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Functional expression in mammalian cells of a full-length cDNA coding for the pp42/MAP kinase ($p42^{mapk}$) protein

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We recently cloned from a mouse 3T3 cell cDNA library a cDNA with sequence similarity to the $p42^{mapk}$ protein and other members of the MAP kinase family. To determine with certainty which member of the family this clone encodes, we have expressed the cDNA in COS cells and characterized the protein product. When the pSV_2MAP plasmid carrying the full-length clone was transfected into COS cells, a protein of 42 000 Da was expressed. This 42 kDa protein displayed chromatographic properties indistinguishable from the endogenous $p42^{mapk}$, and could be separated from the closely related pp44. In addition, upon serum stimulation, the 42 kDa protein became tyrosine-phosphorylated and enzymatically active towards the substrate myelin basic protein. We conclude that this clone codes for a functional $p42^{mapk}$ protein kinase.

Protein kinase; Phosphotyrosine; MAP kinase; COS cell, Transient expression

1. INTRODUCTION

Ray and Sturgill originally identified the pp42/MAP kinase protein ($p42^{mapk}$) as a serine/threonine protein kinase [1–3] which required phosphorylation on both threonine and tyrosine residues to be enzymatically active [4]. The two sites of regulatory phosphorylation were found to reside on a single tryptic peptide, separated by only one amino acid [5].

An apparently similar enzyme was purified and sequenced by Cobb and co-workers, a partial cDNA clone based on this sequence was obtained from a rat brain library [6] and this clone was named ERK1. Since the amino acid sequence surrounding the regulatory phosphorylation sites of $p42^{mapk}$ was 85% identical to the equivalent region in ERK1 [5], we used oligonucleotides based on the ERK1 sequence to screen a 3T3 cDNA library, with the expectation that a $p42^{mapk}$ clone could be obtained [7]. With the ERK1 oligonucleotides we were able to isolate a number of ERK1 clones as well as a full-length cDNA corresponding to a candidate $p42^{mapk}$ clone. The deduced amino acid sequence of this clone predicted a 42 kDa protein exhibiting the characteristic features of a serine/threonine protein kinase and containing a sequence identical to the site of regulatory phosphorylation we had previously determined for $p42^{mapk}$ [5,7].

Although we suspected that this clone encoded the $p42^{mapk}$ protein, this issue was complicated by the fact that $p42^{mapk}$ is a member of a protein kinase family which includes at least four mammalian members [8] and several related proteins in amphibians [9] and invertebrates [10]. The site of regulatory phosphorylation is highly conserved even in the yeast kinases FUS3 [11] and KSS1 [12]. Different family members function variously in intracellular signaling during the $G_0 \rightarrow G_1$ transition [13,14] or during mitosis [9] or upon activation of terminally differentiated cells [15]. Since the $p42^{mapk}$ protein we purified was from EL4 thymoma cells [5] it was by no means certain that the 3T3 cDNA clone we obtained encoded the authentic $p42^{mapk}$ described by Sturgill and collaborators [3], or whether some other family member, with different regulatory or biochemical properties, might have been obtained.

To examine the relationship between te $p42^{mapk}$ protein and the cDNA isolated with ERK1 probes, we constructed a transient expression vector (pSV_2MAP) carrying the full-length cDNA clone and transfected it into mammalian COS cells. This transient expression system has been successfully used to characterize numerous DNA sequences. We show here that a 42 kDa protein was over-expressed and demonstrate that this protein possessed the biochemial, regulatory and enzymatic features expected for the $p42^{mapk}$ protein.

2. MATERIALS AND METHODS

2.1. *Materials*

Fetal calf serum, bovine calf serum and lipofectin reagent were purchased from Gibco BRL, Life Technologies, Inc., Gaithersburg, MD. The phenyl-Superose column and phenyl-Sepharose were from Pharmacia-LKB. [^{125}I]protein A, donkey anti-rabbit horseradish peroxidase (HRP) antibody and ECL (Electro Chemi-Luminescence) kit were all purchased from Amersham, Arlington, IL. [γ-^{32}P]ATP (6000 Ci/mmol) was from NEN-DuPont Research Products, Wilmington, DE. Specific anti-phosphotyrosine antisera were generated in this laboratory as described [13]. The anti-MAP kinase antiserum was generated against a peptide corresponding to the C-terminal amino

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acids of the ERK1 sequence, ARFQPGPEAP [6] using the MAPS procedure [18]. The corresponding predicted sequence in our p42^{mapk} clone is ARFQPGYRS [7]. The IgG fraction of the antiserum was obtained by protein G chromatography.

The pBluescript plasmid was purchased from Stratagene, La Jolla, CA. All other molecular biology reagents were the highest quality commercially available.

2.2 *Construction of a vector for transient expression of recombinant p42^{mapk} cDNA*

The full length p42^{mapk} cDNA was first cloned from a λgt10 cDNA library into pBluescript [7]. For transient expression of p42^{mapk} in COS-7 cells, the 1.3 kb *Hin*dIII/*Sma*I fragment containing the p42^{mapk} gene was further subcloned into pSV$_2$CAT with the chloramphenicol acetyl transferase (CAT) gene being cut out using *Hin*dIII and *Hpa*I restriction enzymes. In thsi vector, the p42^{mapk} cDNA is driven by the SV40 early promoter and transcription is terminated by the SV40 polyadenylation signal downstream from the *Hpa*I/*Sma*I site.

2.3. *Cell culture and transfection experiments*

The cell line COS-7 (ATCC) was grown at 37°C in a 7.5% CO_2 incubator, with Dulbecco's modified Eagle medium (DMEM) supplemented with 5% calf serum + 5% fetal calf serum until 80–90% confluence. The plates were then washed twice with serum-free DMEM before adding fresh serum-free DMEM containing the lipofectin-DNA complex [17]. Complex formation was achieved by diluting 10–15 μg DNA and 30 μg lipofectin into 4 ml DMEM. After 6 h incubation, the solution was aspirated and 10% fetal calf serum-supplemented DMEM was added. The cells were harvested after 36–72 h of expression.

2.4. *Cell lysis, FPLC purication and enzymatic activity*

These procedures were performed as previously described [14,18].

2.5. *Western immunoblotting*

Anti-p42^{mapk} blots were incubated ar 37°C in phosphate-buffer saline (PBS) supplemented with 5% low-fat dried milk (PBS-milk). They were first pre-blocked 0.5 h before probing with anti-p42^{mapk} antiserum (for 2 h). Blots were then washed 3 times in PBS-milk and reacted for 1 h with secondary antibody conjugated to horse radish peroxidase. Blots were washed 3 times again before applying the ECL kit for 1 min at room temperature. Immediately after, X-ray films were exposed for 10 to 1 min. Anti-phosphotyrosine immunoblots were revealed as described elsewhere [14].

3. RESULTS

3.1. *Transient expression levels of p42^{mapk} in whole cell lysates*

The vector pSV$_2$MAP was first constructed (see Materials and Methods) from the transient expression vector pSV$_2$CAT in which the CAT gene was replaced by the full-length cDNA coding for a member of the MAP kinase family, presumably p42^{mapk}. Then, the pSV$_2$MAP plasmid was transfected in the presence of the lipofectin reagent [17] into COS-7 cells for transient expression.

Fig. 1 shows anti-MAP kinase immunoblots of the time-course of induction of p42^{mapk} expression in this mammalian cell line. The only immunoreactive band which was newly expressed after the transfection of the pSV$_2$MAP plasmid had an M_r of 42 000. This expression was detected from 1.5–3 days after transfection, with a peak at 2.5 days. However, when the empty vector pSV$_2$CAT was introduced, no difference, compared to the basal levels, was detectable in the intensity of the p42 band 1.5 days after transfection (see Fig. 1) or 2.5 days (data not shown).

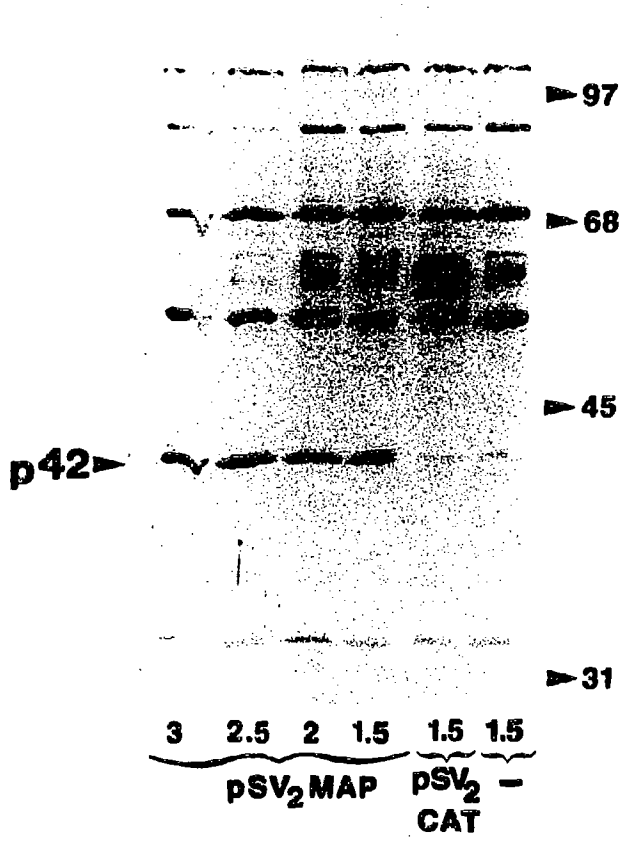

Fig. 1.Time-course of p42^{mapk} protein expression in COS cells. Cells were transfected as described in Methods and were lysed at the indicated times (in days). An anti-MAP kinase immunoblot of whole cell lysates (50 μg protein per lane) is presented and is typical of three independent experiments. pSV$_2$CAT is the empty vector, pSV$_2$MAP is the vector expressing p42^{mapk}.

Fibroblastic cells which have been examined express at least two members of the MAP kinase family, p42^{mapk} and another protein of 44 kDa [19–21], which we refer to as p44^{mapk} [19]. We have previously noticed that whereas p42^{mapk} can readily be detected with anti-phosphotyrosine antibodies in a Western blot of electrophoresed total cell lysates, the p44^{mapk} could not be consistently detected [13,14,18] unless the proteins were first separated on 2-D gels [18]. We speculated that a protein of around 44 kDa (such as actin) could inhibit the interaction of p44^{mapk} with the antibodies. To determine whether a similar explanation could account for our failure to detect the 44 kDa protein in the immunoblots shown in Fig. 1, we took advantage of the fact that p42^{mapk} and p44^{mapk} are capable of binding to hydrophobic chromatography matrices, such as phenyl-Sepharose. When phenyl-Sepharose was utilized to partially purify the MAP kinases, and the eluted proteins were separated by gel electrophoresis, both p42^{mapk} and the 44 kDa protein could be detected with the anti-MAP kinase antiserum (Fig. 2, left panel). Note that when COS cells were

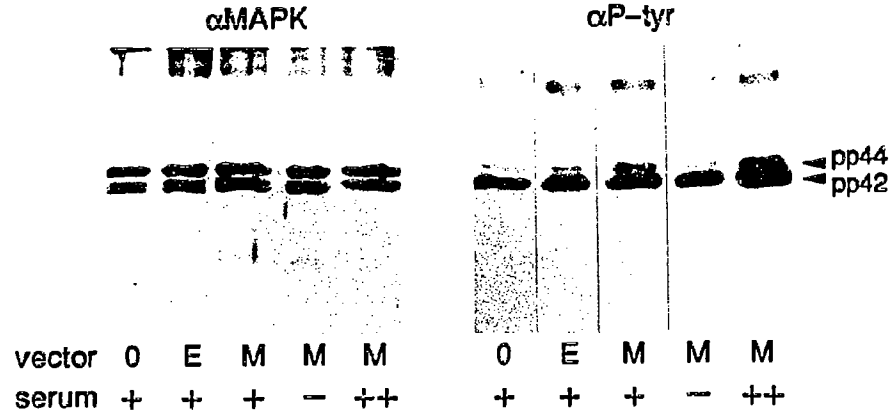

Fig. 2. Expression and tyrosine phosphorylation of partially purified MAP kinases. Cells from 100 mm dishes were lysed by Dounce homogenization in hypotonic buffer and subjected to batch-wise partial purification of MAP kinases on DEAE cellulose and then phenyl-Sepharose, essentially as described [2,3,5] except that the phenyl-Sepharose was washed with 20% ethylene glycol, prior to elution with 60% ethylene glycol. The partially purified preparations were electrophoresed (25% of eluted material per lane), transferred to nitrocellulose and probed either with the anti-MAP kinase antibody or with anti-phosphotyrosine antibody. Vectors: O, untransfected cells; E, empty vector, pSV_2CAT; M, pSV_2MAP. Serum: −, incubated in serum-free DMEM for 2 h; +, cultured in the continuous presence of serum; ++, serum-starved for 2 h then stimulated for 10 min with 10% fetal calf serum.

transfected with the pSV_2MAP plasmid, only the lower band increased in amount, demonstrating that the clone encodes $p42^{mapk}$, not $p44^{mapk}$. In addition, the 42 kDa region contained a doublet of proteins reactive with the anti-MAP kinase antiserum. It si possible that the upper band in the doublet is the phosphorylated form of $p42^{mapk}$, and the lower form is unphosphorylated, since we have observed a similar small mobility shift in $p42^{mapk}$ following serum stimulation of 3T3 cells (Rossomando and Weber, unpublished). Both the $p42^{mapk}$ and the 44 kDa protein could be detected with antibodies against phosphotyrosine (right panel, Fig. 2). When cells were cultured in the continuous presence of serum (+), both proteins displayed a level of tyrosine phosphorylation which was only marginally higher than was seen in serum-starved cells (−). Serum stimulation of quiescent cells resulted in substantially increased tyrosine phosphorylation of both pp42 and pp44 (++). Interestingly, in all these cases the extent of phosphorylation was higher on the $p42^{mapk}$ protein than on the 44 kDa protein. We do not know whether this reflects differences in the ability of serum to induce phosphorylation of these proteins, or whether the phosphorylated 44 kDa protein simple binds less well to phenyl-Sepharose.

3.2. *Expression, phosphorylation and activation of purified* $p42^{mapk}$

Various membranes of the MAP kinase protein family can be separated by FPLC column chromatography, particularly using the hydrophobic interaction resin phenyl-Superose, and a double-gradient of increasing ethylene glycol and decreasing NaCl [2,3,19]. The $p42^{mapk}$ binds exceptionally tightly to this resin, and elutes at approximately 37% ethylene glycol and 100 mM NaCl, whereas $p44^{mapk}$ binds much less tightly and elutes with many other cellular proteins and kinases

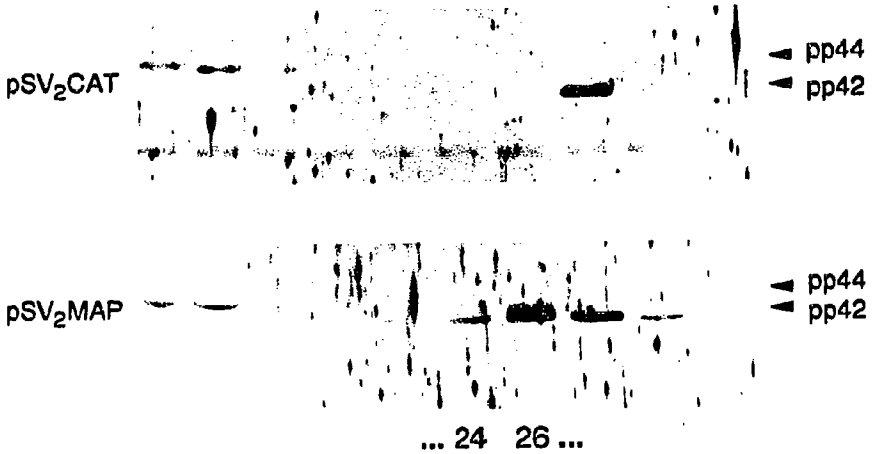

Fig. 3. Chromatography of $p42^{mapk}$ on phenyl-Superose. After 2.5 days of expression, cells transfected with empty vector (pSV_2CAT) or MAP kinase vector (pSV_2MAP) were serum-deprived for 18 h before lysis. Cell lysates were fractionated by DEAE and phenyl-Superose chromatography as described [2,14,18], fractions were subjected to 10% SDS-PAGE, followed by immunoblotting with anti-MAP kinase antibody. Fraction numbers are indicated at the bottom of the figure.

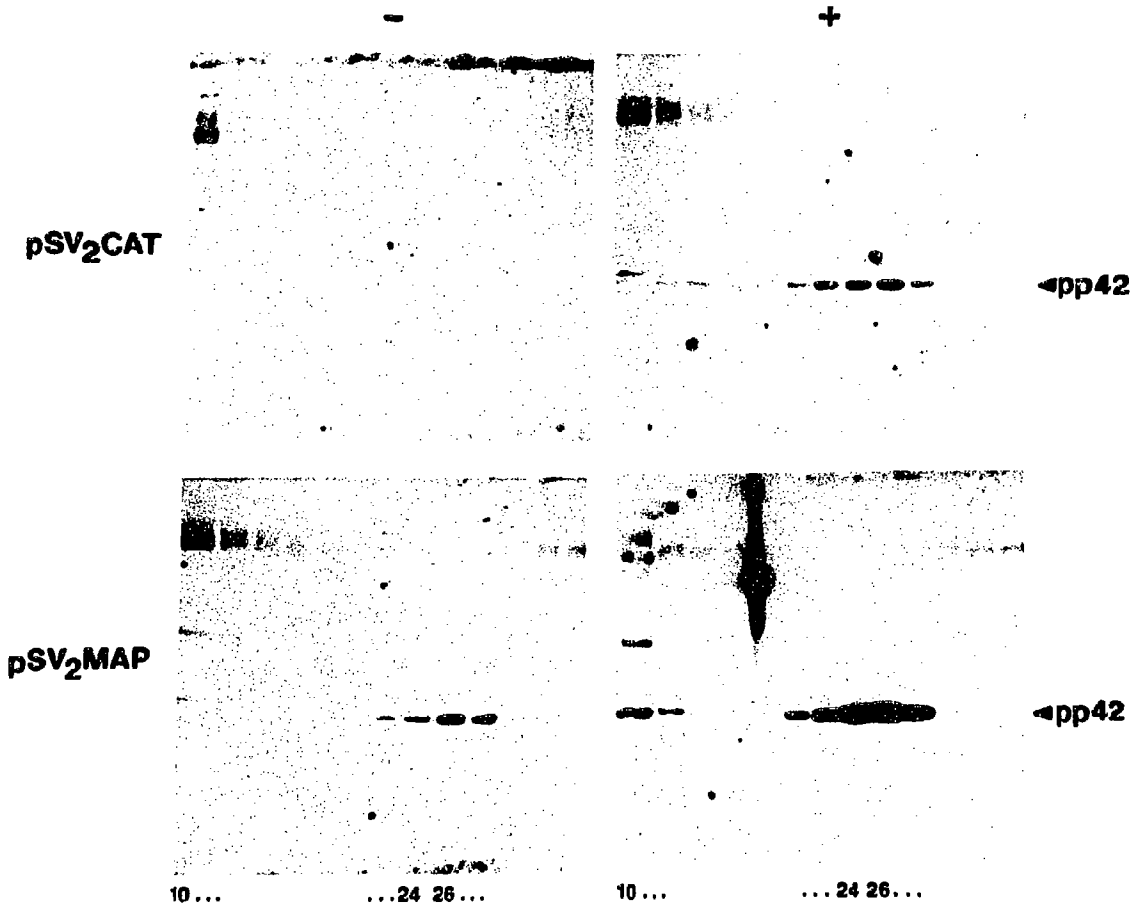

Fig. 4. Phosphotyrosine content of purified p42^{mapk} protein. Cells were transfected with pSV$_2$CAT (two top panels) or pSV$_2$MAP (two bottom panels). After 2.5 days of expression cells were serum-deprived for 18 h and reactivated (two right panels, +) or not (two left panels, –) with 10% fetal calf serum for 10 min. Anti-phosphotyrosine immunoblots from phenyl-Superose purified material (1.5 mg protein per FPLC run) are presented. Fraction numbers are indicated at the bottom of the figure.

during the wash or early in the gradient [14,19]. When lysates of cells transfected with pSV$_2$MAP were subjected to purification on phenyl-Superose, increased expression of p42^{mapk} could be detected by immunoblotting with anti-MAP kinase antibodies, compared to lysates of cells transfected with the empty vector pSV$_2$CAT (Fig. 3). The over-expressed p42^{mapk} eluted from phenyl-Superose at the characteristic elution position of the MAP kinase described by Ray and Sturgill [2,3,19].

Parallel blots of phenyl-Superose-purified p42^{mapk} probed with anti-phosphotyrosine antibodies are shown in Fig. 4. They indicate that the level of tyrosine phosphorylation present on the endogenous p42^{mapk} protein (from cells transfected with the empty pSV$_2$CAT vector) is increased following serum stimulation of the cells. This is the expected result, since tyrosine phosphorylation of p42^{mapk} has been demonstrated in various cell systems in response to serum, growth factors and other agonists. In cells transfected with pSV$_2$MAP vector, the phosphorylation of p42^{mapk} purified from quiescent cells was increased, compared to cells transfected with empty vector, and was further enhanced by serum stimulation. These findings demonstrate that the over-expressed p42^{mapk} also displays enhanced tyrosine phosphorylation in response to serum treatment.

Fig. 5 displays the kinase activity of the fractions shown in Fig. 4 which contained the p42^{mapk}: because other unrelated kinases are present in the early fractions from this column, these were not suitable for assay of p44. The results clearly show that there is an excellent correlation between the levels of tyrosine-phosphorylated p42^{mapk} and the levels of ^{32}P-incorporation onto the substrate myelin basic protein, a characteristic feature of phenyl-Superose-purified p42^{mapk} [3,13,14,18,19,22].

4. DISCUSSION

The functional expression in mammalian cells of a full-length cDNA encoding a member of the recently characterized MAP kinase family is described for the first time in this paper. The over-expressed p42^{mapk} protein can be visualized on whole cell blots (Fig. 1) as well as Western blots from material partially purified on phenyl-Sepharose (Fig. 2), and more highly purified on phenyl-Superose (Fig. 3), thus excluding the possibility

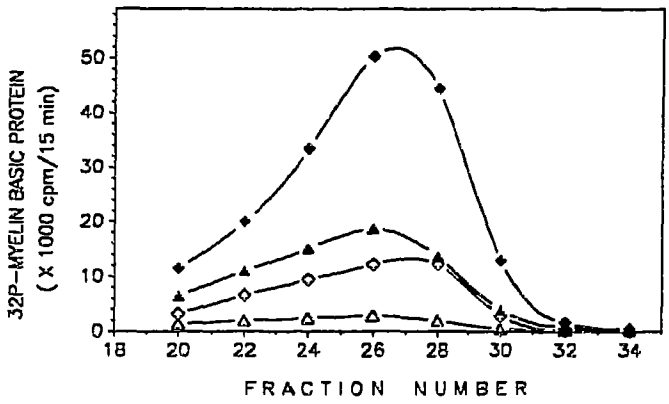

Fig. 5. Purified p42^{mapk} activity in transfected COS cells. Phenyl-Superose fractions from the experiment shown in Fig. 4 (5 μl out of 1 ml for each) were analyzed for their capacity to phosphorylate myelin basic protein [22]. Fraction numbers are indicated at the bottom of the figure. COS cells transfected either with pSV$_2$CAT (△,▲) or pSV$_2$MAP (◇,◆) were serum-deprived for 18 h and reactivated (▲,◆) or not (△,◇) with 10% FCS for 10 min. Results are typical of two kinase assays.

that the enhanced expression is an artefact of the purification procedures. However, the levels of expression appeared to be higher when whole cell lysates were blotted than when purified material was assessed. The reasons for this discrepancy are not understood. However, it is conceivable that a portion of the over-expressed protein does not achieve the appropriate conformation and thus does not behave normally during column chromatography.

We conclude from this study that the clone we isolated previously [7] encodes a completely functional p42^{mapk} protein: the over-expressed protein encoded by our clone migrates at the molecular weight of 42 kDa in SDS-polyacrylamide gel electrophoresis, elutes at 37% ethylene glycol from a phenyl-Superose column, is tyrosine-phosphorylated in response to growth factors, and is enzymatically active towards the relatively specific substrate myelin basic protein. Finally, the presence of the two signals, tyrosine phosphorylation and enzymatic activity, correlate extremely well after purification (see Figs. 4 and 5). All these features are characteristic of the p42^{mapk} protein described in several cell systems [3,13–15,18,19,22].

These results establish that the ERK1-related clone we isolated [7] is full-length and that it codes for the original p42^{mapk} protein described by Sturgill and coworkers. This clone, which corresponds to the ERK2 of Boulton et al. [8] can now be reliably used to elucidate the mechanism of activation of p42^{mapk} and its functions in cell regulation. Transient expression in the COS cell system should be particularly suitable for these studies if over-expression of p42^{mapk} inhibits cell growth or has long-term effects on cellular regulation. Such consequences of over-expression would make it difficult to isolate stable over-expressor cell lines or to unambiguously interpret the results of over-expression. Although COS cells are transformed, we show here that the over-expressed p42^{mapk} undergoes normal agonist-induced phosphorylation and enzymatic activation in these cells. Thus, the COS cell system can be used not only for the identification of members of the MAP kinase family, but also for studies on the regulation of their activity.

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