CELL REGULATION, Vol. 2, 965–978, December 1991

Extracellular signal-regulated kinases: ERKs in progress

Review

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Hormones and other agents with receptors that signal through known heterotrimeric G proteins control the production and release of second messengers (i.e., cAMP, diacylglycerol, intracellular calcium) (Gilman, 1987). These messengers modulate cellular functions in part due to altered protein-serine/threonine phosphorylation catalyzed by second messenger-dependent protein kinases. Many growth factors and cytokines also lead to pronounced changes in protein-serine/threonine phosphorylation. They have some ability to utilize phospholipid- and calcium-dependent pathways, but second messenger-independent protein-serine/threonine phosphorylation is required to elicit their full spectrum of actions. The early steps in signal transduction mechanisms triggered by their receptors to regulate second messenger-independent serine/threonine phosphorylation are unknown. Notable among these factors are the considerable number whose receptors contain or associate with tyrosine kinases (Rosen *et al.,* 1983; Hunter and Cooper, 1985; Veillette *et al.,* 1988; Yarden and Ullrich, 1988; Squinto *et al.,* 1991). Thus, a critical unsolved problem in understanding cellular regulation is how tyrosine kinases induce the major changes in phosphorylation that occur downstream of the receptor on serine/threonine residues. In the last 5 y the insulin- and growth factor-stimulated phosphorylation of ribosomal protein S6 on serine residues has been the object of intense scrutiny in the dissection and reconstruction of signaling mechanisms triggered by factors that activate tyrosine kinases (Erikson and Maller, 1986; Blenis *et al.,* 1987; Ballou *et al.,* 1988; Gregory *et al.,* 1989; Ahn *et al.,* 1990; Price *et al.,* 1990; Chen *et al.,* 1991). Despite the fact that the precise effects of S6 phosphorylation on ribosome function are unclear, the increased phosphorylation of S6 is a rapid consequence of insulin and growth factor action that has been exploited to gain insights into mechanisms by which growth factors regulate serine/threonine phosphorylation networks (Rosen *et al.,* 1981; Novak-Hofer and Thomas, 1984; Tabarini *et al.,* 1985; Cobb, 1986; Erikson and Maller, 1986; Pelech and Krebs, 1987; Bannerjee *et al.,* 1990).

The complexity of protein kinase networks, involved not only in S6 phosphorylation but also in the regulation of phosphorylation of other proteins (Flotow *et al.,* 1990), exceeds the expectations of 5 y ago. Compounding the complexity, a large fraction of cellular proteins, perhaps as many as 25% of the total (Blackshear, personal communication), are phosphorylated and many of these phosphorylations influence protein function. Nevertheless, significant progress has been made in identifying enzymes involved in S6 phosphorylation. A second protein kinase cascade, in addition to the cascade originally discovered by Krebs and associates in 1968 in which cAMP-dependent protein kinase activates phosphorylase kinase (DeLange *et al.,* 1968), has been found. This newly identified cascade (Figure 1) includes three activities acting sequentially: one type of S6 kinase known as rsk for ribosomal protein S6 kinase (Erikson and Maller, 1986; Jones *et al.,* 1988), a kinase that phosphorylates this S6 kinase (Sturgill *et al.,* 1988), and an activator of this S6 kinase kinase (Ahn *et al.,* 1991). Each of these components will be discussed below.

Recently, two enzymes that are S6 kinase kinases and a third related kinase have been cloned and named extracellular signal-regulated kinases (ERKs)[1] 1, 2, and 3 (Boulton *et al.,* 1990b, 1991b) to reflect the great diversity of signals that stimulate them (Table 1). These kinases have been referred to in the literature not only as S6 kinase kinases, but also as

[1] Abbreviations used: EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; GVBD, germinal vesicle breakdown; IGF1, insulin-like growth factor 1; MAP, maturation-activated protein; MAP2, microtubule-associated protein 2; MBP, myelin basic protein; NGF, nerve growth factor; PDGF, platelet-derived growth factor.

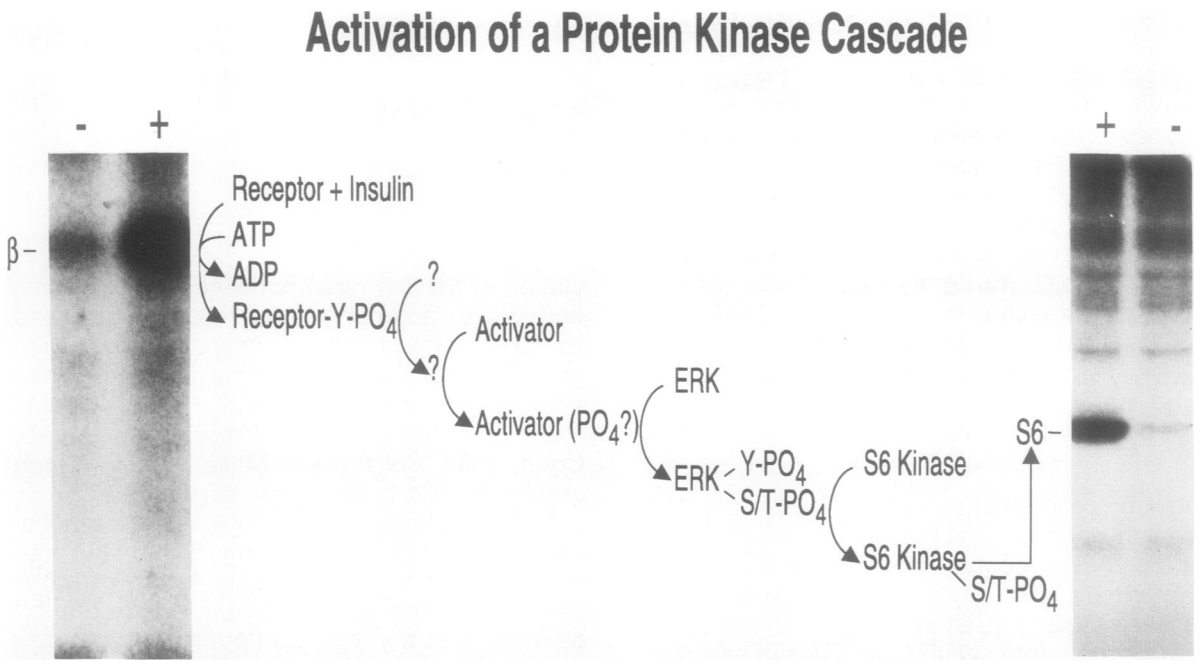

Figure 1. **Activation of a protein kinase cascade through the insulin signaling pathway.** Exposing cells to insulin (+) causes autophosphorylation of the β subunit of the insulin receptor on tyrosine residues. An autoradiogram of the insulin-dependent in vitro autophosphorylation reaction is shown in the left panel. This phosphorylation is the first in a series of events leading to the increase in phosphorylation of ribosomal protein S6. The S6 kinase activity present in supernatants of insulin-treated (+) and untreated (−) cells is shown in the autoradiogram in the right panel. The cascade of intermediate events has been proposed based primarily on the in vitro studies described in the text.

rsk kinases, microtubule-associated protein-2 (MAP2) kinases, myelin basic protein (MBP) kinases, mitogen- or maturation-activated protein (MAP) kinases, and ERKs. This review describes studies that led to the isolation of these kinases, indicates relationships among them where known (Table 2), and discusses their substrates and mechanisms of regulation.

ERKs 1 and 2 (MAP2/MBP kinases) are activated in many cell types by mitogenic and nonmitogenic stimuli

In 1987, Ray and Sturgill characterized a MAP2 kinase from 3T3-L1 adipocytes stimulated by insulin that was distinct from any other insulin or growth factor-sensitive protein kinases that had been described in detail including the S6 kinases (Ray and Sturgill, 1987). Others were also recognizing hormonally regulated activities that we now know are closely related or identical (Table 1). Krebs and coworkers noted the activation of an MBP kinase in *Xenopus* oocytes undergoing maturation (MAK-M) (Cicirelli *et al.*, 1988), after germinal vesicle breakdown (GVBD) in the sea star, and after fertilization of sea urchin eggs (Pelech *et al.*, 1988). Hoshi *et al.* (1988) reported on a MAP2 kinase that was stimulated in 3Y1 cells in response to epidermal growth factor (EGF), phorbol esters, platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF), and inhibited by low concentrations of calcium; they later found that the effect of calcium was lost as the enzyme was further purified (Gotoh *et al.*, 1990b).

Work from these and many other laboratories has now revealed that such an activity can be measured in extracts as a MAP2, MBP, and rsk kinase (see Table 1 for listing of stimuli) that is stimulated by mitogens including EGF (Hoshi *et al.*, 1988, 1989; Rossomando *et al.*, 1989; Smith *et al.*, 1989; Ahn and Krebs, 1990; Gotoh *et al.*, 1990a,b; Miyasaka *et al.*, 1990a,b, 1991; Northwood and Davis, 1990; Tsao *et al.*, 1990; Ahn *et al.*, 1991; Ballou *et al.*, 1991; Chung *et al.*, 1991a; Lee *et al.*, 1991; Takishima *et al.*, 1991), FGF (Hoshi *et al.*, 1988; Tsao *et al.*, 1990; Chung *et al.*, 1991a; Kawakami *et al.*, 1991; Lee *et al.*, 1991), PDGF (Hoshi *et al.*, 1988; Chung *et al.*, 1991a,b; Kawakami *et al.*, 1991; L'Allemain *et al.*, 1991; Lee *et al.*, 1991), insulin-like growth factor 1 (IGF1) (Hoshi *et al.*, 1988; Chung *et al.*, 1991a), and insulin ([Hoshi *et al.*, 1988; Ray and Sturgill, 1987; Boulton *et al.*, 1990a, 1991a;

Haystead *et al.,* 1990; Kyriakis and Avruch, 1990] only mitogenic for 3T3 cells at concentrations sufficient to bind to IGF1 receptors [Haselbacher *et al.,* 1979]). In these cases, increased activity parallels the entry of quiescent cells into G_1. Nonmitogenic stimuli also regulate the activity, indicating that increased MAP2/MBP kinase activity is not uniquely tied to mitogenesis. Ligands for the c-kit (mast cell growth factor) and c-met (hepatocyte growth factor) proto-oncogenes in human melanocytes (Funasaka *et al.*, 1992), bradykinin (Ahn, Robbins, Haycock, Seger, Cobb, and Krebs, unpublished data), the tumor promoter okadaic acid in rat adipocytes (Haystead *et al.,* 1990), phorbol esters (Hoshi *et al.,* 1988, 1989; Smith *et al.,* 1989; Erickson *et al.,* 1990; Ferrell and Martin, 1990; Nel *et al.,* 1990a,b; Northwood and Davis, 1990; Chung *et al.,* 1991a; Kawakami *et al.,* 1991; Lee *et al.,* 1991; Payne *et al.,* 1991), activation of the ros oncogene in Chinese hamster ovary cells (Boulton *et al.,* 1990a), and ligation of CD3 on T cells (Nel *et al.,* 1990a,b; Whitehurst, Owaki, Boulton, Cobb, Geppert, unpublished data) stimulate kinase activity. However, these agents usually require additional factors or must be added in combinations to cause proliferation in the appropriate cell types. Furthermore, activation of MAP2/MBP kinases may accompany the normal differentiated functions of various cells. Insulin, which is not a mitogen for terminally differentiated adipocytes but rather enhances lipogenesis, stimulates MAP2/MBP kinase activity in both differentiated 3T3-L1 adipocytes in culture (Ray and Sturgill, 1987, 1988a,b) and also in adipocytes prepared from rat epididymal fat (Haystead *et al.,* 1990). Nerve growth factor (NGF), which induces neuronal proteins and morphology in PC12 cells (Barde, 1989), increases MAP2 kinase activity having a more prolonged effect on enzyme activity than PC12 mitogens (Smith *et al.,* 1989; Gómez *et al.,* 1990; Gotoh *et al.,* 1990b; Landreth *et al.,* 1990; Miyasaka *et al.,* 1990a,b, 1991; Tsao *et al.,* 1990; Schanan-King *et al.,* 1991; Tsao and Greene, 1991; Robbins and Cobb, unpublished data). Brain, a nondividing tissue, contains very high concentrations of these enzymes (Boulton *et al.,* 1991b), supporting a role for ERKs in neuronal function. Secretagogues, such as nicotine, elevate MAP2 kinase activity in bovine adrenal chromaffin cells (Ely *et al.,* 1990). ERKs are also activated in maturing frog oocytes (Posada *et al.,* 1991; Ferrell *et al.,* 1991; Gotoh *et al.,* 1991a,b) and in clam oocytes (Ruderman *et al.,* 1991) as they approach GVBD. Colchicine and other microtubule-disrupting agents cause a modest increase in kinase activity in 3Y1 cells (Shinohara-Gotoh *et al.,* 1991), although the increase may correlate with the mitogenic potency of these agents.

MAP kinase is pp42

The 3T3-L1 MAP2 kinase (Rossomando *et al.,* 1989) appears to be similar to a protein, pp42, whose phosphotyrosine content is increased in growth factor-treated and virally transformed cells (Kazlauskas and Cooper, 1988). In light of this finding it was dubbed MAP kinase for mitogen-activated protein kinase. This protein was recognized over 10 years ago as a possible target for tyrosine phosphorylation by receptor tyrosine kinases and those of the src family (Cooper and Hunter, 1981; Martinez *et al.,* 1982; Maytin *et al.,* 1984). However, it proved difficult to purify to homogeneity based solely on this property and defied identification until it had been studied as a growth factor-regulated protein kinase. The possibility that it is a substrate of upstream tyrosine kinases will be discussed below.

Purification of MAP2/MBP kinases

As anticipated by Sturgill, Weber, and colleagues in 1989 (Rossomando *et al.,* 1989), purification and cloning of the MAP2 kinase were important milestones to be achieved to understand its regulation. Subsequently, three purifications of 42–44 k MAP2/MBP kinases to near homogeneity have been documented (Sanghera *et al.,* 1990a; Boulton *et al.,* 1991a; Gotoh *et al.,* 1991b). All three of the groups that purified the enzymes succeeded in obtaining protein sequence from their preparations. Sanghera *et al.* (1990a) described the purification of a pp44 MBP kinase from sea star. The specific activity of the enzyme was 0.94 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ with MBP. Boulton *et al.* (1991a) purified a MAP2 kinase, ERK1, from insulin-treated Rat 1 HIRc B cells over 6300-fold to a specific activity of ~ 1 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ with MAP2 and ~ 3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ with MBP as substrate. Gotoh *et al.* (1991b) have also reported the purification of a MAP2 kinase from *Xenopus* oocytes with a specific activity of 0.32 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ with MBP as substrate.

Isolation of cDNAs encoding ERKs

Tryptic peptide sequence from the purified Rat 1 HIRc B MAP2 kinase (Boulton *et al.,* 1991a) was used to design degenerate oligonucleotides as primers for the polymerase chain reaction.

Table 1. Summary of stimuli reported to activate MBP or MAP2 kinase activities attributable to ERKs

Stimulus	Cell/tissue	References
AlF_4^-	BC_3H1 monocytes	Anderson *et al.*, 1991a
	3T3-L1	Anderson *et al.*, 1991a
Bradykinin	PC12	Ahn, Robbins, Haycock, Seger, Cobb, and Krebs, unpublished data
Colchicine	3Y1	Shinohara-Gotoh *et al.*, 1991
EGF	Swiss 3T3	Rossomando *et al.*, 1989; Ahn and Krebs, 1990; Ahn *et al.*, 1991; Ballou *et al.*, 1991; Chung *et al.*, 1991a,b
	Rat 1 HIRcB	Robbins and Cobb, unpublished data
	3Y1	Hoshi *et al.*, 1989; Gotoh *et al.*, 1990b
	PC12	Smith *et al.*, 1989; Gotoh *et al.*, 1990b; Miyasaka *et al.*, 1990a,b, 1991; Tsao *et al.*, 1990
	A431	Northwood and Davis, 1990
	TIG-3	Hoshi *et al.*, 1988
	HIR3.5	Takishima *et al.*, 1991
	3T3-L1	Lee *et al.*, 1991
Electroconvulsive treatment	Rat hippocampus	Stratton *et al.*, 1991
Fertilization	Clam oocytes	Ruderman *et al.*, 1991
FGF	Swiss 3T3	Chung *et al.*, 1991a
	TIG-3	Hoshi *et al.*, 1988
	HIR3.5	Lee *et al.*, 1991
	PC12	Tsao *et al.*, 1990
	3T3-Balb C	Kawakami *et al.*, 1991
Hepatocyte growth factor	Melanocytes	Funasaka, Boulton, Cobb, Yarden, Fan, Lyman, Williams, Anderson, Mishima, and Halaban, unpublished data
IGF-1	TIG-3	Hoshi *et al.*, 1988
	Swiss 3T3	Chung *et al.*, 1991a
Insulin	3T3-L1	Ray and Sturgill, 1987, 1988a,b
	Rat 1 HIRcB	Lee *et al.*, 1991; Boulton *et al.*, 1991a
	Chinese hamster ovary-IRros	Boulton *et al.*, 1990a
	Rat adipocytes	Haystead *et al.*, 1990
	Rat liver	Kyriakis and Avruch, 1990
	TIG-3	Hoshi *et al.*, 1988
	HIR3.5	Lee *et al.*, 1991
KCl	PC12	Tsao *et al.*, 1990
Engagement of T-cell antigen receptor/CD3 complex	Jurkat	Nel *et al.*, 1990a,b; Whitehurst, Owaki, Boulton, Cobb, Geppert, unpublished data
Maturation promoting factor (MPF)	*Xenopus* oocytes	Gotoh *et al.*, 1991a
1-Methyladenine	Sea star oocytes	Pelech *et al.*, 1988; Sanghera *et al.*, 1990b; Posada *et al.*, 1991
Mast cell growth factor	Melanocytes	Funasaka, Boulton, Cobb, Yarden, Fan, Lyman, Williams, Anderson, Mishima, and Halaban, unpublished data
NGF	PC12	Smith *et al.*, 1989; Gómez *et al.*, 1990; Gotoh *et al.*, 1990a,b; Landreth *et al.*, 1990; Miyasaka *et al.*, 1990a,b, 1991; Tsao *et al.*, 1990; Boulton *et al.*, 1991b; Schanan-King *et al.*, 1991; Tsao and Greene, 1991
	A126-1B2 (PC12 subclone)	Tsao and Greene, 1991
	PC12D	Sano *et al.*, 1990
Nicotine	Bovine adrenal chromaffin cells	Ely *et al.*, 1990
Okadaic acid	PC12	Tsao and Greene, 1991; Miyasaka *et al.*, 1990b, 1991
	Rat adipocytes	Haystead *et al.*, 1990
	3Y1	Gotoh *et al.*, 1990a
PDGF	TIG-3	Hoshi *et al.*, 1988
	HIR3.5	Lee *et al.*, 1991
	3T3-Balb C	Kawakami *et al.*, 1991
	Swiss 3T3	Chung *et al.*, 1991a
	3T3-TNR9	L'Allemain *et al.*, 1991
Phorbol 12-myristate 13-acetate	Swiss 3T3	Chung *et al.*, 1991a
	3Y1	Hoshi *et al.*, 1989; Gotoh *et al.*, 1990b
	PC12	Smith *et al.*, 1989
	NIH3T3	Ferrell and Martin, 1990
	Jurkat	Nel *et al.*, 1990a,b
	T cells	Erickson *et al.*, 1990; Payne *et al.*, 1991

Table 1. (Continued)

Stimulus	Cell/tissue	References
Phorbol 12-myristate 13-acetate	Melanocytes	Funasaka *et al.*, 1992
	TIG-3	Hoshi *et al.*, 1988
	A431	Northwood and Davis, 1990
	HIR3.5	Lee *et al.*, 1991
	3T3-Balb C	Kawakami *et al.*, 1991
Progesterone	*Xenopus* oocytes	Cicirelli *et al.*, 1988; Haccard *et al.*, 1990; Gotoh *et al.*, 1991b; Posada *et al.*, 1991
Prostaglandin $F_{2\alpha}$	Swiss 3T3	Chung *et al.*, 1991a
ros (IRros + insulin)	Chinese hamster ovary-IRros	Boulton *et al.*, 1990a
Serum	TIG-3	Hoshi *et al.*, 1988
	3Y1	Hoshi *et al.*, 1989
	NIH-3T3	Ferrell and Martin, 1990
	Swiss 3T3	Ballou *et al.*, 1991; Chung *et al.*, 1991a,b
	HIR3.5	Lee *et al.*, 1991
$pp60^{v\text{-}src}$	CEF	Chung *et al.*, 1991a
Vanadate	PC12	Tsao and Greene, 1991
Vinblastine	3Y1	Shinohara-Gotoh *et al.*, 1991

A rat brain cDNA library was probed with the product, resulting in the isolation of a cDNA encoding the kinase, named ERK1 (Boulton *et al.*, 1990b), and complete cDNAs encoding related enzymes, ERK2 and ERK3 (Boulton *et al.*, 1991b). ERK2 is 83% identical to ERK1, whereas ERK3 encodes a more distantly related kinase that is only 42% identical to ERK1 (Boulton *et al.*, 1991b). Within these three kinases, there are clusters of greater identity that we suggest can be used to define the ERK family of kinases. Subdomains V, VI, VII, IX, and XI (Hanks *et al.*, 1988) are highly conserved even in ERK3 (e.g., ERK3 subdomain V is 83% identical and subdomain IX is 72% identical to those of ERK1), suggesting that the enzymes diverged from a common ancestor. The ERKs are most closely related to KSS1 (Courchesne *et al.*, 1989) and FUS3 (Elion *et al.*, 1990), enzymes that mediate entry into the cell cycle and growth arrest in response to pheromones, and to a lesser extent to cdc2, which regulates cell-cycle progression (Maller, 1991). KSS1 and FUS3 are also most similar to ERKs in the subdomains with ERK-

Table 2. Nomenclature and grouping of ERK homologs based on their chromatographic and enzymatic behavior and their amino acid sequences

	ERK1	ERK2	ERK3	ERK-like
Boulton *et al.*, 1991a,b; Boulton and Cobb, 1991	~43 k	41 k	62 k	ERK4(45 k)
Ray and Sturgill, 1987, 1988a,b; Sturgill and Wu, 1991	$p44^{MAPK}$	$p42^{MAPK}$ MAP kinase		
Ahn and Krebs, 1990	MBP kinase E4 (Mono Q peak 2)	MBP kinase E3 (Mono Q peak 1)		
Cooper *et al.*, 1984; Rossomando *et al.*, 1989; Ferrell and Martin, 1990; Her *et al.*, 1991		pp42		
Gotoh *et al.*, 1991a		MPK1		
Cooper, 1989	pp45			
Sanghera *et al.*, 1990b	$pp44^{MPK}$?	$pp44^{MPK}$?		
Kyriakis *et al.*, 1991				pp54 MAP2 kinase
Tsao and Greene, 1991				HMK
Northwood *et al.*, 1991				ERT

A more extensive list of MAP2/MBP kinases and their activators appears in Table 1. HMK, high-molecular-weight microtubule-associated protein kinase; ERT, EGF receptor Thr^{669} protein kinase.

family features. The 5′ end of the ERK1 cDNA has not been present in clones isolated from several cDNA libraries (Boulton *et al.,* 1991b; Whitehurst, Owaki, Boulton, Cobb, Geppert, unpublished data) and has proven difficult to obtain by primer extension. Immunoblotting with antipeptide antibodies suggests that there is another ERK-related protein tentatively named ERK4 (Boulton and Cobb, 1991), whereas Southern analysis suggests that there may be several other related kinases (Boulton *et al.,* 1991b). ERK homologs have now been identified by cDNA cloning from *Xenopus* (Gotoh *et al.,* 1991a; Posada *et al.,* 1991), human T cells (Whitehurst, Owaki, Boulton, Cobb, Geppert, unpublished data), mouse (Her *et al.,* 1991), *Drosophila* (1 clone is ~80% identical to ERK1 and ERK2 whereas another is less related, ~44% identical, Biggs, and Zipursky, unpublished data), and alfalfa (Jacobs, and Duerr, unpublished data); some of these ERK homologs are compared in Table 3. The ERKs appear to be highly conserved across species, which may attest to their important roles in signal transduction.

Tissue-specific expression of ERKs

Northern analysis using probes specific for each ERK demonstrates that ERKs 1, 2, and 3 are ubiquitously expressed with high levels in brain and spinal cord (Boulton *et al.,* 1991b). In peripheral tissues the ERK3 message is expressed at highest concentrations in muscle. ERK1 mRNA is expressed at highest levels in intestine and placenta and to a lesser extent in lung. ERK2 mRNA is expressed at highest levels in muscle, placenta, and heart. Furthermore, messages for both ERK2 and ERK3, but not ERK1, increase dramatically during differentiation of P19 teratocarcinoma cells into neuronal cells, whereas only the mRNA for ERK3 increases in the pathway leading to muscle differentiation (Boulton *et al.,* 1991b). These studies suggest that each of these enzymes may have unique functions. Western blotting indicates that ERK1 and ERK2 proteins are present in most tissues and cell lines examined. Their relative amounts are roughly proportional to the amounts of their mRNAs and increase in the developing rat brain (Boulton *et al.,* 1991b; Boulton and Cobb, 1991).

MAP2/MBP kinases, ERK1 and ERK2, activate rsk-like S6 kinases

Interest in MAP2/MBP kinase activities was sparked when Sturgill, Maller, and coworkers demonstrated that the 3T3-L1 MAP2 kinase phosphorylated and activated S6 kinase II (Sturgill *et al.,* 1988), a rsk-type enzyme, from *Xenopus laevis* eggs (Erikson and Maller, 1986). Since that important observation, many groups have confirmed this in vitro event. Gregory *et al.* (1989) showed reactivation of a rabbit liver S6 kinase displaying immunological cross-reactivity to both of the catalytic domains and the linking region of *Xenopus* rsk-α (Cobb and Gregory, unpublished data) by a MAP2 kinase from insulin-stimulated Rat 1 HIRc B cells. Ahn and Krebs (1990) obtained independent evidence for this two-enzyme cascade. They mixed Mono Q fractions from stimulated and unstimulated cell supernatants in an 8 × 8 matrix to search for EGF-stimulated activitors of kinases that phosphorylate an S6 peptide and other substrates. They found that two EGF-sensitive MBP kinases (Mono Q fraction E3 that contains ERK2 and

Table 3. Identities among ERK homologs in percent

	rERK1	rERK2	rERK3	hERK1	$p42^{MAPK}$	Xp42	MPK1	Alfalfa ERK
rERK1	100	83	42	97	83	82	83	47
rERK2		100	43	83	100	95	95	49
rERK3			100	42	43	43	43	36
hERK1				100	83	82	83	47
$p42^{MAPK}$					100	95	95	49
Xp42						100	98	49
MPK1							100	40
Alfalfa ERK								100

Sequences were aligned using Microgenie (Beckman, Palo Alto, CA) over their entire lengths except for ERK3, only the first 365 residues of which were used in the comparisons. rERK1, 2, and 3 are the rat proteins (Boulton *et al.,* 1990b, 1991b); hERK1, human ERK1 (Whitehurst, Owaki, Boulton, Cobb, and Geppert, unpublished data); $p42^{MPK}$, mouse (Her *et al.,* 1991); Xp42, *Xenopus* (Posada *et al.,* 1991); MPK1, *Xenopus* (Gotoh *et al.,* 1991a); Alfalfa ERK, alfalfa (Jacobs and Duerr, personal communication).

Mono Q fraction E4 that contains ERK1) enhanced S6 peptide kinase activity. The original observation of Sturgill *et al.* (1988) has also been corroborated by Kyriakis and Avruch (1990), using a rat liver insulin-stimulated MAP2 kinase and S6 kinase II. Further, Blenis and colleagues identified two growth factor-stimulated rsk kinases that have been identified as ERKs by immunoblotting partially purified rsk kinase fractions with antibodies to sea star $p44^{MPK}$ and antibodies to ERK1 (Chung *et al.,* 1991b).

Relationship of MAP2/MBP/rsk kinases to ERK1 and ERK2

Boulton and Cobb (1991) generated antipeptide antibodies to four ERK1 peptides, the C-terminal peptide, a peptide from protein kinase subdomain XI (Hanks *et al.,* 1988), a peptide near the N-terminus, and a peptide from subdomain V conserved among the ERKs. These antibodies recognize ERK1 (~43 kDa), ERK2 (41 kDa), and a fourth ERK (~45 kDa), larger than ERK1 and ERK2 but smaller than ERK3, in extracts of numerous rat, human (Whitehurst, Owaki, Boulton, Cobb, Geppert, unpublished data), and mouse (Boulton and Cobb, 1991) cell lines. One or more ERK-like proteins are identified in frog (Boulton and Cobb, 1991; Ferrell *et al.,* 1991), clam (Ruderman *et al.,* 1991), and *Drosophila* (Briggs and Zipursky, unpublished data).

MAP2/MBP kinase activities are stimulated within 1–5 min of exposure of cells to many extracellular signals (Table 1), concomitant with the appearance of phosphotyrosine on the proteins (Ray and Sturgill, 1988b; Gómez *et al.,* 1990; Nel *et al.,* 1990a,b; Ahn *et al.,* 1991; Boulton *et al.,* 1991a,b; Gotoh *et al.,* 1991b; Miyasaka *et al.,* 1991; Posada *et al.,* 1991; Schanan-King *et al.,* 1991). Their apparent sizes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis range from 38 to 45 kDa. The MAP2/MBP kinase activities from several systems have been resolved into two distinct forms on Mono Q (Ahn and Krebs, 1990; Ahn *et al.,* 1990; Gómez *et al.,* 1990; Boulton and Cobb, 1991; Boulton *et al.,* 1991b), the smaller form (reported as 38–42 kDa) eluting ahead of the larger form (reported as 43–45 kDa). Immunoblotting and immune complex kinase assays indicate that the first peak of growth factor-stimulated MAP2/MBP kinase eluting from Mono Q is ERK2, whereas the second peak of stimulated activity eluting from Mono Q is ERK1. Thus, both ERK1 and ERK2 are insulin- and growth factor-sensitive MAP2 kinases that together account for most of the reports of signal-stimulated MAP2/MBP kinase activity in cell extracts as deduced from immunological cross-reactivity (Ahn *et al.,* 1991; Boulton and Cobb, 1991; Boulton *et al.,* 1991b; Posada *et al.,* 1991), protein sequence (Gotoh *et al.,* 1991a; Her *et al.,* 1991; Posada *et al.,* 1991), and chromatographic behavior (Table 2). Kyriakis *et al.* (1991) have found a larger 54-kDa MAP2 kinase with common features, such as apparent regulation by both tyrosine and serine/threonine phosphorylation, but also unique ones, such as size and relative inability to phosphorylate MBP (Kyriakis and Avruch, 1990), that suggest it is different from the enzymes discussed here. Recently, Northwood *et al.* (1991) identified a 46-kDa kinase in KB cells that phosphorylated Thr-669 of the EGF receptor and appears to be distinct from the MAP2 kinase. They designated it ERT for EGF receptor Thr-669 kinase and suggest this kinase may be similar to the 45-kDa ERK4 that was identified in rat cells by immunoblotting with ERK antibodies (Boulton and Cobb, 1991). The relationships of these proteins to the ERKs are summarized in Table 2.

The recently published sequence of mouse MAP kinase studied by Sturgill and Weber (Her *et al.,* 1991) indicates that it is ERK2 (Table 3). The major frog MAP2 kinase also appears to be an ERK2 homolog. Ferrell *et al.* (1991) found that ERK antipeptide antibodies cross-react with *Xenopus* p42. Posada *et al.* (1991) have isolated a cDNA that encodes *Xenopus* Xp42 that has greatest identity (95%) to ERK2 (Table 3). The cDNA recently isolated from *Xenopus* by Gotoh and colleagues (1991a), named MPK1, is also more similar to ERK2 than ERK1 but slightly different from the other *Xenopus* clone (Posada *et al.,* 1991). On the other hand, tryptic peptide sequence of $pp44^{MPK}$ from sea star (Posada *et al.,* 1991) is ~77% identical with comparable sequences of ERK1 and ERK2. Antibodies to this sea star enzyme and ERK1 cross-react with the 3T3-L1 MAP kinase (Chung *et al.,* 1991b) and identify two rsk kinases in DEAE-Sephacel profiles that almost certainly correspond to ERK1 and ERK2.

The chromatographic properties of ERK3 and the 45-kDa ERK have not yet been examined. However, antipeptide antibodies generated to a peptide near the C-terminus of ERK3 identify a band of ~62 kDa, near the size predicted for ERK3, in rat and human cells (Boulton and Cobb, unpublished data). The relationship, if any, of ERK3 to other kinases that have been studied biochemically is unknown.

Table 4. Substrates of ERK1 and ERK2 and the sequences of the phosphorylation sites where known

Substrate	Phosphorylation site
MAP2	? (S and T)
MBP	VTPRTPPP[a]
Tyrosine hydroxylase, rat	EAVTSPRF[b]
EGF receptor	VEPLTPSG[c]

The residues phosphorylated are MBP threonine 97, tyrosine hydroxylase serin 31, and EGF receptor threonine 669. References: [a](Erickson *et al.*, 1990; Sanghera *et al.*, 1990a,b); [b](Haycock, 1990); [c](Alvarez *et al.*, 1991; Takishima *et al.*, 1991). Additionally S6 kinase II (Sturgill *et al.*, 1988; Gregory *et al.*, 1989; Ahn and Krebs, 1990), raf (Anderson *et al.*, 1991; Lee *et al.*, 1991b), myc (Alvarez *et al.*, 1991; Frost, Boulton, Cobb, and Feramisco, unpublished data), jun (Dutta, Cobb, and Karin, unpublished data), and acetyl CoA carboxylase (Pelech *et al.*, 1991) are also substrates, although the phosphorylation sites have not been reported.

Substrates of ERKs 1 and 2

In addition to phosphorylating MAP2, MBP, and rsk, both ERK1 and ERK2 have been found to phosphorylate a number of other important regulatory proteins (Table 4), demonstrating that the ERKs may participate in the control of diverse cellular functions (Figure 2). Not only is the activity of rsk altered, the properties of some of these other substrates change after phosphorylation by ERKs. Gotoh and colleagues have provided evidence that MAP2 kinase may influence microtubule dynamics inducing a pattern characteristic of metaphase (Gotoh *et al.*, 1991b). Together with work by Greene and associates (Volonte *et al.*, 1989) and Sano *et al.* (1990) these studies raise the possibility not only that one or more MAPs may be physiological ERK substrates but also that ERKs may be important regulators of neuronal morphology. Haycock and coworkers have found that Ser-31 of rat tyrosine hydroxylase is a substrate for both ERK1 and ERK2 (Haycock, Ahn, Cobb, and Krebs, unpublished data), although the consequences for its enzymatic activity have not yet been delineated. Davis and colleagues (Northwood *et al.*, 1991) and Rosner and coworkers (Takishima *et al.*, 1991) find that Thr-669 of the EGF receptor or a peptide derived from it is a substrate for ERKs. Notably, Anderson *et al.* (1991b) have also found that MAP kinase phosphorylates the proto-oncogene, raf, although no changes in raf kinase activity were reported. That observation has been extended by Lee *et al.* (1991b) who found that the two peaks of activity that phosphorylate raf, identified in cells stimulated with insulin (Lee *et al.*, 1991a), cochromatograph with ERK1 and ERK2. Thus, ERKs 1 and 2 have the capacity to control two downstream serine/threonine kinases, rsk and raf, giving the ERKs an apparently unique role as kinase kinases. An ERK-like protein also phosphorylates a peptide from myc (Alvarez *et al.*, 1991; Frost, Boulton, Cobb, and Feramisco, unpublished data) in vitro. This suggests not only an indirect mechanism via downstream kinases but also a direct mechanism by phosphorylating nuclear proteins by which ERKs might participate in the regulation of gene transcription.

The sites of phosphorylation in tyrosine hydroxylase and the EGF receptor contain the same (S/T)P motif found in the site phosphorylated in MBP (Erickson *et al.*, 1990; Sanghera *et al.*, 1990a; Alvarez *et al.*, 1991; Clark-Lewis

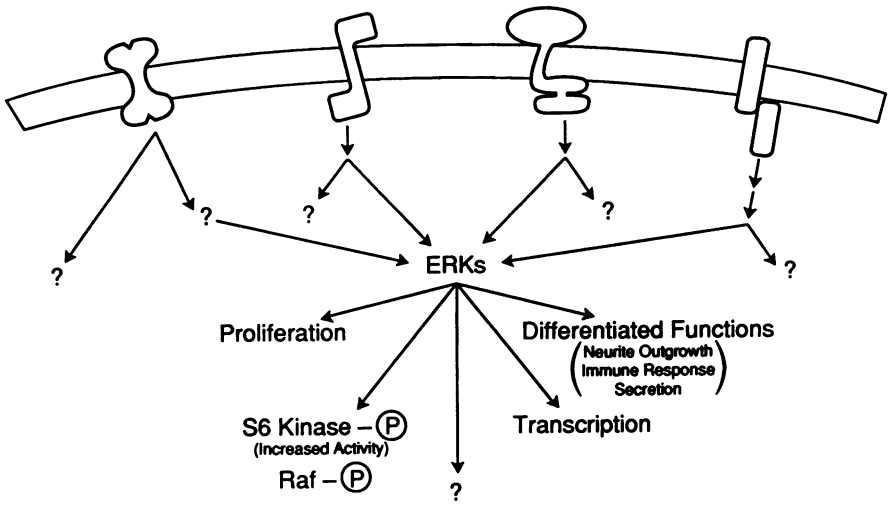

Figure 2. **Possible roles of ERKs in mediating cellular events in response to activation of diverse receptors.**

et al., 1991) by MAP kinase (ERK2) and ERK1 (Table 4). Interestingly, at least at this superficial level, the SP sequence recognized by ERKs 1 and 2 is the same as that phosphorylated by cdc2 (Shenoy *et al.,* 1989). Differences in substrate specificity between ERK1 and ERK2 have not yet been reported.

Regulation of ERKs 1 and 2 by phosphorylation

Phosphatase inhibitors must be included in homogenization cocktails to preserve hormone-stimulated MAP2 kinase activity in cell extracts, providing the first evidence that phosphorylation regulates the activities of these enzymes. Both the serine/threonine-selective phosphatase 2a and the phosphotyrosine-selective phosphatase CD45 inactivate ERK1 and ERK2 (Ahn *et al.,* 1990; Anderson *et al.,* 1990; Gómez *et al.,* 1990; Haystead *et al.,* 1990; Boulton and Cobb, 1991), indicating that phosphorylation of both serine/threonine and tyrosine residues are required to achieve the maximum specific activity of these enzymes. These results are consistent with the observation that activated ERKs bind more tightly to Mono Q (Ahn *et al.,* 1990; Boulton and Cobb, 1991; Boulton *et al.,* 1991b). In some cell types only a portion of each ERK displays retarded Mono Q elution and only that portion is activated, although some fraction of inactive protein may also contain phosphotyrosine (Boulton and Cobb, 1991; Miyasaka *et al.,* 1991; Schanan-King *et al.,* 1991). Perhaps the less phosphorylated forms of ERK1 and ERK2, less tightly bound to Mono Q and inactive, account for the more basic forms of pp42 noted by Rossomando *et al.* (1989) and others (Kazlauskas and Cooper, 1988).

Several groups have been able to activate MAP2/MBP kinases with okadaic acid (Gotoh *et al.,* 1990a; Haystead *et al.,* 1990; Miyasaka *et al.,* 1990b; Tsao and Greene, 1991), an inhibitor of the serine/threonine-selective phosphatases 2a and 1. Haystead *et al.* (1990) determined that this effect depended on the cell type or cell context because the kinases were activated by okadaic acid in rat epididymal adipocytes but not in quiescent 3T3-D1 cells. This suggests that okadaic acid may prevent the dephosphorylation of serine/threonine residues of ERKs; this would only be possible if ERKs were phosphorylated in the absence of an added stimulus in the okadaic acid-sensitive but not in the insensitive cells. Tsao and Greene (1991) find that in addition to its ability to activate MBP kinase, okadaic acid markedly increases the rate of inactivation of the NGF-stimulated enzyme, leading them to hypothesize that there may be additional phosphorylations that are necessary to inactivate the enzyme.

Consistent with effects of phosphatases on activity, Ray and Sturgill (1988b) found that MAP kinase from insulin-treated cells comigrated with a 42-kDa phosphoprotein that contained phosphotyrosine and phosphothreonine. Using immunoprecipitation, Boulton *et al.* (1991b) found that three ERKs, ERK1, ERK2, as well as the 45-kDa ERK, were phosphorylated in insulin- and NGF-treated cells. By phosphoamino acid analysis, ERK1 and ERK2 from either source contain phosphoserine, phosphothreonine, and phosphotyrosine (Boulton *et al.,* 1991b; Robbins and Cobb, unpublished data), although the largest increases in hormone-sensitive phosphate are on tyrosine and threonine residues. The increase in tyrosine phosphate content of ERK1 precedes the increase in phosphothreonine found on the enzyme (Robbins and Cobb, unpublished data), suggesting that the phosphorylation of ERKs may be ordered with tyrosine phosphorylation occurring before threonine phosphorylation. This is consistent with the finding discussed above that some of the inactive enzyme contained phosphotyrosine. An alternate explanation is that the phosphorylations may occur randomly but more rapidly on tyrosine than threonine.

Payne *et al.* (1991) have published tryptic peptide maps of the partially purified MAP kinase revealing one major phosphopeptide. They reported the sequence surrounding two sites phosphorylated in phorbol 12-myristate 13-acetate-stimulated MAP kinase both within one tryptic peptide. The sequence of the peptide (VADPDHDHTGFLT(P)EY(P)VATR) is similar to rat ERK1 but identical to rat ERK2, suggesting that MAP kinase is ERK2 as noted above. Tyrosine and threonine phosphorylation sites separated by only one residue are contained in this single tryptic peptide and are thought to be important for regulation of the kinase. In contrast, maps of tryptic peptides resolved under different conditions showed three to four major spots and as many as four additional minor spots in immunoprecipitated ERK1 and ERK2 (Robbins and Cobb, unpublished data). The major phosphopeptides of immunoprecipitated ERK1 and ERK2 are indistinguishable from each other, indicating that sites of phosphorylation are conserved between the proteins.

Activation of ERKs 1 and 2 in vitro

One property of the ERKs that has generated excitement is that they are phosphorylated on

tyrosine residues in response to growth factors and transforming agents. This finding has led to speculation that these enzymes are substrates for tyrosine kinases, including the insulin and EGF receptors and src. However, evidence from in vitro studies (Seger *et al.,* 1991; Sturgill and Wu, 1991) now suggests that the ERKs are poor substrates for these enzymes and may not be directly activated by them.

Another line of experimentation suggests that the pathway leading to the activation of the ERKs may not be a simple kinase cascade as had previously been anticipated. Using the mixing strategy described earlier, Ahn *et al.* (1991) have identified a soluble EGF-sensitive protein that increased the MAP2/MBP kinase activity of partially purified 3T3 cell extracts. Highly enriched fractions of this ERK activator caused phosphorylation and activation of phosphatase-inactivated, purified ERK1, and that of bacterially expressed ERK2 (Seger *et al.,* 1991). The ERK activator protein partially purified from A431 cells stimulated the activity of recombinant ERK2 over 200-fold, up to a specific activity of 160 $nmol \cdot min^{-1} \cdot mg^{-1}$, and completely restored the activity of dephosphorylated and inactive ERK1. Both phosphothreonine and phosphotyrosine were found on ERK1 and ERK2 after treatment in vitro with the activator (Seger *et al.,* 1991; Ahn, Robbins, Haycock, Seger, Cobb, and Krebs, unpublished data). By tryptic peptide mapping, the sites of phosphorylation on ERK2 in vitro in the presence of activator were a subset of those on ERK2 in intact cells (Seger, Robbins, Cobb and Krebs, unpublished data). As observed for enzymes activated in intact cells (Boulton and Cobb, 1991), in vitro activation is associated with retarded elution of the enzymes from Mono Q (Ahn *et al.,* 1991). Lysates from clam oocytes that have not been stimulated to undergo GVBD do not stimulate ERK2 kinase activity; however, lysates of oocytes that have been stimulated to undergo GVBD also contain an ERK activator that increases ERK2 kinase activity (Ruderman *et al.,* 1991). This is consistent with the observation that clam ERK becomes tyrosine phosphorylated as oocytes approach GVBD (Ruderman *et al.,* 1991).

Autophosphorylation of ERKs 1 and 2

Unlike almost all other protein kinases, as purified in their active forms, ERKs display little or no propensity to autophosphorylate (Boulton *et al.,* 1991a; Sturgill and Wu, 1991). However, the rate of autophosphorylation increases significantly in enzyme that has first been dephosphorylated. Although the stoichiometry of autophosphorylation detected in vitro is small in the absence of the activator protein described above, it is correlated with an increase in protein kinase activity (Seger *et al.,* 1991). Surprisingly, ERK1 and ERK2 autophosphorylate on both threonine and on tyrosine residues (Seger *et al.,* 1991). Peptide maps of autophosphorylated ERK2 contain phosphopeptides found in ERK2 phosphorylated in vivo (Robbins and Cobb, unpublished data). These data suggest that autophosphorylation of ERKs may participate in their activation in vivo.

Hypotheses regarding activation of ERKs 1 and 2

The ERK activator described by Ahn *et al.* (1991) raises the stoichiometry of ERK phosphorylation, apparently by causing phosphorylation of the same peptides (Seger, Robbins, Ahn, Cobb, and Krebs, unpublished data) as are phosphorylated in vivo. It has not been possible to demonstrate kinase activity of the activator (Ahn *et al.,* 1991). Perhaps, the activator increases ERK activity by stimulating ERK autophosphorylation. The finding that the tryptic phosphopeptides from autophosphorylated ERK2 are a subset of the in vivo phosphorylation sites leads us to propose that the ability of ERK1 or ERK2 to autophosphorylate, although limited in vitro, may be the mechanism operating in intact cells (Seger *et al.,* 1991). The mechanism of activation is discussed in more detail elsewhere (Cobb *et al.,* 1991).

Conclusions

Three of the numerous questions that remain to be answered are discussed below. Although we have few facts with which to construct satisfactory models to elaborate answers now, some speculation may provide focal points for future research.

1) If ERKs are activated by so many cellular regulators, how can they participate in pathways unique to a given hormone or factor? The most logical starting point to consider the actions of the ERKs is the exposition of Krebs and Beavo (1979) concerning the means by which cAMP may mediate the actions of many different hormones in many different cell types. That is, as is the case with cAMP, it is likely that the functions of ERKs will be diverse and will be tightly coupled to their localization within cells, the specific combinations of substrates present in the cells, and the cumulative effects of phos-

phorylation and other types of regulation on ERK substrates at the time ERKs are activated (Krebs and Beavo, 1979; Cantley *et al.,* 1991). Studies of the substrates of and localization of ERKs may substantiate this contention.

2) Why are there multiple ERKs that seem to have similar properties? Isoenzymes produced from distinct genes are now recognized as commonplace. As is the case with other isoenzymes, ERK1 and ERK2 may be redundant. If elimination of all ERK activity is lethal for a cell, having two genes encoding similar enzymes would protect the cell from a chance mutation that might delete enzyme function. It is also likely that the enzymes have unique functions, although these may not be easy to discover in cultured cell systems. The fact that ERK2 is expressed so highly in neural tissues, whereas ERK1 appears to be present primarily in neuron-supporting cells, certainly suggests that the enzymes play different roles. Their similarities are sufficient so that they catalyze many or perhaps all of the same reactions in vitro. Efforts to identify substrates unique to one or the other of these enzymes and to find cell-specific ERK functions may illuminate this issue.

3) Are ERKs substrates of conventional tyrosine kinases? If not, how are they activated and what are the functions of the tyrosine kinases in this pathway? Our best guess at this time is that ERKs are not substrates for receptor-linked tyrosine kinases. Continued efforts to identify molecules that can activate the ERKs in vitro, the purification and cloning of such molecules, and the design of strategies to examine the activation process in vivo should eventually allow us to answer this final question that was posed earlier in this review.

Acknowledgments

We thank Natalie Ahn, Rony Seger, and Edwin Krebs at the University of Washington (Seattle, WA) for valuable discussions and significant contributions to work reviewed here, George Yancopoulos and others of Regeneron Pharmaceuticals, Inc. and Tom Geppert for valuable collaborations, and Jo Hicks for preparation of the manuscript. We also thank L. Greene and H. Tsao, J. Haycock, W. Biggs and L. Zipursky, T. Jacobs, B. Duerr, and J. Ruderman for communicating work before publication. Work performed in this laboratory was supported by research grant DK-34128 and Research Career Development Award DK-01918 from the National Institutes of Health.

Received: August 30, 1991.
Revised and accepted: October 8, 1991.

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