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Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase

(signal transduction/growth control/transcription factors/protein kinases)

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ABSTRACT **Phosphorylation of the C terminus of c-Fos has been implicated in serum response element-mediated repression of c-*fos* transcription after its induction by serum growth factors. The growth-regulated enzymes responsible for this phosphorylation in early G_1 phase of the cell cycle and the sites of phosphorylation have not been identified. We now provide evidence that two growth-regulated, nucleus- and cytoplasm-localized protein kinases, 90-kDa ribosomal S6 kinase (RSK) and mitogen-activated protein kinase (MAP kinase), contribute to the serum-induced phosphorylation of c-Fos. The major phosphopeptides derived from biosynthetically labeled c-Fos correspond to phosphopeptides generated after phosphorylation of c-Fos *in vitro* with both RSK and MAP kinase. The phosphorylation sites identified for RSK (Ser-362) and MAP kinase (Ser-374) are in the transrepression domain. Cooperative phosphorylation at these sites by both enzymes was observed *in vitro* and reflected *in vivo* by the predominance of the peptide phosphorylated on both sites, as opposed to singly phosphorylated peptides. This study suggests a role for nuclear RSK and MAP kinase in modulating newly synthesized c-Fos phosphorylation and downstream signaling.**

The product of protooncogene c-*fos* is implicated in cell proliferation (1–4), differentiation (5), and development (6–8). To ensure proper expression during these processes, it is subjected to tight regulation at multiple levels. The c-*fos* gene undergoes rapid and transient transcriptional activation in response to a variety of extracellular stimuli in various cell types (9, 10). The protein synthesis-independent induction is mediated mainly through the serum response element (11), which binds a protein complex composed of a homodimer of serum response factor and $p62^{TCF}$ (12–18). Interestingly, the repression of c-*fos* gene expression following its activation may also be mediated through the serum response element (19) and the newly synthesized c-Fos protein plays a role in repression of its own promoter (20–22). The transforming v-Fos protein, which is truncated at the C terminus with five additional point mutations (23), is defective in the transrepression activity (24, 25). Upon serum stimulation, c-Fos is more extensively phosphorylated than v-Fos, due to this C-terminal region (23, 25–28). Phosphorylation of the C terminus has been shown to be responsible for the transrepression activity (29, 30), and truncation or mutations that block phosphorylation in this region enhance the transforming capacity of c-Fos (30).

c-*fos* gene induction correlates with the agonist-dependent activation of the mitogen-activated protein kinase (MAP kinase)/90-kDa ribosomal S6 kinase (RSK) signal transduction pathway (31–33). In addition, both kinases are nuclear and cytoplasmic (34), which may be a prerequisite for their participation in regulation of gene expression. Although MAP kinases and RSK are activated before initiation of c-*fos* transcription, these enzymes are still active during transcriptional repression of the c-*fos* gene. In this study, we have asked whether these kinases contribute to the phosphorylation of c-Fos in early G_1. We demonstrate that c-Fos is coordinately phosphorylated *in vitro* by MAP kinases and RSK at the major *in vivo* phosphorylation sites within the C-terminal transrepression domain, implicating these kinases as possible contributors to the transrepression activity of c-Fos toward its own promoter. This study raises the possibility that the growth-regulated MAP kinase/RSK signal transduction pathway participates in both the transcriptional activation (35–37) and repression of c-*fos* gene expression.

MATERIALS AND METHODS

Cell Cultures, Cell Lysates, and Immune Complex Kinase Assays. Swiss 3T3 and rat fibroblast F2408 cells were cultured to confluence, serum-starved, and serum-stimulated as described (38). Cell lysates were prepared and immune complex kinase assays for $pp90^{rsk}$ (38), $pp44^{mapk}$ (36), and $pp70^{s6k}$ (39) were completed by using 1 μg of various recombinant rat c-Fos polypeptides (40) as substrates. For cooperation studies, c-Fos was first phosphorylated with either RSK or MAP kinase in the presence of 100 μM (minus radiolabeled) ATP for 30 min. The enzymes were then removed by centrifugation, and the supernatants containing phosphorylated c-Fos were subjected to phosphorylation with the second enzyme in the presence of [γ-^{32}P]ATP. Aliquots from the reaction mixtures were removed and terminated at 0, 2.5, 5, 10, and 15 min. ^{32}P incorporation into c-Fos was determined after resolving the reaction products by SDS/PAGE.

For biosynthetic labeling, serum-starved rat fibroblast F2408 cells were cultured in 4 ml of serum- and phosphate-free medium per 100-mm culture dish for 1 h before being labeled for 2 h with 8 mCi (1 Ci = 37 GBq) of $^{32}P_i$ (ICN). Cells were stimulated with dialyzed calf serum to a final concentration of 10% (vol/vol) for 1 h before preparation of the cell lysates. Three 100-mm plates of labeled cells were washed with STE (38) and scraped in 0.5 ml of RIPA buffer (38). The cell suspension was incubated on ice for 20 min, and the cell lysate was clarified by centrifugation at 10,000 $\times$g for 30 min. Supernatants were then adjusted to 1% SDS, denatured by heating to 85°C–90°C for 5 min, and diluted with 9 vol of SDS-free RIPA buffer. Immunoprecipitation of c-Fos polypeptides was performed with antiserum made against full-length recombinant c-Fos protein (from T. Curran, Roche Institute) and completed as described (38).

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Abbreviations: MAP kinase, mitogen-activated protein kinase; RSK, 90-kDa ribosomal S6 kinase; NGF, nerve growth factor; PKA, protein kinase A.

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Two-Dimensional Phosphopeptide Mapping. Tryptic peptide mapping was performed as described (38). For proteolysis with a second enzyme, tryptic peptides were eluted from TLC plates in water, lyophilized, and dissolved in 50 μl of 0.1 M potassium phosphate (pH 7.5). Proline-directed endopeptidase (BMB) was added to 0.15 unit and the reactions were allowed to proceed overnight at 30°C. The samples were then lyophilized three times in water and resolved on TLC plates.

Site-Directed Mutagenesis. To mutate Ser-362 and/or -374 to Ala, site-directed mutagenesis was performed with overlap extension by PCR as described (41). The mutant proteins were expressed in bacteria as hexahistidine fusion proteins and purified by nickle-affinity chromatography as described (40).

RESULTS

To determine whether the cytoplasmic/nuclear, growth-regulated protein serine/threonine kinases, MAP kinases, RSK, and pp70^{s6k} phosphorylate c-Fos *in vitro*, phosphorylation reactions were performed with enzymes immunoprecipitated from cells, using recombinant c-Fos as a substrate. The protein was phosphorylated by activated RSK and MAP kinase but not by pp70^{s6k} (Fig. 1*A*). The phosphorylation of c-Fos by either enzyme alone gave a large mobility shift in SDS/polyacrylamide gels, as observed *in vivo* (24, 26). Phosphorylation by both enzymes resulted in an even greater mobility shift, consistent with phosphorylation at distinct sites (Fig. 1*B*, compare lane 3 to lanes 1 and 2). Furthermore, c-Fos first phosphorylated by RSK or MAP kinase was a better substrate for subsequent phosphorylation by the other (Fig. 2), indicating a cooperative phenomenon.

We began localizing c-Fos phosphorylation sites by examining several truncated Fos polypeptides (Fig. 3*A*). c-Fos polypeptides containing the C terminus were excellent substrates for both kinases, whereas the C-terminal deletion mutants were poorly phosphorylated, indicating that the major phosphorylation sites were localized to the C terminus (amino acids 321–380). C-terminal truncations generated tryptic phosphopeptides corresponding to minor spots in full-length c-Fos labeled *in vitro* (Fig. 3*Bc* and *f*) and in biosynthetically ^{32}P-labeled c-Fos (Fig. 3*Bh*).

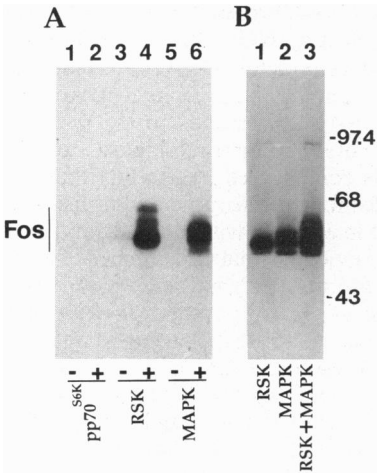

FIG. 1. *In vitro* phosphorylation of recombinant c-Fos protein by RSK and MAP kinase. (*A*) Phosphorylation of recombinant c-Fos protein was performed with pp70^{S6K} (lanes 1 and 2), RSK (lanes 3 and 4), or MAP kinase (lanes 5 and 6) immune complexes from serum-starved (odd-numbered lanes) or serum-stimulated (5 min; even-numbered lanes) Swiss 3T3 cells. (*B*) Comparison of migration of recombinant c-Fos phosphorylated by RSK (lane 1), MAP kinase (lane 2), or both enzymes (lane 3). Migration of molecular size markers (kDa) is indicated on the right.

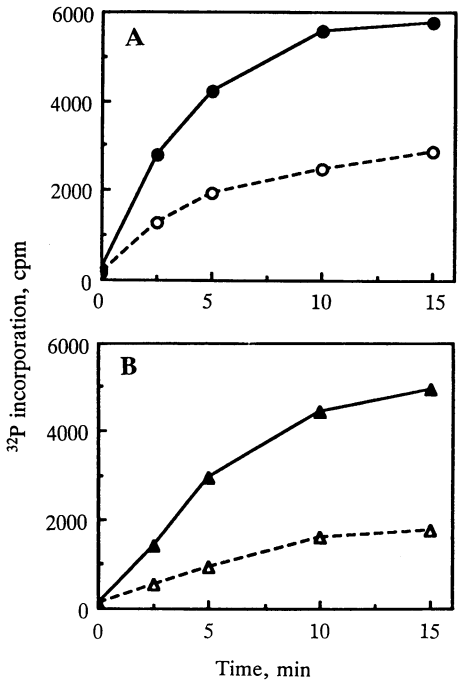

FIG. 2. Coordinate phosphorylation of c-Fos by RSK and MAP kinase. (*A*) Enhanced c-Fos phosphorylation by RSK after prephosphorylation with MAP kinase. c-Fos preincubated with immune complexes of MAP kinase antiserum (●) or of preimmune serum (○) was used as a substrate for phosphorylation with RSK in the presence of [γ-^{32}P]ATP. ^{32}P incorporated into c-Fos was determined at 0, 2.5, 5, 10, and 15 min of the reactions. (*B*) Enhanced c-Fos phosphorylation by MAP kinases after prephosphorylation with RSK. c-Fos preincubated with RSK immune complexes (▲) or with immune complexes of preimmune serum (△) was used as a substrate for MAP kinases. ^{32}P incorporation was determined as in *A*.

Comigration of the major tryptic phosphopeptide arising from phosphorylation by RSK (Fig. 3*Ba*, peptide 1′) or by MAP kinase (Fig. 3*Bd*, peptide 1) indicates that these kinases phosphorylate c-Fos within the same tryptic peptide. Phosphorylation by both enzymes resulted in generation of a new peptide (Fig. 3*Bg*, peptide X) in addition to the peptides phosphorylated by either enzyme alone (peptides 1′/1 and 2), suggesting that peptide X likely comes from phosphorylation of peptide 1′/1 by both kinases. Most importantly, peptide X comigrated with the major tryptic phosphopeptide derived from biosynthetically ^{32}P-labeled c-Fos (Fig. 3*Bh* and *i*). These results suggest that RSK and MAP kinase phosphorylate c-Fos at distinct sites within the same tryptic peptide that is phosphorylated *in vivo*. The low level of peptides 1/1′ in biosynthetically labeled c-Fos indicates that *in vivo* this peptide is usually phosphorylated on two sites instead of one, supporting the notion of cooperative phosphorylation.

Within amino acids 321–380 of c-Fos, a tryptic peptide containing potential RSK (44) and MAP kinase phosphorylation sites (45, 46) was identified. This domain (amino acids 361–380) has also been reported to be required for the transrepression activity (25, 29, 30) of c-Fos. These potential RSK and MAP kinase sites are separated by a single proline residue (Fig. 4*A*). Proteolysis of peptides 1′, 1, and X with a proline-directed endopeptidase gave rise to two distinct phosphopeptides (Fig. 4*B*). One (peptide a) was the result of RSK phosphorylation (Fig. 4*Ba*) and a second (peptide b) was the result of MAP kinase phosphorylation (Fig. 4*Bb*). Both peptides were observed after digestion of peptide X generated from *in vitro* (Fig. 4*Bc*) or *in vivo* (Fig. 4*Bi*) labeling. In addition, *in vitro* phosphorylation of a synthetic peptide containing amino acids 357–367 (AHRKGSSSNEP) by RSK

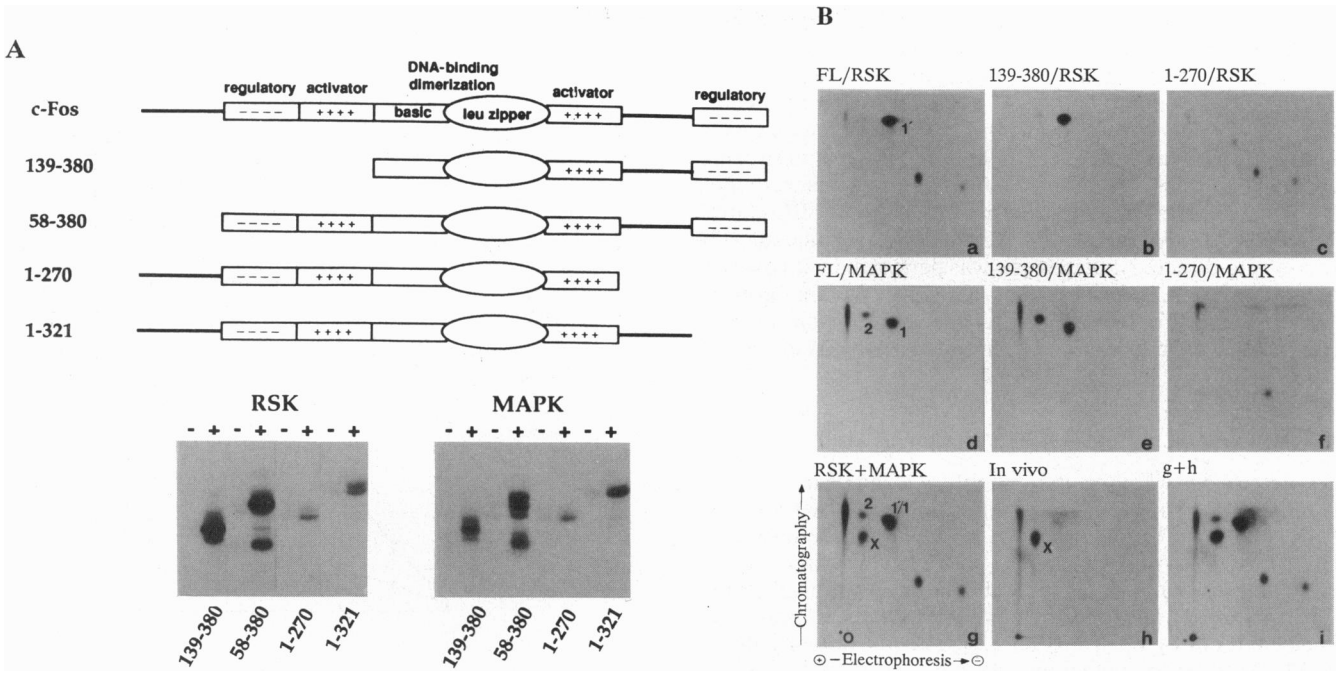

FIG. 3. Phosphorylation of deletion mutants of recombinant c-Fos by RSK and MAP kinase. (*A*) Illustration of the functional domains of c-Fos. Region required for dimerization and DNA binding is contained within the leucine zipper and the adjacent basic region. Fos contains two activator regions that contribute to transcriptional stimulation *in vitro* (++++) and two putative regulatory regions that repress transcription *in vitro* (----) (42, 43). Truncated Fos proteins containing amino acids 139–380, 58–380, 1–270, or 1–321 were phosphorylated by RSK or MAP kinase, which were immunoprecipitated from quiescent (−) or serum-stimulated (+) 3T3 cells. (*B*) Tryptic phosphopeptide maps of *in vitro* versus *in vivo* phosphorylated c-Fos. *In vitro* phosphorylation was performed with RSK (*a–c*), MAP kinases (*d–f*), or both enzymes (*g*). (*a*, *d*, and *g*) Full-length c-Fos. (*b* and *e*) Truncated Fos containing amino acids 139–380. (*c* and *f*) Truncated Fos with amino acids 1–321. (*h*) *In vivo* labeled Fos. (*i*) Mix of equal counts of samples spotted in *g* and *h*. Directions of electrophoresis and chromatography (first and second dimensions, respectively) are indicated. O, origin.

followed by double proteolysis gave the same phosphopeptide map as the recombinant c-Fos phosphorylated only by RSK (Fig. 4*Bf* and *g*). This synthetic peptide was not phosphorylated by MAP kinase. These results are consistent with our prediction that the main phosphorylation sites of c-Fos for RSK and MAP kinase localize within amino acids 362–364 and 371–374, respectively. Sequential Edman degradation of peptides a and b led to identification of Ser-362 and Ser-374 as the RSK and MAP kinase phosphorylation sites, respectively (data not shown).

Peptide 2 (Fig. 3*Bd*) in Fos phosphorylated by MAP kinase was consistently found to coexist with peptide 1 and was also localized within amino acids 321–380. Proteolysis of peptide 2 with proline-directed endopeptidase resulted in a phosphopeptide that comigrated with that from peptide 1 (data not shown), indicating that peptides 1 and 2 are related and likely represent differentially digested peptides. The streaking material (Fig. 3*B*, peptide 3) that was not resolved in our system was also observed in the map from biosynthetically labeled c-Fos. Digestion of this peptide with a second protease did not generate consistent, well defined phosphopeptides (data not shown). It may represent insoluble material present in the sample; however, we cannot rule out the possibility that it comes from minor phosphorylation of the Fos N terminus by MAP kinase.

To confirm the phosphorylation sites for RSK and MAP kinases, c-Fos mutated at Ser-362 and/or -374 to Ala (designated S362A, S374A, and S362/374A in Fig. 5) were tested as kinase substrates. As predicted, S362A and S362/374A mutants phosphorylated *in vitro* by RSK no longer gave peptide 1′, whereas the S374A mutant generated the same map as did the wild-type c-Fos. Upon phosphorylation by MAP kinase, S374A and S362/374A mutants did not give rise to peptides 1 and 2; however, S362A mutant gave the same map as the wild-type protein. The mutagenesis study demonstrated that Ser-362 and -374 are indeed the phosphorylation sites for RSK and MAP kinases, respectively.

DISCUSSION

c-Fos undergoes extensive phosphorylation in early G_1 stage of the cell cycle (23–26). Several protein kinases have been reported to phosphorylate Fos *in vitro*, including $p34^{cdc2}$ (47), protein kinase C (47), protein kinase A (PKA) (25, 47), and a nerve growth factor (NGF)-stimulated Fos kinase activity from rat pheochromocytoma PC12 cells (48). However, it has not been determined whether *in vitro* phosphorylation of Fos by these enzymes corresponds to the *in vivo* situation. In addition, the potential for these kinases to be physiological Fos kinases is complicated by the fact that their regulation does not correlate with the rapid and transient expression of the c-*fos* gene in early G_1 with all mitogens. In this study, we have provided evidence that two families of growth-regulated protein kinases, MAP kinase and RSK, can coordinately phosphorylate c-Fos at its major *in vivo* phosphorylation sites. MAP kinase and RSK are good candidates for physiological Fos kinases on the basis that both enzymes are regulated with rapid and transient kinetics in response to a variety of extracellular signals and are active when the Fos protein is expressed (31, 32, 34) and that both enzymes colocalized with the Fos protein (34).

Phosphorylation by MAP kinase and RSK appears not to be an ordered reaction, since either enzyme can efficiently phosphorylate Fos without the action of the other. However, cooperation between the two phosphorylation events is suggested since Fos previously phosphorylated with one enzyme became a better substrate for the second kinase (Fig. 2). The observed cooperative phenomenon *in vitro* is also reflected *in vivo* as discussed below.

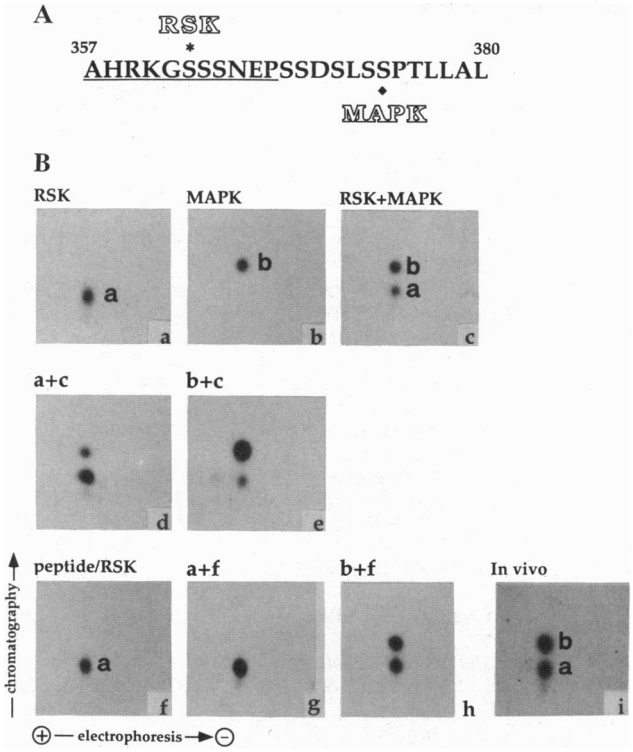

FIG. 4. Localization of phosphorylation sites of RSK and MAP kinase to the extreme C terminus of c-Fos. Sequence of amino acids 357–380 at C terminus of c-Fos is shown in *A*. A synthetic Fos peptide was made according to the sequence underlined (residues 357–367). (*B*) Two-dimensional peptide maps of proline-directed endopeptidase cleavage of isolated tryptic peptides. The phosphorylation sites for RSK and MAP kinase are indicated above and below the sequence, respectively, in *A*. These were determined by sequential Edman degradation of peptides a and b.

With several biochemical and molecular approaches, we have localized the major phosphorylation sites for RSK and MAP kinase to Ser-362 and -374, respectively. These sites reside within the two C-terminal serine-rich regions that were previously mutated and shown to result in reduction of transrepression activity of c-Fos at the c-*fos* promoter (25). Importantly, these residues correspond to the major *in vivo* phosphorylation sites for c-Fos. The small amount of peptide 1′/1 in the tryptic maps of *in vivo* labeled protein suggests that c-Fos is phosphorylated in the cell predominantly on both serines instead of one, consistent with the notion that phosphorylation by MAP kinase and RSK is cooperative.

Ser-362 resides in a RSK and PKA consensus recognition sequence (49). An earlier study has shown that PKA can phosphorylate Fos *in vitro* at Ser-362, and this phosphorylation contributed to part of the *in vivo* phosphorylation (30). A more prominent, uncharacterized tryptic phosphopeptide present in the *in vivo* labeled protein has been suggested to contain two phosphates, with one of them the result of phosphorylation at position 362. However, the second phosphorylation site and the enzyme that contributes to the phosphorylation were not determined. Nevertheless, these results are consistent with our observation that in the cell c-Fos is predominantly phosphorylated on two serines in the C-terminal tryptic peptide. The fact that PKA is not activated by serum growth factors suggests that RSK is more likely the physiological kinase responsible for mitogen-stimulated phosphorylation at Ser-362 of c-Fos in early G_1.

In PC12 cells a NGF-stimulated kinase, which is distinct from MAP kinase, RSK, casein kinase II, protein kinase C, calmodulin-dependent kinase, and PKA, was recently reported to phosphorylate Fos at Ser-362 (48). This Fos kinase was activated in PC12 cells induced with NGF or epidermal growth factor (EGF), but not with fibroblast growth factor (FGF), phorbol 12-myristate 13-acetate (PMA), cAMP, or membrane depolarization. Addition of NGF, EGF, FGF, or PMA to PC12 cells stimulates rapid and transient activation of MAP kinase and RSK (50) as well as c-Fos expression and phosphorylation (51). The isolation of different kinase activities that can phosphorylate Fos at Ser-362 indicates that multiple, alternative mechanisms might contribute to the

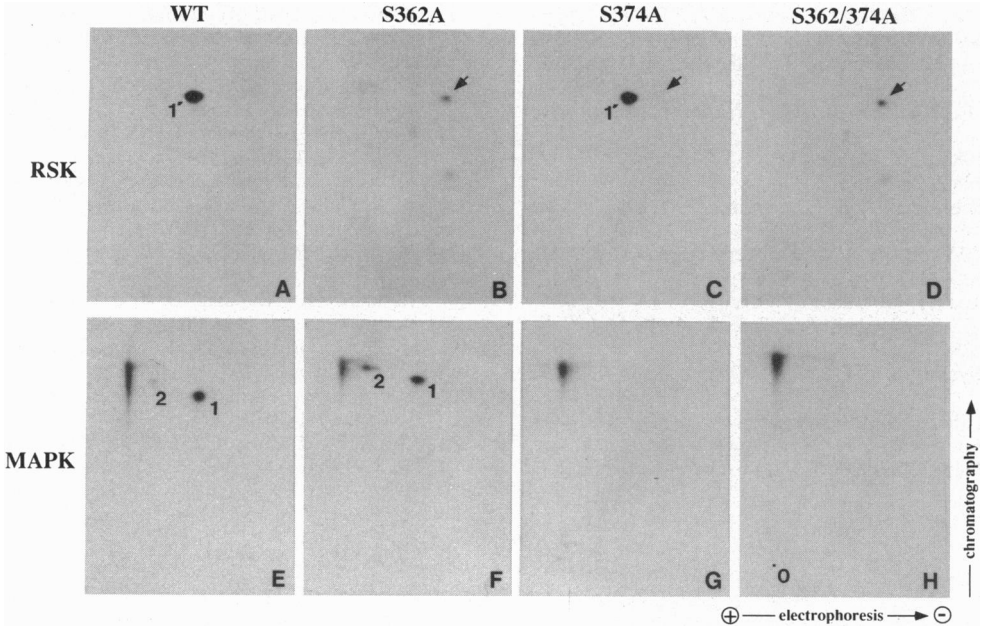

FIG. 5. Tryptic peptide mapping of mutant c-Fos proteins phosphorylated by RSK (*A–D*) and MAP kinase (*E–H*) *in vitro*. The following substrates were used in each experiment: *A* and *E*, wild-type (WT) c-Fos proteins; *B* and *F*, S362A mutants with Ser-362 mutated to Ala; *C* and *G*, S374A mutants with Ser-374 mutated to Ala; *D* and *H*, S362/374A mutants with both Ser-362 and -374 mutated to Ala. Equal counts of samples were applied onto TLC plates, and autoradiograms with the same exposure time were presented for phosphorylation with the same enzyme. Arrows in *B–D* point to the minor RSK phosphorylation site, which migrates differently from peptide 1′ and is present at a significant level only when Ser-362 is mutated.

phosphorylation and transrepression activity of Fos, possibly in a cell- and ligand-specific manner.

One of the components involved in serum induction of the c-*fos* gene, $p62^{TCF}$, was recently shown to be phosphorylated by MAP kinase. This phosphorylation is required for recruitment of $p62^{TCF}$ into a ternary complex at the serum response element (37) or enhances its transactivation activity (36). Serum response factor is phosphorylated by RSK *in vitro* at an *in vivo* serum-stimulated phosphorylation site (35). Combined with our results, this raises the interesting possibility that the MAP kinase/RSK signaling pathway participates in both transcriptional activation and subsequent repression of c-*fos* gene expression. Failure to activate the transrepression activity of c-Fos by phosphorylation might be expected to enhance its transforming capacity, as in the case of v-Fos. Further investigation, including structure/function studies with mutations specifically at the MAP kinase and RSK phosphorylation sites, is necessary to support this model.

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