Docking Sites on Substrate Proteins Direct Extracellular Signal-regulated Kinase to Phosphorylate Specific Residues*

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Mitogen-activated protein (MAP) kinases such as extracellular signal-regulated kinase (ERK) are important signaling proteins that phosphorylate (S/T)P sites in many different protein substrates. ERK binding to substrate proteins is mediated by docking sites including the FXFP motif and the D-domain. We characterized the sequence of amino acids that can constitute the FXFP motif using peptide and protein substrates. Substitutions of the phenylalanines at positions 1 and 3 had significant effects, indicating that these phenylalanines provide substantial binding affinity, whereas substitutions of the residues at positions 2 and 4 had less effect. The FXFP and D-domain docking sites were analyzed in a variety of positions and arrangements in the proteins ELK-1 and KSR-1. Our results indicate that the FXFP and D-domain docking sites form a flexible, modular system that has two functions. First, the affinity of a substrate for ERK can be regulated by the number, type, position, and arrangement of docking sites. Second, in substrates with multiple potential phosphorylation sites, docking sites can direct phosphorylation of specific (S/T)P residues. In particular, the FQFP motif of ELK-1 is necessary and sufficient to direct phosphorylation of serine 383, whereas the D-domain directs phosphorylation of other (S/T)P sites in ELK-1.

The MAP[1] kinase superfamily is composed of several subfamilies including ERK, c-Jun N-terminal kinase, and p38 (1, 2). These MAP kinases can be activated by a remarkably diverse set of stimuli that function through a variety of signaling pathways. MAP kinase activation is regulated by two upstream protein kinases; a MAP kinase kinase kinase, such as Raf, phosphorylates and thereby activates a MAP kinase kinase (3, 4). The MAP kinase kinase that regulates ERK is called MEK (MAP kinase kinase or ERK kinase). MEK is a dual specificity protein kinase that phosphorylates a threonine and tyrosine in a TXY motif of ERK, resulting in a significant increase in ERK

kinase activity. ERK is inactivated by dual specificity phosphatases that dephosphorylate the TXY motif. Many different stimuli can activate the protein kinase cascade that activates ERK. One extensively characterized activation pathway is initiated by a secreted growth factor, such as epidermal growth factor, that leads to the activation of a receptor tyrosine kinase, Ras, and Raf (5).

Signaling pathways that include ERK mediate a remarkably diverse set of responses during the development and homeostasis of organisms such as *Caenorhabditis elegans*, *Drosophila*, and vertebrates. The mechanisms that enable highly conserved signaling pathways to elicit cell type-specific responses are being actively investigated, yet remain poorly understood. In principle, any protein in a signaling cascade could function differently in different cell types and, thus, contribute to a specific response. However, ERK is likely to play an important role in generating cell type-specific responses (6). By contrast to Raf and MEK, whose only well documented physiological substrates are MEK and ERK, respectively, ERK has been reported to phosphorylate more than 50 different substrates (7–10). These include signaling proteins likely to function upstream of ERK, such as son-of-sevenless guanine nucleotide exchange factor, kinase suppressor of Ras (KSR), and MEK, and signaling proteins likely to function downstream of ERK, such as the protein kinase pp90^{rsk}, phosphodiesterase, and phosphatases. These findings suggest that ERK plays a central role in feedback regulation and signal propagation. ERK substrates also include many different transcription factors such as c-Fos, c-Myc, and ETS proteins including ELK-1, LIN-1, and AOP/YAN, suggesting ERK is a transition point between signaling proteins and regulators of gene expression and differentiation. ERK substrates also include proteins that may regulate cell fate through non-transcriptional mechanisms, such as proteins that regulate programmed cell death and cytoskeletal organization. The large number of ERK substrates that affect differentiation suggests the model that ERK phosphorylates different groups of substrates in different cell types and thereby contributes to cell type-specific responses.

The biological role of ERK raises important mechanistic questions as follows: 1) how does ERK specifically recognize a large number of different substrates, and 2) how does ERK phosphorylate specific residues in these proteins? The initial steps in answering these questions came from studies that identified the residue(s) in substrate proteins that are phosphorylated by ERK and analyzed peptides that can function as ERK substrates (7, 11). These studies demonstrated that ERK phosphorylates serine or threonine followed by proline ((S/T)P). In addition, a proline at position −2 is favorable, whereas a proline at position −1 is unfavorable (the phosphoacceptor S/T is position 0). However, this information was not sufficient to explain how ERK recognizes specific proteins as substrates, because many proteins that contain (S/T)P sequences are not

phosphorylated by ERK. Furthermore, it did not explain fully how ERK phosphorylates specific sites in substrates with multiple (S/T)P motifs. More recently, some ERK substrates have been shown to contain motifs that mediate high affinity interactions with ERK; we refer to such motifs as docking sites (12). Sharrocks and colleagues (13–15) showed that the D-domain, a conserved motif in the Elk subfamily of ETS transcription factors, is a docking site for ERK. The D-domain is characterized by a cluster of basic residues followed by an LXL motif. Related motifs mediate ERK binding to phosphatases (16–19), MEK (20–23), phosphodiesterase (24), and the ribosomal S6 kinase pp90^{rsk} (25, 26). Structural studies have not yet revealed how ERK interacts with docking sites. However, mutagenesis of ERK has identified a groove in ERK that is necessary for interacting with the basic residues in these docking motifs (19, 27, 28). We reported that FXFP, a motif that is conserved in ETS transcription factors of the Elk subfamily, is a docking site for ERK (15, 29, 30). The FXFP docking site also mediates ERK binding to KSR proteins and phosphodiesterase (24, 30). We proposed that the D-domain and FXFP form a modular system of docking sites (30). The domain of ERK that interacts with the FXFP motif has yet to be defined. These findings indicate that ERK recognizes specific substrate proteins by binding to docking sites and that there are at least two different classes of ERK docking sites.

Here we investigate the function of ERK docking sites by creating a series of mutant versions of human ELK-1 and *C. elegans* KSR-1 that contain different numbers and types of docking sites arranged in different positions and combinations. Our results indicate that the modular system of docking sites is highly flexible and accomplishes two significant goals. First, the number, position, and type of docking sites determine the affinity of a substrate protein and ERK. Second, the position and type of docking sites direct ERK phosphorylation of specific (S/T)P residues. These findings address how many unrelated proteins may have evolved to become ERK substrates that are phosphorylated on specific residues.

EXPERIMENTAL PROCEDURES

Protein Production and Peptide Synthesis—Plasmids that express GST:Elk-1 and GST:KSR-1 were constructed from cloned cDNAs (31–33) and pGEX vectors (Amersham Pharmacia Biotech). Plasmids that express GAL4:Elk-1 were constructed from pcDNA3a-GAL4-Elk1-COOH (31). These plasmids were modified by *in vitro* mutagenesis using standard techniques (34). The DNA sequence of the polymerase chain reaction-amplified region was determined to confirm the presence of the engineered mutation and the absence of extraneous mutations. GST fusion proteins were expressed in *Escherichia coli* BL21, partially purified using glutathione-Sepharose (Amersham Pharmacia Biotech) essentially according to the manufacturer's instructions, and dialyzed into kinase assay buffer. The amount of intact protein was estimated by comparison to known amounts of purified bovine serum albumin present in adjacent lanes of Coomassie Blue-stained SDS-polyacrylamide gels. Our purified extracts usually contained some proteins that are larger or smaller than the intact fusion protein and appear to be endogenous *E. coli* proteins and fragments of the fusion protein, respectively. Purified myelin basic protein was from Life Technologies, Inc. Peptides were synthesized using standard Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry and purified using high pressure liquid chromatography by the Tufts Core Facility (Boston, MA).

Assays of ERK Activity—Purified, recombinant murine ERK2 (New England Biolabs) is produced in *E. coli* containing constitutively active MEK and, thus, is phosphorylated and active. To calculate V_{max}, we defined 1 unit of ERK2 activity as the ability to transfer 200 pmol of PO$_4$/min to myelin basic protein; the activity was determined by the manufacturer. Assays were performed as described previously (30, 35). A 50-μl reaction contained 100 μM ATP to which was added 0.79 μCi of [^{32}P]ATP (4500 Ci/mmol) and 1 unit of ERK2. Reactions were terminated after 15 min at 30 °C, at which point ^{32}P incorporation was linear with respect to time. SDS-polyacrylamide gel electrophoresis and autoradiography were used to establish that intact fusion protein contained most or all of the incorporated ^{32}P. To quantify phosphorylation, we measured radioactive protein bound to phosphocellulose paper (P81, Whatman) using a scintillation counter. To compare the K_m, V_{max}, or IC$_{50}$ values of two substrates, we employed a one-sided t test. Values were judged to be significantly different when the p value was ≤0.05 and not significantly different when the p value was ≥0.1.

Cell Culture and Luciferase Assays—Human embryonic kidney epithelial 293 cells and mouse fibroblast NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Summit Biotechnology), 2.5 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were plated in 6-well dishes (5×10^5, 293 cells/well, and 1.5×10^4, NIH 3T3 cells/well) and transfected the following day by calcium phosphate precipitation using standard techniques (34). Luciferase was assayed in 293 cells by cotransfecting one pcDNA3a-GAL4-Elk-1-COOH plasmid (1 ng/well), the UAS::luciferase reporter plasmid (630 ng/well) (pFR-LUC, Stratagene), the CMV-β-galactosidase plasmid (68 ng/well), and in some cases the constitutively active MEK plasmid (25 ng/well) (pFC-MEK1, Stratagene). Two days after transfection, cells were grown in media containing 0.1% fetal bovine serum. Three days after transfection, cells were resuspended in 300 μl of β-galactosidase enzyme assay lysis buffer (Promega) according to the manufacturer's protocol (Promega Technical Bulletin No. 097). Luciferase activity was measured by automated injection of 50 μl of luciferin (0.4 mM) into 50 μl of cell lysate using a Tropix TR717 Microplate Luminometer. β-Galactosidase activity was measured by incubating 50 μl of cell lysate with 50 μl of β-galactosidase reaction buffer (120 mM Na$_2$HPO$_4$, 80 mM NaH$_2$PO$_4$, 2 mM MgCl$_2$, 100 mM β-mercaptoethanol, and 1.33 mg/ml *o*-nitrophenyl-β-galactopyranoside) at 37 °C until a faint yellow color appeared. Reactions were stopped by addition of 150 μl of 1.0 M sodium carbonate and analyzed using a spectrophotometer at 420 nm. Luciferase was assayed in NIH 3T3 cells using the same procedure except that pFC-MEK1 was not used, and ERK activity was stimulated by the addition of fibroblast growth factor to a final concentration of 10 ng/ml (Collaborative Biomedical Products) 2 days after transfection.

Western Blots—Partially purified GST fusion proteins were phosphorylated by ERK, resolved by SDS-polyacrylamide gel electrophoresis, and transferred to a nitrocellulose membrane. α-Phospho-ELK-1 Ser-383 (New England Biolabs) was incubated with the membrane, and chemiluminescent detection of horseradish peroxidase-conjugated secondary antibody was performed using standard techniques (34). This antibody was raised against residues 379–392. The mutations that alter the FQFP motif and the D-domain do not affect residues 379–392. Thus, this antibody should be able to recognize these mutant forms of ELK-1 if they are phosphorylated on serine 383. 293 cells were plated in 6-well dishes, cotransfected with GAL4:Elk-1 fusion construct (100 ng/well) and pFC-MEK1 (25 ng/well). Two days after transfection, cells were grown in media containing 0.1% fetal bovine serum. Three days after transfection, cells were washed twice in phosphate-buffered saline and resuspended in boiling 2× Laemmli Sample Buffer (Bio-Rad). Genomic DNA was sheared with a 28-gauge needle, and cellular debris was removed by centrifugation. Western blots were prepared and analyzed as described above and treated with an α-ELK-1 antibody (New England Biolabs) or an α-phospho-ELK-1 Ser-383 antibody.

RESULTS

Determination of the Primary Sequence of the FXFP Docking Site—FXFP motifs in physiological substrates have been demonstrated to mediate high affinity interactions with ERK in the following cases: ETS transcription factors of the Elk subfamily including *C. elegans* LIN-1, human ELK-1 and SAP-1, and *Drosophila melanogaster* AOP/YAN; KSR protein kinases including *C. elegans* KSR-1, *D. melanogaster* KSR, and murine KSR; and human phosphodiesterase PDE4D (Table I). These motifs all contain phenylalanine in positions 1 and 3 (the first phenylalanine is position 1). Position 2 usually contains glutamine, but there are examples of leucine, serine, and asparagine. Position 4 usually contains proline, but there are examples of histidine and glutamic acid. These observations provide information about primary sequences that can function as FXFP motifs. However, this group of motifs may not represent the full range of sequences that can function as a docking site because, except for the motif in LIN-1 that was identified by random mutagenesis and functional screening, all of these motifs were identified based on their similarity to the FQFP

Docking Sites for ERK Direct Phosphorylation of (S/T)P Sites

TABLE I

Physiological ERK substrates that contain FXFP motifs that have been demonstrated to mediate interactions with ERK

Gene (species)	Protein	FXFP motif	Ref.
lin-1 (C. elegans)	ETS transcription factor	QV FQFP PV	30
ELK-1 (Homo sapiens)	ETS transcription factor	LS FQFP SS	30
SAP-1 (H. sapiens)	ETS transcription factor	TL FQFP SV	15
Aop/Yan (D. melanogaster)	ETS transcription factor	DK FQFH PL	30
ksr-1 (C. elegans)	Protein kinase	PA FQFP DT	30 and this study
		KK FLFP DT	
ksr-1 (Mus musculus)	Protein kinase	SR FSFP DI	30
Ksr (D. melanogaster)	Protein kinase	SQ FNFP NV	30
PDE4D (H. sapiens)	Phosphodiesterase	EK FQFE LT	24

sequence of LIN-1 or other members of this group. It is important to determine the sequence requirements of this docking site in order to identify additional ERK substrates and to understand the mechanism of interaction between ERK and this docking site.

To investigate how amino acid substitutions of the FQFP motif affect binding to ERK, we used *in vitro* mutagenesis and peptide synthesis to generate a series of mutant versions of human ELK-1. ELK-1 protein contains 428 amino acids including an ETS DNA binding domain and two docking sites for ERK, the D-domain (residues 310–323) and FQFP (residues 395–398) (Fig. 1*a*) (13, 30, 36). To establish an assay system that would facilitate characterization of the FQFP motif, we modified ELK-1 in four ways. First, we chose to analyze ELK-1 residues 307–428; this region of ELK-1 contains nine (S/T)P motifs that are potential sites of ERK phosphorylation (Fig. 1). Seven of these (S/T)P sites have been shown to be phosphorylated by ERK (37–40). Second, ELK-1 was fused to glutathione *S*-transferase (GST) to facilitate affinity purification. Third, to eliminate binding mediated by the D-domain and reveal the role of the FQFP motif, we mutated the D-domain by changing the conserved LXL motif to AEA. This change significantly reduces the function of the D-domain (13). This protein is designated GST:Elk-1(307–428 −/FQFP) (Fig. 1*b*). Fourth, one or more residues in the FQFP motif were mutated.

Proteins were produced in *E. coli*, partially purified by affinity chromatography, and assayed as substrates using purified, recombinant, murine ERK2. Increasing the concentration of substrate protein resulted in saturation of phosphate incorporation (Fig. 2*a*). These data were used to determine K_m and V_{max} values and the relative acceptor ratio (RAR, V_{max}/K_m) (Fig. 2*c*). To analyze additional mutants, we synthesized a series of pentadecapeptides based on the primary sequence of ELK-1 (residues 387–399) (Fig. 1*a* and 2*d*). Each peptide contains a single SP acceptor site that corresponds to serine 389. These peptides were assayed as substrates for ERK as described above. Furthermore, these peptides can inhibit ERK. The concentration of peptide that was necessary to inhibit phosphorylation of a protein substrate by 50% (IC_{50}) was measured for each peptide (Fig. 2*b*). The peptides appear to function as competitive inhibitors that bind to ERK and, thus, interfere with ERK phosphorylation of substrate proteins. Thus, IC_{50} is a measure of the binding affinity between the peptide and ERK.

GST:Elk-1(307–428 −/FQFP) had a K_m of 7.3 μM (Fig. 2*c*). Changing the phenylalanine at position 1 to alanine increased the K_m about 7-fold to 56 μM ($p \leq 0.025$) and had no significant affect on the V_{max} ($p \geq 0.3$). Because tyrosine is structurally related to phenylalanine, we tested the effect of tyrosine at position 1; GST:Elk-1(307–428 −/YQFP) had a K_m of 7.1 μM, similar to wild type, although it had a significantly lower V_{max} ($p \leq 0.01$). The same mutations were analyzed in peptides. The wild-type peptide containing FQFP had a K_m of 51 μM (Fig. 2*d*). This is substantially higher than the K_m of the protein sub-
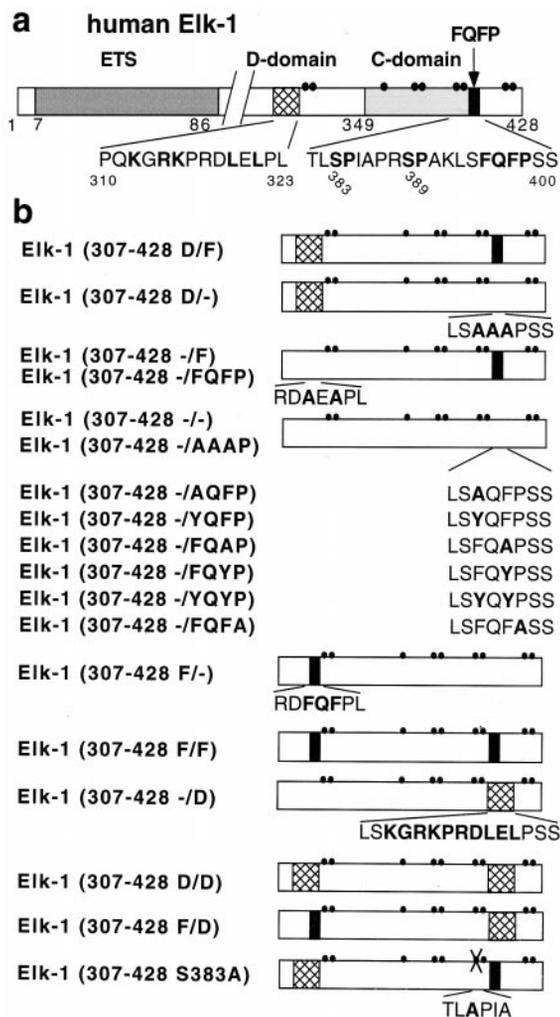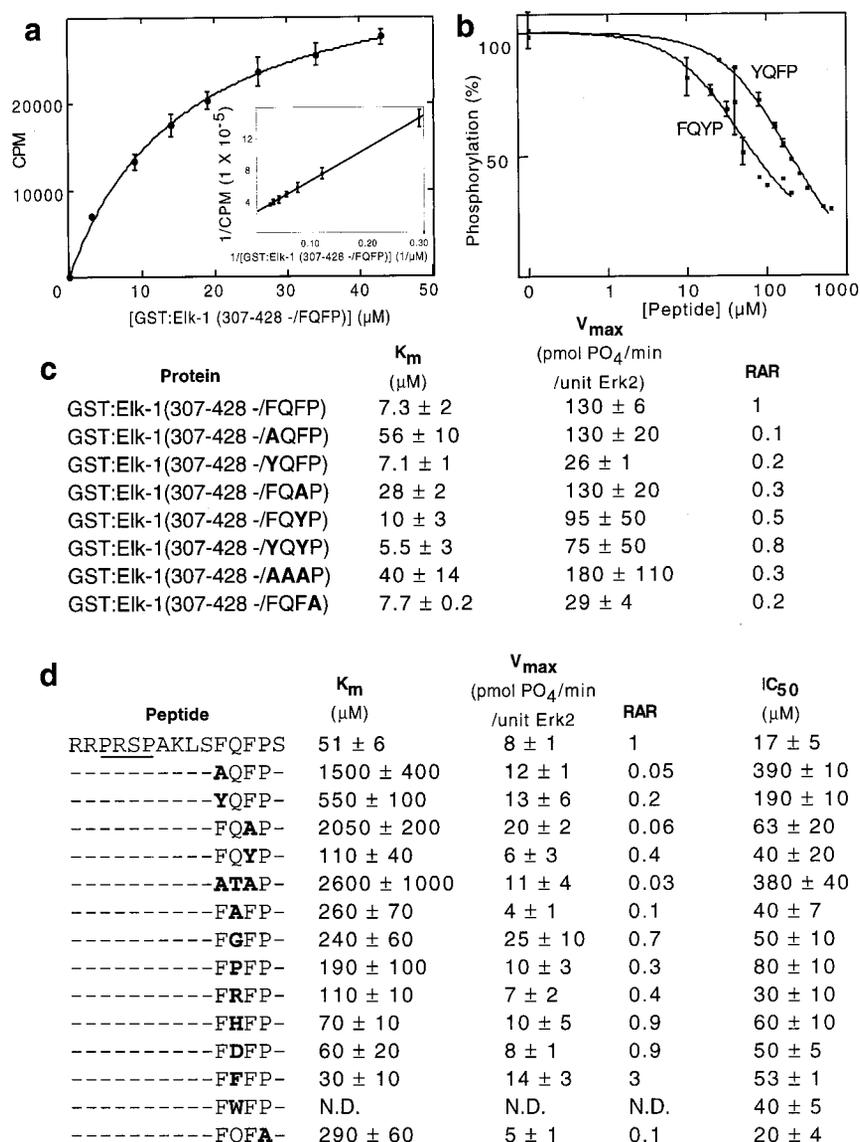

FIG. 1. **Wild-type and mutant versions of human ELK-1.** *a*, numbers indicate the first and last residue of the protein domains (36). The amino acid sequence of the D-domain (*hatched*) including the FQFP motif (*black*) are shown. Highly conserved residues and SP sites are *bold*. *Black circles* indicate (S/T)P sites. *b*, ELK-1 residues 307–428 were fused to GST and expressed in *E. coli* or fused to GAL4 and expressed in vertebrate cultured cells. The names in the *left-hand column* describe only the ELK-1 portion of these fusion proteins. Wild-type ELK-1 is designated *D/F* to indicate that it contains an N-terminal D-domain and a C-terminal FQFP. Mutagenesis was used to eliminate the activity of the D-domain (−) or to convert it to an FQFP motif (*F*). The altered amino acids are shown in *bold type* and depicted as *no box* (−) or a *black box* (*F*). Similarly, mutagenesis was used to eliminate the activity of the FQFP motif (−), alter a single residue (AQFP, etc.), or convert it to a D-domain (*D*). The names Elk-1(307–428 −/F) and Elk-1(307–428 −/FQFP) refer to the same protein. Similarly, Elk-1(307–428 −/−) and Elk-1(307–428 −/AAAP) designate the same protein.

strate that contains FQFP, indicating that residues other than 387–399 contribute to the affinity of the protein substrates for ERK. Changing the first position phenylalanine to alanine

c

Protein	K_m (μM)	V_{max} (pmol PO_4/min /unit Erk2)	RAR
GST:Elk-1(307-428 -/FQFP)	7.3 ± 2	130 ± 6	1
GST:Elk-1(307-428 -/**A**QFP)	56 ± 10	130 ± 20	0.1
GST:Elk-1(307-428 -/**Y**QFP)	7.1 ± 1	26 ± 1	0.2
GST:Elk-1(307-428 -/FQ**A**P)	28 ± 2	130 ± 20	0.3
GST:Elk-1(307-428 -/FQ**Y**P)	10 ± 3	95 ± 50	0.5
GST:Elk-1(307-428 -/**Y**Q**Y**P)	5.5 ± 3	75 ± 50	0.8
GST:Elk-1(307-428 -/**AAA**P)	40 ± 14	180 ± 110	0.3
GST:Elk-1(307-428 -/FQF**A**)	7.7 ± 0.2	29 ± 4	0.2

d

Peptide	K_m (μM)	V_{max} (pmol PO_4/min /unit Erk2)	RAR	IC_{50} (μM)
RRPRSPAKLSFQFPS	51 ± 6	8 ± 1	1	17 ± 5
---------**A**QFP-	1500 ± 400	12 ± 1	0.05	390 ± 10
---------**Y**QFP-	550 ± 100	13 ± 6	0.2	190 ± 10
---------FQ**A**P-	2050 ± 200	20 ± 2	0.06	63 ± 20
---------FQ**Y**P-	110 ± 40	6 ± 3	0.4	40 ± 20
---------**ATA**P-	2600 ± 1000	11 ± 4	0.03	380 ± 40
---------F**A**FP-	260 ± 70	4 ± 1	0.1	40 ± 7
---------F**G**FP-	240 ± 60	25 ± 10	0.7	50 ± 10
---------F**P**FP-	190 ± 100	10 ± 3	0.3	80 ± 10
---------F**R**FP-	110 ± 10	7 ± 2	0.4	30 ± 10
---------F**H**FP-	70 ± 10	10 ± 5	0.9	60 ± 10
---------F**D**FP-	60 ± 20	8 ± 1	0.9	50 ± 5
---------F**F**FP-	30 ± 10	14 ± 3	3	53 ± 1
---------F**W**FP-	N.D.	N.D.	N.D.	40 ± 5
---------FQF**A**-	290 ± 60	5 ± 1	0.1	20 ± 4

FIG. 2. **Mutagenesis of the FXFP docking site.** Each protein listed in *c* and each peptide listed in *d* were analyzed using the same experimental procedures; *a* and *b* show examples of data. *a,* a kinetic analysis showing incorporated [32P] measured by filter binding and scintillation counting (*cpm*) using increasing concentrations of GST:Elk-1(307–428 −/FQFP). Values are the average of two samples; a *bar* indicates the range. The *inset* shows a Lineweaver-Burk plot of the data. *b,* inhibition of ERK2 phosphorylation of a protein substrate was measured by adding increasing concentrations of the FQYP peptide or the YQFP peptide to reactions containing [32P]ATP and 3 μM GST:Elk-1(307–428 D/F). [32P] incorporation was measured by filter binding and scintillation counting. Values were normalized by assigning a value of 100% to reactions containing no peptide and plotted on a logarithmic scale. Values are the average of two samples; a *bar* indicates the range. *c,* K_m and V_{max} values were calculated from kinetic analyses like that shown in *a*; in each case a Lineweaver-Burk plot of the data closely approximated a *straight line*. Values are the mean ± S.D. of two to five separate experiments. To determine V_{max}, we calculated total phosphate incorporation using the measured counts/min and the specific activity of the [32P]ATP, and we factored in the assay time and amount of ERK2 (expressed in units). RAR is V_{max}/K_m; values were normalized by assigning a value of 1.0 for GST:Elk-1(307–428 −/FQFP). *d,* each peptide contained 15 amino acids including a PRSP phosphoacceptor site (*underlined*). Peptides in the *2nd* to *15th* lines had the sequence shown in the *1st line* except for residues in the FQFP docking site that are *bold*. K_m and V_{max} values were calculated from kinetic analyses like that shown in *a*. In each case, a Lineweaver-Burk plot of the data closely approximated a *straight line*. Values are the mean ± S.D. of two separate experiments. RAR values were normalized by assigning a value of 1.0 to the peptide in the *1st line*. IC_{50} values (the concentration of peptide that reduced by 50% ERK phosphorylation of GST:Elk-1(307–428 D/F) were obtained from curves like that shown in *b*. Values are the mean ± S.D. of at least three separate experiments. The FWFP peptide functioned as an inhibitor but when measured as a substrate did not display linear phosphate incorporation using increasing peptide concentrations.

increased the K_m 30-fold to 1500 μM ($p ≤ 0.025$) and increased the IC_{50} from 17 to 390 μM ($p ≤ 0.005$). Substituting tyrosine for phenylalanine increased the K_m 11-fold to 550 μM ($p ≤ 0.01$) and increased the IC_{50} to 190 μM ($p ≤ 0.005$). These data indicate that the phenylalanine at position 1 is a critical determinant of affinity and that a tyrosine in position 1 can function to some extent.

Changing the phenylalanine at position 3 to alanine in GST:Elk-1(307–428 −/FQAP) increased the K_m about 4-fold to 28 μM ($p ≤ 0.005$) (Fig. 2*c*). Changing position 3 to tyrosine had a

small effect. In the peptides, changing position 3 to alanine increased the K_m about 40-fold to 2050 μM ($p ≤ 0.005$) and increased the IC_{50} about 4-fold ($p ≤ 0.05$) (Fig. 2*d*). Changing position 3 to tyrosine had smaller effects. Thus, the phenylalanine at position 3 is important for affinity, and tyrosine can function effectively in position 3.

The effect of simultaneously changing positions 1 and 3 was investigated. Changing both phenylalanine residues to alanine in GST:Elk-1(307–428 −/AAAP) resulted in a K_m of 40 μM (Fig. 2*c*). This value is similar to the K_m of a mutant with alanine at

position 1 ($p \geq 0.1$). Changing positions 1 and 3 to alanine in the peptide substrate increased the K_m to 2600 μM and the IC$_{50}$ to 380 μM (Fig. 2d); these values are similar to mutants with alanine at position 1 or 3. Changing positions 1 and 3 to tyrosine in GST:Elk-1(307–428 –/YQYP) resulted in a K_m of 5.5 μM, similar to the values of ELK-1 with phenylalanine in positions 1 and 3 ($p \geq 0.2$) (Fig. 2c). Thus, a motif with tyrosines in both of these positions displayed relatively high affinity.

The second position of the motif displays variation in physiological substrates (Table I). To determine the effect of different residues at position 2, we generated a series of nine peptides that differ only at position 2 (Fig. 2d). Alanine, glycine, or arginine at position 2 resulted in a small but significant increase in the K_m of about 2–5-fold ($p \leq 0.05$, $p \leq 0.025$, and $p \leq 0.001$). Proline, histidine, aspartic acid, and phenylalanine in position 2 were not significantly different than glutamine. These findings indicate that many residues can function in position 2.

Position 4 contains proline in most but not all physiological substrates (Table I). Missense mutations in *C. elegans lin-1* that change proline to leucine or serine appear to reduce the interaction with ERK, since they cause a vulval defective phenotype (29). Changing proline to alanine in GST:Elk-1(307–428 FQFA) did not increase the K_m value significantly ($p \geq 0.4$), but it did reduce the V_{max} value significantly ($p \leq 0.005$), and the relative acceptor ratio was 0.2 (Fig. 2c). Changing proline to alanine increased the K_m of a peptide about 6-fold ($p \leq 0.025$) but did not significantly effect IC$_{50}$ ($p \geq 0.2$) (Fig. 2d). Thus, in these assays the proline at position 4 makes a small contribution to affinity.

Docking Sites Are Important for Phosphorylation of ELK-1 in Cultured Cells—By using purified extracts of ELK-1 and ERK, we previously demonstrated that the D-domain and FQFP can function independently and that together they function additively to mediate phosphorylation by ERK (30). To evaluate how these docking sites mediate interactions with ERK in a more complex cellular environment, we used a well established assay that directly measures the ability of the C-terminal region of ELK-1 to stimulate transcription in cultured cells (38). This assay is an indirect measure of ELK-1 phosphorylation, since phosphorylation by ERK increases ELK-1 transactivation. This assay was conducted by treating cells as follows. 1) A chimeric transcription factor composed of the DNA-binding domain of GAL4 and the C-terminal transactivation domain of ELK-1, residues 307–428, was expressed by transient transfection of an expression plasmid. 2) A luciferase gene that is regulated by a basal promoter and upstream activating sequences that bind the GAL4 DNA-binding domain was introduced by transient transfection of a reporter plasmid. 3) ERK activity was stimulated by transient transfection of a plasmid encoding constitutively active MEK or by addition of fibroblast growth factor to the culture media. A plasmid encoding β-galactosidase regulated by a constitutively active promoter was cotransfected, and β-galactosidase activity was used to control for transfection efficiency. Two different cultured cell lines were used, 293 human embryonic kidney cells and NIH 3T3 murine fibroblasts. ERK was activated by transfection of MEK in 293 cells and by exogenous fibroblast growth factor in NIH 3T3 cells. Similar results were obtained with both cell lines.

Expression of the GAL4 DNA-binding domain alone did not result in significant luciferase expression irrespective of ERK activation (Fig. 3). Expression of GAL4 fused to wild-type ELK-1, GAL4:Elk-1(307–428 D/F), increased luciferase expression in unstimulated cells; stimulation of ERK further increased luciferase 21-fold in 293 cells and 36-fold in NIH 3T3
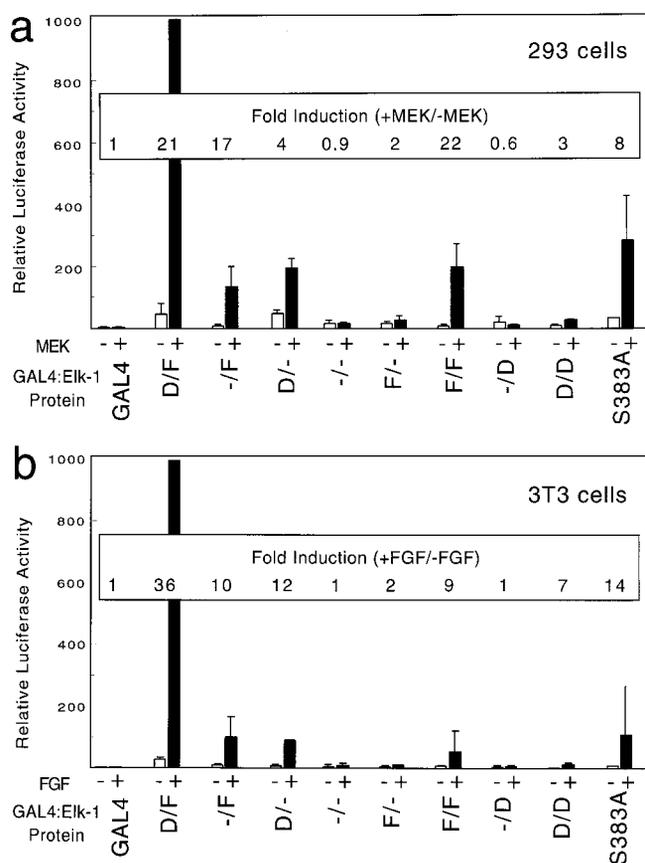
FIG. 3. **Docking sites are necessary for ELK-1 activity in cultured cells.** *a,* 293 human embryonic kidney cells were transiently transfected with an expression plasmid that encoded the GAL4 DNA binding domain and no ELK-1 (GAL4) or the GAL4 DNA binding domain fused to a fragment of ELK-1 that is shown in Fig. 1 and abbreviated below. For example, GAL4:Elk-1(307–428 D/F) is labeled *D/F*. Some cells were cotransfected with a plasmid that expresses constitutively active MEK (+). All cells were cotransfected with a luciferase reporter plasmid and a control plasmid that expresses β-galactosidase. Cell extracts were assayed for luciferase and β-galactosidase activities. Relative luciferase activity was determined by assigning a value of 1000 to the luciferase activity of GAL4:Elk-1(307–428 D/F) + MEK and adjusting other luciferase values based on β-galactosidase activity to control for variation in transfection efficiency. The values are the mean of 5–12 independent transfections; a *bar* indicates the standard deviation. The fold induction is the ratio of the relative luciferase activity in the presence and absence of MEK. Small amounts (1 ng) of expression plasmids were transfected because this optimized the transcriptional regulatory activity of GAL4:Elk-1(307–428 D/F) (data not shown). By using these transfection conditions, we were not able to detect GAL4:Elk-1 protein using Western blotting. However, transfection of 100 ng of expression plasmids resulted in GAL4:Elk-1 protein expression that could be detected by Western blotting, and all the mutant proteins displayed similar expression levels (see Fig. 5). Thus, we conclude that the reduced activity of mutant proteins is not a result of reduced expression. *b,* NIH 3T3 mouse fibroblasts were treated and analyzed as described above, except that fibroblast growth factor (*FGF*) was used to stimulate ERK. The values are the mean of 6–9 independent transfections. It is notable that the pattern of activity displayed by the mutant proteins was similar in the two cell lines.

cells (Fig. 3). These results are similar to previous reports and indicate that the C-terminal region of ELK-1 is a transactivation domain, and phosphorylation by ERK significantly increases transactivation (38–40). The transactivation observed in unstimulated cells may be caused by unphosphorylated ELK-1 that is weakly active and/or a fraction of ELK-1 that is phosphorylated, as there is likely to be some active ERK in unstimulated cells.

To determine the role of docking motifs, we expressed mutant versions of ELK-1 that lacked the D-domain (GAL4:Elk-

TABLE II

The FQFP and D-domain docking sites can function in novel positions

GST:Elk-1 fusion proteins are described in Fig. 1. Partially purified fusion proteins were phosphorylated by murine ERK2 as described in Fig. 2. K_m and V_{max} values were calculated from kinetic analyses; in each case a Lineweaver-Burk plot closely approximated a straight line. Values are the mean ± S.D. of three to five separate experiments. RAR values were normalized by assigning a value of 1.0 to V_{max}/K_m of GST:Elk-1 (307–428 D/F).

Protein	K_m	V_{max}	RAR
	μM	$pmol\ PO_4/$ $min/unit$ $ERK2$	
GST:Elk-1 (307–428 D/F)	0.8 ± 0.3	100 ± 3	1
GST:Elk-1 (307–428 −/F)	7.3 ± 2	130 ± 6	0.1
GST:Elk-1 (307–428 D/−)	3.7 ± 1.4	140 ± 30	0.3
GST:Elk-1 (307–428 −/−)	40 ± 14	180 ± 110	0.04
GST:Elk-1 (307–428 −/D)	2.1 ± 0.3	100 ± 10	0.4
GST:Elk-1 (307–428 D/D)	2.3 ± 0.7	210 ± 40	0.7
GST:Elk-1 (307–428 F/−)	5 ± 3	150 ± 50	0.2
GST:Elk-1 (307–428 F/F)	2 ± 0.4	110 ± 30	0.4
GST:Elk-1 (307–428 F/D)	5.5 ± 3	150 ± 10	0.2
GST:Elk-1 (307–428 S383A)	0.8 ± 0.2	110 ± 10	1

1(307–428 −/F)), the FQFP motif (GAL4:Elk-1(307–428 D/−)), or both motifs (GAL4:Elk-1(307–428 −/−)). Mutation of the D-domain decreased transactivation significantly in unstimulated and stimulated cells; the fold induction was also reduced, but ERK stimulation still had a significant effect (Fig. 3). Similarly, mutation of the FQFP motif reduced transactivation significantly in unstimulated and stimulated cells; the fold induction was reduced but not eliminated. While multiple (S/T)P sites in ELK-1 are phosphorylated by ERK and can contribute to transactivation, a mutation of serine 383 reduces transactivation more than a mutation of any other (S/T)P site (38, 39). We found that a mutant of ELK-1 with alanine at position 383, GAL4:Elk-1(307–428 S383A), displayed reductions in transactivation in stimulated and unstimulated cells that were similar to reductions caused by mutations in one of the docking sites (Fig. 3). Mutation of both the D-domain and the FQFP motif reduced transactivation dramatically in unstimulated and stimulated cells and eliminated the induction caused by ERK. These results are very similar to the findings with ERK and ELK-1 in purified extracts; they indicate that the D-domain and the FQFP motif can function independently, and together they function additively to mediate ERK phosphorylation of ELK-1. Galanis *et al.* (15) recently reported comparable findings with SAP-1, an ETS transcription factor that is similar to ELK-1.

The FQFP and D-domain Docking Sites Can Function in Novel Positions—To investigate whether the D-domain and the FQFP motif are capable of functioning in novel positions and how position affects the docking site function, we generated mutant versions of ELK-1 that contain the D-domain in the position normally occupied by FQFP, GST:Elk-1(307–428 −/D) and GST:Elk-1(307–428 D/D), or FQFP in the position normally occupied by the D-domain, GST:Elk-1(307–428 F/−) and GST:Elk-1(307–428 F/F), or both substitutions, GST:Elk-1(307–428 F/D) (Fig. 1b). GST:Elk-1(307–428 −/D) had a K_m of 2.1 μM, and GST:Elk-1(307–428 F/−) had a K_m of 5 μM (Table II, 5th and 7th lines); these values are significantly lower than the 40 μM K_m value of GST:Elk-1(307–428 −/−) ($p \le 0.05$ and $p \le 0.025$), which has no docking sites, demonstrating that both motifs are sufficient to function effectively in novel positions. All three substrates containing combinations of docking sites in novel positions were good substrates that had K_m values that were similar to or slightly lower than substrates containing a single docking site (Table II, 6th, 8th, and 9th lines).

To investigate how these novel positions and combinations of docking motifs affect ELK-1 activity in cultured cells, we generated the same mutants fused to GAL4 and measured transcriptional activation in the presence and absence of ERK stimulation. An FQFP motif in the native D-domain position had no significant effect in GAL4:Elk-1(307–428 F/−) or GAL4:Elk-1(307–428 F/F) (Fig. 3). A D-domain in the native FQFP position had no significant effect in GAL4:Elk-1(307–428 −/D) and reduced transcriptional activation in GAL4:Elk-1(307–428 D/D) (Fig. 3). Thus, although ectopically positioned docking domains increase the affinity of ELK-1 for ERK and promote phosphorylation in purified extracts, they do not increase transcriptional activation of ELK-1 by ERK in cultured cells. It is possible that the inserted docking motifs interfere with transcriptional activation by ELK-1 or result in phosphorylation of (S/T)P motifs that do not contribute to transcriptional activation.

KSR Proteins Use a Modular System of FXFP Docking Sites—The KSR protein kinase is likely to have a conserved function as a physiological ERK substrate based on the following observations. 1) *C. elegans*, *Drosophila*, and murine KSR contain FXFP docking sites (Table I). 2) These three proteins function as high affinity ERK substrates in purified extracts (30, 41, 42). 3) Murine KSR is phosphorylated on (S/T)P residues in cultured cells, and this phosphorylation correlates with ERK activity (41, 43). *C. elegans* KSR-1 contains two FXFP motifs that flank a TP phosphorylation site (Fig. 4a). By contrast, *Drosophila* and murine KSR contain a single FXFP motif that is homologous to the C-terminal FXFP motif of *C. elegans* KSR-1. Thus, KSR proteins have diverged during evolution and display two different arrangements of docking motifs.

To characterize the function of the two FXFP motifs in *C. elegans* KSR-1, we analyzed KSR-1 residues 281–479; this region contains both FXFP motifs and a single TP acceptor site. This region was fused to GST to facilitate affinity purification. *In vitro* mutagenesis was used to remove either one or both motifs (Fig. 4b). GST:KSR-1 fusion proteins were produced in *E. coli*, partially purified by affinity chromatography, and assayed as substrates with purified, murine ERK2. Removing the C-terminal FLFP motif did not significantly affect the K_m ($p \ge 0.1$), whereas removing the N-terminal FQFP increased the K_m from 1.2 to 3.4 μM ($p \le 0.05$) (Fig. 4c). Removing both FXFP motifs increased the K_m further to 25 μM ($p \le 0.025$). These findings indicate that the two FXFP motifs can function independently. However, they do not display significant additivity. It is notable that the N-terminal motif, which is not evolutionarily conserved, mediates stronger affinity.
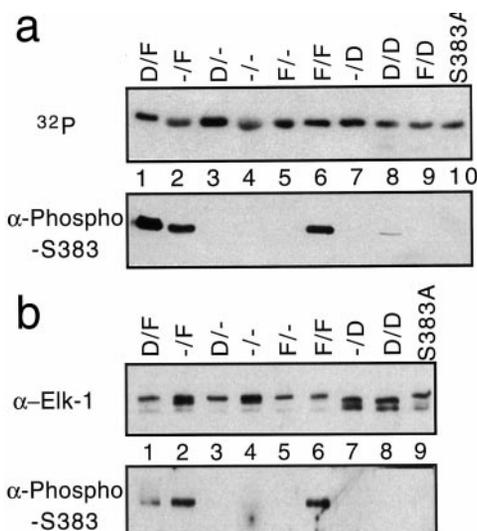
The D-domain can function in an ectopic position in ELK-1. To investigate whether the D-domain could function in additional positions, we used *in vitro* mutagenesis to engineer GST:KSR-1(281–479) proteins containing N- or C-terminally positioned D-domains (Fig. 4b). Introducing a D-domain in the N-terminal position had little effect on K_m, since the 18 μM K_m value of GST:KSR-1(281–479 D/−) was similar to the 25 μM K_m value of GST:KSR-1(281–479 −/−) ($p \ge 0.2$), and the 3.6 μM K_m value of GST:KSR-1(281–479 D/F) was similar to the 3.4 μM K_m value of GST:KSR-1 (281–479 −/F) ($p \ge 0.4$) (Fig. 4c). However, it significantly increased the V_{max} values of these substrates ($p \le 0.005$) and ($p \le 0.05$). Introducing a D-domain in the C-terminal position mediated binding to ERK since the 3.1 μM K_m value of GST:KSR-1(281–479 −/D) was lower than the 25 μM K_m value of GST:KSR-1(281–479 −/−) ($p \le 0.025$) (Fig. 4c). By contrast to the D-domain in the C-terminal position, which resulted in a high V_{max}, the D-domain in the N-terminal position resulted in a very low V_{max}. A substrate with two D-domains, GST:KSR-1(281–479 D/D), displayed K_m and
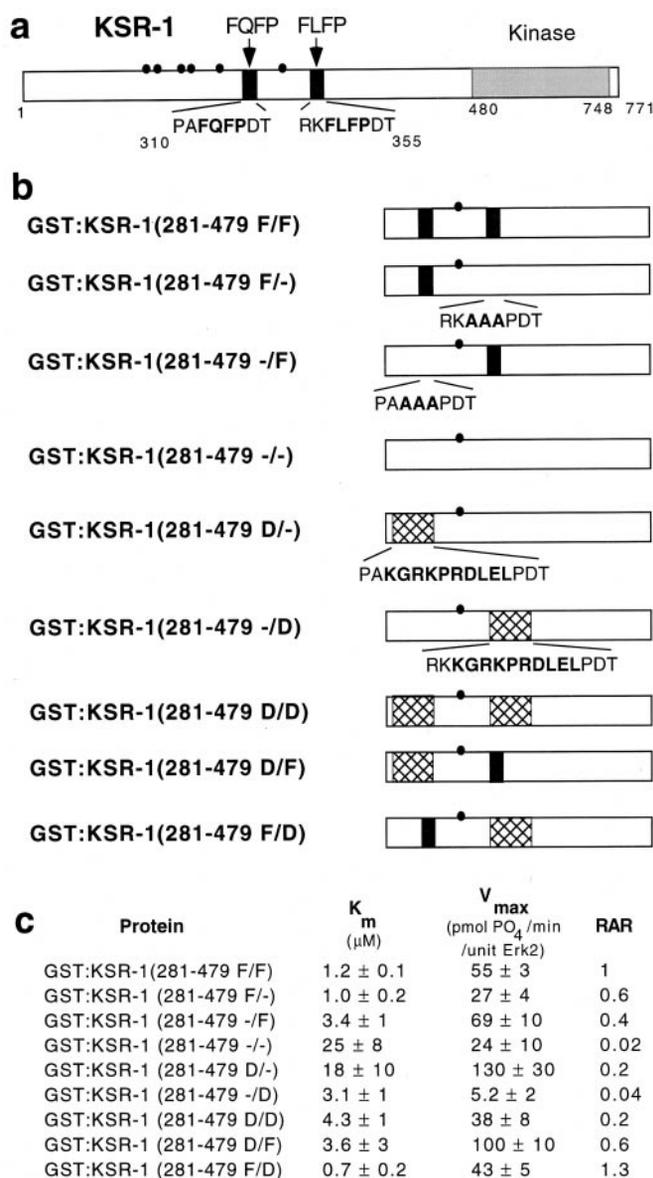
FIG. 4. **KSR-1 has a modular system of FXFP docking sites.** *a,* numbers indicate the first and last residue of the protein and domains (32). The sequences of the two F*X*FP docking sites (*black boxes*) are shown. *Black circles* indicate (S/T)P sites. *b,* KSR-1 residues 281–479 were fused to GST. Wild-type KSR-1 is designated *F/F* to indicate that it contains an N-terminal and a C-terminal F*X*FP motif. Mutagenesis was used to eliminate the activity of the F*X*FP motifs (−, *no black box*) or to convert F*X*FP motifs to D-domains (*D, hatched box*). The altered amino acids are shown in *bold type. c,* GST:KSR-1 fusion proteins were expressed in *E. coli*, partially purified, and phosphorylated by murine ERK2. K_m and V_{max} values were calculated from kinetic analyses; in each case a Lineweaver-Burk plot closely approximated a *straight line*. Values are the mean ± S.D. of three to five separate experiments. RAR values were normalized by assigning a value of 1.0 to GST:KSR(281–479 F/F).

FIG. 5. **Docking sites direct phosphorylation of specific (S/T)P sites**. *a,* GST:Elk-1 fusion proteins were produced in *E. coli*, partially purified, and phosphorylated by ERK2 using an amount of substrate equal to one-half of the K_m value of that substrate (Table II) and [^{32}P]ATP. Phosphate incorporation was measured by filter binding and scintillation counting. Protein samples with approximately equal phosphate incorporation were resolved by gel electrophoresis, transferred to a nitrocellulose membrane, and analyzed by autoradiography (^{32}P, *upper panel*) and Western blot analysis using an antibody that reacts with ELK-1 protein phosphorylated at serine 383 (α-phospho-Ser-383, *lower panel*). The GST:Elk-1 fusion proteins that were analyzed in *lanes 1–10* are shown in Fig. 1 and abbreviated names are given *above* the lanes. For example, GST:Elk-1(307–428 D/F) is called *D/F*. The autoradiogram demonstrates that similar amounts of phosphorylated substrates were loaded in each lane. The *lower panel* demonstrates that only the proteins containing FQFP in its native position displayed significant reactivity with the anti-phospho-Ser-383 antibody. The stoichiometry of phosphate incorporation for the GST:Elk-1(307–428 D/F) is 0.4 PO4/molecule, and it is lower for the other proteins, since they have higher K_m values. To control for the possibility that large amounts of unphosphorylated protein might interfere with antibody detection, we mixed phosphorylated GST:Elk-1(307–428 D/F) with each mutant protein and analyzed the protein mixtures on a Western blot. These samples displayed strong immunoreactivity, suggesting that the presence of unphosphorylated proteins did not cause the observed differences in immunoreactivity (data not shown). These results are representative of three separate experiments. *b,* GAL4:Elk-1 fusion proteins were expressed in 293 cells by transient transfection of 100 ng/well of expression plasmids. Protein extracts were analyzed by Western blotting using an antibody that reacts with ELK-1 protein (α-ELK-1, *upper panel*), and blots were then analyzed with the anti-phospho-Ser-383 antibody (*lower panel*). The names of GAL4:Elk-1 fusion proteins are abbreviated as in *a*. The *upper panel* demonstrates that approximately equal amounts of GAL4:Elk-1 were loaded in each lane. Although the amount of cell extract loaded in each lane was adjusted somewhat, in general each mutant protein was expressed at similar levels 3 days after transient transfection. The *lower panel* demonstrates that only GAL4:Elk-1 proteins with FQFP in the native position display significant immunoreactivity. These results are representative of three separate experiments.

V_{max} values that were between the values of mutants containing a single D-domain (Fig. 4*c*). These findings indicate that the D-domain can function in additional ectopic positions and that it can significantly affect both the K_m and V_{max} values of ERK.

Docking Sites Direct Phosphorylation of Specific (S/T)P Sites—The fragment of ELK-1 used in our assays, residues 307–428, contains nine (S/T)P motifs. Kinetic analysis of phosphorylation using purified extracts and measurements of transactivation in cultured cells do not reveal which of these site(s) are phosphorylated. To investigate whether different combinations of docking sites result in different patterns of phosphorylation, we analyzed the pattern of ELK-1 phosphorylation by using a commercially available antibody that specifically reacts with ELK-1 phosphorylated at serine 383. Ten different GST:Elk-1 fusion proteins were produced in *E. coli*, partially purified, and phosphorylated by purified murine ERK2. Incorporation of ^{32}P was measured, and this information was used to apply protein samples with similar phosphate incorporation to a Western blot (Fig. 5*a, upper panel*). This blot was treated with the anti-phosphoserine 383 antibody (Fig. 5*a, lower panel*). The wild-type protein reacted strongly with the antibody, whereas unphosphorylated GST:Elk-1(307–428 D/F) and a mutant with alanine replacing serine 383, GST:Elk-1(307–428 S383A), gave no detectable signal (Fig. 5*a, lanes 1* and *10* and data not shown). This is consistent with the re-

ported specificity of the antibody.

Mutation of the D-domain in GST:Elk-1(307–428 −/F) had little effect on phosphorylation of serine 383 (Fig. 5*a, lane 2*). By contrast, mutation of the FQFP motif eliminated detectable phosphorylation of serine 383 (Fig. 5*a, lane 3*). These findings indicate that FQFP in its native position is both necessary and sufficient to direct phosphorylation of serine 383, whereas the D-domain in its native position is neither necessary nor sufficient to direct phosphorylation of serine 383. However, the D-domain in its native position is sufficient to direct phosphorylation of other (S/T)P residues. Insertion of the D-domain in the C-terminal position of the FQFP motif in GST:Elk-1(307–428 −/D) or GST:Elk-1(307–428 D/D) was not sufficient to direct significant phosphorylation of serine 383, although GST:Elk-1(307–428 D/D) displayed faint reactivity (Fig. 5*a, lanes 7* and *8*). Insertion of the FQFP motif in the N-terminal position was not sufficient to cause phosphorylation of serine 383 in GST:Elk-1(307–428 F/−) and GST:Elk-1(307–428 F/D) (Fig. 5*a, lanes 5* and *9*). GST:Elk-1(307–428 F/F) was phosphorylated on serine 383 (Fig. 5*a, lane 6*). Of the nine proteins with different combinations of docking sites, all three that had FQFP in its native position displayed phosphorylation of serine 383, whereas none of the six that lacked FQFP in its native position displayed significant phosphorylation of serine 383.

To investigate how these ELK-1 mutants are phosphorylated in a cellular environment, we expressed GAL4:Elk-1 fusion proteins and constitutively active MEK in 293 cells and prepared protein extracts. A commercially available anti-ELK-1 antibody reacted with all the samples, indicating that all the mutant proteins were expressed. Approximately equal amounts of immunoreactivity were applied to each lane of a Western blot (Fig. 5*b, upper panel*). The blot was then probed with anti-phosphoserine 383 antibody; GAL4:Elk-1(307–428 D/F), GAL4:Elk-1(307–428 −/F), and GAL4:Elk-1(307–428 F/F) reacted with the antiserum, whereas the other six proteins did not (Fig. 5*b, lower panel*). These results are very similar to the findings with purified proteins and support the conclusion that FQFP in its native position is necessary and sufficient to direct phosphorylation of serine 383.

DISCUSSION

Primary Sequence Requirements of the FXFP Docking Site—Information about the amino acid sequences that are sufficient to function as FXFP docking sites can be obtained by analyzing physiological ERK substrates with FXFP motifs (Table I). These motifs resulted from random mutagenesis and natural selection during evolution. The characterization of mutant motifs provides a second source of information. Here we analyzed mutations affecting the FQFP motif of ELK-1 that were generated by site-directed mutagenesis. The information provided by physiological substrates and mutagenesis is limited, since a relatively small number of physiological substrates have been characterized, and only a subset of possible mutant variations was tested. However, taken together these two sources of information provide substantial evidence about the primary sequence requirements of this motif.

All the motifs in physiological substrates contain phenylalanines at positions 1 and 3. Substituting alanine at either position significantly decreased the affinity for ERK, although position 1 appears somewhat more important than position 3. Tyrosine, which has a structure similar to phenylalanine, was highly functional in position 1 or 3. Thus, there may be motifs in physiological substrates that contain tyrosine in position 1 or 3.

Position 2 is glutamine in most physiological substrates, but serine, leucine, and asparagine have each been observed once. Eight other residues were investigated including aspartic acid (acidic), arginine (basic), alanine (small hydrophobic), phen-

ylalanine (large hydrophobic), histidine, glycine, proline, and tryptophan. In general, these substitutions affected the affinity of the motif by less than 2-fold, suggesting that the residue at position 2 provides only a small amount of affinity.

Position 4 is proline in most physiological substrates, but glutamic acid and histidine have each been observed once. Mutations of *C. elegans* LIN-1 that substitute leucine or serine at position 4 cause a vulvaless phenotype that indicates a reduced interaction between LIN-1 and MPK-1 ERK MAP kinase (29). Furthermore, substitution of position 4 proline with leucine in full-length LIN-1 increases the K_m for ERK about 4-fold. Here we show that substitution of position 4 proline with alanine reduces the effectiveness of the motif.

These findings indicate how the F*X*FP motif interacts with a binding pocket of ERK. This binding pocket has yet to be defined. The phenylalanines at positions 1 and 3 probably contact ERK directly and provide the majority of binding energy. The residue at position 2 probably contacts ERK directly as well but provides less binding energy. The proline at position 4 may not bind ERK directly. Instead, this proline may play a structural role that facilitates the direct binding of residues in positions 1–3. This is suggested by the structure of proline and the finding that some physiological motifs lack position 4 proline. These substrates may have alternative mechanisms that orient the residues in positions 1–3. The characterization of the amino acid sequences that can function as an F*X*FP docking site is also important because it facilitates identification of docking sites in proteins that are known ERK substrates and the prediction of additional ERK substrates.

Docking Sites Direct Phosphorylation of Specific (S/T)P Residues—ERK phosphorylates at least seven different (S/T)P residues in the ELK-1 C-terminal region (37–40). It is not known whether these residues are phosphorylated in a specific order and, if so, how that order is regulated. The ability of docking sites to regulate phosphorylation of specific (S/T)P residues was investigated using purified extracts and cultured cells. Purified extracts were used to analyze the initial phosphorylation events (*i.e.* the ELK-1 substrate was phosphorylated to low stoichiometry). The cultured cells expressed ELK-1 and activated MEK for about 3 days and, thus, are likely to have achieved a steady state level of phosphorylation. Although the experimental conditions were quite different, the results were almost identical. All three ELK-1 substrates containing FQFP in the native position showed significant phosphorylation of serine 383, whereas all five ELK-1 substrates lacking the FQFP in the native position did not show significant phosphorylation of serine 383. These observations indicate that an FQFP motif in its native position is both necessary and sufficient for significant phosphorylation of serine 383. Because phosphorylation of serine 383 is important for ELK-1 transcriptional activation, this function of the FQFP docking site is likely to be functionally significant. By contrast, neither an FQFP motif in an ectopic position nor a D-domain in the native position of the FQFP was sufficient to direct phosphorylation of serine 383. Instead, these other arrangements of docking sites were sufficient to direct phosphorylation of non-Ser-383 sites.

These observations led us to propose the model illustrated in Fig. 6. ERK can dock on ELK-1 by binding to either the FQFP motif or the D-domain. Docking on the D-domain directs phosphorylation of non-Ser-383 sites but not serine 383. Docking on the FQFP motif directs phosphorylation of serine 383. The FQFP motif likely directs phosphorylation of non-Ser-383 sites as well, since FQFP promotes phosphorylation of serine 389 in peptide substrates.

Docking sites for ERK have not been shown previously to direct phosphorylation of specific sites. However, this issue has
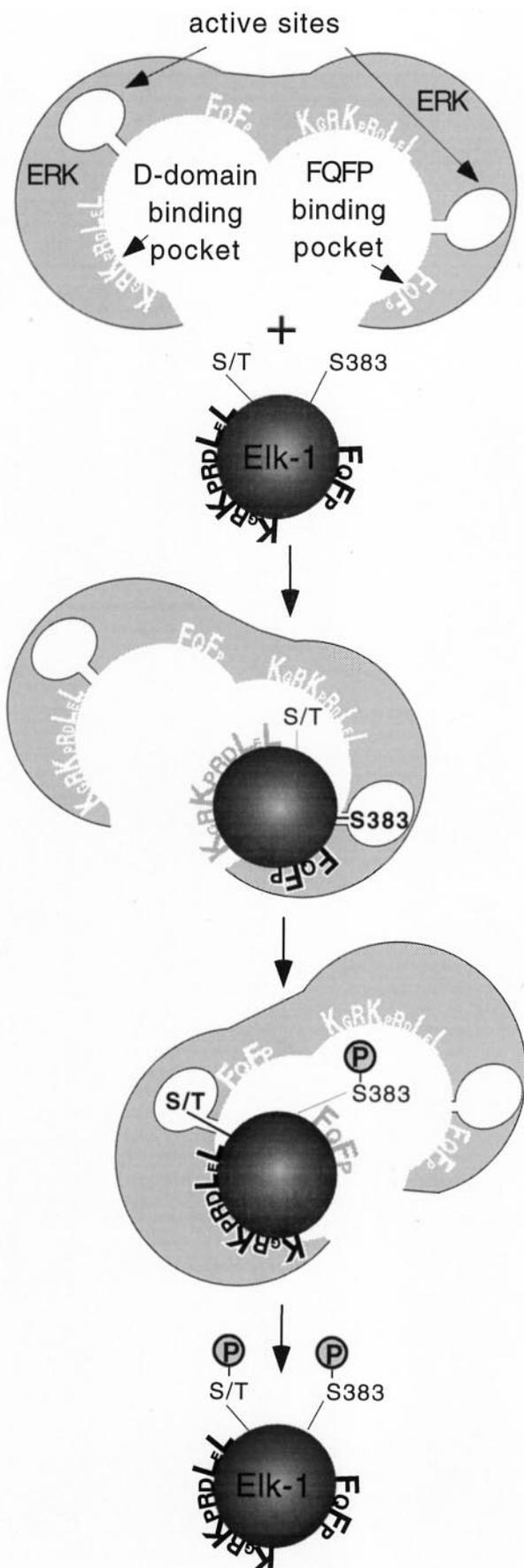
FIG. 6. **A model of ERK phosphorylation of ELK-1.** ERK is shown as a dimer; this model is supported by crystallographic and functional studies (47, 48). The active site has been identified by crystallography (49); the binding pocket for the D-domain has been identified by mu-

been investigated for the MAP kinase c-Jun N-terminal kinase. In particular, mutation of the delta domain docking site in c-Jun affects the pattern of c-Jun phosphorylation by c-Jun N-terminal kinase (44). Thus, this function of docking sites is likely to be quite general. Considering the question of how ERK phosphorylates substrates with multiple (S/T)P sites more generally, we propose there are two main determinants that control the order of phosphorylation as follows: 1) differences between the local environment of individual (S/T)P sites, such as accessibility to the ERK active site and nearby proline residues; and 2) docking sites that mediate binding between ERK and substrates with a particular geometry such that certain (S/T)P sites are directed to the active site, whereas other (S/T)P sites are excluded from the active site. The effects of docking sites will be combined with the effects of the local environment of (S/T)P sites to generate a preference hierarchy.

FXFP and D-domain Docking Sites in Substrate Proteins Form a Flexible, Modular System That Mediates ERK Binding and Phosphorylation of Specific (S/T)P Residues—Physiologic ERK substrates display different types, numbers, and arrangements of docking sites. Members of the MEK, phosphatase, and ribosomal S6 kinase protein families have been demonstrated to contain a motif similar to the D-domain that mediates binding to ERK (16–26). These proteins have only been shown to contain a single docking motif. ETS proteins of the Elk subfamily and phosphodiesterase contain an N-terminal D-domain and a C-terminal FXFP flanking one or more (S/T)P sites (15, 24, 30). Our analysis of ELK-1 using purified extracts and cultured cells indicates that the D-domain and the FXFP motif can function independently, and together these motifs function additively to mediate ERK phosphorylation (30). SAP-1 displays similar properties, and in phosphodiesterase both motifs are necessary for normal regulation by ERK (15, 24).

In this report we document a second combination of docking motifs; *C. elegans* KSR-1 contains two FXFP motifs that flank a TP acceptor site. Our mutational analysis indicates that both FXFP motifs can mediate binding to ERK, although they display little additivity. It is interesting that murine and *Drosophila* KSR contain only one FXFP motif, corresponding to the C-terminal position in *C. elegans* KSR-1. Thus, within the KSR protein family there are different arrangements of docking sites. Overall, experimentally documented arrangements of docking sites include a single D-domain, a single FXFP, a D-domain and an FXFP, and two FXFP motifs.

To explore the flexibility of this system, we generated many possible arrangements of FXFP motifs and D-domains using human ELK-1 and *C. elegans* KSR-1. These experiments demonstrate that the FXFP and D-domain motifs are sufficient to function in ectopic positions. This indicates that the primary sequences contain the necessary information to mediate binding to ERK. However, the position of the motifs in a substrate can affect the affinity for ERK, the velocity of ERK phosphorylation, and the residues that are phosphorylated.

tagenesis (27, 28), and the binding pocket for FQFP has yet to be defined. The ELK-1 substrate is shown with the D-domain docking site (KGRKPRDLEL), the FQFP docking site, serine 383 (*S383*), and (S/T)P sites other than serine 383 (*S/T*). When ERK docks on FQFP, serine 383 is directed to the active site and phosphorylated. When ERK docks on the D-domain, a non-Ser-383 site is directed to the active site and phosphorylated. Our data do not indicate which docking event occurs first, and the order that is shown was selected arbitrarily. We speculate that docking can initiate on either site. The model shows that ELK-1 is phosphorylated on multiple sites after its encounter with ERK. Our data do not address this issue directly, but our findings raise the possibility that an ERK dimer that encounters an ELK-1 substrate that contains two docking sites and nine (S/T)P sites will undergo multiple docking and phosphorylation cycles before disassociating.

The modular system of docking sites has intriguing similarities to modular systems of enhancers that regulate transcription (45, 46). Enhancers are short DNA elements that mediate transcription factor binding to promoters. Enhancers are autonomous units that can function at a variety of positions relative to the transcription initiation site. The ERK docking sites are also short autonomous units that can function at a variety of positions in substrate proteins. Different numbers and types of enhancers can be arranged in a variety of combinations to confer complex regulatory properties on promoters. ERK docking sites possess a similar capacity to form a modular system. These similarities are likely to reflect an effective engineering solution to a related problem. Transcription factors need to bind the promoters of multiple genes, and during evolution they need to acquire the ability to regulate new genes. ERK needs to phosphorylate multiple substrates, and during evolution ERK needs to acquire the ability to phosphorylate new substrates. In both cases, the solution is to mediate binding with short, autonomous units that can function at various positions relative to the site of enzyme activity (transcriptional initiation or phosphorylation). Furthermore, in both cases the individual units can be combined to generate a system with greater affinity and more complex regulatory properties.

As multicellular animals became more complex during evolution and used Ras-ERK pathways to specify an expanding number of cell fates, it is likely that ERK acquired the ability to phosphorylate many additional substrates. Our observations suggest a speculative model about how this occurred and how the properties of ERK facilitated this process. First, the striking lack of specificity of the ERK active site is important. ERK can phosphorylate (S/T)P sites in almost all contexts, and about 90% of all proteins contain such sites. ERK probably phosphorylates many cellular proteins at a low frequency. Such proteins are, in a sense, just a docking site away from being a reasonably good ERK substrate. Second, the observation that docking sites are short and can function in many positions suggests they can be readily created by missense mutations. For example, since FXF can function as a minimal ERK docking site, a protein with a phenylalanine is just a missense mutation away from having a reasonably good ERK docking site. Once a protein begins to function as an ERK substrate, selection for higher affinity or more precise regulation probably improves the initial docking site and generates additional docking sites. The hypothesis that ERK docking sites have evolved repeatedly to generate new ERK substrates predicts that the FQFP docking site in LIN-1 and the FQFP docking site in KSR-1 are examples of convergent evolution.

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