

ERK phosphorylation drives cytoplasmic accumulation of hnRNP-K and inhibition of mRNA translation

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Heterogeneous nuclear ribonucleoprotein K (hnRNP-K) is one of a family of 20 proteins that are involved in transcription and post-transcriptional messenger RNA metabolism. The mechanisms that underlie regulation of hnRNP-K activities remain largely unknown. Here we show that cytoplasmic accumulation of hnRNP-K is phosphorylation-dependent. Mitogen-activated protein kinase/extracellular-signal-regulated kinase (MAPK/ERK) efficiently phosphorylates hnRNP-K both *in vitro* and *in vivo* at serines 284 and 353. Serum stimulation or constitutive activation of ERK kinase (MEK1) results in phosphorylation and cytoplasmic accumulation of hnRNP-K. Mutation at ERK phosphoacceptor sites in hnRNP-K abolishes the ability to accumulate in the cytoplasm and renders the protein incapable of regulating translation of mRNAs that have a differentiation-control element (DICE) in the 3' untranslated region (UTR). Similarly, treatment with a pharmacological inhibitor of the ERK pathway abolishes cytoplasmic accumulation of hnRNP-K and attenuates inhibition of mRNA translation. Our results establish the role of MAPK/ERK in phosphorylation-dependent cellular localization of hnRNP-K, which is required for its ability to silence mRNA translation.

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are predominantly nuclear RNA-binding proteins that function in a wide array of cellular activities, ranging from transcription and pre-mRNA processing in the nucleus to cytoplasmic mRNA translation and turnover^{1–3}. In human cells, hnRNPs consist of a set of 20 principal proteins, which are formally defined as proteins that bind to hnRNA *in vivo* but are not stable components of other classes of RNP complexes^{1–3}. Along with histones, hnRNPs are the most abundant nuclear proteins in higher eukaryotes.

The hnRNP-K (K) protein has been implicated in diverse molecular and cellular functions, including nuclear–cytoplasmic shuttling and RNA transcription and translation⁴. The K-homology (KH) motifs, which were originally identified in hnRNP-K, are almost completely conserved between *Xenopus laevis* and mammals and have been implicated in RNA binding^{4,5}. Import and export of K protein have been shown to be mediated through the hnRNP-K nuclear-shuttling (KNS) domain (amino acids 323–390), which confers bidirectional transport across the nuclear envelope and represents a new shuttling pathway⁶.

The mammalian^{5,7–10} and *Drosophila melanogaster*¹¹ homologues of K protein have been implicated in transcription. Transactivation by K protein involves an increase in transcription of c-Myc⁵ and of Sp1 and Sp3 (ref. 8). Interconversion of double- and single-stranded

DNA¹² and association with C/EBPβ⁹ are among the mechanisms that may explain the effect of K protein on transcription.

K protein has also been shown to regulate translation in the cytoplasm. Together with hnRNP-E1, it binds to a C/U-rich DICE in the 3' UTR of 15-lipoxygenase (LOX) mRNA and silences its translation in immature erythroid precursor cells^{13,14}. Inhibition of mRNA translation by K protein could be mediated through different DICE elements found in alternative forms of LOX RNAs¹⁵ and has also been reported for human papillomavirus type 16 L2 mRNA¹⁶.

We do not yet understand the mechanism(s) underlying regulation of K-protein activities. The carboxy terminus of K protein consists of the SH3-binding cluster, which is required for binding to Vav¹⁷. K protein has also been shown to associate with c-Src^{17,18}. Src, as well as protein kinase Cδ and an interleukin-1-responsive K-protein kinase, has been shown to phosphorylate hnRNP-K, although the significance of this phosphorylation is not known^{19–21}.

In searching for possible new substrates of Jun amino-terminal kinase (JNK), we modified JNK by mutating it within the ATP-acceptor site, allowing selective use of a modified form of ATP^{22–24}. Microsequencing analysis of proteins that were phosphorylated by JNK *in vitro* led to the identification of hnRNP-K. hnRNP-K (K protein) was efficiently phosphorylated *in vitro* by JNK and ERK, but not by p38. To identify the JNK/ERK phosphoacceptor sites in K protein, we mutated the proline-driven serines at amino acids 116, 216, 284 and 353. Of the four resulting mutants, S284A and S353A were identified as the phosphoacceptor sites for ERK phosphorylation (data not shown).

To confirm that *in vivo* phosphorylation of K protein is carried out by MAPK/ERK, we carried out orthophosphate labelling of cells that had been co-transfected with hnRNP-K and a constitutively active form of MEK (MEK-EL) and then treated them with PD98059, a pharmacological inhibitor of ERK. Efficient phosphorylation of K protein upon expression of MEK-EL *in vivo* was inhibited in a dose-dependent manner by the ERK inhibitor (Fig. 1a). Whereas ultraviolet irradiation also induced phosphorylation of K protein, a constitutively active form of JNK elicited weak activation (data not shown). In light of the substantially greater phosphorylation by ERK as compared with JNK, we further characterized K-protein phosphorylation by focusing on ERK kinases.

One of the primary specific functions attributed to the K protein relates to its ability to inhibit translation of mRNAs bearing DICES within their 3' UTRs¹⁴. To assay for this function, we used the 3' UTR DICE element linked to a luciferase reporter gene (Luc-2R) and its mutant counterpart (Luc-2Rmut)¹⁴. Forced expression of K protein led to 55% inhibition of luciferase activity mediated from Luc-2R sequences in a DICE-dependent manner

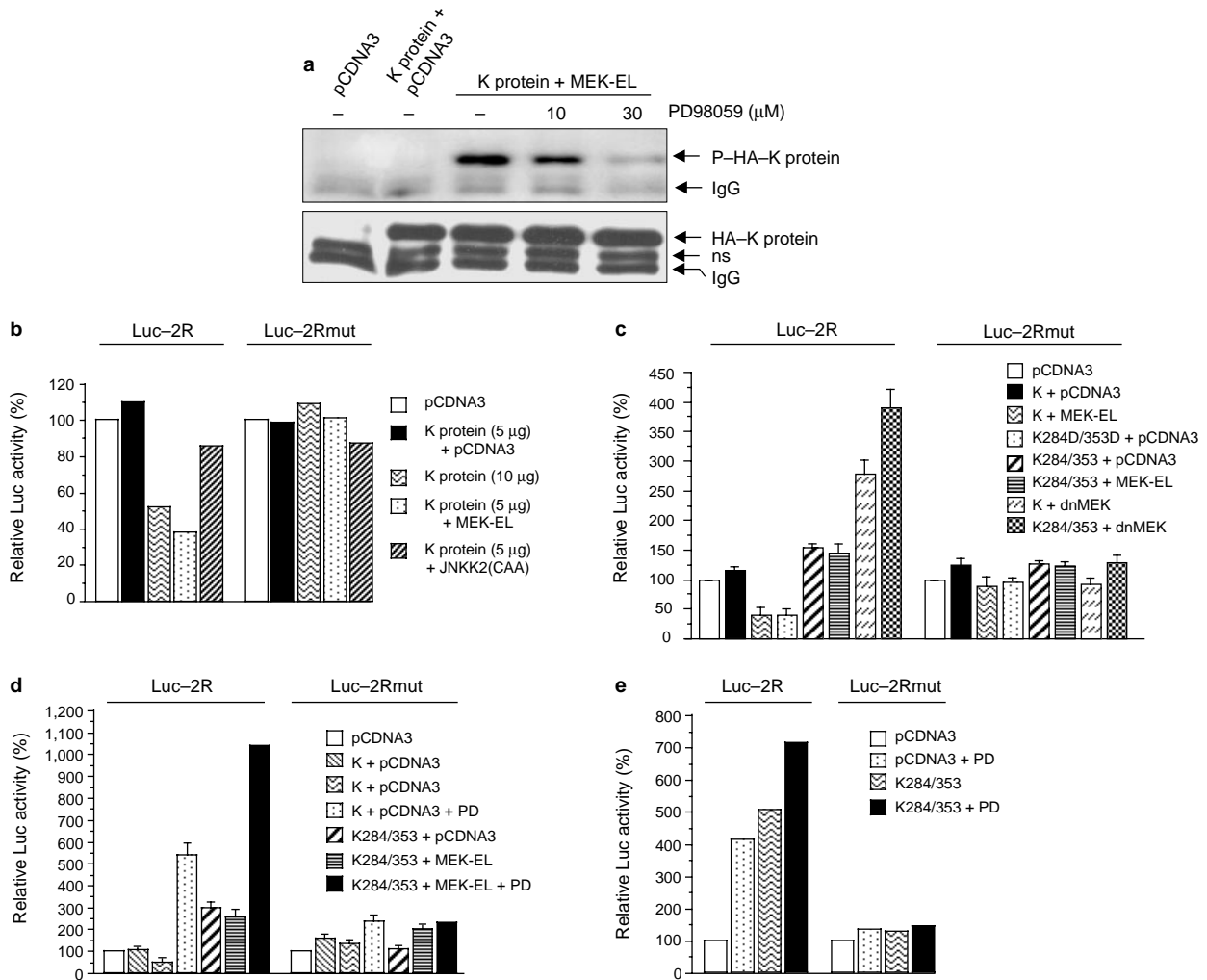


Figure 1 *In vivo* phosphorylation of HA-hnRNP-K by ERK is required for translational inhibition of Luc-2R. **a**, HeLa cells were co-transfected with hnRNP-K and MEK-EL, and subjected 24 h later to *in vivo* labeling with [³²P]orthophosphate for 2 h before protein extraction, in the presence or absence of PD98059 at the indicated concentrations. HA-hnRNP-K labelled with ³²P was immunoprecipitated and washed extensively before separation by SDS-PAGE, transfer to nitrocellulose membrane and subsequent analysis using autoradiography (upper panel). Western blotting with anti-HA antibodies (lower panel) was used to confirm equal loading. Arrows show the positions of K protein, immunoglobulin G (IgG) and a non-specific HA-crossreacting band (ns). **b**, HeLa cells were transfected using the calcium phosphate method with Luc-2R or Luc-2Rmut (5 μg) forms of DICE-bearing LOX 3' UTR sequences, together with SV-40-β-gal (5 μg) and wild-type hnRNP-K (5 or 10 μg) in the presence or absence of MEK-EL or JNKK2(CAA) as indicated. Proteins were prepared 30 h after transfection and were analysed for luciferase and β-gal activities.

Values are normalized to show relative increase in luciferase activity (%). **c**, Luc-2R or Luc-2Rmut was co-transfected with 5 μg of wild-type (K), phosphomutant (K284/353) or constitutively active mutant (K284D/353D) hnRNP-K in the presence or absence of MEK-EL. Luciferase activities were determined as in **b**. **d**, Reporter genes were co-transfected with 5 μg of wild-type or mutant (K284/353) hnRNP-K in the presence or absence of MEK-EL. After 16 h the transfection medium was replaced with low-serum medium (0.2%) for 20 h before fresh medium containing 10% FBS was added for 4 h. Where indicated, 50 μM PD98059 (PD) was added to the medium 2 h before addition of fresh medium, and was present in the fresh medium. Open and thick diagonally hatched columns represent data obtained without serum stimulation. **e**, HeLa cells were co-transfected with reporter genes and either empty pCDNA3 or phosphomutant hnRNP-K to examine the effect on translational inhibition mediated by endogenous hnRNP-K.

(Fig. 1b). Forced expression of MEK-EL in the presence of small quantities of the K-protein construct, which were not themselves capable of inhibiting DICE-Luc activity, led to >60% inhibition of the wild-type DICE element, as compared with 4% inhibition of the mutant DICE form (Fig. 1b). These findings indicate that inhibition of Luc-2R translation by K protein may depend on its phosphorylation by ERK.

Contrary to the inhibition of Luc-2R activity by wild-type K protein, forced expression of the S/A phosphomutants of K protein (K284/353) increased Luc-2R activity (Fig. 1c). Furthermore, forced expression of K-protein phosphomutants attenuated the ability of MEK-EL to inhibit Luc-2R activity (Fig. 1c). These

findings indicate that expression of phosphomutant K protein may be sufficient to override the ability of endogenous K protein and to attenuate the translational inhibition mediated by K protein that has been phosphorylated by ERK. Expression of dominant negative MEK (dnMEK) led to a marked increase in Luc-2R activity, which was further increased upon co-expression of phosphomutant K protein (Fig. 1c). Expression of K protein that was mutated to have constitutive charge at phosphoacceptor sites (S284D/S353D), led to inhibition of Luc-2R translation (Fig. 1c); this inhibition was not affected by PD98059 (data not shown), which further supports the idea that K protein has an ERK-phosphorylation-dependent effect on RNA translation.

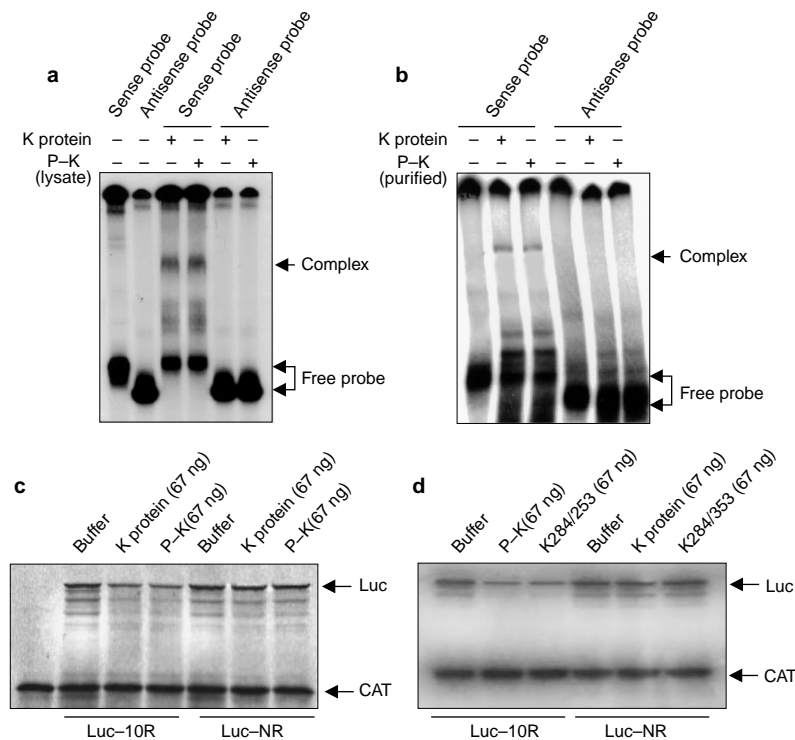


Figure 2 Association of LOX mRNA with K protein and inhibition of its translation *in vitro* is not phosphorylation-dependent. **a**, RNA-protein complex analysis was carried out as a gel-shift assay for hnRNP-K binding, using an mRNA probe that was synthesized from three repeats of the LOX 3' UTR DICE. Lysates (25 µg) from 293T cells that were co-transfected with hnRNP-K in the presence or absence of MEK-EL (P-K) was incubated with ³²P-labelled (0.1 ng) sense (specific) or antisense (non-specific) probe and were then separated on 6% polyacrylamide gel with 0.5 × Tris/boric acid/EDTA buffer. **b**, HA-hnRNP K (20 ng) that was immunopurified on an HA-affinity column from cells that were co-transfected with HA-hnRNP-K and MEK-EL or with control pCDNA3 was bound to labelled RNA (see **a**), after which complexes were separated by PAGE and visualized using autoradiography. **c**, Luc mRNA bearing

the 15-LOX 3' UTR regulatory region (Luc-10R) or the non-regulatory region (Luc-NR) and chloramphenicol acetyltransferase (CAT) mRNA (as a control) were *in vitro* transcribed before incubation with immunopurified hnRNP-K and subsequent *in vitro* translation. K protein was purified from MEK-EL-expressing cells in parallel to its purification from cells expressing control vector (unphosphorylated conditions); complexes were separated by PAGE and visualized by autoradiography. Arrows show the positions of Luc and CAT products. **d**, Analysis was carried out as in **b**; proteins purified from MEK-EL-expressing cells include the wild-type and phosphomutant (K284/353) forms of hnRNP-K. K proteins were purified on an HA-affinity column before incubation with Luc-10R or Luc-NR mRNA that was transcribed and purified *in vitro*; the mixture was then subjected to an *in vitro* translation reaction and was analysed by autoradiography

Analysis of Luc-2R translation in serum-stimulated cells revealed a 50% reduction in Luc-2R activity (Fig. 1d). Such inhibition was reversed upon treatment of cells with PD98059. Moreover, addition of PD98059 to the culture medium 2 h before serum stimulation efficiently increased Luc-2R activity (more than fivefold; Fig. 1d). These observations indicate that endogenous K protein may extensively inhibit translation of sequences bearing LOX 3' UTRs, and that this inhibition may be ERK-dependent. The idea that ERK phosphorylation is important for inhibition of RNA translation by K protein is further supported by the observation that forced expression of phosphomutant K protein elicited a threefold increase in RNA translation, which was further increased (sixfold) upon treatment with PD98059 (Fig. 1d). Thus, through inhibition of endogenous ERK activity, PD98059 has an effect that is additive to that mediated by exogenously expressed phosphomutant K protein.

Treatment with PD98059 was sufficient to increase Luc-2R activity in serum-stimulated cells, which was mediated by endogenous K protein (Fig. 1e). Forced expression of phosphomutant K protein in PD98059-treated and serum-stimulated cells efficiently increased Luc-2R activity (more than fivefold); this increase was greater (sevenfold) upon inhibition of ERK with PD98059 (Fig. 1e). These observations provide direct support for a role of ERK in the ability of K protein to inhibit translation through the 3' UTR DICE.

To assess whether phosphorylation alters the association of K protein with RNA, we incubated ³²P-labelled LOX 3' UTR RNA, in its sense or antisense form, with cellular extracts prepared from cells that were transfected with K protein in the presence or absence of MEK-EL. K protein-RNA complexes were detected with sense, but not with antisense, forms of sequences bearing LOX 3' UTR elements, and were not affected by expression of MEK-EL (Fig. 2a). Incubation of ³²P-labelled DICE sequences, in sense or antisense forms, with K protein immunopurified from MEK-EL (phosphorylated) or control (unphosphorylated) transfected cells revealed that the sense, but not the antisense, form of LOX 3' UTR RNA bound to K protein, and that such association was not altered upon phosphorylation of K protein (Fig. 2b). Association of K protein with labelled DICE RNA was inhibited by the sense, but not by the antisense, form of this RNA fragment, which demonstrates the specificity of this association (data not shown). Together, these findings indicate that binding of K protein to DICEs is not affected by ERK phosphorylation.

We monitored inhibition of RNA translation *in vitro* using *in vitro*-synthesized luciferase reporter RNA bearing DICEs, which we assayed for *in vitro* translation in the presence of K protein. K protein efficiently elicited dose-dependent inhibition of RNA translation from the LOX regulatory region (10R), but not from a non-regulatory region (data not shown). Such inhibition was not affected

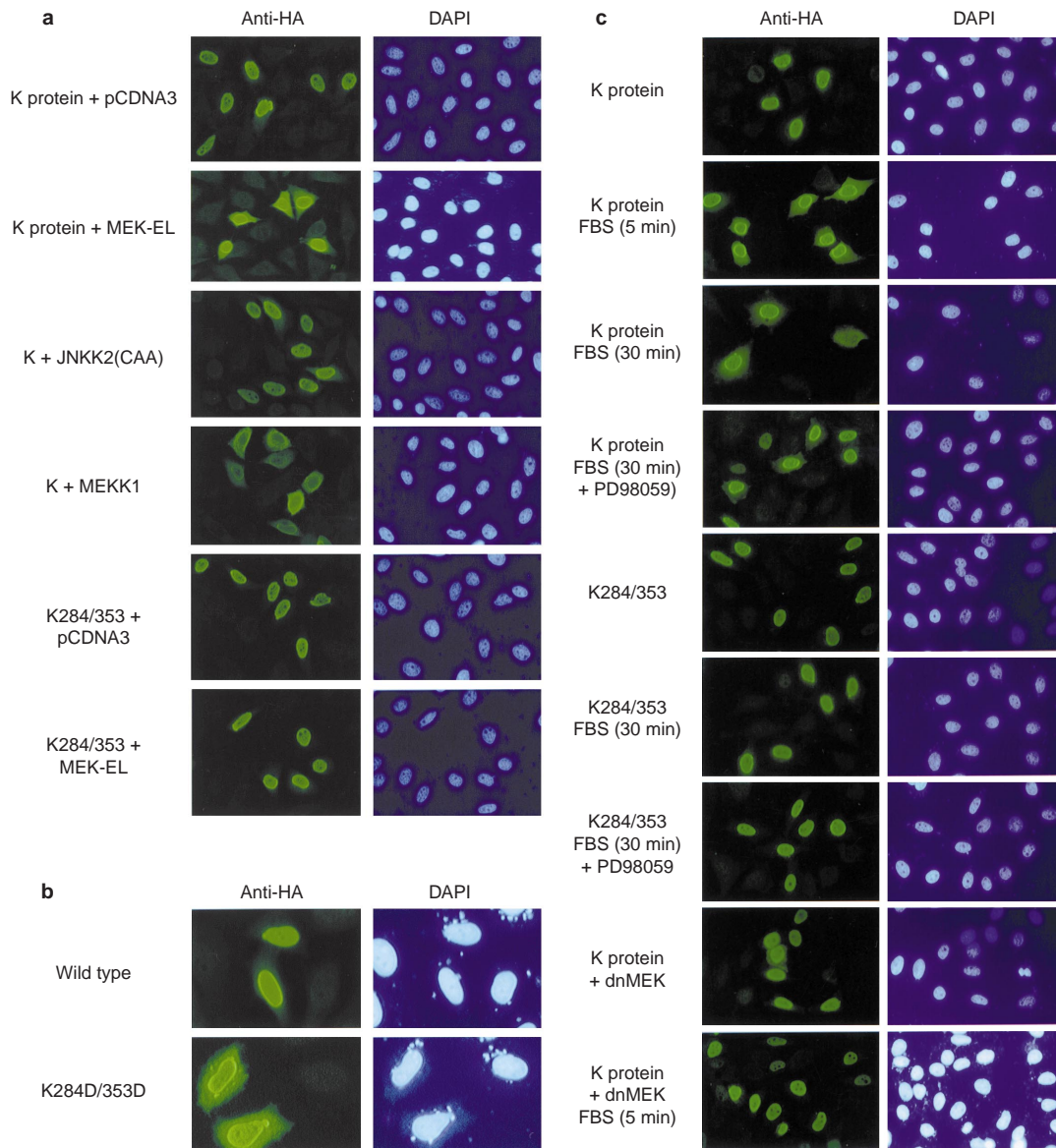


Figure 3 Cytoplasmic accumulation of hnRNP-K upon ERK phosphorylation and serum stimulation. **a**, HeLa cells cultured on glass coverslips were transfected with wild-type or phosphomutant (K284/353) HA-hnRNP-K in the presence or absence of constitutively active forms of upstream MAP kinases (MEK-EL, JNKK2(CAA), Δ MEKK1). HA-hnRNP K was visualized 24 h later after cell fixation followed by immunostaining with anti-HA monoclonal antibody (left panels). Right panels show control DAPI staining. **b**, HeLa cells grown on coverslips were transfected with wild-type or mutant (K284/353D) forms of K protein in which S284 and S353

were mutated to aspartic acid D. Cells were fixed and stained with anti-HA antibody 24 h after transfection. **c**, HeLa cells grown on coverslips were transfected with wild-type or phosphomutant HA-hnRNP-K and were then incubated in low-serum medium for 16 h; medium containing 10% FBS was then added for the indicated time periods. Where indicated, PD98059 was added to the medium 2 h before and during serum stimulation. Panels labelled 'dnMEK' show the effect of dominant negative MEK on cytoplasmic accumulation of K protein after serum stimulation.

upon ERK phosphorylation of K protein (Fig. 2c). Wild-type and phosphomutant K protein, immunopurified from MEK-expressing cells, inhibited *in vitro* RNA translation to equal extents (Fig. 2d). These observations indicate that phosphorylation of K protein does not affect the rate of RNA translation *per se*.

Whereas exogenous K protein is primarily localized within the nucleus, wild-type, but not phosphomutant, K protein was also present within the cytoplasmic compartment when MEK-EL was expressed (Fig. 3a). The constitutively active form of the upstream kinase MEKK1 (Δ MEKK1) was capable of eliciting even more efficient accumulation of K protein within the cytoplasm (Fig. 3a). Conversely, JNK did not affect cytoplasmic accumulation of K

protein (Fig. 3a).

Further support for the importance of phosphorylation at amino acids 284 and 353 for cytoplasmic accumulation of K protein comes from the use of K-protein mutants in which these residues are replaced with acidic residues, thereby generating a negative charge that mimics the phosphorylated form of the protein. Expression of S284D/S353D-mutant K protein led to cytoplasmic accumulation without the need for external stimuli (Fig. 3b). We monitored further the changes in K-protein localization that occur in response to serum stimulation. Rapid accumulation of haemagglutinin (HA)-tagged wild-type, but not phosphomutant, K protein in the cytoplasm was observed within 5 min of addition of

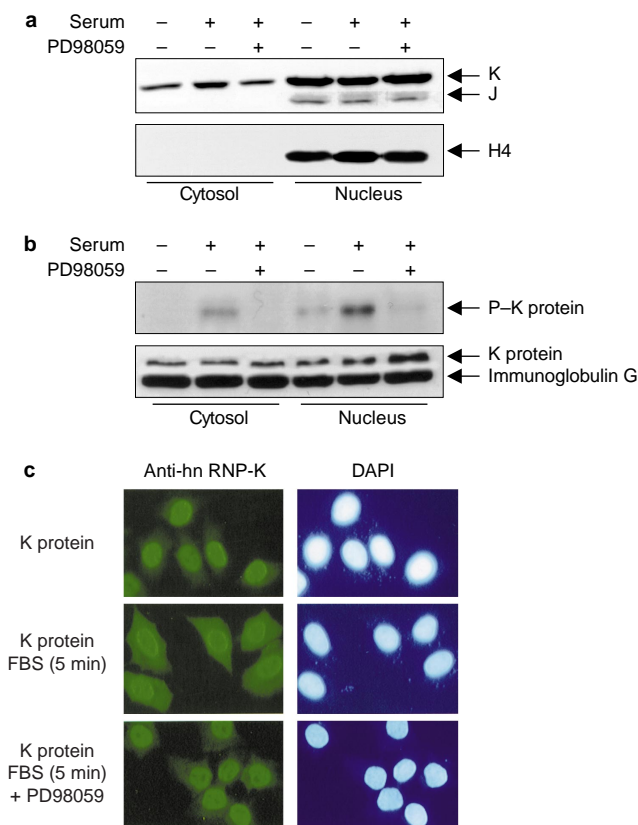


Figure 4 Cytoplasmic accumulation of ERK-phosphorylated endogenous hnRNP-K upon serum stimulation. **a**, Nuclear and cytoplasmic proteins were prepared from HeLa cells 5 min after serum stimulation (cells were maintained in serum for 20 h at 1% FBS). Where indicated, PD98059 was added 2 h before serum stimulation. Western blotting was carried out using monoclonal antibodies (12G4) against K protein, which allowed the levels of endogenous K protein in the respective cellular compartments to be monitored (upper panel). Analysis using antibodies against histone 4 (lower panel) reveals the separation of nuclear and cytoplasmic proteins. Arrows indicate the positions of the K and J members of hnRNP protein family recognized by these antibodies. **b**, HeLa cells were maintained in low-serum (1% FBS) medium for 20 h before addition of serum (10% FBS) and [³²P]orthophosphate (1 mCi per 100-mm plate) for 30 min. Nuclear and cytoplasmic proteins were prepared 30 min after serum stimulation (three plates per experimental condition) and were subjected to immunoprecipitation (1 mg cytosolic and 0.3 mg nuclear extract per immunoprecipitation) using the monoclonal 12G4 antibody against endogenous K protein. Immunoprecipitated material was washed extensively, separated by SDS-PAGE and electrotransferred onto nitrocellulose membrane. Upper panel shows a representative autoradiograph; phosphorimager analysis allowed the respective signals to be quantified. Lower panel shows a western blot of the same membrane probed with 12G4 antibody. **c**, HeLa cells grown on coverslips were serum-starved (20 h at 1% serum) and then serum-stimulated (10% FBS). Cells were fixed for immunostaining 5 min after serum stimulation. PD98059 was added to the medium 2 h before (and was present during) serum stimulation. Immunostaining was carried out using monoclonal anti-hnRNP-K (12G4) antibody (left panels). Right panels show DAPI staining.

serum to starved cells (Fig. 3c). Cytoplasmic accumulation of K protein after serum stimulation was blocked both in cells that were also treated with PD98059 and in cells that were forced to express a dominant negative form of MEK (Fig. 3c). These findings demonstrate the requirement for ERK phosphorylation to allow cytoplasmic accumulation of K protein under physiological growth conditions.

Immunoblotting revealed an increase in the amount of endogenous K protein in the cytoplasmic fraction prepared after serum stimulation. Such an increase was no longer observed when cells were pretreated with PD98059 (Fig. 4a). These findings indicate that ERK phosphorylation of endogenous K protein may contribute to its cytoplasmic accumulation. Pulse-chase analysis of K protein after serum stimulation revealed no significant change in the half-life of K protein (data not shown), indicating that the ERK-mediated increase in amount of K protein in the cytoplasm could be attributed either to nuclear export or to cytoplasmic accumulation.

Orthophosphate labelling of cells showed that a relatively small fraction of overall K protein was phosphorylated and was selectively localized within the nuclear compartment of normal growing cells (Fig. 4b). In response to serum stimulation, there was a marked increase (fivefold) in the amount of phosphorylated K protein within the nuclear compartment. Phosphorylated K protein was detected in the cytoplasmic fraction of cells only after serum stimulation (Fig. 4b). Addition of PD98059 before serum stimulation attenuated K-protein phosphorylation in the nuclear compartment and abolished K-protein phosphorylation in the cytoplasm (Fig. 4b). These results support the idea that ERK phosphorylates endogenous K protein and indicate that such phosphorylation may occur primarily in the nucleus, after which phosphorylated K protein would be translocated to the cytoplasm. These data also shown that only a fraction of overall K protein is phosphorylated, and that external stimulation, such as the serum stimulation used here, efficiently increases phosphorylation of endogenous K protein. As western blotting may not always reflect the actual subcellular protein distribution, we assessed further the effect of ERK phosphorylation on cellular localization of the K protein by immunohistochemistry analysis. Specific monoclonal antibodies against K protein revealed the cytoplasmic accumulation of endogenous K protein in response to serum stimulation (Fig. 4c). Furthermore, treatment with PD98059 efficiently blocked this cytoplasmic accumulation after serum stimulation (Fig. 4c). These data confirm that ERK affects cytoplasmic accumulation of endogenous K protein.

We have shown, for the first time, that the MAPK signal-transduction pathway can directly control the cellular distribution of hnRNP-K and consequently influences the ability of K protein to regulate translation. Our data establish that partitioning of hnRNP-K between the nucleus and the cytoplasm is subject to modulation by extracellular signals. The MEK/ERK pathway, which is activated in response to stress, is both required and sufficient for accumulation of phosphorylated hnRNP-K in the cytoplasm, and therefore for the ability of this protein to inhibit RNA translation.

Cytoplasmic accumulation of K protein upon ERK phosphorylation could be explained either by inhibition of import of K protein to the nucleus by phosphorylation by ERK, thereby causing it to accumulate in the cytoplasm, or by export of K protein, which is predominantly localized in the nucleus, to the cytoplasm upon phosphorylation by ERK. Although our *in vivo* phosphorylation data support the latter possibility, further experiments are required to distinguish between these two models. Only a fraction of the overall amount of K protein expressed in the cell is phosphorylated in response to serum stimulation, an observation that highlights the regulatory nature of K-protein phosphorylation.

Earlier reports revealed *in vitro* phosphorylation hnRNP-K by PKC δ and Src¹⁸⁻²¹, although the significance of this phosphorylation for the activities of K protein *in vivo* is not clear. p38, which has no effect on K protein, has recently been implicated in phosphorylation of hnRNP-A1, which consequently affects its alternative splicing activity²⁵. Although hnRNP-A1 may not be a direct substrate for p38, these findings, together with our observations, indicate the role of MAPK family members in the localization and activities of hnRNPs. The finding that phosphorylation of the phosphorylated adaptor for RNA export (PHAX) is essential for export of U-rich small nuclear RNA provides another example of

