nuclear translocation.

Plotnikov et al. demonstrated that TBB treatment of HeLa cells abolishes the TPA-stimulated binding of ERK2 to importin α-7 [178]. Using co-immunoprecipitation, these workers showed that ERK2 and CK2 interact in TPA-stimulated HEK 293T cells but not in unstimulated cells. The ERK2 (Ser244Ala) mutant exhibits somewhat diminished nuclear translocation kinetics while the Ser246Ala mutant exhibits greatly decreased translocation kinetics when compared with wild-type ERK2. They found that CK2 catalyzes the phosphorylation of both serine residues within the kinase insert domain, but Ser244 can be phosphorylated only after Ser246 phosphorylation. They reported that ERK2 catalyzes the phosphorylation of Ser244 both in vitro and in vivo. Recall that Chen et al. observed in 1992 that nuclear ERK1/2 have more phosphoserine relative to phosphothreonine and phosphotyrosine after cellular stimulation (Section 6.1) [166], and the finding of dual phosphorylation of the Ser-Pro-Ser sequence in the kinase insert domain provides an explanation for this early observation.

In summary, ERK2 nuclear translocation occurs by passive and active mechanisms [168], by energy-independent processes involving the nuclear pore complex [165], and by a phosphorylation-dependent process involving a nuclear translocation sequence within the kinase insert domain [170]. In addition to the cytoplasm and nucleus, MEK1/2 and ERK1/2 associate with the mitochondria, endosomes, plasma membrane, cytoskeleton, and Golgi apparatus [23]. Because the nuclear translocation of ERK1/2 is an integral part of the Raf-MEK-ERK signaling pathway, inhibition of nuclear translocation represents a potential anti-tumor drug target. However, such a strategy may prove to be too toxic owing to the large number of macromolecules that undergo nuclear translocation.

6.5. Nuclear import and the ERK1/2 dimerization state

The role of ERK2 dimerization and nuclear transport is intricate. Khokhlatchev et al. reported that bisphosphorylated rat His_6-tagged ERK2 is translocated into the nucleus of quiescent rat embryo fibroblast REF52, mouse Swiss 3T3, and human foreskin fibroblast cells following microinjection into the cytoplasm [179]. Nuclear translocation, which was detected with anti-phospho-ERK antibodies, was evident within 1 min after injection and the duration of the translocation was short. Prior to its purification for these uptake studies, the phosphorylation and activation of His_6-tagged rat ERK2 occurred in *Escherichia coli* co-expressing rat ERK2 and human MEK1 [180]. Unphosphorylated wild-type or unphosphorylated kinase-deficient rat Myc-ERK2 remained in the cytoplasm following microinjection into quiescent cells. However, these constructs were translocated into the nucleus following PDGF treatment.

As with phosphorylated ERK2, Khokhlatchev et al. found that thiophosphorylated ERK2 was translocated from the cytoplasmic microinjection site into the nucleus; moreover, it remained there for at least 6 h [179]. Similar results were obtained with ERK1 and with a kinase-impaired rat ERK2 (K52R) mutant. Thiophosphorylated proteins are useful because they are not readily dethiophosphorylated in reactions catalyzed by cellular protein phosphatases. They prepared the bisthiophosphorylated wild-type and mutant rat ERK2 enzymes in vitro by using recombinant rat ERK2 as the substrate, recombinant GST-MEK2 as the enzyme catalyst, and ATPγS (where the oxygen atoms on the γ-phosphate are replaced by sulfur) as the thiophosphoryl donor. Following injection into the nucleus, Khokhlatchev et al. found that the thiophosphorylated wild-type or the kinase-dead K52R mutant remained the nucleus for at least 2 h [179]. In contrast, unphosphorylated Myc-ERK2 (K52R) remained for less than 5 min before redistributing to the cytoplasm. The data obtained from the thiophosphorylated ERK (K52R) mutant indicate that ERK protein kinase activity is not required for nuclear accumulation.

Khokhlatchev et al. found that mutant rat Myc-ERK2 (T183A/Y185F) remained in the cytoplasm following microinjection into quiescent rat 52 fibroblasts [179]. This mutant lacks the phosphorylation sites in the activation lip. After stimulating the cells with TPA, the microinjected mutant was translocated from the cytoplasm into the nucleus. When unphosphorylated Myc-ERK2 (K52R) and phosphorylated wild-type His_6-ERK2 were injected, kinase-defective Myc-ERK2 (K52R) was translocated into the nucleus. This translocation was short-lived, consistent with the dephosphorylation of wild-type ERK2. When unphosphorylated Myc-ERK2 (K52R) was injected with thiophosphorylated kinase-deficient His_6-ERK2 (K52R), nuclear accumulation of Myc-ERK2 (K52R) was observed for up to 3 h. These studies indicate that

ERK2 protein kinase activity per se is not a requirement for nuclear uptake. These experiments suggest, however, that phosphorylated ERK2 facilitates the formation of ERK2 complexes that can be imported.

Khokhlatchev et al. showed by gel filtration that unphosphorylated ERK2 migrates as a monomer [179]. They observed that phosphorylated ERK2 elutes as two peaks consistent with the existence of monomers and dimers (however, see Ref. [181] for an alternative interpretation). Khokhlatchev et al. found that dephosphorylation of activated ERK2 as catalyzed by protein phosphatase 2A (a serine/threonine phosphatase) or by PTP1 (a tyrosine phosphatase) converted the faster eluting dimer into the more slowly eluting monomer [179]. They concluded that the interconversion of these two species is mediated by phosphorylation and dephosphorylation. They calculated the K_D for the phosphorylated dimer as 7.5 nM and that for the unphosphorylated dimer as 20,000 nM. They reported that phosphorylated wild-type rat ERK2 forms dimers with rat (i) unphosphorylated ERK2, (ii) kinase-impaired Myc-ERK2 (K52R), and (iii) the phosphorylation site Myc-ERK2 (T183A/Y185F) mutant.

Khokhlatchev et al. demonstrated that microinjected rat Myc-ERK2 (T183A/Y185F) remained in the cytoplasm when injected alone or with unphosphorylated wild-type His_6-ERK2 [179]. On the other hand, microinjected Myc-ERK2 (T183A/Y185F) was translocated into the nucleus when injected with thiophosphorylated kinase-deficient His_6-ERK2 (K52R). They concluded that the nuclear uptake of phosphorylation-defective ERK2 mutants occurs as a consequence of the formation of dimers with the phosphorylated protein.

To test the dimerization–translocation hypothesis, Khokhlatchev et al. studied the nuclear transport of two dimerization-defective rat ERK2 mutants [179]. The rat H176E L_4A mutant contains an ERK2 (H176E) substitution along with four leucine-to-alanine substitutions (rat ERK2 residues L333A, L336A, L341A and L344A). The Δ4 mutant results from the deletion of rat H176 and three adjacent amino acids (rat 174–177). These investigators demonstrated that both phosphorylated mutants failed to form dimers by co-immunoprecipitation studies and by sedimentation equilibrium analysis. Although phosphorylated and activated, these mutants were unable to move into the nucleus under their experimental conditions. These studies suggested that dimerization is a requirement for nuclear translocation in the rat embryo fibroblast cell line. Moreover, Adachi et al. reported that a *Xenopus* β-galactosidase-ERK2 (H176E L_4A) fusion protein was not translocated into the nucleus of a mouse liver cell line following the stimulated expression of an inducible estrogen-receptor/B-Raf fusion protein that leads to ERK activation [168]. However, these workers found that the *Xenopus* H176E L_4A mutant is translocated into the nucleus of NIH 3T3 cells following microinjection, but at a rate slower than that of wild-type ERK2. The studies of both Khokhlatchev et al. [179] and Adachi et al. [168] suggest that rat or *Xenopus* ERK2 dimerization enhances or is required for its nuclear uptake in cells from multiple species.

Galli et al. reported that ERK1 dimers occur within the mitochondria and nuclei of HeLa cells while ERK1 monomers are found in the cytosol [182]. These dimers were converted to monomers following treatment with β-mercaptoethanol, indicating that the monomers were linked by disulfide bridges. Thus, these are not the type of dimer that results from hydrophobic contacts and salt bridges between adjacent monomers that are based upon X-ray crystallographic structural studies [70,179]. Galli et al. reported that ERK1 increases mitochondrial gene transcription thus indicating a function that parallels that in the nucleus [182]. This novel finding warrants follow up and confirmation.

Casar et al. used siRNAs against several ERK1/2 scaffolds including IQGAP1, KSR1, β-arrestin1, β-arrestin2, and Sef in human embryonic kidney (HEK) 293T cells [130]. Following the knockdown of these scaffolds, the activation of cytosolic phospholipase A_2 ($cPLA_2$) and the release of arachidonate is decreased following EGF treatment when compared with control cells expressing normal amounts of these scaffolds. Using co-immunoprecipitation, they observed that EGF treatment leads to an increased association of endogenous KSR1 and ERK2 that is blocked by pre-treatment with U0126, a MEK1/2 inhibitor. This result indicates that the association of KSR1 and ERK2 requires ERK2 phosphorylation, which is catalyzed by MEK1/2. They reported that $cPLA_2$ is not found in KSR1 immunoprecipitates under basal conditions, but it is found after cells are treated with EGF. KSR1 possesses an F-docking motif and binds to the ERK2 FRS. Using cells expressing a rat FRS FLAG-ERK2 mutant (rat Tyr261Ala) that cannot bind directly to KSR1, they confirmed that the FLAG-ERK2 mutant is absent from KSR1 immunoprecipitates derived from resting cells. However, the FLAG-ERK2 mutant is present in KSR1 immunoprecipitates derived from EGF-stimulated cells, which suggests that the FLAG-ERK2 binds to wild-type endogenous ERK1/2 associated with KSR1. FLAG refers to DYKDDDDK; adding this sequence to a protein provides a means for its recognition by anti-FLAG. Casar et al. also studied a second rat mutant, ERK2 (H176E L_4A), which fails to form ERK2 dimers with itself or with wild-type ERK2 [130]. These workers observed that the quantity of ERK2 dimers present in proliferating HEK 293T cells is reduced in cells expressing the ERK2 (H176E L_4A) mutant. Decreased dimer formation is associated with decreased EGF-stimulated arachidonate production and decreased $cPLA_2$ in anti-ERK2 immunoprecipitates thereby implicating the requirement that ERK2 dimers must bind to KSR1 scaffolds in order to produce this $cPLA_2$-mediated response.

Casar et al. found that $cPLA_2$ binds to the ERK2 FRS (F-site recruitment site) [130]. Following EGF treatment of the HEK 293T cells, they reported that KSR1 binds to ERK2 dimers whereas in unstimulated cells KSR1 binds to ERK2 monomers. These investigators found that the ERK2 (H176E L_4A) dimerization-deficient mutant blocks the binding of ERK2 dimers to KSR1, and it prevents the association of $cPLA_2$ with KSR1. This result suggests that ERK2 dimers are required for the interaction of scaffold complexes with $cPLA_2$ and with $cPLA_2$ activation. They also found that RSK1 and PDE4 associate with ERK2 dimers, but not with ERK2 monomers. And they reported that the IQGAP1 and MP1 scaffolds exhibited behavior that parallels that of KSR1 indicating that ERK2 substrates bind to these scaffolds indirectly by interacting with scaffold-linked ERK2 dimers. Thus, the binding of $cPLA_2$ to the KSR1 scaffold is indirect, which leads to the suggestion that the complex may have the following form: KSR1-ERK2-ERK2-$cPLA_2$. These experiments indicate that ERK dimers play an essential role in the activation of cytoplasmic substrates.

Casar et al. observed that depletion of the KSR1, IQGAP1, and MP1 scaffolds fails to inhibit the EGF-stimulated Elk1 expression, a process that requires the nuclear translocation of ERK1/2 [130]. Expression of the ERK2 (H176E L_4A) dimerization-deficient mutant fails to decrease Elk1 phosphorylation and activation. Surprisingly, the mutant increases this process. They found that only monomeric ERK2 is observed in immunoprecipitates of the endogenous transcription factors (Elk1, c-Fos, Fra) in EGF-stimulated cells. They concluded that ERK1 or ERK2 dimerization is not a prerequisite for their nuclear translocation.

Casar et al. found that ERK2 dimerization is important for cell proliferation and tumor progression [130]. When mouse fibroblast NIH 3T3 cells were transfected with H-RasV12, v-Src, or Dbl, they found that the ERK2 (H176E L_4A) dimerization-deficient mutant decreases the number of foci induced by these three oncogenes when compared with control cells lacking the dimerization-deficient mutant. They also investigated the relevance of ERK2 dimerization on cellular proliferation using three human tumor cell

lines: T24 (bladder), HCT116 (colorectal), and H1299 (lung). They found that the ERK2 (H176E L_4A) dimerization-deficient mutant decreases their proliferation rates by 50% or more. Injecting the mutant cell lines into athymic hairless nude mice, which are unable to launch an immune response against the cells, decreases the time at which the tumors became evident and reduces the tumor mass and size when compared with animals that received cells not expressing the dimerization-deficient mutant. These investigators propose that ERK1/2 monomers are bound to scaffold proteins such as KSR1/2 and to anchor proteins such as MEK1/2. Following stimulation, ERK1/2 bound both to scaffolds and to anchors become phosphorylated by MEK1/2. The phosphorylated-ERK1/2 that dissociate from MEK1/2 may be transported to the nucleus as a monomer. Alternatively, monomeric phosphorylated-ERK1/2 from the anchor binds to the monomeric phosphorylated-ERK1/2 that is complexed with the scaffold to form a dimer. The dimer now binds to a cytosolic substrate and mediates its phosphorylation and activation. The studies of Casar et al. imply that cytosolic dimer formation is important in mediating cell proliferation and tumorigenesis [130], which is a concept that warrants confirmation.

Burack and Shaw demonstrated that the mouse dimerization defective ERK2 (H176E L_4A) mutant fused to yellow fluorescent protein is imported normally into the nucleus of human embryonic kidney HEK 293 cells [183]. These workers could not demonstrate ERK2 dimerization in live cells through FRET (fluorescent resonance energy transfer) measurements between co-expressed yellow fluorescent protein-ERK2 and cyan fluorescent protein-ERK2. To demonstrate the effectiveness of this methodology, these investigators observed that mouse yellow fluorescent protein-ERK2 formed a complex with mouse cyan fluorescent protein-MEK1 in the HEK293 cells. For a review of FRET methodologies, see Ref. [184].

Using a mouse embryo fibroblast *erk1*$^{-/-}$ cell line expressing human ERK1, Lidke et al. reported that serum stimulation increased the nuclear content of ERK1 within 5 min and this nuclear accumulation persisted for several hours as determined by an anti-ERK1 antibody that does not cross react with ERK2 [185]. The level of nuclear ERK1 was maximal 2 h after stimulation. They also reported that a dimerization-deficient ERK1-Δ4 mutant behaved similarly. Both wild-type and ERK1-Δ4 failed to accumulate in the nucleus 2 h after serum stimulation in cells treated with the protein synthesis inhibitor cycloheximide. This is consistent with the idea that serum treatment and ERK1/2 activation leads to the synthesis of nuclear anchors that sequester ERK1/2 in the nucleus [186].

Using this mouse embryo fibroblast *erk1*$^{-/-}$ cell line expressing a human GFP-ERK1 construct along with real time fluorescence microscopy and fluorescence correlation spectroscopy, Lidke et al. reported that GFP-ERK1 nuclear accumulation increases upon serum stimulation just as wild-type ERK1 (non-GFP-ERK1) accumulates [185]. The average cellular concentration of GFP-ERK1 was on the order of 1 μM, similar to that observed by Fujioka et al. [38] for endogenous ERK1/2, thereby indicating that this cellular system reflects the physiological condition. The nuclear translocation of GFP-ERK1 occurred within minutes of serum stimulation and lasted for several hours mimicking wild-type transfected ERK1 [185] and endogenous ERK1/2 in CCL39 Chinese hamster lung fibroblasts [186]. Endogenous ERK1/2 and GFP-ERK1 are retained in the nucleus after dephosphorylation [185,186]. This observation indicates that active and inactive ERK1/2 can be sequestered in the nucleus, presumably by binding to nuclear anchoring proteins such as the MAP kinase phosphatases [186].

Following growth factor stimulation of the cells, ERK1/2 become activated by phosphorylation [186]. After translocation into the nucleus, they catalyze the phosphorylation and activation of transcription factors leading to the formation of numerous mRNAs including those of class I MAP kinase phosphatases (Table 7). After biosynthesis in the cytosol and translocation into the nucleus, the class I phosphatases catalyze the dephosphorylation and inactivation of ERK1/2 and sequester them in the nucleus [186].

Lidke et al. found that a dimerization-deficient mutant (GFP-ERK1-Δ4) accumulates in the nucleus to the same extent as wild-type GFP-ERK1, confirming that dimerization of ERK1 is not required for nuclear entry and retention [185]. Using fluorescence correlation spectroscopy and energy migration Förster resonance energy transfer measurements, they observed no dimerization of wild-type GFP-ERK1 in the cytoplasm or nucleus of living cells upon activation. These investigators suggested that ERK or GFP-ERK is released from cytoplasmic anchors such as MEK1/2 after activation and each diffuses to the nucleus. Although wild-type GFP-ERK1 and a GFP-ERK1-Δ4 mutant accumulate in the nucleus to the same extent, the kinetics of phosphorylation and nuclear accumulation of the GFP-ERK1-Δ4 mutant were slower than that of wild-type GFP-ERK1. They reported that the differential shuttling behavior of the mutant results from the delayed phosphorylation of GFP-ERK1-Δ4 by MEK1/2. In contrast to the work of Khokhlatchev et al. [179], the studies of Casar et al. [130], Burack and Shaw [183], and Lidke et al. [185] indicate that nuclear translocation of ERK1/2 does not require ERK1/2 dimerization. See Ref. [187] for a review of fluorescence correlation spectroscopy and Ref. [188] for a review of energy migration Förster resonance energy transfer where energy migration can be measured by monitoring changes in the polarization of emitted photons.

The notion that physiological, native, activated ERK2 assembles into dimers has been challenged by Kaoud et al. [181]. They reported that a rat ERK2 construct containing a thrombin cleavable His_6 tag (Met-Gly-Ser-Ser-$(His)_6$-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-ERK2) exhibits substantial dimerization [181], while previous work from this laboratory indicated that a construct containing a non-cleavable His_6 tag (His-Ala-$(His)_6$-Ala-ERK2) failed to form dimers [189,190]. Thus, the nature of the His_6 tag on ERK2 may influence its dimerization. Following thrombin catalyzed proteolysis after the arginine residue, they obtained a derivative of rat ERK2 that lacks a His_6 tag but contains three additional residues (Gly-Ser-His) upstream from the physiological polypeptide chain-initiating methionine. Although Wilsbacher et al. reported that dimerization of bisphosphorylated rat ERK2 with an Ala-$(His)_6$-tag requires divalent cations [191], Kaoud et al. reported that their bisphosphorylated construct lacking the His_6 tag following thrombin-catalyzed proteolysis shows little tendency to dimerize with or without calcium or magnesium ions as determined by light scattering, analytical ultracentrifugation, and NMR [181].

Kaoud et al. [181] noted that the procedure employed by Casar et al. [130] for the identification of ERK dimers in cells requires incubation of the proteins under non-reducing conditions where they may undergo cross-linking via disulfide bond formation as described by Galli et al. [182]. They noted that most of the evidence supporting the importance of homodimerization in solution uses the mutants originally formulated by Khokhlatchev et al. [179]. These mutants were designed to break the homodimer interface that was observed in the crystal structure. Kaoud et al. note [181] that caution is needed when interpreting experiments with these mutants as their translocation into the nucleus (which is not dependent upon dimerization) is impeded [192]. Kaoud et al. point out that a similar dimer interface mutation in ERK1 binds less tightly to a phosphospecific antibody, and they suggest that ERK2 mutations designed to prevent dimerization may thus alter the ability of such mutants to interact with other proteins [181]. This suggestion implies that the ERK1/2 dimerization deficient mutants behave abnormally in ways that are unrelated to possible dimer formation.

It has been difficult to confirm or rule out the existence of homodimers formed from activated native ERK1/2 or activated

Table 7
General features of dual specificity MAP kinase phosphatases.[a]

Class	Name	Human gene	Subcellular location	ppMAP kinase family substrate preference		
				ERK	P38	JNK
I	MKP1	*DUSP1*	Nuclear	X	X	X
I	PAC-1	*DUSP2*	Nuclear	X	X	
I	MKP2	*DUSP4*	Nuclear	X	X	X
I	hVH3	*DUSP5*	Nuclear	X		
II	MKP3	*DUSP6*	Cytoplasmic	X		
II	MKPX	*DUSP7*	Cytoplasmic	X		
II	MKP4	*DUSP9*	Cytoplasmic	X	S	
III	M3/6	*DUSP8*	Cytoplasmic/nuclear		X	X
III	MKP5	*DUSP10*	Cytoplasmic/nuclear		X	X
III	MKP7	*DUSP16*	Cytoplasmic/nuclear		X	X

[a] Adapted from Ref. [107]. X, preferred substrate; S, some detectable activity toward this kinase family.

native ERK1/2 constructs. The discrepant results may be due to subtle differences in the preparation and activation of the ERK2 enzymes, which may be difficult to sort out. Kaoud et al. first purified their ERK2 and then used purified MEK to mediate ERK2 activation [181]. In contrast, Wilsbacher et al. co-expressed MEK1 and ERK2 in *E. coli* and then purified activated ERK2 [191]. However, Khokhlatchev et al. from the same laboratory prepared their activated thiophosphorylated ERK2 using recombinant MEK1 and ATPγS [180], a procedure similar to that used by Kaoud et al. to prepare phosphorylated ERK2 [181]. Perhaps the differences in isolating and preparing the enzymes modify their ability to dimerize.

We are faced with two dilemmas. It is unclear whether or not ERK1/2 dimerize in vitro and whether or not they dimerize in cells. The first dilemma is a biochemical problem, and the second is the more important physiological problem. Burack and Shaw [183] were unable to demonstrate dimerization in cells using fluorescent resonance energy transfer measurements between co-expressed yellow fluorescent protein-ERK2 and cyan fluorescent protein-ERK2. Moreover, Lidke et al. observed no dimerization of wild-type GFP-ERK1 using fluorescence correlation spectroscopy and energy migration Förster resonance energy transfer measurements in living cells [185]. At this time the evidence in favor of ERK1/2 dimerization in cells is weak. However, determining the mechanisms for non-activated and activated ERK1/2 nuclear translocation in various cell types continues to be an intricate and vexing problem. Additional experimentation is required to address these issues.

6.6. Nuclear export

Ranganathan et al. studied the nuclear export of rat ERK2 in permeabilized HeLa cells and BJ fibroblasts [193]. They used a 68-kDa GFP-ERK2 construct and found that its export, following nuclear uptake, is inhibited by wheat germ agglutinin indicating the involvement of the nuclear pore complex in the process. They reported that export occurs by at least two mechanisms. The rate of export was greatest in the presence of cytosolic factors and energy (an ATP-generating system). The absence of cytosol decreases export, but the absence of energy decreases it even further. However, the nuclear export of the GFP-ERK2 construct occurs in the absence of either cytosol or an energy source. These workers showed that phosphorylated rat ERK2 (not the GFP-ERK2 construct) is exported from the nucleus of BJ cells in the absence of energy and cytoplasm. These data indicate that more than one process is responsible for the translocation of ERK2 from the nucleus.

The classical nuclear export mechanism involves CRM1 (chromosome region maintenance 1), or exportin-1, a receptor that links nuclear export signal (NES)-containing proteins with the nuclear pore complex. CRM1 is the human homologue of a yeast protein that is a receptor for leucine-rich export signals. The nuclear export sequence, which ERK1/2 lack, consists of hydrophobic-amino-acid-rich sequences with conserved spacing among the residues [96]. Φ-X_{2-3}-Φ-X_{2-3}-Φ-X-Φ represents the canonical export signal where Φ is L, I, V, F, M and X is any amino acid [194].

Ranganathan et al. examined the possible role of CRM1 in the ERK1/2 export process [193]. After using CRM1 siRNA to knockdown the expression of CRM1, they observed a 45% reduction in nuclear export of the GFP-ERK2 construct. CRM1-dependent export depends upon energy and Ran. When energy and the cytoplasm were omitted, no energy-independent export was observed. Leptomycin B is a fungal metabolite that blocks CRM1-mediated export, which leads to the retention or accumulation of proteins within the nucleus. Ranganathan et al. reported that leptomycin B inhibits the nuclear export of GFP-ERK2 by about 50% [193]. However, these authors reported that ERK2, which lacks a nuclear export signal, fails to bind the CRM1.

Adachi et al. suggested that ERK1/2 binds to proteins such as MEK1/2 that contain an NES and is exported in a piggyback-like process [195]. However, Burack and Shaw reported that the co-export of ERK1/2 with MEK1/2 is unlikely [183]. They were unable to detect an association of ERK2 and MEK1 in the nucleus based upon FRET experiments. Moreover, NES-dependent export requires energy, and they were unable to detect a change in GFP-MEK1 localization after energy depletion in HEK293 cells.

6.7. Summary of nuclear import and export mechanisms

The nuclear import and export of ERK1/2 are intricate processes, and this is the main area of ERK1/2 experimentation where discrepancies have arisen. Unphosphorylated ERK2 enters the nucleus by an energy-independent mechanism facilitated by direct interaction with nucleoporins [164,165]. This appears to be the primary mechanism for the nuclear uptake of ERK2 lacking activation loop phosphorylation. Cytoplasmic proteins that bind ERK2 prevent its entry into the nucleus by anchoring it to the cytoplasm or by inhibiting its interaction with nucleoporins. ERK2 can also exit from the nucleus by an energy-independent process involving the nucleoporins, most likely as the reverse of the nucleoporin import mechanism. A second export process for unphosphorylated ERK2 requires energy and operates with the CRM1 nucleoporin receptor. The details of this process are unclear owing to the absence of a nuclear export signal in ERK1/2.

Phosphorylated ERK2 can enter and exit the nucleus by energy- and carrier-independent mechanisms. An energy-dependent component increases the rate and extent of nuclear import of phosphorylated ERK2 when compared with the unphosphorylated form. Classical nuclear uptake requires a nuclear localization signal (NLS) composed of protein segments containing groups of acidic residues, which ERK2 lacks. ERK1/2 contain a Ser-Pro-Ser segment that is phosphorylated under the aegis of casein kinase 2,

and this phosphorylated enzyme participates in nuclear import and requires the participation of importin α-7 [178]. Based upon the X-ray crystallographic structure [70], phosphorylated ERK2 is approximately 4.0 nm × 4.4 nm × 7.0 nm in size. Jamali et al. reported that molecules with diameters up to 9.0 nm are able to diffuse through the nuclear pore complex [100]. Thus simple diffusion can account for a portion of ERK1/2 nuclear import and export, which has been experimentally confirmed [168]. Other processes involving interactions with nucleoporins, Ran, and importins also play a role in ERK1/2 nuclear translocation. Moreover, the participation of active nuclear translocation mechanisms that are regulated following cellular stimulation is consistent with the physiological effects produced by the ERK1/2 MAP kinase cascade.

7. Phosphoprotein-phosphatase-catalyzed dephosphorylation of activated ERK1/2

7.1. MAP kinase phosphatases

The dephosphorylation of bisphosphorylated and activated MAP kinases plays a key role in regulating the magnitude and duration of kinase activation and also the nature of the physiological responses [196]. As noted in Section 2.3, removal of only one of the two phosphates that occur within the activation lip of ERK2 results in an inactive kinase [74]. The MAP kinase phosphatases (MKPs) are divided into three major categories depending on their preference for catalyzing the dephosphorylation of tyrosine, serine/threonine, or both tyrosine and threonine (dual specificity) [197,198]. The tyrosine-specific MKPs include PTP-SL, STEP, and HePTP. The serine/threonine-specific MKPs include protein phosphatase 2A and 2C. The ten human dual specificity MKPs are listed in Table 7. The MAP kinase phosphatases catalyze the following unidirectional reaction:

$$\text{Protein–OPO}_3^{2-} + \text{HO : H} \rightarrow \text{protein–OH} + \text{HO : PO}_3^{2-}$$

The operation of kinases and phosphatases make phosphorylation and dephosphorylation a reversible process.

The dual-specificity mitogen-activated protein kinase phosphatases act as negative regulators of MAPK activity; these can be subdivided into three classes [107]. The class I enzymes are inducible phosphatases localized within the nucleus. With the exception of hVH3 (DUSP5), which is specific for ERK1/2, these phosphatases display a rather broad specificity for inactivation of the bisphosphorylated ERK, p38, and JNK MAP kinase families. The class I enzymes are induced by many of the same stimuli that activate the MAP kinases. These dual specificity phosphatases thus play an important role in the feedback control of MAP kinase signaling. After activation in the cytoplasm, the MAP kinases are translocated into the nucleus and then catalyze the phosphorylation of transcription factors that lead to phosphatase mRNA biosynthesis. The class I enzymes, which contain nuclear localization signals in their amino-termini (Fig. 10), are translocated from cytoplasmic ribosomes into the nucleus where they catalyze the dephosphorylation and inactivation of the MAP kinases that led to their transcriptional activation.

The class II enzymes, which are three closely related ERK-specific phosphatases, are located in the cytoplasm [107]. The class III enzymes, all of which preferentially inactivate the stress-activated p38 and JNK MAP kinase families, are located in both the nucleus and cytoplasm. The dual-specificity phosphatases play pivotal regulatory roles under physiological conditions, and they may participate in pathological conditions such as cancer [199]. These are probably the most important phosphatases that regulate the MAP kinases. However, other phosphatases can play a role in MAP kinase regulation as noted in Section 7.3.

7.2. Dual specificity MAP kinase phosphatases

7.2.1. The MAP kinase phosphatase mechanism

All of the dual specificity MKPs possess an amino-terminal MAPK binding domain containing about 110 amino acid residues, a carboxyterminal phosphatase domain of nearly 200 amino acid residues, and a short linker of about 40 residues between them (Fig. 10). The MAPK binding domain, also called the kinase interaction motif (KIM), plays a major role in determining substrate specificity through docking interactions with their substrate MAP kinases. This interaction motif contains a cluster of positively charged amino acids that interact with the D-site recruitment site of the ERK, p38, and JNK families [200]. The MAPK binding domain also contains clusters of hydrophobic residues that participate in binding to MAP kinases. ΦΦ-X-RRΦ-XX-G represents a canonical sequence found in the MAP kinase binding domains that promotes their association with the MAP kinase D-site recruitment sites, where Φ represents a hydrophobic residue, R represents arginine, G represents glycine, and X can be any amino acid [197].

The dual specificity phosphatase domain contains a His-Cys-$(Xxx)_5$-Arg signature sequence along with a conserved aspartate that acts as a general acid/base during catalysis [197]. The active site cleft of the dual specificity MAP kinase phosphatases is shallow with a depth of about 5.5 Å that can accommodate either phosphotyrosine or phosphothreonine. PTP1B, which is a protein-tyrosine phosphatase, possesses a much deeper 10 Å active site cleft that accommodates phosphotyrosine [201]. The enzymatic mechanism for the dual-specificity phosphatases and the tyrosine-specific phosphatases are similar and both involve catalytic cysteines and aspartates. Moreover, an important arginine binds the substrate phosphate. After the phosphorylated MAP kinase binds to the phosphatase, the catalytic cysteine thiolate anion attacks the phosphate forming a covalent P–S linkage while displacing the unphosphorylated MAP kinase with aspartate donating a proton and acting as an acid (Fig. 11). Next, aspartate functions as a base by abstracting a proton from water while the resulting hydroxyl group attacks phosphorous displacing the cysteinyl thiolate and releasing inorganic phosphate and readying the MAP kinase phosphatase for another round of dephosphorylation.

Zhao and Zhang reported that ERK2/pTpY dephosphorylation catalyzed by MKP3 involves an ordered, distributive mechanism in which MKP3 binds the bisphosphorylated ERK2/pTpY, mediates the hydrolytic dephosphorylation of phosphotyrosine, and releases monophosphorylated ERK2/pT [202]. The latter binds to the same or another MKP3 that catalyzes the dephosphorylation of the phosphothreonine yielding fully dephosphorylated ERK2. The bisphosphorylated ERK2 is a highly specific substrate for MKP3 with a k_{cat}/K_m value of $3.8 \times 10^6\ M^{-1}\ s^{-1}$, which is more than 6 orders of magnitude higher than that for (i) an ERK2-derived 13-residue phosphopeptide encompassing the pT-E-pY motif or (ii) p-nitrophenylphosphate.

PAC-1 (DUSP2), MKP3 (DUSP6), and MKP4 (DUSP9) lack p-nitrophenylphosphate phosphatase activity in their resting state, and they become catalytically active only after binding to their MAP kinase substrates [203,204]. p-Nitrophenylphosphate is a convenient chromogenic non-specific phosphatase substrate that yields yellow p-nitrophenol upon hydrolysis whose rate of formation can be followed spectrophotometrically at 405 nm. The interaction of the phosphatase MAPK binding domain with its MAP kinase substrate induces phosphatase activity. Camps et al. reported that the phosphatase activity of rat MKP3 is stimulated 30-fold after binding to recombinant ERK2 [203]. They demonstrated that MKP3 and ERK2 form a complex, and phosphatase activation occurs even when an ERK2 kinase-dead mutant is used. They

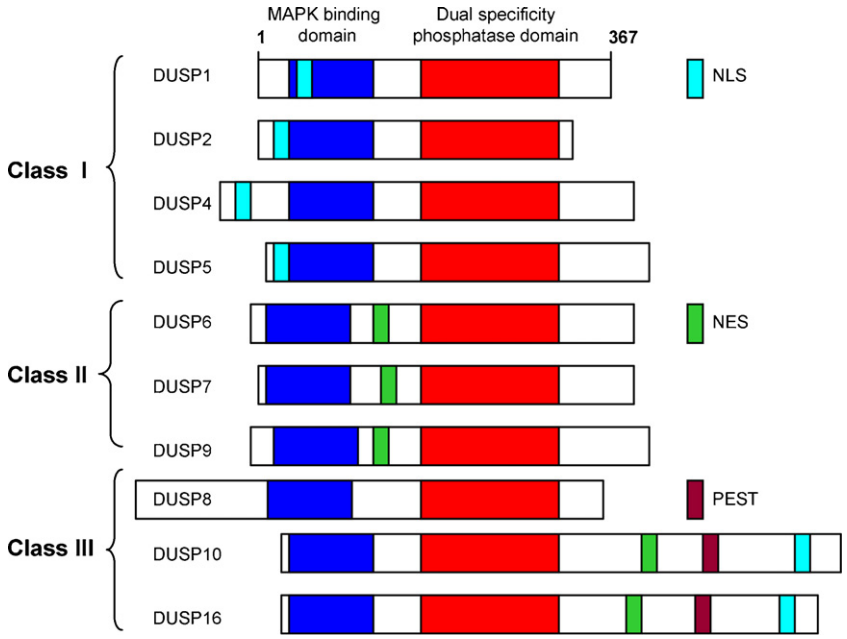

Fig. 10. Architecture of the three classes of dual-specificity MAP kinase phosphatases (DUSPs). The numbers are those of the amino acid residues. The MAP kinase binding domain is the same as the kinase interaction motif (KIM). NLS, nuclear localization signal; NES, nuclear export signal; PEST, sequences that are rich in proline (P), glutamate (E), serine (S), and threonine (T).

Adapted from Ref. [200].

found that activation does not occur when an MKP3 mutant lacking the amino-terminal MAP kinase binding domain was tested. MKP3 phosphatase activation and kinase binding fails to occur when mouse p38α or human JNK2 were tested. MKP3 activation fails to occur when a rat D-site recruitment site ERK2 mutant (rat D319N) was used. These experiments indicate that the interaction of the amino-terminal D-docking site of MKP3 and the DRS of ERK2 are required for the stimulation of MKP3 phosphatase activity. Muda et al. reported that rat MKP3 also forms a tight complex with (human) ERK1 [205].

7.2.2. Interaction of MAP kinase phosphatases and ERK2

Zhang et al. observed that mouse PAC-1 catalyzes the dephosphorylation of bisphosphorylated rat ERK2 but exhibits almost no phosphatase activity toward bisphosphorylated mouse p38α or human JNK2 [204]. PAC-1 forms a complex with ERK2 and p38α but not JNK2 as determined with GST pull-down assays. They observed that Arg62 in the amino-terminal KIM of the phosphatase plays a key role in forming the MKP3/ERK2 complex and that Arg60, Arg61, and Arg62 participate in forming the MKP3/p38α complex. They examined the role of ERK2 binding

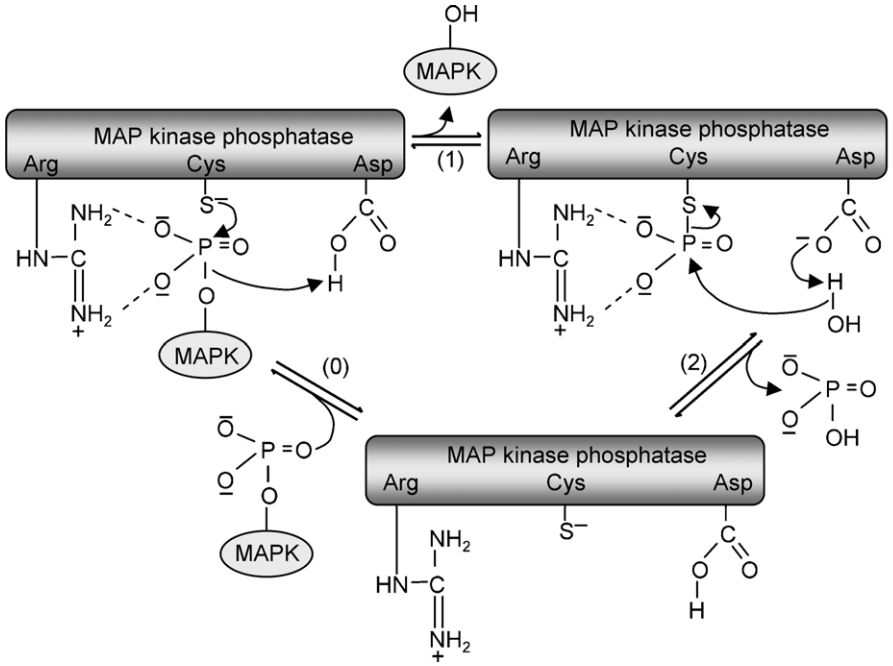

Fig. 11. The two-step process for mediating the dephosphorylation of MAP kinase by dual-specificity MAP kinase phosphatase.

Adapted from Ref. [197].

to MKP3 on the dephosphorylation of the bisphosphorylated ERK2 peptide (DHTGFL-pT-E-pY-VATR). In the absence of ERK2, MKP3 exhibits little phosphatase activity toward this peptide. However, the addition of ERK2 leads to a dramatic stimulation of phosphatase activity. Although p38α binds to MKP3, the kinase fails to induce p-nitrophenylphosphate phosphatase activity. This result indicates that the binding of the kinase to the phosphatase is necessary but not sufficient to induce phosphatase catalytic activity.

MKP3 is an ERK2 specific phosphatase that is one of the enzymes that terminate ERK2 signaling. Zhou et al. examined the interaction of a catalytically inactive rat MKP3 Cys293Ser mutant with rat ERK2 [206]. The use of the mutant lacking the catalytic cysteine maintains the initial concentration of bisphosphorylated ERK2 during the course of the experiment. The affinity of the mutant for unphosphorylated ERK2 was similar to that of the wild-type phosphatase (both K_D values ≈0.15 μM). The K_D of the mutant for bisphosphorylated ERK2 was 31 nM showing that the phosphatase has greater affinity for the substrate than for the product of the reaction. These workers showed that deletion of the MKP3 KIM abolishes the formation of an MKP3/ERK2 complex.

Using X-ray crystallography, Liu et al. observed that the rat ERK2 binding site for a rat MKP3 KIM peptide corresponds to the D-site recruitment site that consists of a highly acidic CD patch (Glu81, Asp162, Asp318, and Asp321 in L_{16}, L_5, β8, and αE) and a hydrophobic groove (Thr110, Leu112, Leu115, Leu121, Phe129, and Leu157 in αE, β7–β8, and αD) (human residue numbers) [207]. The ERK2 CD domain engages the basic Arg64 and Arg65 residues in the KIM sequence, and the ERK2 hydrophobic patch binds to the Leu71-Pro72-Val73 residues in the KIM sequence.

Zhou et al. studied the binding and interaction of rat MKP3 to rat ERK2 using hydrogen/deuterium exchange mass spectrometry [206]. Their C-terminally His_6-tagged MKP3 (Cys293Ser) mutant contains 387 amino acids with 365 exchangeable amide hydrogens, and the N-terminally His_6-tagged ERK2/pYpT has 364 residues with 343 exchangeable amide hydrogens. These differences are related to the number of proline residues, which lack exchangeable backbone hydrogens. After incubation of MKP3, ERK2, or both with D_2O, they subjected their samples to pepsin digestion and analyzed the resulting peptides by mass spectrometry. Peptide segments showing altered hydrogen/deuterium exchange after complex formation could be involved in either ERK2-MKP3 binding or binding-induced backbone conformational changes.

The results of Zhou et al. with full-length MKP3 using hydrogen/deuterium exchange [206] were consistent with the X-ray studies of Liu et al. [207]. Zhou et al. reported that the ERK2 CD domain binds to the β4–α4 MKP3 loop [207]. Although the CD domain of rat ERK2 participates in binding to MKP3, it does not play a role in the activation of latent MKP3 phosphatase activity. This activation involves the ^{364}Phe-Thr-Ala-Pro^{367} sequence of MKP3, which is also essential for binding to ERK2. MKP3 activation appears to require interaction with Arg191, Trp192, Glu220, Arg225, Lys231, and His232 (human residue numbers) in the ERK2 protein substrate binding site.

7.3. Protein-tyrosine and protein-serine/threonine MAP kinase phosphatases

PTP-SL is another regulator of ERK1/2 MAP kinase activity [208]. PTP-SL is a STEP-like protein-tyrosine phosphatase, where STEP is the striatal-enriched tyrosine phosphatase. There is an unusual reciprocal interaction within the complex formed between PTP-SL and ERK2. Pulido et al. reported that ERK2 catalyzes the phosphorylation of Thr 360 of rat PTP-SL, and PTP-SL catalyzes the dephosphorylation of a regulatory phosphotyrosine (pTyr 185) in the activation loop of rat ERK2 [209]. The rat PTP-SL Thr360 residue number represents the UniProtKB correction (Uniprot ID: G3V6L5) of Thr253 reported by Hendricks et al. [210]. Dephosphorylation of pTyr185 inactivates ERK2 and leads to its retention in the cytoplasm [211]. The association of PTP-SL with ERK2, the phosphorylation of Thr360 on PTP-SL, and the dephosphorylation of pTyr 185 on ERK2 are strictly dependent on a novel, 16-amino-acid-long, PTP-SL KIM [209]. Co-expression of ERK2 with catalytically active PTP-SL in COS-7 cells impaired the EGF-induced activation of ERK2, whereas a PTP-SL mutant, lacking phosphatase activity, increased the ERK2 response to EGF. These effects were dependent on the PTP-SL kinase interaction motif. Furthermore, ERK1/2 activity was down regulated in 3T3 cells stably expressing PTP-SL. These experiments demonstrate the existence of a conserved ERK1/2 interaction motif within the cytoplasmic non-catalytic domain of PTP-SL, which is required for the regulation of ERK1/2 activity and for phosphorylation of the protein-tyrosine phosphatases by these kinases. These findings suggest that PTP-SL acts as a physiological regulator of the ERK1/2 signaling pathway.

The kinase interaction motif, which is situated between residues 331 and 346 of rat PTP-SL [210,211, UniProtKB ID: G3V6L5] and is highly conserved among all members of the PTP-SL sub-family [208], binds to the D-site recruitment site of ERK2. This motif promotes binding of all members of the KIM-containing protein-tyrosine phosphatases (PTP-SL, PTPBR7, STEP, and HePTP). The sequence carboxyterminal to KIM, termed the kinase-specificity sequence (KIS), provides binding specificity to ERK2 and p38. Thus, PTP-SL preferentially binds ERK2 whereas STEP and HePTP (hematopoietic protein-tyrosine phosphatase) selectively bind p38α [208].

Because protein-tyrosine phosphatases reverse the actions of protein-tyrosine kinases and lead to the inhibition of the ERK1/2 signaling cascade, these enzymes may function as tumor suppressors [212]. Down regulation of MKP3 (DUSP6) is associated with ovarian cancer. Large scale genetic analyses of human tumors suggest that protein-tyrosine phosphatases may play a role as either tumor suppressors or oncoproteins [212]. Counter-intuitively, over expression or amplification of the non-transmembrane protein-tyrosine phosphatase N1 (PTPN1) is associated with breast, ovarian, gastric, pancreatic, and prostate cancer [212,213]. This phosphatase targets the Ras-MAP kinase and AKT pathways. Over expression of PTP4A, which targets ERK1/2 signaling, is implicated in the pathogenesis of pancreatic and colorectal cancer. This phosphatase enhances cell proliferation and cell motility, and it promotes invasive activity and metastasis. See Ref. [212] for a comprehensive summary of the relationship of protein-tyrosine phosphatases and cancer.

Anderson et al. demonstrated that protein phosphatase 2A, a serine/threonine phosphatase, inactivates bisphosphorylated ERK2 in vitro [74]. Because many proteins in the Ras-Raf-MEK-ERK cascade contain phosphoserine and phosphothreonine, protein phosphatase 2A has the potential to regulate several steps in the overall pathway. Alissi et al. found that the dephosphorylation of rat ERK2 Thr183 by protein phosphatase 2A is the rate-limiting step for ERK inactivation in EGF-stimulated rat PC12 cells [214]. Following EGF stimulation, ERK activation is maximal at 5 min and returns to basal levels in 15–30 min. Following NGF stimulation of PC12 cells, ERK activation is rapid but is sustained for hours. NGF, but not EGF, leads to neurite outgrowth. This difference in the duration of signaling may be the key to the different outcomes following stimulation of the Raf-MEK-ERK cascade [215]. Regulation by protein phosphatase 2A is not cell-type specific; Letourneux et al. reported that phosphatase 2A is the major serine/threonine phosphatase that mediates the rapid inactivation of ERK in Chinese hamster ovary cells [216].

8. ERK1/2 small-molecule inhibitors

8.1. General properties of clinical protein kinase inhibitors

There are currently 15 low molecular weight protein kinase inhibitors that have been approved for use by the FDA in the United States (Table 8). There are also four large molecule monoclonal antibodies that target protein-tyrosine kinases, which include bevacizumab (binds VEGF, a VEGFR family protein-tyrosine kinase activating ligand), cetuximab (targets EGFR, or HER1), panitumumab (targets EGFR, or HER1), and trastuzumab (targets ErbB2, or HER2). See http://www.accessdata.fda.gov/scripts/cder/drugsatfda/ for a list of U.S. FDA approved drugs with supporting documentation including structural formulas, mechanisms of action, and disease indications. See Refs. [217–219] for reviews of the EGF and VEGF families of receptor protein-tyrosine kinases.

The gatekeeper residue is an important landmark for characterizing protein kinase inhibitors owing to its ability to influence drug binding. This residue lies between the ATP adenine binding-site and an adjacent hydrophobic pocket (hydrophobic pocket II [67], or the back pocket [220]) (Fig. 12). The gatekeeper is the residue that occurs immediately before the hinge region, and it lies next to the 6-amino group of adenine when ATP is bound. The ATP binding site is equivalent to the front pocket of the kinase [67]. Many protein kinase inhibitors form (i) 1–3 hydrogen bonds with the hinge region, (ii) hydrophobic bonds with the residues that make up the adenine binding-site, and (iii) hydrophobic bonds with the hydrophobic pockets [220]. The gatekeeper residues for human ERK1/2 are Gln122/105. When the gatekeeper residue is small (Gly, Ala, Ser, Cys, Thr, or Val), hydrophobic pocket II tends to be large and readily accessible [220]. When the gatekeeper residue is of medium size (Ile, Leu, Met, Gln) as in the case of ERK1/2, hydrophobic pocket II is medium-sized and accessible. When the gatekeeper residue is large (Phe, Tyr), the pocket is small and marginally accessible.

Dar and Shokat reviewed protein kinase inhibitors and divided them into three classes: Types I, II, and III [221]. Type I inhibitors bind reversibly to the ATP-binding site of protein kinases and exhibit steady-state enzyme competitive inhibition with respect to ATP; when ATP is bound to the enzyme Type I inhibitors cannot bind. They do not require a specific orientation of the αC-helix or the DFG Mg^{2+}-binding loop for their effectiveness (Table 9).

Type II inhibitors occupy the adenine site and a region adjacent to it called the back pocket [220] or hydrophobic pocket II [67]. Unlike type I inhibitors, Type II inhibitors bind to or induce a DFG-aspartate out configuration. The aspartate or D residue of the DFG conserved sequence is rotated nearly180° relative to the active state DFG-aspartate in conformation. This rotation then leaves a hydrophobic site (hydrophobic pocket III), which is available for drug binding. The peptide N-H group of the DFG-aspartate (Asp167 in human ERK2) is available to form a hydrogen bond with a ligand that occupies hydrophobic pocket III. The DFG-aspartate out configuration is illustrated in Fig. 12. The conserved β6-strand Glu71 of human ERK2 is also able to form a hydrogen bond with a donor residue within hydrophobic pocket III. Zuccotto et al. introduced the concept of Type I ½ inhibitors, which is a hybrid of the Type I and II classes [220] (Table 9). These inhibitors bind to the hinge region, the ATP-binding site, and the back pocket, and the DFG-aspartate in conformation (not the DFG-aspartate out conformation of Type II inhibitors). However, enzymes that are constitutively active cannot readily assume the DFG-Asp out conformation.

Because Type I ½ and II inhibitors occupy part of the ATP site, ATP prevents their binding, and these inhibitors exhibit steady-state competitive inhibition with respect to ATP. Type I, I½, and II inhibitors typically form 1–3 hydrogen bonds with the hinge region. ATP typically forms hydrogen bonds with the first and the

Table 8
Classes of protein kinase inhibitors.

	Type I	Type I 1/2	Type II	Type III	Type IV	Type V
Description	Reversible	Reversible	Reversible	Allosteric	Irreversible	Blocks protein–protein interaction
Requites DFG-Asp out conformation	No	No; only in	Yes	No	No	No
Applies to every kinase	Yes	No; small/medium gatekeeper preferred	No; must be able to assume DFG-Asp out conformation	Yes	No; only those with appropriate target nucleophiles	No; requires docking-site recruitment sites
Binding region	ATP site	ATP and hydrophobic sites	ATP and hydrophobic sites	Allosteric sites only	ATP, hydrophobic, and/or allosteric sites	Protein–protein interaction sites
Hinge H-bonding	Yes	Yes	Yes	No	Possible	No
ATP competitive	Yes	Yes	Yes	No	Possible	No
Selectivity	Usually low but very selective inhibitors identified	Targeting the hydrophobic site may increase selectivity	Targeting the hydrophobic site may increase selectivity	Targeting the allosteric site may increase selectivity	Targeting the hydrophobic and allosteric sites may increase selectivity	Potentially high
Examples	Sunitinib, erlotinib, getifinib, dasatinib, lapatinib	AP23464 (a Src inhibitor)	Imatinib, sorafinib, nilotinib	PD318088 (a MEK1 inhibitor)	FR148083 (an ERK2 inhibitor)	3-(2-aminoethyl)-5-(4-ethoxybenzylidene)-1,3-thiazolidine-2,4-dione (an ERK2 inhibitor)

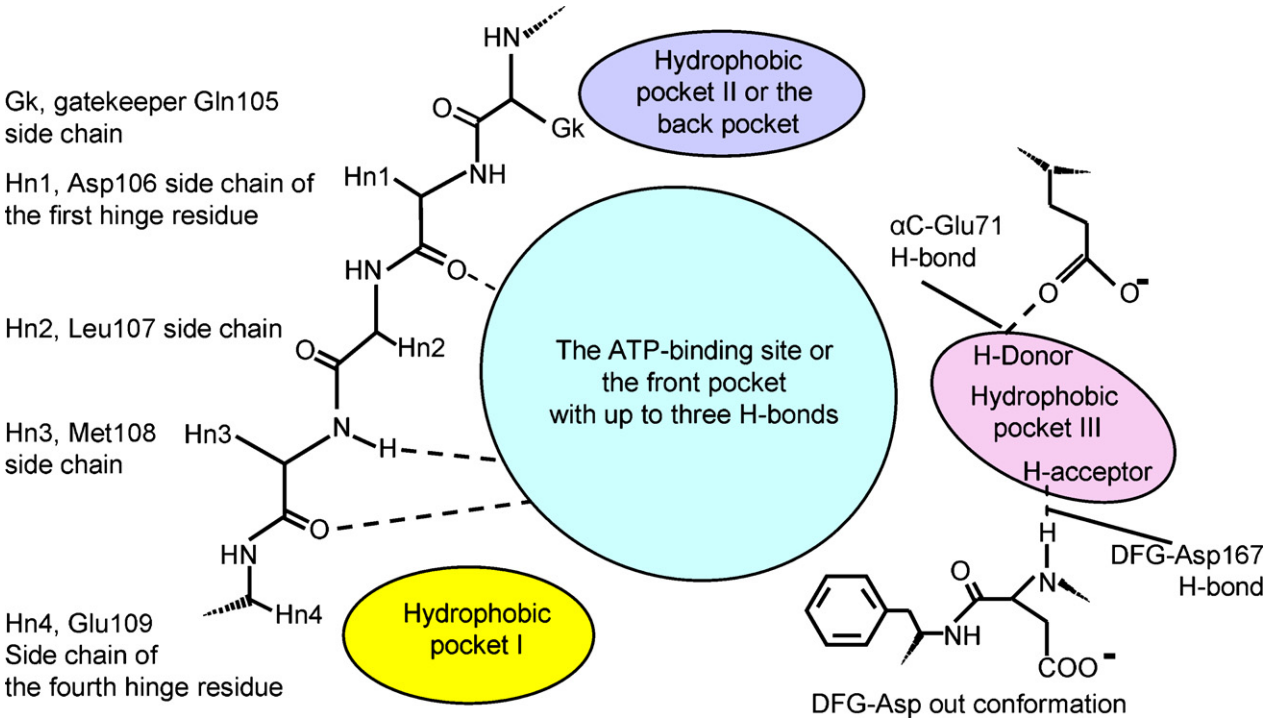

Fig. 12. Potential interaction sites of human ERK2 with inhibitory drugs. In the "DFG-aspartate in" structure, the aromatic ring of phenylalanine (F) occupies hydrophobic pocket III. Hydrophobic pocket III becomes a potential interaction site only after the enzyme assumes the DFG-aspartate out conformation. Dashed lines indicate hydrogen bonds. Positions of hydrogen-bond donors and acceptors in hydrophobic pocket III are depicted.

third residues of the hinge region. The 6-amino group functions as a hydrogen bond donor with the first hinge backbone residue, and the N-1 nitrogen functions as a hydrogen bond acceptor with the third hinge backbone residue.

Historically, allosteric sites refer to other sites, i.e., sites other than the active site [222]. In the case of protein kinases, allosteric sites refer to locations outside of the ATP-binding region. Type III inhibitors are those that occupy an allosteric site. These inhibitors block kinase activity while having no effect on ATP binding, and they do not hydrogen bond with the protein kinase hinge region. ATP is unable to displace allosteric inhibitors, and such inhibitors are expected to demonstrate steady-state non-competitive inhibition. In principle, there are several potential allosteric sites in each protein kinase.

Table 9
FDA approved small molecule protein kinase inhibitors[a]

Generic name	Formula	MW	Brand name	Number	Year approved	Known targets	Disease
Axitinib	$C_{22}H_{18}N_4OS$	386	Inlyta	AG-013736	2012	VEGFRs	RCC
Crizotinib	$C_{21}H_{22}Cl_2FN_5O$	450	Xalkori	PF-0234106	2011	ALK, HGFR or Met	ALK positive NSCLC
Dasatinib	$C_{22}H_{26}ClN_7O_2S$	488	Sprycel	BMS-35482	2006	BCR-Abl, Src, Lck, Yes, Fyn, Kit, EphA2, and PDGFR-β	CML
Erlotinib	$C_{22}H_{23}N_3O_4$	393	Tarceva	OSI-774-01	2004	EGFR	NSCLC and pancreatic cancer
Everolimus	$C_{53}H_{83}NO_{14}$	958	Afinitor, Zortress	RAD001	2009	mTOR	Progressive neuroendocrine tumors of pancreatic origin, RCC, subependymal giant cell astrocytoma
Gefitinib	$C_{22}H_{24}ClFN_4O_3$	447	Iressa	ZD1839	2003	EGFR	NSCLC
Imatinib	$C_{29}H_{31}N_7O$	493	Gleevec/Glivec	STI571	2003	BCR-Abl, c-Kit, PDGFR	CML, acute lymphoblastic leukemia, aggressive systemic mastocytosis, GIST
Lapatinib	$C_{29}H_{26}ClFN_4O_4S$	580	Tykerb	GW572016	2007	EGFR, ErbB2	Breast cancer
Nilotinib	$C_{28}H_{22}F_3N_7O$	529	Tasigna	AMN107	2007	BCR-Abl	CML
Pazopanib	$C_{21}H_{23}N_7O_2S$	437	Votrient	GW786034	2009	VEGFRs, PDGFRs, FGFR, Kit, Lck, c-Fms, Itk	RCC
Ruxolitinib	$C_{17}H_{18}N_6$	306	Jakafi	INCB018424	2011	JAK-1/2	Myelofibrosis
Sorafenib	$C_{21}H_{16}ClF_3N_4O_3$	464	Nexavar	BAY 43-9006	2005	C-Raf, B-Raf, mutant B-Raf, Kit, Flt-3, Ret, VEGFRs, and PDGFR-β	Hepatocellular carcinoma and RCC
Sunitinib	$C_{22}H_{27}FN_4O_2$	398	Sutent	SU11248	2006	PDGFRs, VEGFRs, Kit, Flt-3, CSF-1R, and RET	RCC, GIST, pancreatic neuroendocrine tumors
Vandetanib	$C_{22}H_{24}BrFN_4O_2$	474	Caprelsa	ZD6474	2011	EGFRs, VEGFRs, RET, Brk, Tie-2, EphRs, Src family	Medullary thyroid cancer
Vemurafenib	$C_{23}H_{18}ClF_2N_3O_3S$	489	Zelboraf	PLX4032	2011	B-Raf (V600E)	Melanoma with the $BRAF^{V600E}$ mutation

[a] CML, chronic myeloid leukemia; GIST, gastrointestinal stromal tumor; Itk, interleukin-2 receptor inducible T-cell kinase; JAK, janus associated kinase; MW, molecular weight; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma.

Type IV, or irreversible protein kinase inhibitors, characteristically form covalent bonds with their target enzyme (Table 9) [223]. Owing to safety and toxicity concerns, covalent irreversible drugs are disfavored as a drug class [224]. However, irreversible enzyme inhibitors have been in the medical therapeutic armamentarium for more than a century. Aspirin is the most widely used medication in the world with an estimated 80 billion tablets being consumed annually in the United States alone. Roth and coworkers discovered that aspirin exerts its therapeutic effect by covalently modifying Ser530 of cyclooxygenase 1 by acetylation [225,226]. Selegiline and rasagiline, which are FDA approved for the treatment of Parkinson disease, are additional examples of irreversible enzyme inhibitors [227]. These drugs, which are used as monotherapy in early Parkinson disease or as an adjunct therapy in more advanced cases, inhibit type B monoamine oxidase. Covalent proton pump inhibitors such as omeprazole, esomeprazole and lansoprazole have been shown to be safe and effective for the millions of people that receive them [224]. Neratinib and afatinib, which possess α, β-unsaturated carbonyl groups (see Section 8.2.2), are irreversible covalent inhibitors of the EGFR family of receptor protein-tyrosine kinases that are in stage III clinical trials [224].

As noted in Section 4, ERK1/2 use their D-site and F-site recruitment sites to interact with many of their substrates. This property has fostered the notion that blocking protein–protein interaction (P–PI), or ERK1/2–protein interaction, represents yet another mechanism for inhibiting the ERK1/2 MAP kinase cascade. Drugs designed to inhibit protein kinase signaling by blocking kinase–protein interaction are herein called Type V inhibitors (Table 9). This class of drugs might be grouped with allosteric inhibitors because they involve interactions with locations other than the active site, but the strategies used to identify and study them are so different from the study of allosteric inhibitors that they are classified separately. The mechanism of action of monoclonal antibodies represents a special case of P–PI inhibitors [228]. For example, bevacizumab binds to and blocks the action of VEGF; cetuximab, panitumumab, and trastuzumab are directed against the members of the EGFR family. Peptides corresponding to ligands that bind to P–PI sites represent important experimental tools, but they are limited in their potential therapeutic use owing to metabolic degradation and impaired cellular uptake. The large sizes of protein–protein interaction sites when compared with enzyme–substrate binding sites (1500–3000 versus 300–1000 Å^2) have raised doubt on the possibility of the development of potent small molecules that bind to P–PI sites and that meet the requirements required for therapeutic use such as bioavailability [228]. Owing to the importance of the ERK1/2 MAP kinase cascade in the pathogenesis of numerous maladies, steps have been taken to develop Type V inhibitors as noted in Section 8.2.3.

8.2. Small-molecule inhibitors of ERK1/2

8.2.1. ATP-competitive inhibitors

Ohori et al. identified FR180204 (Fig. 13), which is a pyrazolopyridazine derivative, from a chemical library that inhibits the phosphorylation of myelin basic protein catalyzed by human ERK1 (IC_{50} = 0.51 μM) and ERK2 (IC_{50} = 0.33 μM) [229]. This drug is a steady-state competitive inhibitor with respect to ATP, and the compound blocks the activation of the AP1 transcription factor in TGF-β-stimulated mink lung epithelial Mv1Lu cells. The authors solved the crystal structure of FR180204 bound to human ERK2 and confirmed that the drug occupies the ATP-binding site (PDB ID: 1TVO). This small molecule makes three hydrogen bonds with residues in the hinge region: Gln105 (the gatekeeper), Asp106, and Met108. Aronov et al. used X-ray crystallographic studies and structure-guided optimization to develop Type I pyrazolylpyrrole- [230] and pyrimidylpyrrole-based [231] ERK2 inhibitors with nanomolar affinities.

8.2.2. Irreversible inhibitors

Ohori et al. identified FR148083 (Fig. 13), which is a benzoxacyclotetradecine derivative, as a human ERK2 inhibitor (IC_{50} = 80 nM) [232]. The drug blocks TGF-β-induced AP1-dependent luciferase expression in Mv1Lu cells (IC_{50} = 50 nM). X-ray crystallography of the human ERK2–FR148083 complex reveals that the inhibitor binds to the ATP-binding site by making a hydrogen bond with Met108 in the hinge region (PDB ID: 2E14). It also forms a hydrogen

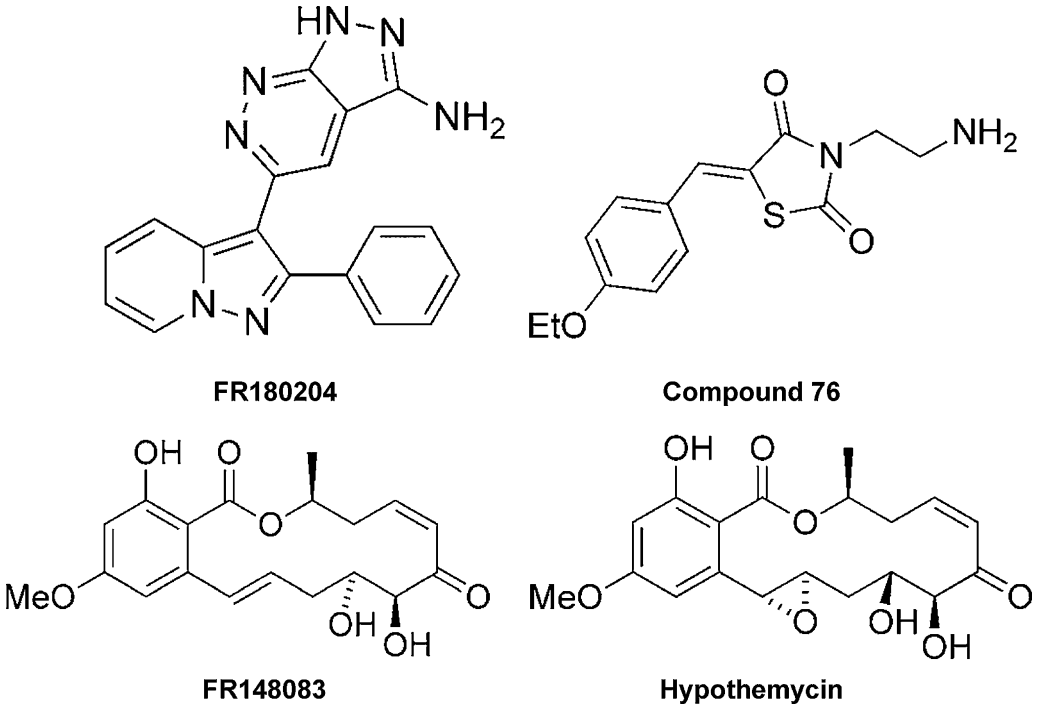

Fig. 13. Structures of selected ERK1/2 inhibitors.

bond with the carbonyl group of Ser153 and with the side-chains of Lys114 and Asn154. FR148083 forms hydrophobic contacts with Ile31, Val39, Ala52, and Leu156. Note that the last three of these residues form part of the catalytic spine of human ERK2 (Table 3). Moreover, a covalent bond forms between the drug and the thiol group of C166. JNK1/2 and the four p38 isoforms are not inhibited by FR140083 owing to a lack of a cysteine residue in the ATP-binding site. In contrast, MEK1 and MKK7 are inhibited by the drug owing to cysteine residues that correspond to C166 in ERK2. It is likely that non-covalent interactions position the small molecule in a productive orientation that allows the covalent modification to proceed as the enzyme attacks the electrophilic portion of the drug to form a Michael adduct where a Michael reaction represents the addition of a nucleophile to an α, β-unsaturated carbonyl compound [63].

Rastelli et al. characterized the inhibition of rat ERK2 with hypothemycin (Fig. 13) [233]. This compound first binds reversibly to the ATP-binding site with the earmarks of a classical Type I inhibitor. The compound contains an alkene moiety that undergoes nucleophilic attack by the thiol group of rat C164 (human C166) to form a covalent Michael adduct. These investigators determined the crystallographic structure of the ERK2-hypothemycin complex (PDB ID: 3C9W) and found that the drug forms two hydrogen bonds with the hinge region (the rat Asp104 carbonyl group and the Met106 N-H backbone). Moreover, hypothemycin interacts with rat A50, L154, L153, and M106 of the catalytic spine (Table 3), providing additional evidence that the drug functions as an ATP mimetic. Leproult et al. performed a systematic analysis of cysteine residues present in the nucleotide binding site of protein kinases, which could be targeted for irreversible inhibition, taking into consideration the different kinase conformations [234]. Their review represents an analysis of 1043 crystal structures of 205 human protein kinases.

8.2.3. ERK1/2 D-site recruitment site blockers

Hancock et al. used computer-aided drug design (CADD) algorithms to identify potential D-docking site inhibitors [235]. They identified compound 76, or 3-(2-aminoethyl)-5-(4-ethoxybenzylidene)-1,3-thiazolidine-2,4-dione (Fig. 13), which produced greater than 50% inhibition of RSK1 activation in EGF-stimulated human cervical carcinoma (HeLa) cells at a 100 μM concentration. Based upon the fluorescence quenching titration of rat ERK2, this drug binds with a K_D of 5 μM, but it fails to significantly inhibit enzyme-catalyzed phosphorylation of myelin basic protein with concentrations up to 150 μM. The latter finding is consistent with the notion that its inhibition of RSK1 phosphorylation is unrelated to its kinase activity per se, but rather to its inhibition of ERK2–protein interactions. However, note that clinically effective protein kinase inhibitors generally have affinities in the low nanomolar range.

Boston et al., from the same laboratory, demonstrated that treatment with compound 76 (100 μM) for seven days inhibited HeLa (cervical), SUM159 (breast), SKMEL-28 (skin) and Panc-1 (pancreatic) cancer cell proliferation more than 75% [236]. These investigators reported that compound 76 (50 μM) inhibited ERK-catalyzed caspase-9 (a pro-apoptotic protease) phosphorylation in HeLa cells and in vitro. Boston et al. reported that somewhat more potent congeners of compound 76 inhibited ERK-mediated phosphorylation of RSK1 and Bad [236]. RSK1 is a MAPKAP kinase downstream from ERK1/2, and Bad is a pro-apoptotic protein that is inhibited following RSK1-catalyzed phosphorylation. Both caspase-9 and RSK1 contain D-domains that are presumed to form contacts with the CD domain of ERK proteins, the region that is targeted by the test compounds.

The development of Type V protein kinase inhibitors is at an early developmental stage. P–PI inhibitors of non-protein kinases are under investigation [228]. For example, ABT-263 (avitoclax), which is in early clinical trials, is a low molecular weight BH3 α-helical proteomimetic that antagonizes pro-apoptotic Bcl-XL [237,238].

As noted in Section 4.1, the DRS of ERK1/2 is accessible in both active and inactive conformations of ERK2. In contrast, the FRS is accessible in only active bisphosphorylated ERK1/2. This observation implies that it may be more difficult to identify FRS protein–protein interaction inhibitors. However, since activated ERK1/2 is implicated in driving proliferation in cancer cells, blocking the ERK1/2 FRS may represent a more effective cancer therapeutic target.

9. Epilog

9.1. Phosphorylated proteins and protein kinases

Casein and phosvitin (vitellinic acid, vitellin) are two of the earliest known phosphoproteins [239]. Casein occurs in milk and contains about 3% phosphorus by weight [240]. Phosvitin occurs in egg yolk and contains about 10% phosphorus by weight, which is among the most highly phosphorylated proteins in nature (about one phosphate group for every two amino acid residues). Following acid hydrolysis, Lipmann and Levine identified serine phosphate as the phosphorylated component in phosvitin in 1932, which is thus the first identified phosphorylated protein residue [240]. Threonine was unknown at the time; it was discovered by W.C. Rose and two of his graduate students in 1935 [241]. de Verdier isolated and characterized phosphothreonine from casein in 1953 [242].

Burnett and Kennedy, in 1954, were the first to characterize protein kinase activity [243]. They used an extract of a mitochondrial fraction isolated from rat liver by differential centrifugation as a source of their kinase activity. They chose casein as a test substrate because it was a known phosphoprotein, which however the liver lacks. They generated [^{32} P]-ATP in their assays by including $^{32}P_i$ and a freshly isolated and oxidative-phosphorylation active mitochondrial fraction. Under aerobic conditions mitochondria convert ADP and $^{32}P_i$ into [γ-^{32}P]-ATP, which is translocated from the mitochondria where it can participate in the ATP-dependent protein kinase reaction. They included the mitochondrial [γ-^{32}P]-ATP-generating system, casein, and the protein kinase activity derived from mitochondrial extracts for their assays. They carried out their reaction in a final volume of 3.0 mL containing 5 mM $MgC1_2$, 33 mM sodium glutamate (a substrate for oxidative phosphorylation and ATP production), 33 mM of Tris buffer (pH 7.4), 1 μC $^{32}P_i$, freshly isolated rat liver mitochondria (containing about 20 mg of protein), 50 mg of rat liver protein (the source of protein kinase activity), and 10 mg casein. The reaction proceeded for 60 min at 30 °C in ambient air. They isolated and identified [^{32}P]-phosphoserine following acid hydrolysis of the casein product (2 N HCl for 20 h). More recently, radiometric protein kinase assays are performed in a volume from 20 to 50 μL with nanogram amounts of purified recombinant kinases and microgram amounts of kinase substrates along with commercially available reagents (e.g., [γ-^{32}P]-ATP) and shorter incubation times [80,99].

The enzyme that Burnett and Kennedy characterized was most likely casein kinase 1, casein kinase 2, or both. These proteins occur in the cytoplasm, and it is probable that their mitochondrial fraction contained cytoplasmic components as the source of their protein kinase activity. This was the only paper that these authors published on protein kinases leaving subsequent work to other investigators. Eugene P. Kennedy spent a short time in the laboratory of Fritz Lipmann, the discoverer of protein–phosphoserine, at the Massachusetts General Hospital as a post-doctoral associate [244]. Kennedy made seminal contributions to the understanding of complex lipid biosynthesis.

In 1979 Eckhart et al. identified phosphotyrosine in polyoma T antigen precipitates [245]. In 1980, Sefton and Hunter reported that the transforming gene product of Rous sarcoma virus (Src) catalyzes the phosphorylation of tyrosine, which represents the initial paper on a protein-tyrosine kinase [246]. The ratio of protein phosphoserine/phosphothreonine/phosphotyrosine in normal animal cells is about 3000/300/1 [246]. Despite the paucity of protein–phosphotyrosines, they play key roles in signal transduction as has been emphasized in this review. Protein-serine/threonine kinases typically catalyze the phosphorylation of various substrates, and this may be associated with amplification (one protein kinase molecule catalyzes the phosphorylation of many substrate molecules). In contrast, protein-tyrosine kinases per se are not usually associated with extensive amplification. They undergo autophosphorylation and catalyze the phosphorylation of a few exogenous substrate molecules. In growth-factor stimulated cells, the most abundant phosphotyrosines occur in the stimulated receptor itself. The resulting protein-tyrosine phosphates serve as docking sites for molecules that transmit downstream signals, which often include the activation of protein-serine/threonine kinases as described for the Ras-Raf-MEK-ERK cascade. Amplification occurs after protein-tyrosine phosphorylation and accounts in part for the high ratio of phosphoserine and phosphothreonine to phosphotyrosine in cells. As noted in Section 1, ERK1/2 activation requires its phosphorylation at both tyrosine and threonine residues. Dual specificity kinases were unknown at the time (≈1990), and it was hypothesized that this dual phosphorylation resulted from the collaborative interaction of two different protein kinases: a protein-threonine and a protein-tyrosine kinase [74]. Now we know that MEK1 or MEK2 are able to catalyze the phosphorylation of tyrosine and then threonine in ERK1/2.

9.2. Therapeutic Inhibitors of the Ras-Raf-MEK-ERK signaling cascade

9.2.1. Prenyltransferase inhibitors of Ras function

The Ras-Raf-MEK-ERK pathway has attracted considerable attention as a target for anti-cancer and other therapies. However, inhibition of Ras GTPase activity has proven intractable. However, H-Ras, K-Ras, and N-Ras undergo post-translational prenylation reactions that are required for their biological activity [247,248]. Prenylation involves the addition of the 15-carbon farnesyl group ($C_{15}H_{25}$) or the 20-carbon geranylgeranyl group ($C_{20}H_{33}$) to protein-cysteines near the carboxytermini of substrates [249]. The prenyl, or isoprenoid, lipid donors are the respective farnesyl diphosphate or geranylgeranyl diphosphate. The protein farnesyltransferase (FTase) and protein geranylgeranyltransferase (GGTase-I and II) reactions are illustrated by the following chemical equations:

$$\text{Farnesyl diphosphate} + \text{HS-protein} \rightarrow \text{farnesyl-}S\text{-protein} + PP_i$$

$$\text{Geranylgeranyl diphosphate} + \text{HS-protein} \rightarrow \text{geranylgeranyl-}S\text{-protein} + PP_i$$

The isoprenoid groups become linked to polypeptidic cysteines through thioether (C—S—C) bonds. The prenyltransferases are heterodimers consisting of α- and β-subunits. The α-subunits of FTase and GGTase-I are the same, and the β-subunits differ [249].

FTase and GGTase-I catalyze the prenylation of substrates with a carboxyterminal tetrapeptide sequence called a CaaX box, where C refers to cysteine, a refers to an aliphatic residue, and X typically refers to methionine, serine, alanine, or glutamine for FTase or to leucine for GGTase-I [249]. Following prenylation of physiological substrates, the terminal three residues (aaX) are removed by a CaaX endoprotease and the terminal cysteine is methyl esterified. GGTase-II, or Rab geranylgeranyltransferase, catalyzes the geranylgeranylation of Rab proteins that terminate in CC or CSC sequences. FTase and GGTase-I can catalyze the prenylation of tetrapeptides, polypeptides, and proteins containing the appropriate CaaX boxes.

The importance of the prenyltransferases is underscored by the nature of their substrates, including H-Ras, K-Ras, and N-Ras, many of which participate in signal transduction pathways related to cell growth and differentiation [249]. H-Ras, K-Ras, and N-Ras are farnesylated under physiological conditions. Following the inhibition of FTase in cells, K-Ras and N-Ras become geranylgeranylated as catalyzed by GGTase-I; they thereby become functional [247]. Since most human *RAS* mutations occur in *KRAS*, the use of farnesyltransferase inhibitors has not been as effective as initially expected owing to adventitious K-Ras geranylgeranylation. Four farnesyltransferase inhibitors have been evaluated in at least 75 clinical trials since 2000: tipifarnib, lonafarnib, BMS-214662 and L-778123 [247]. In human clinical trials, monotherapy with farnesyltransferase inhibitors shows limited anti-tumor activity in hematopoietic cancers, and generally no or very little activity in solid tumors.

When used in combination with other agents, farnesyltransferase inhibitors have fared better [247]. For example, phase I studies based on a combination of tipifarnib with gemcitabine and cisplatin have shown some promise in advanced solid tumors (33% complete response rate or 26% partial response rate). However, farnesyltransferase inhibitors are effective in inhibiting the growth of mutant *KRAS*-harboring tumors in athymic hairless nude mice and transgenic mouse models, suggesting that the inhibition of K-Ras prenylation is not required for farnesyltransferase inhibitor anti-tumor activity, and that, in these models, tumors are dependent upon farnesylated proteins other than K-Ras. Similar considerations apply to N-Ras, because it can also escape farnesyltransferase inhibitor-mediated blockade resulting from geranylgeranylation. H-Ras, conversely, is not alternatively geranylgeranylated in cells treated with farnesyltransferase inhibitors. Therefore, the inhibition of H-Ras farnesylation can still contribute to farnesyltransferase inhibitor anti-tumor activity in malignancies that are dependent upon mutant or wild-type H-Ras. Thus, it may be worthwhile to design clinical trials that involve farnesyltransferase inhibitors for people with *HRAS*-mutant bladder and other cancers.

The prenyltransferase inhibitors have been much more effective in tumorigenic animal models that in the clinic [247]. Berndt et al. reported that 587 human genes encode proteins that bear a carboxyterminal CaaX motif [247]. Hundreds of proteins are known to contain either a farnesyl group or a geranylgeranyl group. One major unknown is the identification of the prenyltransferase substrates that are crucial for the proliferation or survival of different cancer types. Although the prenyltransferase inhibitors were initially developed to block the farnesylation of the Ras oncoproteins, it is likely that other prenylated proteins participate in the pathogenesis and maintenance of the tumorigenic phenotype. Berndt et al. suggest that the identification of subsets of prenylated proteins that are affected by FTase or GGTase-I inhibitors will help in the design of better clinical trials [247].

9.2.2. Raf, MEK1/2, and ERK1/2 inhibitors

Davies et al. reported that B-Raf somatic missense mutations occur in 40–60% of malignant melanomas and at lower frequency in a wide range of human cancers including those of the thyroid, ovary, and colon [26]. A single substitution (Val600Glu) accounts for 80% of all mutations, which occur within the kinase domain. Raf kinases thus represent an attractive cancer drug target [250]. To better understand Raf kinase inhibition and the paradoxical increase in

ERK1/2 activation following treatment with Raf kinase inhibitors, a description of Raf kinase activation and regulation is necessary.

Rushworth et al. reported that the activation of wild-type Raf kinases requires the formation of Raf homodimers and heterodimers [251]. Following transfection of COS-1 cells with human HA–B-Raf and FLAG–C-Raf, they found that HA–B-Raf/FLAG–C-Raf heterodimers are more active that either homodimer. Following transfection with wild-type HA–B-Raf or an HA–B-Raf (T753A) mutant, Rushworth et al. observed that phosphorylation of HA–B-Raf is induced by EGF and blocked by the MEK inhibitor U0126. Importantly, no phosphorylation of the HA–B-Raf (T753A) mutant was detectable. To investigate the contribution of ERK1/2 phosphorylation of HA–B-Raf to dissociation of the HA–B-Raf/FLAG–C-Raf heterodimer, they transiently transfected COS-1 cells with either wild-type HA–B-Raf or HA–B-Raf (T753A) along with FLAG–C-Raf and monitored heterodimer formation in response to EGF over a time course of 0 to 120 min. In response to stimulation, wild-type HA–B-Raf bound to FLAG–C-Raf, but this association declined quickly and was back to basal levels between 30 and 60 min. FLAG–C-Raf binding to HA–B-Raf (T753A) was also stimulated by EGF. However, the heterodimer persisted much longer and was still present at 120 min. This finding indicates that ERK1/2 phosphorylation of HA–B-Raf at Thr753 exerts negative feedback on the persistence of the HA–B-Raf/FLAG–C-Raf heterodimer. Phosphorylation of HA–B-Raf at Thr753 as catalyzed by ERK1/2 destabilizes heterodimer formation with FLAG–C-Raf and decreases kinase activity; this ERK1/2-catalyzed phosphorylation turns off the response following Raf kinase activation.

Rajakulendran et al. demonstrated that residues occurring within the αC helix of C-Raf participate in dimer formation, and the αC helix is an important determinant of active and inactive enzyme conformations [252]. These investigators expressed wild-type and mutants of *Drosophila* Raf in *Drosophila* Schneider S2 cells, and they found that three mutations that occur within the side-to-side interface domain abolish stimulated downstream MEK phosphorylation while two mutations outside of the interface domain do not. These studies indicate that dimer formation is necessary for the expression of wild-type Raf kinase activity and that the wild-type Raf monomer is inactive. Activation occurs even when one monomer is kinase-dead indicating that dimerization is the key to activation and is not the result of kinase-mediated phosphorylation of one protomer by the other. Ras-GTP, which is localized chiefly on the inner leaflet of the plasma membrane, leads to the activation of the Raf kinase family (A-, B-, and C-Raf) in part by inducing the formation of side-to-side homodimers and heterodimers.

Heidorn et al. found that the B-Raf specific inhibitor 885-A produces an unexpected increase in ERK1/2 phosphorylation in four wild-type B-Raf human melanoma cell lines bearing activated *NRAS* mutations [253]. How can a Raf kinase inhibitor lead to the paradoxical increase in Raf kinase activity and ERK1/2 phosphorylation? Heidorn et al. showed that depletion of N-Ras and C-Raf by siRNAs blocks the ability of 885-A to activate ERK1/2, indicating that N-Ras and C-Raf participate in the paradoxical response [253]. They reported that 885-A binding to wild-type B-Raf or to a kinase-dead B-Raf mutant drives their binding to C-Raf in COS-7 cells. The experimental introduction of a gate-keeper mutation in B-Raf abolishes the ability of B-Raf inhibitors (i) to bind to B-Raf and (ii) to induce the binding of B-Raf to C-Raf. This result indicates that compounds that bind to the ATP-binding site induce Raf dimer formation and enzyme activation. Inhibitor binding to B-Raf in the presence of activated Ras induces B-Raf binding to C-Raf leading to C-Raf activation and increased downstream ERK1/2 phosphorylation and activation thereby providing a mechanism for the Raf inhibitor paradox.

Poulikakos et al. reported that six ATP-competitive Raf inhibitors induce ERK1/2 activation in cells with activated Ras and wild-type B-Raf, but they inhibit signaling in mutant B-Raf (V600E) cells [254]. The Raf inhibitor vemurafenib induces ERK1/2 signaling in human SKBR3 breast adenocarcinoma cells in which Ras activation is HER2-dependent. The HER2 (EGFR2) inhibitor lapatinib abolishes basal and vemurafenib-induced ERK1/2 signaling. These results imply that activated Ras is required for MEK-ERK activation by Raf inhibitors. Poulikakos et al. reported that the binding of an ATP-competitive inhibitor to C-Raf is sufficient for the induction of MEK-ERK signaling. These workers also showed that a mutation that blocks C-Raf homodimer formation prevents transactivation and downstream MEK-ERK activation.

The studies performed by Poulikakos et al. indicate that the binding of an inhibitor to the ATP-binding site of C-Raf leads to the formation of a C-Raf homodimer and C-Raf activation, which promotes downstream MEK-ERK activation [254]. Another possible, but not mutually exclusive, mechanism is that binding of an inhibitor to B-Raf leads to the formation of a B-Raf/C-Raf heterodimer and C-Raf activation. The experiments of Hatzivassiliou et al. suggest that A-Raf heterodimers may also participate in the paradoxical response [255].

The inhibition of Raf kinases as a therapeutic modality has uncovered the paradoxical activation of ERK1/2 signaling by Raf inhibitors in *BRAF*-wild-type, *RAS* mutant cancer cells [33,253–255]. In such cells, the persistently GTP-bound Ras associates with B-Raf/C-Raf heterodimers. The binding of a B-Raf inhibitor (including vemurafenib) to wild-type B-Raf inhibits it, but causes the transactivation of C-Raf leading to MEK1/2 and ERK1/2 activation rather than inhibition. The use of MEK1/2 inhibitors represents one possible strategy to prevent the paradoxical activation of the ERK1/2 cascade. To summarize, the treatment of tumors with mutant (and perhaps wild-type) *RAS* and wild-type Raf kinases with ATP-mimetic Raf kinase inhibitors is ineffective owing to the paradoxical activation of the ERK1/2 signaling cascade. What about tumors that contain mutant B-Raf (V600E)?

As noted above, wild-type Raf family kinases are active only as homo- or heterodimers [252,256]. In contrast, monomeric B-Raf (V600E) is active [257]. This mutant contains an acidic negatively charged residue in its activation loop adjacent to Thr599 and near Ser602, which are the B-Raf physiological activation phosphorylation sites. Vemurafenib (PLX4032), which has higher affinity for the oncogenic B-Raf (V600E) than wild-type B-Raf (31 nM versus 100 nM) [258] was approved by the U.S. FDA for the treatment of melanomas harboring the $BRAF^{V600E}$ mutation (Table 9). Although the initial response rate is about 50% with significant survival benefit, tumor resistance usually occurs within 8–12 months after initial treatment [259].

Multiple mechanisms of resistance to vemurafenib have been described. These include mutational activation of *NRAS* and over expression of PDGFRβ or insulin-like growth factor receptor leading to Ras activation [260,261]. Ras activation in turn leads B-Raf (V600E)/C-Raf- or C-Raf/C-Raf-dependent activation of MEK1/2-ERK1/2 signaling (Fig. 14). Johannessen et al. reported that COT (MAP3K8), which is upstream from MEK1/2, is amplified in some (i) $BRAF^{V600E}$ cell lines and (ii) two of three human clinical samples obtained from relapsing patients following vemurafenib treatment [262]. COT signaling bypasses the Raf kinase family (Fig. 14). Furthermore, Shi et al. reported that vemurafenib-resistance in four of twenty human melanomas is due to the over expression of B-Raf (V600E) [263].

Poulikakos et al. have uncovered another mechanism for vemurafenib resistance that is mediated by the dimerization of aberrantly spliced B-Raf (V600E) (Fig. 14(D)) [257]. These investigators exposed melanoma cells carrying B-Raf (V600E) mutants to vemurafenib and derived five drug-resistant clones. Three of these lines expressed a truncated form (molecular weight of 61 kDa) of B-Raf (p61B-Raf (V600E)) that results from the aberrant splicing

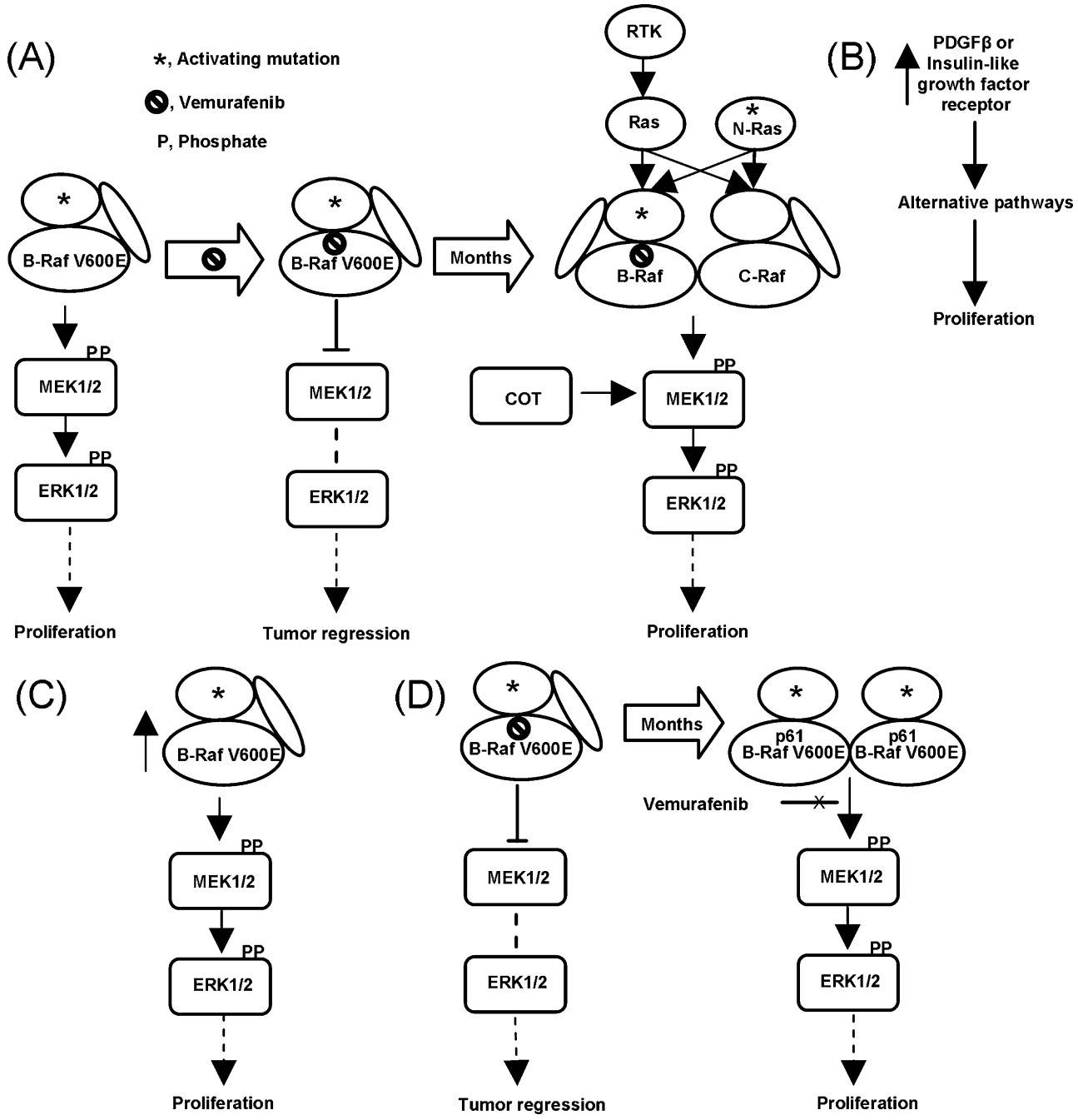

Fig. 14. Mechanisms of B-Raf inhibitor resistance in malignant melanomas. (A) After inhibiting tumor proliferation for months, some tumors over express COT which leads to the activation of MEK1/2 bypassing B-Raf (V600E). Alternatively activated *NRAS* mutations drive tumor growth by activating B-Raf/C-Raf heterodimers or C-Raf homodimers. (B) Over expression of PDGFRβ or insulin-like growth factor receptor leads to the activation of pathways such as PI3 kinase/AKT that drive tumor growth. (C) Over expression of B-Raf (V600E) leads to vemurafenib resistance. (D) Alternative splicing of B-Raf (V600E) hnRNA leads to the formation of p61 B-Raf (V600E), which forms dimers that are not susceptible to the action of vemurafenib. RTK, receptor protein-tyrosine kinase.

of B-Raf hnRNA. The authors note that the sequences missing in the truncated p61B-Raf (V600E) encompass a region that normally suppresses B-Raf dimerization, and they showed that the truncated mutant exhibits increased dimerization. They prepared dimerization-deficient mutants of p61B-Raf (V600E) and made two surprising observations. As noted above, monomeric full-length and truncated B-Raf are as active as their dimeric versions. Secondly, monomeric p61B-Raf (V600E) was inhibited by vemurafenib whereas dimeric p61B-Raf (V600E) was not. These workers reported that about one third (6 of 19) of clinically obtained drug-resistant samples expressed a truncated form of B-Raf (V600E), which accounts for the observed drug resistance in these six cases.

The elucidation of the mechanisms of B-Raf inhibitor drug resistance is in its early stages. Only a few dozen clinical drug resistant samples have been studied. Although it is too early to make definitive conclusions, it appears that resistance is unrelated to the generation of B-Raf mutants with gatekeeper mutations. This is in contrast to the resistance of BCR-Abl protein kinase in chronic myelogenous leukemia that is due to gatekeeper mutations that prevent drug binding [264]. As noted by Winer et al. “Biologically, the cancer cell is notoriously wily; each time we throw an obstacle

in its path, it finds an alternate route that must then be blocked" [265].

That chemotherapy can both cure and cause cancer has long been known to contribute to the development of secondary cancers that can arise a decade or more after completion of successful therapy. Thus, the risk of developing a second malignancy is estimated to range from 8% to 12% by 20 years after the diagnosis and treatment of a first cancer [266,267]. Leukemia is the most frequent secondary neoplasm [266,268]. Such malignancies are secondary to the DNA damaging and carcinogenic properties of standard cytotoxic therapies. Targeted cancer therapies also contribute to the development of secondary cancers. A side effect of vemurafenib treatment is the rapid appearance (within a few months) of well-differentiated squamous cell skin carcinomas and keratoacanthomas in 15–30% of melanoma patients [269]. These tumors are easily identified, simple to excise, and are not metastatic. However, the possibility that non-epidermal tumors may result from vemurafenib treatment is of concern. Su et al. analyzed 35 cutaneous tumors from a total of 23 vemurafenib-treated melanoma patients, and they identified *RAS* mutations in 60% of them, the most prevalent being *HRAS Q61L* [269]. Similarly, Oberholzer et al. found a 30% frequency of *HRAS* mutation in 10 tumors from vemurafenib-treated patients [270].

Using a carcinogenesis mouse model, Su et al. studied the effect of vemurafenib on the two-stage 7,12-dimethylbenz[*a*]anthracene (DMBA)- and TPA-induced skin cancers [269]. Vemurafenib alone fails to produce these skin cancers. However, they found that vemurafenib accelerated the time of onset of the skin tumors. These investigators found that the combination treatment of vemurafenib and a MEK1/2 inhibitor (PD184352) decreased tumor formation by 91% in the mice. The authors suggest that the combined use of Raf and MEK1/2 inhibitors may prevent the accelerated appearance of cutaneous squamous-cell carcinomas or keratoacanthomas.

Several inhibitors of MEK1/2 and ERK1/2 have been developed, but none have achieved FDA approval for patient treatment [24]. Sorafinib, an FDA-approved drug for the treatment of renal carcinoma that was initially developed as a Raf inhibitor, most likely exerts its therapeutic effects by inhibiting vascular endothelial growth factor receptors and angiogenesis [24]. Many groups have made considerable effort to design ERK1/2 inhibitors, but none of these endeavors have yet been clinically successful.

9.3. Future developments

Deciphering the mechanisms of Ras-Raf-MEK-ERK signaling continues to be an important and challenging task. As noted in Section 8.1, although fifteen protein kinase inhibitors have been approved by the U.S. FDA for the treatment of a variety of cancers, these inhibitors target only a few kinases. The cost of the targeted protein kinase inhibitors (thousands of dollars per month) has limited their use in many countries. Except for the treatment of Philadelphia-chromosome BCR-Abl positive chronic myelogenous leukemia, the other anti-cancer kinase inhibitors prolong life for only a few weeks or months. Designing therapeutic approaches to improve the length of survival is an important theoretical and practical undertaking.

Chronic myelogenous leukemia is characterized by the activation of the Abl kinase following a translocation with the breakpoint cluster region (BCR) with the attendant formation of the Philadelphia chromosome [264]. Blockade of a single enzyme is thus efficacious in the treatment of this malady. Most cancers, however, result from the dysregulation of multiple signaling pathways. It is unlikely that targeting a single kinase or pathway will be effective in treatment. To obviate this dilemma, the use of protein kinases that target more than one pathway or the use of multiple protein kinase inhibitors is under investigation. Yet another strategy is to combine protein kinase inhibitors with traditional cytotoxic drugs. Surely more inhibitors that target more protein kinases will become available in the future, including inhibitors of the ERK1/2 MAP kinase cascade.

Conflict of interest

The author is unaware of any affiliations, memberships, or financial holdings that might be perceived as affecting the objectivity of this review.

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