The extracellular signal-regulated kinases (ERKs)2 are mitogen-activated protein kinases (MAPKs) that play a key role in the intracellular transmission of variety of extracellular signals such as growth factors and hormones (1–5). ERKs are the product of two distinct genes; Erk1 (MAPK3) and Erk2 (MAPK1) (6), which are transcribed into two main proteins (p44ERK1, and p42ERK2 (6)), as well as several alternatively spliced forms (ERK1b (7), ERK1c (8) and ERK2a (9)). ERKs operate within a signaling cascade containing several tiers of protein kinases, including Rads, MEKs, ERKs, and MAPKAPKs (5). The transmission of signals via the cascade is mediated by a sequential phosphorylation and activation of these components, which allows a rapid and tightly regulated signal transmission. ERKs are phosphorylated by their upstream kinases MEK1 and MEK2 (MEKs) on Tyr and Thr residues within a Tyr-Glu-Thr (TEY) motif in their activation loop, which results in their full activation (10, 11). Upon activation, ERKs can either translocate into the nucleus to phosphorylate a large number of nuclear substrates or remain in the cytoplasm where they phosphorylate another set of cytoplasmic substrates. In addition, ERKs can phosphorylate RSK or other MAPKAPKs, which in turn can increase the magnitude of ERK-induced phosphorylation events (1). These activities finally culminate into the regulation of a large number of stimulated cellular processes including proliferation, differentiation, and survival (5).

As a central component in the regulation of many stimulated cell functions, ERKs are spatially and temporally regulated (5). The temporal regulation, which is important for the specificity of ERKs signals (4), is mediated primarily by removal of phosphates from the regulatory Thr or Tyr residues of ERKs (12). This inactivation could occur through the action of Ser/Thr phosphatases (PPs) (13), protein Tyr kinases, such as PTP-SL (14), or a subgroup of dual specificity phosphatases, termed MAPK phosphatases (MKPs) (15). Interestingly, the PTPs and MKPs interact with the ERKs via a D domain, similar to that of MEKs (16). Upon binding, PTPs remove only the phosphate from the pTyr residues, which is sufficient to induce complete inactivation of the ERKs (17). However, this initial dephosphorylation is usually followed by removal of the phosphate from the pThr residue by the PPs (18). PPs inactivate ERKs by removing the phosphate from the pThr, followed by removal of the

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[2] The abbreviations used are: ERK, extracellular signal-regulated kinase; Ab, antibody; CRS, cytoplasmic retention sequence; CD, common docking motif; MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatases; MBP, myelin basic protein; DAPI, 4’,6-diamidino-2-phenylindole; GFP, green fluorescent protein; HA, hemagglutinin; FBS, fetal bovine serum; GST, glutathione S-transferase; PBS, phosphate-buffered saline; RIPA, radiolmmune precipitation assay buffer; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; DEF, docking site for ERK-FXF.
phosphate from the pTyr. However PPs are not known to bind any specific docking domain in ERKs. MKPs inactivate ERKs by an essentially simultaneous removing of the phosphates from the pTyr and pThr residues (19).

In addition to MEKS and phosphatases, ERKs are regulated by their interaction with scaffold proteins and substrates (20). Multiple domains on ERKs are involved in docking interactions with its regulators. These docking interactions contribute to the efficiency and selectivity of the ERK signaling. One of these domains is the cytoplasmic retention sequence (CRS)/common docking (CD) motif (16, 21). This domain is composed of acidic and hydrophobic residues that allow interaction of ERKs with a variety of proteins by binding to their D domain, which contains basic and hydrophobic residues (16). Other regions in ERKs that can contribute to MEKS binding are the hydrophobic β7–β8 and αd-αE grooves (22), several residues within the kinase insert domain (KID) (23), and possibly also a stretch of amino acid in the N terminus of ERKs (24). Another important docking site is the docking site for ERK-FXF (DEF) binding domain (25), which binds through hydrophobic interactions to the DEF domain, mainly in ERKs interacting substrates (26). The binding through these docking domains is reversible, as upon stimulation, phosphorylation of the Thr and Tyr residues in the activation loop induces conformational change (11), which releases the bound proteins. Interestingly, ERKs can also bind to some proteins in a non-reversible manner, leading to sequestration from regulatory proteins (e.g. Vimentin (27)) or to specific localization (e.g. tubulin (28)). This binding seems to be mediated by loop L6 in the small lobe of ERKs (29), probably together with residues in the kinase insert domain (30) and the phosphorylated regulatory Thr and Tyr (29, 30).

Residues in the activation loop of ERKs beside the regulatory Thr and Tyr residues are important for the regulation of ERK activity as well (31, 32). The activation loop is a part of the activation segment, which is defined as the region between the conserved DFG and APE motifs in the big lobe of kinases (31). In ERK2 the activation loop encompass residues 173 to 185 and contains the two regulatory residues Thr183 and Tyr185. In ERK2 the activation loop encompass residues 173 to 185 and conserved DFG and APE motifs in the big lobe of kinases (31). The activation loop is a part of the phosphorylated regulatory Thr and Tyr residues (29, 30). The activation loop is a part of the phosphorylated regulatory Thr and Tyr residues (29, 30). The activation loop is a part of the phosphorylated regulatory Thr and Tyr residues (29, 30). The activation loop is a part of the phosphorylated regulatory Thr and Tyr residues (29, 30). The activation loop is a part of the phosphorylated regulatory Thr and Tyr residues (29, 30).

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show that residues 176–8 are important for the catalytic activity of ERK2 toward Elk1. Finally, combination of activation loop and CRS/CD motif mutations revealed that these two regions cooperate in determining the net phosphorylation of ERK2, but the role of the CRS/CD predominates that of the activation loop residues. Overall, our results implicate several residues within the activation loop of ERK2 in regulating its inactivation rate, subcellular localization and activity, which together are important in determining the ERK2 signal specificity.

EXPERIMENTAL PROCEDURES

Reagents—EGF, protein A/G-Sepharose, 4,6-diamino-2-phenylindole (DAPI), and myelin basic protein (MBP) were obtained from Sigma. Antibodies (Abs) against doubly phosphorylated ERK (pERK), general ERK (gERK) were from Sigma. Anti-Elk1 antibody (Ab) (I-20) and anti-pElk1 Ab were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-green fluorescent protein (GFP) and anti-hemagglutinin tag (HA) Abs were from Roche Diagnostics (Indianapolis, IN). Alkaline phosphatase-conjugated secondary Ab and the developing substrate nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate were purchased from Promega (Madison, WI). ECL was from Amersham Biosciences. Rhodamine-conjugated secondary Ab was from Jackson Immunoresearch (West Grove, Pa.). Elk1 fusion protein was from Cell Signaling (Beverly, MA).

Buffers—Buffer A contains 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, and 0.1 mM sodium vanadate. Buffer H (homogenization buffer) contains 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM sodium vanadate, 1 mM benzamide, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A. Buffer R (reaction mixture for phosphorylation in 3-fold final concentration) contains 75 mM β-glycerophosphate, pH 7.3, 30 mM MgCl₂, 4.5 mM dithiothreitol, 0.15 mM sodium vanadate, 3.75 mM EGTA, and 30 μM calmodulin. Buffer RMP (reaction mixture for phosphorylations at 3-fold final concentration), contains 90 mM HEPES pH 7.4, 450 mM NaCl, 10 mM dithiothreitol, and 3 mM EDTA. RIPA buffer contains 137 mM NaCl, 20 mM Tris, pH 7.4, 10% (v/v) glycerol, 1% Triton X-100, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, 2 mM EDTA, 1 mM phenethylsulfonyl fluoride, and 20 μM leupeptin.

DNA Constructs and Mutations—WT-GFP-ERK2, GFP-173A, GFP-176A and GFP-316A were previously described (21, 32). GFP-173,5,7A, GFP-174A, and GFP-177A were prepared using site-directed mutagenesis kit (Stratagene, La Jolla, CA) and were sequenced to verify their proper structure. GFP-176A and GFP-316A were prepared by ligating MEK1 or MKP3-glutathione S-transferase (GST) into the C-terminus of pGFlex, HA-MEK1, and HA-MKP3 were prepared by ligating MEK1 or MKP3 into the 3' end of the HA tag followed by insertion into the EcoRI and Xhol sites of pCDNA3 (Invitrogen).

Cell Culture and Transfection—COS7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). These cells were transfected using the DEAE-dextran method. CHO cells were grown in F12-DMEM
supplemented with 10% FBS and transfected using Lipofectamine (Invitrogen).

**Protein Expression and Purification**—The MKP3-GST was expressed in *Escherichia coli* BL21 (DE3), and purified as a GST fusion protein essentially as described (33). Thus, the cells were lysed by sonication followed by addition of 1% Triton X-100 (30 min at 4 °C), spun down (30,000 rpm 30 min), and the supernatant was combined with 2 ml of 50% slurry of glutathione-Sepharose 4B (Amersham Biosciences) equilibrated with the lysis buffer. After incubation the matrix was washed, and the fusion protein was eluted with 1 bed volume of reduced glutathione.

Subcellular Localization Studies—CHO cells were co-transfected with the various GFP-ERK2 constructs together with HA-MEK1. The GFP-ERK2 mutants and the HA-MEK1 were transfected in a ratio of 1:3, respectively. Serum-starved cells were washed with PBS and fixed (20 min in 3% (w/v) paraformaldehyde in PBS). The cells were then permeabilized by 0.1% (v/v) Triton X-100 in PBS (23 °C, 5 min), washed with PBS, and stained with monoclonal anti-HA Ab and rhodamine-conjugated goat-anti-rat Ab. The nuclei were visualized using DAPI.

Preparation of Cell Extracts and Western Blotting—Cells were grown to confluence and serum-starved for 18 h (0.1% FBS). After treatments, the cells were rinsing twice with ice-cold PBS and once with ice-cold Buffer A, scraped into Buffer H and disrupted by sonication (2 pulses for 7 s of 70 watts) on ice. The extracts were centrifuged (15,000 rpm, 15 min at 4 °C) and the supernatants were boiled for 5 min in sample buffer. The samples were then subjected to 10% SDS-PAGE, and Western blotting with the appropriate Abs, which were detected using alkaline phosphatase or ECL according to the manufacturer.

Immunoprecipitation—One day after transfection, cells were serum-starved (0.1% FBS) for 16 h, and then stimulated with EGF (50 ng/ml, Sigma) or left untreated. After treatments, the cells were washed, lysed and centrifuged as described above. The extracts were incubated (2 h, 4 °C) with a G/G-conjugated monoclonal anti-GFP antibody (Ab), after which the beads were washed twice with RIPA, once with 0.5 M LiCl in 0.1 M Tris pH 8.0, and either once with PBS for Western blot analysis, or twice with Buffer A for the in vitro kinase assay or RMP for the in vitro phosphatase assay. The immunoprecipitates were subjected to either Western blotting as described above, or to an in vitro kinase assay/ in vitro phosphatase assay as described below.

Partial Purification of MEKs from COS7 Cells Using DE52 Column—Partial MEKs purification on a DE52 column was performed as described previously (34) in EGF-stimulated (50 ng/ml, 5 min) COS7 cells. After harvesting as above, the lysate was loaded onto a DE52 column, followed by washing with 400 μl of Buffer A that eluted the partially purified active MEKs.

In Vitro Kinase and Phosphatase Assays—The immunoprecipitated GFP-ERK2 proteins were mixed with partially purified MEKs and Buffer RMP in a final volume of 30 μl. The phosphorylation reaction was allowed to proceed at 30 °C for 30 min with constant shaking and either terminated by sample buffer followed by boiling for 5 min and Western blotting or the active GFP-ERKs were washed as described below. For MBP and Elk1 phosphorylation, the active GFP-ERK2 constructs were washed once with RIPA, twice with 0.5 M LiCl in 0.1 M Tris, pH 8.0 and twice with Buffer A, and mixed with either MBP (5 μg) or Elk1 (0.25 μg) and Buffer RMK containing 100 μM \( [\gamma-^3P]ATP \) (1–2 cpm/fmol, 30 μl, 30 °C, 30 min with constant shaking). The reaction was terminated by boiling in sample buffer, and the proteins were resolved by SDS-PAGE, and subjected to autoradiography and Western blot analysis as indicated. For phosphatase assay the phosphorylated active ERK2s were immunoprecipitated and washed once with RIPA, twice with 0.5 M LiCl in 0.1 M Tris pH 8.0 and twice with RMP. The immunoprecipitates were incubated with purified MKP3-GST and Buffer RMP (30 μl, 30 °C, 30 min, with shaking). The reaction was terminated by boiling in sample buffer, and subjected to Western blotting.

**RESULTS**

In a previous study (32), we found that several activation loop residues besides Thr\(^{183}\) and Tyr\(^{185}\) participate in ERKs regulation. Here we studied the molecular mechanisms by which these additional residues participate in the regulation of ERK2 activity and localization. For this purpose, we substituted either residues 173–175 (GFP-173A) or 176–178 (GFP-176A) to alanines (Fig. 1) in constructs that were GFP-fused (21). To study the acidic cluster within this region, we substituted either residues Asp\(^{316}\), Asp\(^{319}\), Asp\(^{320}\); GFP-316A) to construct double mutants (GFP-173,5,7A, Pro\(^{174}\) (GFP-174A), or Asp\(^{177}\) (GFP-177A) to alamines. To study the role of these residues in conjunction with the CRS/CD motif, we combined the activation loop mutants together with the CRS/CD motif mutants (Asp\(^{316}\), Asp\(^{319}\), and Glu\(^{320}\); GFP-316A) to construct double mutants (GFP-316/173,5,7A and GFP-316/173,5,7A).

**Residues 173–175 of ERK2 Regulate Its Thr and Tyr Phosphorylation**—To test the effect of the activation loop residues of ERK2 on its phosphorylation by MEKs, the constructs were co-transfected with HA-MEK1 into COS7 cells, which were then serum-starved and stimulated with EGF. Anti pERKs Ab used in a Western blot revealed that the stimulated phosphorylation of GFP-173A was much lower than that of WT-GFP-ERK2. This effect was mainly due to substitution of Pro\(^{174}\), as similar effect was detected also with GFP-174A. However, the acidic residues Asp\(^{316}\) and Asp\(^{317}\) might have a supporting role because GFP-173,5,7A exhibited a slightly reduced phosphorylation without any effect of GFP-177A. GFP-176A showed similar phosphorylation to WT-GFP-ERK2, suggesting that residues 176–178 are not involved in TEY phosphorylation.
To examine the interrelationships of the activation loop residues with another regulatory region, namely the CRS/CD motif, we used combined CRS/CD-activation loop mutants. Interestingly, when the CRS/CD alone mutant (GFP-316A) was used, we found that despite the reported reduced interaction of this mutant with MEK1 (21), the phosphorylation of GFP-316A in our system was not reduced as expected, but was in fact elevated both in resting and in stimulated COS7 cells. This effect could have been achieved by a residual CRS/CD-independent phosphorylation of GFP-316A by MEKs that cannot be suppressed by phosphatases, which do not interact with CRS/CD motif-lacking ERKs as well (16). This tilted equilibrium suggests that the CRS/CD motif is required to phosphatase association more than it is required for MEK interaction. Combined activation loop with CRS/CD mutations showed a slight reduction in their phosphorylation as compared to GFP-316A. These results indicate that the two regions operate additively in regulating the phosphorylation of ERK2. Moreover, the effects detected here are not restricted to EGF-stimulated COS7 cells, as similar results were obtained with TPA-treated CHO cells (data not shown) verifying the general role of residues 173–175 in ERK2 phosphorylation.

The Interaction of ERK2 with MEK1 Is Affected by Asp177 but Not by Residues 173–175 of ERK2—Although the direct phosphorylation by MEK1 was not reduced, it was still possible that MEKs do play a role in altering the in vivo stimulated phosphorylation of the activation loop mutants by differentially influencing their subcellular localization (21). To examine this possibility we studied the localization of GFP-ERK2 constructs when coexpressed with MEK1 that serves as a cytoplasmic anchor for ERKs (21, 35), in CHO cells. This experiment also serves as a sensitive tool to measure the ability of the two proteins to interact. As expected (32), when overexpressed in CHO cells, GFP-ERK2 constructs were

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Residues 173–175 Are Involved in ERK2 Substrate Recognition—Besides the role of activation loop residues in determining ERK2 phosphorylation, we undertook to study whether these residues play a role in ERK2 catalytic activity. One of the best-studied substrates of ERKs is Elk1 (36), and this transcription factor was used as a readout for this purpose. To this end, we immunoprecipitated GFP-ERK2 constructs from non-stimulated COS7 cells, and activated them in vitro using partially purified active MEKs. The phosphorylated ERK2 constructs were then incubated in three kinase concentrations to confirm linearity. We found that both GFP-173A and to a lesser extent also GFP-174A and GFP-176A but not GFP-177A demonstrated a reduced Elk1 phosphorylation (Fig. 5). In addition, GFP-316A, which was less phosphorylated by MEKs, had only a residual activity toward Elk1. These effects on Elk1 were extended toward cell proliferation, as COS7 cells expressing GFP-173A and GFP-174A, but not GFP-176A or GFP-316A, showed a reduced proliferation that was almost as slow as the rate of cells expressing KA-MEK1 that served as a positive control (supplemental Fig. S1). On the other hand, when the nonspecific substrate MBP was used to examine the effect on the catalytic kinase activity of ERK2, the activity of GFP-173A and GFP-174A was not significantly altered, while GFP-176A showed a reduce activity. GFP-316A and GFP-316/173,5,7A showed only a weak activity as expected from their phosphorylation levels. Taken together, our results indicate that these mutants are much more sensitive to phosphatases than the WT-GFP-ERK2. As expected (16), GFP-316A was less phosphorylated, while the two mutants completely lost their phosphates (Fig. 6, A and B). Therefore, these results may indicate that these mutants are much more sensitive to phosphatases than the WT-GFP-ERK2. As expected (16), GFP-316A was much less sensitive to phosphatases as compare with WT-GFP-ERK2, and therefore its activity was more sustained over the time tested. The inclusion of 173A (GFP-316/173A) only slightly accelerated the inactivation rate of 316A, indicating that the CRS/CD motif is the predominant phosphatase responsive site of ERK2.

The hypersensitivity of the GFP-173A and GFP-174A to phosphatases was supported by experiments with sodium vanadate, which inhibits protein Tyr phosphatases and MKPs. This inhibitor had only a slight activatory effect on the EGF-induced phosphorylation of WT-GFP-ERK2 (Fig. 6C), probably because this phosphorylation was already maximal at the time point used. However, the phosphorylation of GFP-173A and GFP-174A was significantly recovered upon treatment with the phosphatase inhibitor. Thus the rescue of activation by phosphatase inhibitors further indicates that the reduced phospho-
rylation of the GFP-173A and GFP-174A mutants is caused mainly by their hypersensitivity to phosphatases.

**Residues 173–175 Are Negative Regulators of ERK2 Dephosphorylation by MKP3**—To further assess the role of the activation loop residues in ERK2 regulation by phosphatases, we examined the sensitivity of the ERK2 constructs to HA-MKP3 in COS7 cells. As expected (37), elevated amount of MKP3 reduced the EGF-stimulated activity of WT-GFP-ERK2 (Fig. 7A). However, this reduction was much more pronounced when the GFP-173A and GFP-174A constructs were used, indicating that as predicted in the previous experiments, residues 173–175 serve as negative regulators of phosphatase action on ERK2, and therefore substitution of these residues induces a much faster dephosphorylation. However, substitution of residues 173–175 did not change the predicted resistance of 316A to MKP3 (Fig. 7A), corroborating the conclusion that the CRS/CD motif predominate the sensitivity of ERK2 to phosphatases. We also performed an in vitro phosphatase assay by MKP3 using immunoprecipitated phosphorylated GFP-ERK2 under the in vitro conditions used (Fig. 7B). These results support the importance of residues 173–175 in ERK regulation by phosphatases. Interestingly, these mutants had no effect on dephosphorylation by the phosphatase CPG21 (DUSP5; supplemental Fig. S2), indicating that the high affinity of the phosphatase to ERK2 is required for the effect.

**FIGURE 5. Activity of GFP-ERK2 and its mutants.** A, COS7 cells were transfected with each of the various constructs, serum-starved, and stimulated with EGF (50 ng/ml, 10 min). Cell lysates were prepared and subjected to immunoprecipitation with monoclonal anti-GFP Ab. The catalytic activity of the different GFP-ERKs was tested by an in vitro kinase assay using Elk-1 as the substrate. Each mutant was tested for its catalytic activity in three different concentrations. The concentrations were in a ratio of 1:4:12. Samples were separated by SDS-PAGE and subjected to Western blotting with the indicated Abs. B, COS7 cells were transfected with each of the various constructs, serum-starved, and harvested. The cell lysates were subjected to immunoprecipitation with monoclonal anti-GFP Ab. The GFP-ERK2 immunoprecipitates were subjected to in vitro kinase assay using partially purified MEKs as the kinases. The catalytic activity of the different activated GFP-ERKs was tested by an in vitro kinase assay using MBP as a substrate. The phosphorylation of MBP was detected by autoradiography on an x-ray film. The amounts of the GFP-ERKs were determined by a Western blot analysis with the indicated Abs.

**FIGURE 6. Inactivation of GFP-ERK2 mutants.** A, COS7 cells were transfected with each of the various constructs, serum-starved, and then were either stimulated with EGF (50 ng/ml) for the indicated time or left untreated. Cell extracts were subjected to SDS-PAGE followed by determination of the phosphorylation and amount of the GFP-ERKs using Western blot analysis with the indicated Abs. B, phosphorylation of the different constructs in A, 40 min after stimulation is presented as fold intensity of each construct per the intensity after 10 min of treatment. The bar graphs represent mean and S.E. of two experiments. C, inactivation of the GFP-ERK2 mutated in their activation loop is partially overcome by the phosphatase inhibitor, sodium vanadate. COS7 cells were transfected with each of the various constructs. The cells were serum-starved, incubated with sodium vanadate (1 mM, 30 min), before EGF treatment (50 ng/ml, 10 min). Cell extracts were subjected to SDS-PAGE and Western blotting to determine the phosphorylation of the GFP-ERK2 constructs.
DISCUSSION

The activity of ERKs is mainly regulated by MEKs and phosphatases that determine the levels of phosphate incorporation to the Thr and Tyr residues within the ERKs activation loop. The dual phosphorylation causes a significant conformational change that enables substrate binding to the catalytic pocket. Although the molecular consequences of the Thr and Tyr phosphorylation and the conformational changes of the activation loop induced by it were extensively examined (11, 38), much less is known about the role of other activation loop residues in regulating ERK interactions and activity. In a previous study we found that residue 176 plays a role in the release of ERK2 from its interaction with MEKs. In the current study we continued to examine the role of residues 173–177 in the activation loop in regulating ERK2 activity. We found that substitution of residues 173–175, and in particular Pro174 to alanine significantly reduces the phosphorylation of the mutated ERK2 upon extracellular stimulation (Fig. 2 and Ref. 32). The reduced activation could have been derived from either reduced phosphorylation by MEKs, or by accelerated dephosphorylation by phosphatases. These two possibilities were examined, and it was found that the sensitivity of GFP-173A to MEKs in vitro is not different from that of WT-GFP-ERK2 (Fig. 3). Moreover, the interaction of this mutant with MEK1 was not altered, as determined by its proper cytosolic anchoring when coexpressed with HA-MEK1 in CHO cells (Fig. 4). On the other hand our results indicate that the substitution of 173–175 makes ERK2 more susceptible to dephosphorylation by phosphatases (Fig. 6) such as MKP3 (Fig. 7). Therefore, it is likely that these residues protect phosphorylated ERKs from a premature or too fast inactivation, and therefore are important in determination of the duration of ERKs activity (4, 5). Because substitution of Pro174 mimicked the effect of 173–175 substitution, while substitution of Asp177 and Asp175 (within GFP-173,5,7A) had only a small effect, it is likely that the important residue in mediating this effect is Pro174 of ERK2.

Besides the effect of Pro174 and its adjacent Asp173 and Asp175 identified here, as well as His176 identified in our previous publication (32), we found that also Asp177 plays a role in regulating ERK2. Although substitution of residues 176–178 (GFP-176A) or Asp177 (GFP-177A) had no effect on the sensitivity of ERK2 to EGF-induced phosphorylation, it significantly affected the MEK1-dependent subcellular localization of ERK2 in CHO cells (Ref. 32 and Fig. 4). As mentioned in our previous publication, the fact that these mutants had a reduced interaction with MEK1 is best explained by their ability to enhance dissociation of ERK2 from MEK1. Substitution of Asp177 to alanine (Fig. 4) reduced the cytoplasmic retention of ERKs in similarity to the effect of His176 that was previously described (32), indicating that Asp177 may assist His176 in the MEK1-ERK2 dissociation process. The full phosphorylation of GFP-177A upon EGF stimulation (Fig. 2) is not clear since GFP-177A is localized in the nucleus and should be spatially separated from the cytoplasmic MEK1. It is possible that the phosphorylation is mediated by MEKs that translocate to the nucleus (33, 39), but this should be further investigated.
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The CRS/CD motif of ERKs has previously been implicated in allowing the interaction and activation of ERKs with MEKs (16, 21, 40, 41). Indeed, the rate of GFP-316A phosphorylation by active MEK1 in vitro was significantly reduced as compared with that of WT-GFP-ERK2 (Fig. 3). Therefore, the hyperphosphorylation of GFP-316A observed upon mitogenic stimulation (Fig. 2) needs an explanation. It was previously reported that the CRS/CD motif plays a role in the docking interaction of ERKs with phosphatases (16, 42), and therefore it was possible that the elevated phosphorylation of GFP-316A occurs due to impaired interaction with the phosphatases. Nonetheless, the results presented here indicate that MEKs can still phosphorylate CRS/CD-deficient ERK2 to considerable levels upon stimulation, although the rate of this phosphorylation is severely impaired as determined by the in vitro kinase assay. The differences between MEKs and the phosphatases in the outcome of their interaction with the CRS/CD motif might be explained by the necessity of the phosphatases to interact with additional docking moieties in ERK2, such as the DEF binding domain (43, 44), which are not required for MEK1 activity. Thus, it is possible that the mutation of the CRS/CD motif modify also the conformation of the additional regions (e.g. DEF binding, activation loop) that are important for activity of the phosphatases, while the additional regions required for MEK1 activity are not affected. In addition, it is possible that some other proteins in the cell compensate the CRS/CD deficiency of ERK2 by forming a productive complex whereby MEKs can efficiently activate ERKs. This type of altered interactions may lead to the tilted equilibrium between MEKs and phosphatases in regulating the GFP-316A construct, resulting in its hyperphosphorylation upon stimulation.

Interestingly, addition of activation loop mutants to the GFP-316A slightly reduced the hyperphosphorylation obtained by CRS/CD substitution upon EGF stimulation (Fig. 2), indicating that the two sites have an additive effect in regulating stimulation. However, because the rate of long-term inactivation of GFP-316A and GFP-316A-173A by phosphatases was similar to each other (Figs. 6 and 7), it can point out CRS/CD motif as a predominant domain in regulating the ERK2 sensitivity to phosphatases and also can raise the possibility that the CRS/CD motif beside its role to increase the cytoplasmic anchoring. Regarding the activity of ERKs we found that residues 173-5 play a role in the activity toward Elk1, while residues 176-8 are important for the catalytic activity. Finally we found that CRS/CD motif plays a bigger role in ERK2 dephosphorylation than in its phosphorylation. Combining CRS/CD with activation loop mutation revealed that these two regions cooperate in determining the net phosphorylation of ERK2, but the role of the CRS/CD motif predominates that of the activation loop residues. Thus, we show here that residues 173–177 of ERK2 join other regulatory regions of ERKs in governing the output of ERK activity upon stimulation.

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