Selective in Vivo Inhibition of Mitogen-activated Protein Kinase Activation Using Cell-permeable Peptides

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The extracellular signal-regulated kinase (ERK), a member of the mitogen-activated protein kinases (MAPKs), is essential for cellular proliferation and differentiation, and thus there exists great interest to develop specific and selective inhibitors of this enzyme. Whereas small molecule inhibitors PD098095 and U0126 have been used to study MAPK/ERK kinase (MEK), their target selectivity has been questioned recently. The cross-reactivity of ATP-directed inhibitors with other protein kinases prompted us to develop structure-based selective peptide inhibitors of ERK activation. Based on a MEK1-derived peptide, we developed inhibitors of ERK activation in vitro and in vivo. The inclusion of either an alkyl moiety or a membrane-translocating peptide sequence facilitated the cellular uptake of the peptide inhibitor and prevented ERK activation in 4-phorbol 12-myristate 13-acetate-stimulated NIH 3T3 cells or nerve growth factor-treated PC12 cells in a concentration-dependent manner. In addition, cell-permeable peptides inhibited ERK-mediated activation of the transcriptional activity of ELK1. The peptides did not have an inhibitory effect on the activity of two other closely related classes of MAPKs, c-Jun amino-terminal kinase or p38 protein kinase. Thus, these peptides may serve as valuable tools for investigating ERK activation and for selective investigation of ERK-mediated responses. With the knowledge of other kinase interacting domains, it would be possible to design cell-permeable inhibitors for investigating diverse cellular signaling mechanisms and for possible therapeutic applications.

Protein phosphorylation plays a critical role in cellular signaling in response to a variety of hormones, growth factors, neurotransmitters, and a wide range of stimuli. Mitogen-activated protein kinases (MAPKs) play a pivotal role in these processes, particularly in stimulus-mediated cellular responses (1–3). The activation of these enzymes requires a cascade-like mechanism in which each MAPK is phosphorylated on two amino acid residues (Thr/Tyr) by an upstream protein kinase, MAPKK (MEK), and the latter in turn is phosphorylated on two amino acid residues (Ser/Thr) by a third protein kinase, MAPKK kinase (MEKK). There are at least three such protein kinase modules in mammalian cells as follows: extracellular signal-regulated kinases (ERKs), c-Jun amino-terminal kinases (JNKs), and the p38 MAP kinases (p38). The dual phosphorylation of MAPKs by MEKs is necessary for their activation (4) and is considered an essential step in the signaling pathways in response to growth factors and mitogenic stimuli, stress-causing agents, and cytokines.

For phosphorylation-dependent activation of MAPKs to occur, MAPK must first associate with its cognate upstream kinase, MEK. Thus, disrupting this interaction using a peptide derived from an association domain of either enzyme (5, 6) would be predicted to block the activation of the downstream protein kinase. To test this hypothesis, we evaluated the ability of a peptide corresponding to the amino-terminal 13 amino acids of MEK1, which are intimately involved in the association of ERK with MEK (7, 8), to inhibit selectively our target enzyme, ERK, and inhibit ERK activation in cultured mammalian cells.

For a peptide to inhibit ERK activation within cultured mammalian cells, the peptide must have access to ERK within the cells. To allow for efficient entry of peptide into cells in culture, we modified the peptide with membrane-translocating moieties. The first modification was the alklylation (myristoylation or stearation) of the inhibitor peptide in order to increase its hydrophobicity and hence its cellular uptake (9). The second modification was to link a membrane-translocating peptide to facilitate the cellular delivery of the peptide. Several MTPs, capable of transporting peptides or even large proteins, have been described recently (10). These include peptides derived from the Drosophila melanogaster antennapedia (Antp) homeotic transcription factor (11), the human immunodeficiency virus-TAT (TAT) protein (12), the h region of the signal sequence of Kaposi fibrolast growth factor (MTS) (13), and the protein PreS2 of hepatitis B virus (HBV) (14).

Here we report that although the free peptide inhibited ERK activation by active MEK in vitro, it did not inhibit ERK activation in vivo presumably because of its inability to cross cellular membranes. Alklylation of the peptide inhibitor facilitated its ability to inhibit ERK activation in vivo with NGF or PMA as activating ligands. Furthermore, linking the peptide inhibitor to a membrane-translocating peptide was also effective in facilitating cellular uptake of the peptide inhibitor and inhibiting ERK activation in vivo.

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EXPERIMENTAL PROCEDURES

Peptide Synthesis—All peptides listed in Table I were synthesized using commercially available reagents and high pressure liquid chromatography purified to >90% purity by SynPep Corp. (Dublin, CA). The mass of each peptide was confirmed by electrospray mass spectrometry. Peptide 1 has the sequence of the first 13 amino acids of human MEK1, listed in Table I. Peptides 2 and 3 are synthesized with stearic acid and myristic acid, respectively, linked directly to the amino terminus of the peptide by an amide bond. Peptides 4–6 are the combination of membrane-translocating sequences of Antp, Tat, and MTS, respectively, at the amino terminus of the sequence of peptide 1 with an intervening glycine amino acid. Peptides 7 and 8 are the combination of the membrane-translocating sequence HBV at the amino terminus and carboxyl terminus, respectively, of the sequence of peptide 1 with an intervening glycine amino acid. Peptides 1, 4, and 5 were synthesized without a linker. Peptides 1, 4, and 5 were also synthesized without amino-terminal modification. Complete sequences of these peptides are listed in Table I.

Materials and General Methods—Tissue culture reagents were from HyClone (Logan, UT), Sigma, and In VitroGen. NIH 3T3 and PC12 cell types were from American Type Culture Collection (Manassas, VA). PathDetect HeLa luciferase reporter (HRL-ELK1) cells were from Stratagene (La Jolla, CA). 4-Phorbol 12-myristate 13-acetate (PMA) was from Biomol (Plymouth Meeting, PA). Epidermal growth factor (EGF), U0126, luciferase lysing buffer, and luciferase assay buffer were from Promega Corp. (Madison, WI). PC12 cell lines were grown in RPMI 1640 with 20.5 mM glutamate, 10% horse serum, 5% fetal bovine serum, and 2 mM L-glutamine. NIH 3T3 cells were grown in DMEM containing 10% FBS, 100 µg/ml G418, and 100 µg/ml hygromycin B. Further purification of recombinant active MEK1, SKK3, and p38 from Eastman Kodak Co. 4,6-Diamidino-2-phenylindole, Toto-3, and fluorescein from Tropix (Foster City, CA) and Biomax Light II photographic film were used in Western blot analysis using anti-active ERK, JNK, or p38 antibodies, respectively. Anti-pan-MAPK antibodies and other reagents were from Cell Signaling Technology. Complete sequences of these peptides are listed in Table I.
RESULTS

Peptide Binding to ERK2—We have demonstrated that the peptides derived from the amino terminus of MEK1 are capable of binding to ERK2 in vitro by two approaches, fluorescence anisotropy and coprecipitation. First, the fluorescence anisotropy of fluorescein-labeled peptide 7, a measure of the rotational freedom of the peptide, increases with increasing concentrations of ERK2 (Fig. 1A). This anisotropy change is consistent with the formation of a slower rotating complex of peptide 7 with ERK2 in solution. The value of the equilibrium dissociation constant \( K_d \) was determined by nonlinear regression analysis of the data using Equation 1 for a single-site saturation binding event,

\[
A = \frac{\Delta \lambda \times [\text{ERK2}]}{[\text{ERK2}] + K_d} + A_{\text{min}} \tag{1}
\]

where \( A_{\text{min}} \) is the anisotropy of the free peptide and \( \Delta \lambda \) is the difference in anisotropy of fully bound peptide and free peptide. By using Equation 1, we obtained a value of \( K_d \) of 77 nM (see “Discussion”).

Second, fluorescein-labeled peptide 5 bound to and coprecipitated ERK2 upon addition of anti-fluorescein antibodies and protein G-agarose (Fig. 1B, lane 2). The binding is specific because peptide 5 that was not labeled with fluorescein did not coprecipitate ERK2 in the presence of the antibodies and protein G-agarose (Fig. 1B, lane 3).

In Vitro Inhibition of ERK2 Activation—The in vitro inhibitory potency of each peptide was determined by monitoring ERK2 activation using antibodies specific for the activated (dually phosphorylated) form of ERK. As shown in Fig. 2A, the addition of NGF stimulated ERK activation without alteration in the total ERK protein (lane 2 versus lane 1). It is also evident that peptide 2 inhibited the activation of ERK in a concentration-dependent manner without noticeable change in the amount of total ERK (compare lanes 7, 6, 5, 4, and 3 with lane 2). The bands in Fig. 2A were quantitated and plotted against the peptide concentration added to the medium. The value of IC\(_{50}\) for each peptide was determined from these data by nonlinear least squares regression analysis using Equation 2 for a single-site binding inhibitor,

\[
I_{\text{band}} = \frac{\Delta \lambda \times [\text{peptide}]}{I_{\text{IC50}} + [\text{peptide}]} + I_{\text{Inhibited}} \tag{2}
\]

where \( I_{\text{band}} \) is the value of intensity of each band; \( I_{\text{Inhibited}} \) is the value of band intensity for inactive ERK (background); \( \Delta \lambda \) is the difference of the values of the band intensity for fully activated ERK and inactive ERK, and IC\(_{50}\) is the peptide concentration required to decrease band intensity by 50%. An example using peptide 2 is shown in Fig. 2B, where an IC\(_{50}\) value of 2.5 \( \mu \)M was obtained. The values of IC\(_{50}\) for the other peptides we tested are listed in Table I. The lack of in vitro potency of these same peptides for inhibition of JNK and p38 activation was demonstrated in an activation assay analogous to the one performed for ERK2 but using the appropriate MAPKK and anti-active antibodies for the activated forms of JNK and p38 protein kinases. Neither the stearated peptide 2 (Fig. 2C) nor the MTP-linked peptide 7 (Fig. 2D) inhibited the activation of JNK or p38 protein kinases at the maximal concentrations tested (100 \( \mu \)M, Fig. 2C) and (25 \( \mu \)M, Fig. 2D).

Cellular Uptake of MTP-linked Peptides—Fluorescein-labeled peptides 4 and 5 were used to monitor cellular uptake by microscopy. The images obtained by confocal microscopy of peptide 4-treated NIH 3T3 cells (see Supplemental Material Fig. S1A and S1B) show the peptide appears to have a localization pattern that is similar to that of ERK but different from that of active ERK. The peptide was also absent from the nucleus. The cellular uptake of the latter (see Supplemental Material Fig. S1C and S1D). Whereas cells treated with peptides 4 and 5 were 50–100-fold more fluorescent than untreated cells, no increase in relative fluorescence of cells treated with peptides 1 and 6–8 was observed indicating lack of cellular uptake of the latter peptides.

The extent of cellular uptake of peptide 4 by PC12 corresponded to the concentration of the peptide in the medium and reached saturation at 50–100 \( \mu \)M (see Supplemental Material Fig. S2A) which represents about 2–4 pmol of peptide per \( \mu \)g of cellular proteins. Similar results were obtained with peptide 5. The binding was specific because pretreatment of the cells with non-fluorescent peptides 5 before the fluorescently labeled peptide 5 hindered the cellular uptake of the latter (see Supplemental Material Fig. S2A and S2B).

![Fig. 1. Binding of MEK1-derived peptides to ERK. A, fluorescence anisotropy of fluorescein-labeled peptide 7 (5 nM) increases with increasing concentrations of ERK2. This increase in anisotropy is consistent with the increase in molecular volume of the peptide as it binds to ERK2. B, ERK2 coimmunoprecipitates with fluorescein-labeled peptide 5 using anti-ERK antibody and protein G-coated agarose. Lane 1 contains the ERK standard; lane 2 contains the immunoprecipitated ERK using cell lysate treated with fluorescein-labeled peptide 4, anti-ERK antibodies, and protein G-agarose; lane 3 contains the immunoprecipitate of cell lysate treated with peptide 4, anti-ERK antibodies, and protein G-agarose. It is apparent that the ERK bound to fluorescein-labeled peptide 4 and is immunoprecipitated with the anti-ERK antibodies.](http://www.jbc.org/content/journal/jbc/283/21/8743.full.html)
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**A**

| Lane | [Peptide 2] | 0 | 0 | 50 | 5 | 0.5 | 0.05 | 0.005 µM |
|------|-------------|---|---|----|---|-----|------|---------|
|      | NMF        | + | + |    | + |     |      |         |
|      |            |   |   |    |   |     |      |         |

**B**

![Western blot](image)

**C**

| Lane | [Peptide 2] | 0 | 0 | 50 | 100 µM |
|------|-------------|---|---|----|--------|
|      | SKK         |   |   |    |        |
|      |            |   |   |    |        |

**D**

| Lane | [Peptide 2] | 0 | 0 | 50 | 100 µM |
|------|-------------|---|---|----|--------|
|      | MAPKK       |   |   |    |        |
|      |            |   |   |    |        |

**Figure 2. Effect of MEK1-derived peptides on ERK activation in vitro.**

- **A**, the stearated peptide 2 inhibits ERK2 activation by MEK in a dose-dependent manner. The sample loaded in lane 1 contains inactive ERK2, whereas the sample in lane 2 contains ERK2 activated by MEK1; lanes 3–7 contain samples of ERK2 activated by MEK1 in the presence of decreasing concentrations of peptide 2. The top Western blot is probed with anti-active ERK antibody, and the bottom blot is probed with anti-pan-ERK to demonstrate equal loading of total ERK. The quantitation of intensity of the bands corresponding to the active ERK allowing the determination of values of IC50 for inhibition of ERK activation in vitro (see text). C, peptide 2 does not inhibit the activation of JNK or p38 by their respective SKK below a concentration of 100 µM. Samples loaded in lane 1 are JNK and p38 without the activation by their respective SKK. Samples loaded in lane 2 are of JNK and p38 activated by their respective SKK. Samples in lanes 3 and 4 are JNK and p38 activated by their respective SKK in the presence of 50 and 100 µM peptide 2. D, the MTP-linked peptide 7 inhibits the activation of ERK2 in a dose-dependent manner without inhibiting activation of JNK or p38 at similar concentrations of peptide 7. Samples loaded in lane 1 of each blot contain ERK, JNK, or p38 that have not been activated. Samples loaded in lane 2 contain ERK, JNK, and p38 activated by their respective MAPKK; samples loaded in lanes 3–6 contain ERK, JNK, and p38 activated by their respective MAPKK in the presence of decreasing concentrations of peptide 7. Each pair of blots was probed with antibodies reactive with the active and total (active and inactive) forms of ERK, JNK, or p38.

**Table 1. Amino acid sequences and IC50 values of peptides tested for their inhibition of ERK activation in vitro and in vivo.**

| Peptide sequence | Uptake by Cells | IC50 µM |
|------------------|-----------------|---------|
|                  | M               | F.C.    | in cell | in vitro |
| 1. MPKKKPTPiqQLNP | –               | –       | >100    | 30 ± 5   |
| 2. Ste–MPKKKPTPiqQLNP | ND             | ND      | 13 ± 3  | 2.5 ± 0.1 |
| 3. Myr–MPKKKPTPiqQLNP | ND             | ND      | 13 ± 5  | 10 ± 4   |
| 4. RiQKWPQNRMRKQKEGMPPKKPTPiqQLNP | +     | +       | 45 ± 13 | 1.5 ± 0.3 |
| 5. GyGRKRRQRRGMPKKKPTPiqQLNP | +             | +       | 29 ± 7  | 0.21 ± 0.07 |
| 6. AAVLLPAVLALLAPGMPPKKPTPiqQLNP | –             | –       | >100    | 8.5 ± 2  |
| 7. PLSSIFSRIGDPMPKKKPTPiqQLNP | –             | –       | >100    | 7 ± 1    |
| 8. MPKKKPTPiqQLNPGLSIFSRIGDP | –             | –       | >100    | >100     |

**In Vivo Inhibition of ERK2 Activation**—To demonstrate the potency of the peptides in inhibiting ERK activation in vivo, cells were treated with or without peptides prior to stimulation with the appropriate ligand, NGF for PC12 cells and PMA for NIH 3T3 cells. Cells were then lysed, and the lysates were applied to SDS-PAGE followed by Western blotting to determine the extent of ERK activation. As shown in Fig. 3A, ERK activation was robust when NGF was added for 5 min compared with serum-starved PC12 (lanes 2 and 1). The addition of the stearated peptide inhibitor peptide 2 attenuated the activation of ERK in a concentration-dependent manner without altering the total amount of ERK (lanes 7, 6, 5, 4, and 3 compared with lane 2). The bands corresponding to the various peptide concentrations were quantitated and plotted as shown in Fig. 3B, and a value of IC50 was calculated in the same manner as the in vitro analysis using Equation 2 (Table 1). It is noteworthy that the addition of stearated Ht 31-derived peptide, which is shown to inhibit selectively cAMP-dependent protein kinase anchoring in cells (9), had no effect on the activation of ERK in vivo (results not shown). Thus the inhibition of ERK activation by peptide 2 and 3 is specific, and stearation of the peptide had no effect by itself on ERK activation but facilitated the cellular uptake of the MEK-derived peptide, resulting in inhibition of ERK activation. Similar studies on the effect of the MTP-linked peptide inhibitor (peptide 4) in PMA-treated NIH 3T3 cells were carried out (Fig. 3C). As
shown in Fig. 3C, PMA stimulated the activation of ERK (lane 2), and the MEK inhibitor U0126 inhibited it (lane 3), when compared with serum-starved cells (lane 1), and without noticeable changes in the total amount of ERK. The addition of the peptide for 30 min prior to stimulation with PMA resulted in inhibition of ERK activation in a concentration-dependent manner with no alteration in the amount of total ERK (compare lanes 7, 6, 5, and 4 with lane 2 of Fig. 3C). To determine the time required for the peptide to manifest its inhibitory effect in vivo, we treated cells with peptide for varying times before the addition of NGF to PC12 cells (Fig. 3D and E). As shown in Fig. 3D, peptide 4 (100 μM) inhibited the activation of ERK in NGF-stimulated cells to a greater extent when preincubated with the cells for 20 min or longer prior to the addition of NGF. The quantitated band intensities of each incubation time were fitted by non-linear least squares regression analysis to Equation 3 for single exponential decay (Fig. 3E),

\[ I_{\text{band}} = I_{\text{active}} \times e^{-kt} \]  

(Eq 3)

where \( I_{\text{band}} \) is the value of band intensities of the measured bands; \( I_{\text{active}} \) is the value of the fully active ERK; \( k \) is the value of the rate at which ERK activation is inhibited by the peptides; \( t \) is the incubation time of cells with the inhibitor peptide. By using Equation 3, we obtained values of \( k \) for peptides 4 and 5 of 0.036 and 0.052 min\(^{-1} \), respectively. Therefore, the time required to achieve half-inhibition (\( t_{1/2} \)) for peptides 4 and 5 are 19 and 13 min, respectively.

**MTS-linked Peptide Effect on Activation of Transcription Factor ELK1**—Because we successfully demonstrated the inhibition of MEK phosphorylation of ERK by the cell-permeable peptides, we hypothesized that downstream effectors of ERK such as transcription factor ELK1 would therefore be affected by these inhibitors. To test this hypothesis, we used the PathDetect HeLa luciferase reporter (HLR-ELK1) cell line. This stably transfected cell line features constitutive expression of the GAL4-ELK1 fusion protein and the firefly luciferase gene that is controlled by a promoter that responds to GAL4 fusion. When activated by phosphorylation, the fusion protein binds to the promoter and induces luciferase expression. Therefore, luciferase activity reflects the activation status of the ERK signaling pathway, and thus the effect of extracellular stimuli such as PMA, EGF, serum, etc. that converge on ERK activa-
Experimental Procedures.

Serum-free media for 30 min and then induced with inducing media for labeled peptide demonstrated direct binding of the peptide to under the fourth condition, cells were treated peptide under the third condition, cells were treated with 200 ng/ml EGF, and 10% FBS after peptide treatment indicating the stability of peptide for at least that period of time (data not shown). Thus peptide stimulation and subsequent phosphorylation and activation of the transcriptional activity of ELK1. studies for protein kinases. Furthermore, if such inhibitors can be interesting in the cell signaling field. The use of such specific protein-protein interactions and the discovery of protein motifs have been questioned. The recent advances made in understanding the sequence of peptide sequence of peptide the HBV-derived MTP is linked to the amino terminus of the inhibitory peptide, the resulting peptide esterase, when this HBV-derived MTP is linked to the amino terminus of the inhibitory peptide, the resulting peptide esterase, when this HBV-derived MTP is linked to the amino terminus of the inhibitory peptide, the resulting peptide imaging. To demonstrate further the interaction of ERK2 with these peptides, we show that these peptides were able to coimmunoprecipitate ERK2 with fluorescein-labeled peptide using an anti-fluorescein antibody.

Although peptide 1 inhibited ERK2 activation by MEK1, this peptide was not capable of inhibiting ERK activation in cultured mammalian cells (Table I), presumably because of its inability to cross the cellular membrane. Fluorescein-labeled peptide 1 was not detected (by either microscopy or flow cytometry) in cells treated with 100 µM labeled peptide 1 (Table I). To investigate further the effect of this peptide on ERK activation in vivo, it was necessary to modify the peptide to facilitate its cellular uptake.

**Alkylated Peptides**—Conjugation of the peptide to stearic acid did not adversely affect the inhibitory potency of the peptide in vitro (Fig. 2A). The stearated peptide 2 inhibited ERK2 phosphorylation in a concentration-dependent manner with an IC_{50} value of 2.5 µM (Table I and Fig. 2B), which is equally effective or more effective than the MEK1 inhibitors PD098059 or U0126. Similar results were obtained when the peptide was myristoylated (data not shown). The alkylated forms of the peptide were also selective, as no inhibition of p38 and JNK was observed, even at 100 µM of peptide 2 (Fig. 2C).

Alkylated MEK-derived peptides 2 and 3 entered cells and potently inhibited the activation of ERK1 and ERK2 with IC_{50} values of 13 µM; the non-alkylated peptide 1 had no effect on the activation of ERK1 and ERK2 in NGF-stimulated PC12 cells (Table I). When the alkylated peptides were incubated for periods of 15 and 30 min before the addition of NGF, both the stearated peptide 2 and myristoylated peptide 3 inhibited the activation of ERK1 and ERK2; however, the stearated peptide was more effective than the myristoylated peptide at shorter duration (15 min) (data not shown). It is noteworthy that stearation of unrelated peptide such as a cAMP-dependent protein kinase anchoring protein-derived peptide did not inhibit ERK activation in vivo.

**MTP-linked Peptides**—Because MTPs have been successfully used as carrier of other cargo peptides (10–14, 16–18), it was of interest to compare the effectiveness of alkylation as a mean to deliver peptide inhibitors to cellular targets with the effectiveness of ligation to MTPs. Toward this goal, we synthesized peptides containing four different MTPs combined with the sequence of peptide 1, and we tested these peptides for their ability to enter cells as well as for their inhibitory potency in vitro and in vivo. All MTP-linked peptides were inhibitory in vitro with IC_{50} values between 0.21 and 30 µM, except peptide 8 (Table I), indicating that the fusion of the carrier peptides did not adversely affect its potency to inhibit ERK activation by MEK. The latter peptide (peptide 8) consists of the HBV-derived MTP peptide linked to the carboxy terminus of the inhibitory peptide and had an IC_{50} value of over 100 µM. Interestingly, when this HBV-derived MTP is linked to the amino terminus of the inhibitory peptide, the resulting peptide 7 is a 4-fold more effective inhibitor (7 µM) than the free peptide 1, suggesting that the orientation of the HBV-derived carrier peptide in relation to the peptide inhibitor can alter its potency. Peptide 7 is a potent inhibitor of ERK activation in vitro but...
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Inhibition of ERK Activation by MTP-linked Peptides in Vivo—To demonstrate that peptides 2, 4, and 5 inhibit ERK activation in both NIH 3T3 and PC12 cell lines, cells were treated with a range of peptide concentrations prior to stimulation. Peptide 2 inhibited the activation of ERK in NGF-stimulated PC12 cells in a concentration-dependent manner with an IC50 value of 13 μM, with no effect on the total amount of ERK. Similarly, peptides 4 and 5 inhibited ERK activation in cells with IC50 values of 45 and 29 μM, respectively; however, other peptide conjugates 6 and 7 that did not permeate cells (see above) were not able to inhibit MAPK activation (Table I). Thus, the effectiveness of these peptides in inhibiting ERK activation in vivo correlates well with their ability to enter the cells.

Similar to the alkylated peptides 2 and 3, inhibition of ERK activation by the MTP-linked peptide 4 or 5 shows a time dependence. Inhibition of ERK activation is more significant with longer preincubation times. PC12 cells were incubated with peptide 4 for varying times prior to a 5-min stimulation with NGF. Inhibition by peptide 4 or 5 gradually increases with time reaching a maximum after 40 min, with a half-inhibition time of ~15 min. However, the increase in fluorescence in cells treated with peptides 4 or 5 occurred under 5 min. This lag period before inhibition of ERK activation may be required for the diffusion of the peptide to ERK and/or the disruption of pre-existing MEK-ERK complexes.

MTP-linked Peptide Effect on the Activation of Transcription Factor ELK1 in Vivo—Finally, we have shown that the MTP not only inhibited ERK activation in vivo, but it also inhibited ERK-mediated effects such as the phosphorylation of the transcription factor ELK1. The doubly transfected cells contain GAL4 DNA binding domain fused to ELK1 transactivation domain, and firefly luciferase as reporter that is controlled by a promoter that responds to GAL4/ELK1 fusion protein. These cells respond to extracellular stimuli such as PMA, EGF, and serum that affects the ERK activation by increasing the phosphorylation of ELK1 and luciferase activity. Elegant studies using this approach demonstrated a strong correlation between phosphorylation of ELK1 by ERK and ELK1 transcriptional activity (15). The addition of peptide 5 to cells before stimulation with a combination of PMA, EGF, and FBS abrogated the effect of these stimuli to activate ERK and luciferase expression (Fig. 4). In fact, peptide 5 was as effective as the commonly used inhibitor U0126. It was proposed that MEK functions not only as a direct activator of ERK but also as a cytoplasmic anchoring protein for ERK (19, 20). The phosphorylation of ERK by MEK leads to the weakening of ERK/MEK interaction and dissociation of the two enzymes followed by translocation of ERK to the nucleus (19, 20). Based on our data (Supplemental Material Fig. S1A and S1B and Fig. 4), it is likely that the MEK-derived peptide binds to ERK preventing its association with MEK and thus inhibiting ERK phosphorylation. Our results also show that phosphorylation of ERK, which is a prerequisite for its activity is necessary for phosphorylation of ELK1 and ELK1 transcriptional activity. It appears that the peptide not only inhibited ERK phosphorylation in vitro but it also inhibited its translocation to the nucleus to activate ELK1 transcriptional activity. Thus, the peptide is not only specific for inhibition of ERK activation by MEK (as shown by lack of inhibition of JNK and p38), but it also inhibits downstream effectors that are dependent on activated ERK.
Because commercially available organic inhibitors are directed toward the ATP-binding site of protein kinases, the specificity of these inhibitors has been questioned recently (21). Peptide 5, however, was designed to inhibit specifically the interaction between ERK and MEK, and thus it can be used to study selectively the effect of downstream effects of ERK activation. We are currently pursuing similar studies where ERK activation is required. These peptide inhibitors can be used as a reliable diagnostic tool to study cellular signaling pathways where ERK activation is involved.

Conclusions—we have demonstrated that peptide 1, derived from the amino terminus of MEK1 in the ERK-interacting domain, served as a basis for the design of selective inhibitors of ERK activation in vivo. Peptide inhibitors have the added benefit of providing information about biologically relevant interactions. Our approach of designing inhibitors of ERK activation using cell-permeable, domain-specific interacting peptides offers several advantages over existing approaches. The selectivity of small molecule inhibitors has been questionable, and some are found not to be useful when tested rigorously (21, 22). The use of in vivo expression of transfected genes as a tool to interfere with signaling pathways has been severely limited by inability to quantitatively deliver the exact amount of inhibitor. Furthermore, the cell-permeable peptide inhibitor approach makes it feasible for applications in large scale studies when compared with microinjection of peptide inhibitors into cells individually.

The cell-permeable peptide inhibitors we have developed can be used to dissect cellular responses that involve ERK activation in response to extracellular stimuli. It is noteworthy that other protein kinase inhibitors that are directed to the ATP-binding site do not have the desired specificity (21, 22). Our approach can be used to develop similar cell-permeable inhibitors of other MAPK pathways such as JNK and p38. In fact, during the preparation of this work for publication, others (23) used a similar approach to develop an inhibitor of JNK activation composed of a TAT-linked peptide derived from JNK-interacting protein. Several anchoring proteins that have specific recognition motifs to protein kinases in the MAPK-signaling pathways have been reported (24, 25). Such motifs can be used to design and develop selective and specific inhibitors for individual MAPK pathways. We are currently undertaking efforts to screen peptide libraries for a more potent inhibitor based on this inhibitor prototype.

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