Multisite phosphorylation of Erk5 in mitosis

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Summary

The MAP kinase Erk5 plays important roles in cellular proliferation, and has recently been implicated in the regulation of mitosis. The classic pathway of Erk5 activation involves dual phosphorylation at its TEY microdomain by the upstream regulating kinase MEK5. Here we describe a second pathway that controls Erk5 phosphorylation. This pathway is activated in mitotic cells and involves kinase activities distinct from MEK5. Studies aimed at identifying these kinases suggested that CDK1 activity is required to sustain Erk5 phosphorylation in mitosis, as treatment with RO3306, a CDK1 inhibitor, reversed mitotic phosphorylation of Erk5. Moreover, CDK1 co-precipitated with Erk5 in mitotic cells. The mitotic phosphorylation of Erk5 occurs at multiple sites located at its unique C-terminal region, within an Erk5 subdomain that has formerly been implicated in the control of the subcellular location of Erk5. Furthermore, molecular studies indicated that phosphorylation at these sites may participate in the control of the transit of Erk5 between the cytosol and the nucleus, in addition to regulating its transcriptional activity. Together, our results demonstrate the existence of a second Erk5 phosphorylation pathway, that is activated in mitosis, and that may participate in the regulation of Erk5 functions.

Key words: Erk5, Mitosis, Phosphorylation

Introduction

The mitogen-activated protein kinases (MAPKs) routes are highly conserved signaling pathways that have been implicated in multiple cellular responses, including the control of cell proliferation, differentiation and survival (Robinson and Cobb, 1997). The MAPK cascades are composed of modules including three classes of protein kinases: the MAPK itself is phosphorylated and activated by a MAPK kinase (MAPKK), which in turn is also phosphorylated and activated by a MAPKK kinase (MAPKKK). Several MAPK routes have been described in mammals. The extracellular signal-regulated kinases 1 and 2 (Erk1/2) are activated by growth factors and environmental stresses, and participate in the transduction of proliferative signals (Widmann et al., 1999). Two other MAPKs subfamilies, the p38 and the Jun N-terminal kinases, are activated by cytokines or cytotoxic drugs and have mainly been linked to the regulation of cell survival (Nebreda and Porras, 2000; Weston and Davis, 2002). More recently another MAPK, termed Erk5 or BMK1 (big MAP kinase 1) has also been associated to the control of cellular proliferation in response to proliferative signals, cytokines and cellular stresses (Kato et al., 1997; Kato et al., 1998). Hyperactivation of the Erk5 pathway has been associated with highly aggressive forms of breast and prostate cancers (Esparis-Ogando et al., 2002; McCracken et al., 2008; Mehta et al., 2003; Montero et al., 2009).

Erk5/BMK1 is a MAPK whose N-terminus kinase domain is highly homologous to the prototypical MAPKs Erk1/2 and differs from them in having a large, unique C-terminal half not found in other MAPK family members (Nishimoto and Nishida, 2006). This unique region has two proline-rich domains as well as regions that control the nucleo-cytoplasmic trafficking of Erk5 (Borges et al., 2007; Buschbeck and Ullrich, 2005), and shares no homology with other proteins. In addition, this region contains a potent transcriptional activation domain (Kasler et al., 2000). A recent report indicated that Erk5 undergoes autophosphorylation on this C-terminal region and this process could be important in regulating gene expression, since it would enhance the AP-1 activity (Morimoto et al., 2007). In addition, this C-terminal zone has been implicated in the nucleocytoplasmic trafficking of Erk5 (Borges et al., 2007). Thus, deletion of the C-terminal Erk5 region, beyond amino acid 699, provokes retention of Erk5 in the nucleus, and this confers resistance to death-receptor-induced apoptosis.

Erk5 is activated by oxidative stress, hyperosmolarity and several growth factors including epidermal growth factor (EGF) and the neuregulins (Esparis-Ogando et al., 2002; Kato et al., 1997). MEK5 is the upstream MAPKK that specifically phosphorylates and activates Erk5 at the consensus phosphorylation microdomain TEY within the Erk5 activation loop (English et al., 1995; Wang et al., 2006). Although the Erk5 N-terminal domain is very similar to Erk1/2, Erk5 has unique biological functions that cannot be compensated for by Erk1/2, as demonstrated by the fact that Erk5 deficiency in mice results in lethality. Thus, both Erk5- (Regan et al., 2002; Sohn et al., 2002; Yan et al., 2003) and MEK5- (Wang et al., 2005) deficient animals die during embryonic development from angiogenic failure and cardiovascular defects. However, in the adult, Erk5 is also required for endothelial cell survival and maintenance of blood vascular integrity (Hayashi et al., 2004; Pi et al., 2005).

It has recently been reported that Erk5 might have a role in the regulation of cell cycle progression through the control of the G2–M transition (Cude et al., 2007; Girio et al., 2007). Cude and collaborators showed that when both constitutively active MEK5 (MEK5DD) and Erk5 are overexpressed, the mitotic index is increased and this effect is mediated by the NFκB downstream signaling pathway. Girio et al. showed that Erk5 is constitutively phosphorylated in mitosis, when compared with G1 and S phases of the cell cycle, and showed that phosphorylated Erk5 binds to BIM, a BCL2 mediator of cell death, suggesting involvement of Erk5 in the survival of cells along mitotic transit.

There is also evidence linking the other MAPKs routes to the control of mitosis. Thus, inhibition of MEK1/2 by the use of dominant negative forms, or by drugs, has been shown to delay cell cycle

progression (Wright et al., 1999). The molecular mechanisms behind such action may be various. Thus, active Erk1/2 seems to phosphorylate the spindle checkpoint protein MPS1, and this phosphorylation is essential for the mitotic checkpoint function (Zhao and Chen, 2006). In addition, Erk1/2 have also been implicated in the phosphorylation of CENP-E motor protein (Zecevic et al., 1998), histone H3 (Dyson et al., 2005), SWI-SNIF (Sif et al., 1998) and Plk3 (Xie et al., 2004), and may also regulate the function of Plk1, cdc2/CDK1 (Liu et al., 2004) or Myt1 (Palmer et al., 1998). Furthermore, an alternatively spliced form of Erk1, termed Erk1c, has also been detected in the Golgi complex of mitotic cells and is required for the proper segregation of this apparatus to the two daughter cells (Shaul and Seger, 2006). In addition, the Erk1/2 pathway and the p38 route have been implicated in controlling mitotic progression both in normal and stress conditions, modulating CDC25B activity (Astuti et al., 2009; Cha et al., 2007).

In this report we show that Erk5 is phosphorylated in mitosis through a novel pathway that does not involve the canonical MEK5-dependent route. Instead, inhibitor and biochemical studies point to a role of CDK1 in controlling Erk5 phosphorylation in mitosis. Erk5 phosphorylation occurs at multiple sites within its C-terminal tail. Moreover, Erk5 phosphorylation at these sites may also participate in the control of some biological properties of Erk5, such as its subcellular location or transcriptional activation. We propose that Erk5 may act as an important regulator of the proliferation of mammalian cells by nucleating signals that emanate from growth factor receptors in interphase, but also by receiving inputs from kinases that control mitotic progression.

Results

It has recently been described that Erk5 from mitotic HeLa cells was detected as a more slowly migrating band in western blots than Erk5 from interphase cells (Girio et al., 2007; Cude et al., 2007). To analyze whether this was a general phenomenon, different cell lines were treated with nocodazole, and Erk5 was analyzed by immunoprecipitation and western blotting with an antibody raised to the C-terminus of Erk5 (Esparis-Ogando et al., 2002). The mitotic subpopulation of the distinct cell lines expressed an Erk5 that migrated slower than Erk5 isolated from interphase cells (Fig. 1A). These results indicate that the slower migrating form of Erk5 was a widespread phenomenon in mitotic mammalian cells. The change in the migration induced by nocodazole was a reversible process: when cells exited mitosis, Erk5 recovered its normal migration pattern, as demonstrated in experiments in which cells where synchronized in M phase by nocodazole treatment, and then released into normal medium (Fig. 1B). It was possible that the retardation in the mobility of Erk5 from cells treated with nocodazole was due to an action of nocodazole unrelated to its effect on mitosis. To explore this possibility, we decided to treat HeLa cells with different drugs that also interfere with the mitotic transition by targeting microtubules dynamics, and that have been used in cancer therapy. Treatment with vincristine or taxotere induced a band shift in Erk5 analogous to that of nocodazole (Fig. 1C).

In addition, when cells were synchronized in G1–S phase by double thymidine treatment and released into normal medium, the slower migrating form of Erk5 was found in cells transiting through

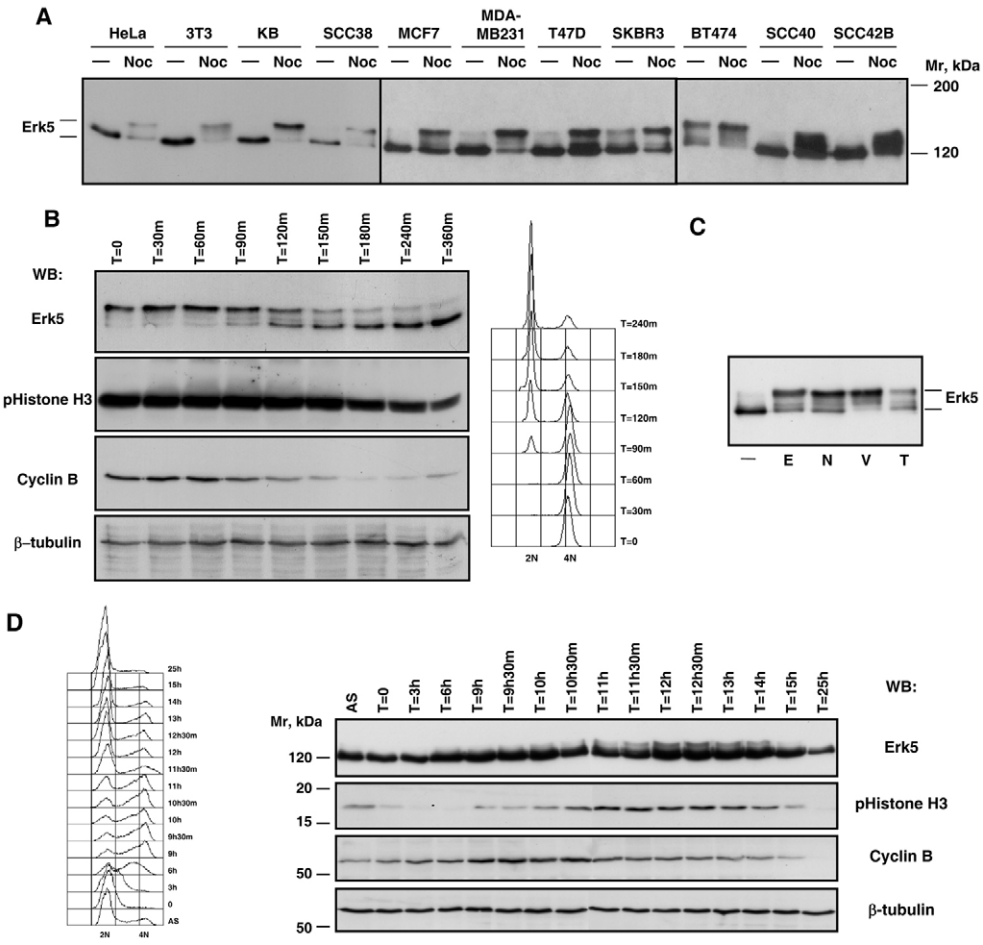

Fig. 1. Erk5 undergoes a bandshift in mitosis. (**A**) The analysis of Erk5 in a panel of cell lines of different origin demonstrates that Erk5 migrated slower after treatment with 200 ng/ml nocodazole (Noc) for 14 hours. Analysis of Erk5 in the mitotic cells was performed as described in the Materials and Methods section. (**B**) HeLa cells were blocked in G2–M by nocodazole treatment and released in normal medium. Cells were harvested at the indicated times after the release (in minutes). Part of the sample was fixed for DNA content determination by flow cytometry (cell cycle progression shown on the right) and the other part lysed for protein preparation. Erk5 was immunoprecipitated and analyzed by western blotting. 50 μg of protein were probed with different cell cycle markers such as phospho-histone H3 or cyclin B. β-tubulin was used as a loading control. (**C**) Cells treated with the indicated agents [EGF (E), nocodazole (N), taxotere (T) or vincristine (V)] were lysed, the immunoprecipitates separated and the western blots probed with the anti-Erk5 antibody. (**D**) An Erk5 bandshift is detected at the beginning of mitosis and disappears at its end. HeLa cells were synchronized at the G1–S phase of the cell cycle by a double thymidine block and released into normal medium and analyzed as in B. β-tubulin was used as a loading control. AS, asynchronous population.

mitosis, as verified by studying their cell cycle profile by flow cytometry and western blotting with the markers phospho-histone H3 and cyclin B (Fig. 1D). Cyclin B is normally used as a marker of the G2–M phases of the cell cycle, because of its cyclical expression, accumulating in G2 and at the beginning of mitosis and being degraded at the metaphase to anaphase transition (Norbury and Nurse, 1992; Pines and Hunter, 1991). Histone H3 is specifically phosphorylated during mitosis and for that reason is used as a bona-fide mitotic marker (Goto et al., 1999; Hendzel et al., 1997; Preuss et al., 2003). These time-course experiments indicated that Erk5 mobility shift in mitosis was maximal at a time that was coincident with the peak of histone H3 phosphorylation.

Erk5 is phosphorylated in mitosis at sites distinct from the TEY microdomain

We then explored the causes of the different mobility of Erk5 from mitotic cells when compared with interphase Erk5. Analogous changes were previously reported to be provoked by changes in the phosphorylation of Erk5, mainly triggered by the activation by growth factor receptors (Esparis-Ogando et al., 2002; Kato et al., 1998). In fact, treatment with EGF caused a mobility shift analogous to that provoked by mitotic arrest of HeLa cells (Fig. 2A). For this reason, we first analyzed whether phosphorylation was responsible for the change in Erk5 mobility. To this end, Erk5 immunoprecipitated from EGF- or nocodazole-treated samples was subjected to in vitro phosphatase treatment, followed by western blot assay. Treatment with lambda phosphatase augmented the electrophoretic mobility of mitotic Erk5, making it indistinguishable from interphase Erk5 from unstimulated cells. This phosphatase treatment also caused increased mobility of Erk5 from EGF-treated interphase HeLa cells (Fig. 2A). Together, these findings indicated that phosphorylation of Erk5 in mitotic cells was the probable cause of its delayed mobility. We noticed that when EGF- or nocodazole-treated samples were carefully compared, the slower migrating bands from both treatments seemed to be slightly different (arrows in Fig. 2B). This suggested that possibly the two treatments affected Erk5 phosphorylation differently. To address this, EGF- or nocodazole-stimulated cells were lysed and the extracts immunoprecipitated with anti-Erk5 or antibodies that recognize Erk5 at the phosphorylation sites located in the TEY microdomain (anti-T-*P*EY-*P* antibody). The slower migrating band appearing after EGF treatment was recognized by the anti-T-*P*EY-*P* antibody. However, this antibody failed to react with Erk5 from mitotic cells (Fig. 2C), indicating that Erk5 phosphorylation in mitosis does not occur at the consensus phosphorylation microdomain TEY. To further verify this finding, we used a form of Erk5 in which the TEY residues had been substituted with the non-phosphorylatable residues, AEF (Erk5^{AEF}) (Esparis-Ogando et al., 2002). In HeLa cells expressing this mutant (HA-Erk5^{AEF}), treatment with nocodazole caused the typical mitotic bandshift of HA-tagged Erk5 (Fig. 2D). By contrast, EGF treatment was unable to cause phosphorylation or bandshift of HA-Erk5^{AEF} (data not shown) (Esparis-Ogando et al., 2002). Together, these results indicate that the phosphorylation of Erk5 in mitosis occurs at noncanonical sites outside the TEY microdomain.

Erk5- and MEK5-independent phosphorylation of Erk5 in mitosis

Two kinase activities have been implicated in the direct phosphorylation of Erk5. MEK5 is responsible for the phosphorylation of Erk5 at the TEY microdomain (Zhou et al., 1995; Nishimoto and Nishida, 2006). In addition, a report indicated that once Erk5 in phosphorylated at the T-*P*EY-*P* sites, it is then able to autophosphorylate at residues located in its C-terminal region (Morimoto et al., 2007). We explored the participation of both kinases as the potential Erk5 mitotic kinase. We initially took advantage of mouse embryonic fibroblasts (MEFs) derived from animals deficient in MEK5. Even though MEK5-deficient animals do not survive above mid-gestation because of cardiac defects and angiogenic failure, MEFs derived from those animals have, however, been isolated (Wang et al., 2005). *Mek5*$^{-/-}$ MEFs were treated with either EGF or nocodazole, and Erk5 analyzed by western blotting. These MEFs did not respond to EGF treatment with a band shift as MEFs derived from their wild-type littermates, demonstrating a role of MEK5 in Erk5 phosphorylation in response to activation of growth factor receptors. Nevertheless, the shift in Erk5 mobility was still detectable in both MEFs after nocodazole

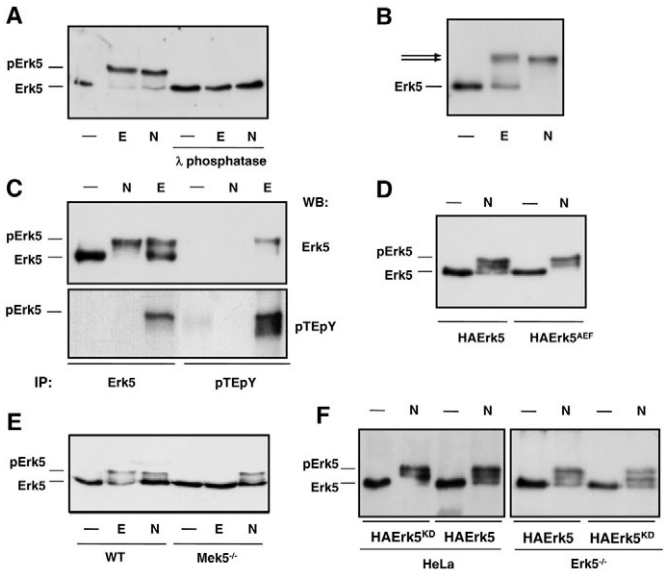

Fig. 2. Erk5 is phosphorylated in mitosis through a novel, MEK5-independent route. (**A**) HeLa cells were treated with nocodazole (N) or EGF (E), and Erk5 immunoprecipitated as described. Half of the sample was then treated with lambda phosphatase where indicated, and the western blot probed with the anti-Erk5 antibody. (**B**) Different migration of Erk5 after treatment with EGF or nocodazole. Extracts from HeLa cells treated with EGF (10 nM, 15 minutes; E) or nocodazole (200 ng/ml, 14 hours; N) were immunoprecipitated with an anti-Erk5 antibody, and the western blot probed with the same antibody. (**C**) Erk5 mitotic phosphorylation does not occur at the TEY microdomain. Extracts from HeLa cells treated with EGF or nocodazole were immunoprecipitated with anti-Erk5 or anti-Erk5-*P* antibodies, and the western blot probed with the indicated antibodies. (**D**) A dominant negative form of Erk5 (HA-Erk5^{AEF}) in which the residues that are normally phosphorylated (Thr218 and Tyr220) had been changed to non-phosphorylatable ones (Ala, Phe) was transfected into HeLa cells and the ability of the exogenous Erk5 to be phosphorylated in mitosis was assessed as described above. (**E**) MEK5 is not involved in the mitotic phosphorylation of Erk5. Wild-type or MEK5-deficient MEFs (*Mek5*$^{-/-}$) were treated with EGF or nocodazole. Extracts were prepared and Erk5 analyzed as above. (**F**) Erk5 kinase activity is not necessary for its mitotic phosphorylation. A mutant form of Erk5 in which the lysine residues involved in its kinase activity had been substituted by other residues (Erk5^{KD}) was transiently transfected into HeLa cells or *Erk5*$^{-/-}$ MEFs and its ability to be phosphorylated in mitosis determined as described.

treatment, indicating that, in fact, this was a MEK5-independent event (Fig. 2E).

To analyze whether Erk5 kinase activity was necessary in a mitotic autophosphorylation reaction, a kinase-dead mutant of Erk5 ($Erk5^{KD}$) was generated. This form, in which lysines 83 and 84 were mutated to other residues was transfected into HeLa cells. $Erk5^{KD}$ still suffered the characteristic mitotic shift when treated with nocodazole (Fig. 2F, left panel). Moreover, in Erk5-deficient cells (Wang et al., 2006), $Erk5^{KD}$ also suffered mitotic phosphorylation (Fig. 2F, right panel). Together, these data indicate that the mitotic phosphorylation of Erk5 was independent of Erk5 kinase activity. Therefore, and based in all the previous data we can conclude that Erk5 is phosphorylated while cells transit through mitosis, and such phosphorylation is independent of MEK5 or Erk5 kinase activity.

CDK1 activity sustains Erk5 phosphorylation in mitosis

One of the main kinases orchestrating mitosis is the cyclin-dependent kinase CDK1. Thus, to further identify the moment at which Erk5 phosphorylation occurs, as well as the possible implication of CDK1 on Erk5 phosphorylation, we took advantage of the drug RO-3306, a specific inhibitor of CDK1 (Vassilev et al., 2006). Treatment of interphase HeLa cells with the inhibitor provoked an increase in cyclin B (Fig. 3A), in accordance to its reported action as an inhibitor of CDK1 that would block cell cycle progression at the end of G2, just before entering mitosis. RO3306 completely blocked Erk5 phosphorylation suggesting either a role of CDK1 in Erk5 phosphorylation, or that Erk5 phosphorylation required entry in mitosis (Fig. 3A). In another type of experiment, the inhibitor was added to the cells once they had been blocked in mitosis by nocodazole, but without eliminating the microtubule-disrupting agent. Under these conditions, Erk5 dephosphorylation was detected as soon as 10 minutes after inhibiting CDK1 activity (Fig. 3B). The inhibitor also rapidly triggered a decrease in phospho-histone H3 and cyclin B levels, indicative of forced exit from mitosis. Nevertheless, HeLa cells had not completed cytokinesis, as verified by flow cytometry, since they still showed a G2–M peak in that assay (Fig. 3C). The above data indicated that CDK1 activity was necessary for Erk5 phosphorylation, and opened the possibility that CDK1 could directly interact with Erk5. To investigate such a possibility, we explored whether Erk5 and CDK1 interacted in mitosis. Interphase and mitotic HeLa cells extracts were immunoprecipitated with anti-Erk5 antibodies, and the blots probed with anti-CDK1. As shown in Fig. 3D, CDK1 and Erk5 co-precipitated, but only from extracts of mitotic cells. This together with the data obtained with

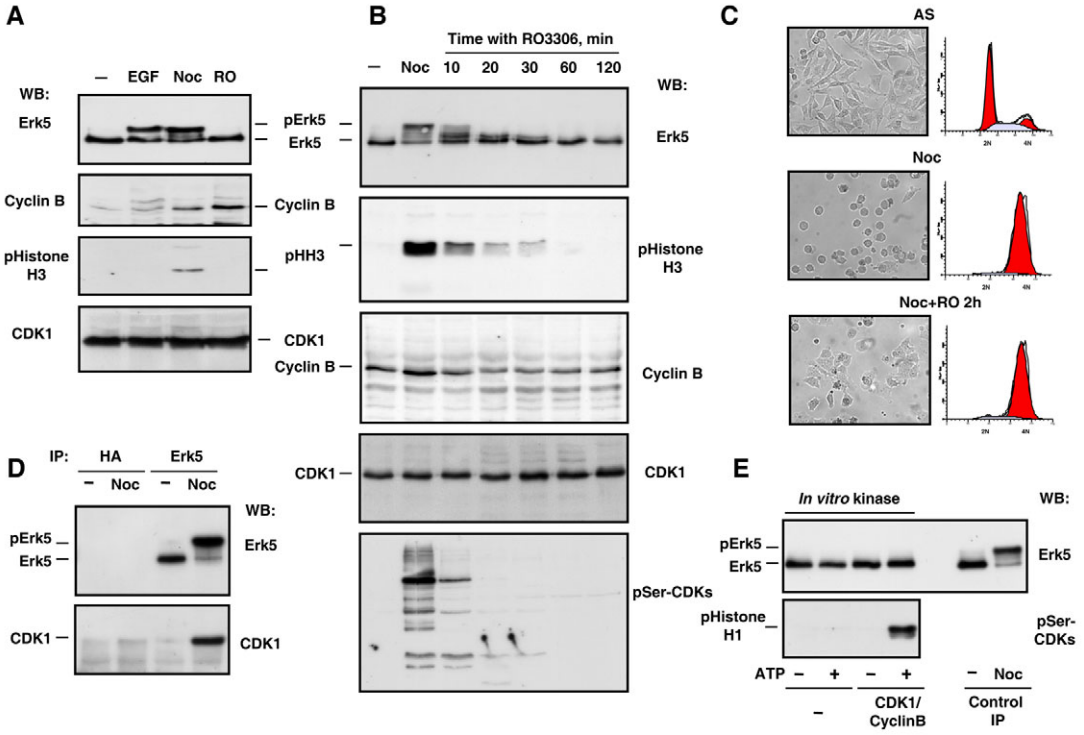

Fig. 3. CDK1 activity is required to sustain Erk5 phosphorylation in mitosis. (**A**) Cells were treated with EGF (10 minutes) or with nocodazole (14 hours; Noc) or RO-3306 (9 μM; RO). Erk5 immunoprecipitation and cyclin B, phospho-histone H3 and CDK1 blots were analyzed. (**B**) Nocodazole-treated cells were treated with RO-3306. At the indicated times, cells were harvested, part of the sample was fixed for cytometry and the other part lysed. 1 mg protein was immunoprecipitated with anti-Erk5 antibodies and 50 μg extract analyzed for the indicated proteins. CDK1 activity inhibition was demonstrated with an anti-Ser-*P*-CDK (pSer-CDK)-specific antibody (bottom panel). (**C**) Photomicrographs and flow cytometry analysis of the cells in B. (**D**) Erk5 co-immunoprecipitates in vivo with the CDK1–cyclin B complex. Asynchronous or nocodazole-treated HeLa cells were harvested in CHAPS lysis buffer (see Materials and Methods section). After 2 hours of immunoprecipitation either with anti-Erk5 or an unrelated antibody (HA), beads were washed, proteins separated by SDS-PAGE and membranes probed with the indicated antibodies. (**E**) CDK1 does not phosphorylate Erk5 in vitro. Erk5 from interphase HeLa cells was immunoprecipitated and used as a substrate in an in vitro kinase assay in the presence or absence of ATP and active CDK1–cyclin B complex. The appearance of the Erk5 phosphorylated band (pERK) was assessed by western blotting with the anti-Erk5 antibody (top panel). The bandshift of mitotic Erk5 is shown as a control in the rightmost lane. A control kinase reaction using histone H1, a known CDK1 substrate was run in parallel and phosphorylation assessed with the anti-Ser-*P*–CDK specific antibody (bottom panel).

the CDK1 inhibitor pointed to the possibility that CDK1 could directly phosphorylate Erk5. To analyze this, in vitro kinase experiments were performed. Active CDK1–cyclin B complexes failed to in vitro phosphorylate Erk5, as analyzed by a mobility shift with the anti-Erk5 antibody (Fig. 3E). However, these complexes did induce phosphorylation of histone H1, a known substrate of CDK1 (Santamaria et al., 2007), as measured with an anti-Ser-*P*–CDK substrate antibody.

Multisite phosphorylation of Erk5 in mitosis occurs in its C-terminus

As Erk5 was not phosphorylated at the canonical sites, our next goal was to identify the residues in Erk5 that are phosphorylated in mitosis. Erk5 is a molecule with an N-terminal half highly homologous to that of Erk1/2 and that differs from those MAPKs in having a unique, long C-terminal tail that includes different domains (Fig. 4A) (Nishimoto and Nishida, 2006; Wang and Tournier, 2006). Nevertheless, Erk1/2 did not change its mobility in mitosis (Fig. 4B). This could be due to the differences in the primary sequence of Erk5 and Erk1/2, or to the fact that phosphorylation was occurring at the unique Erk5 C-terminus. To study the latter possibility, we analyzed whether a truncated form of Erk5 lacking the N-terminal half of the molecule (Erk5-tail) behaved analogously to full length Erk5. As shown in Fig. 4C,

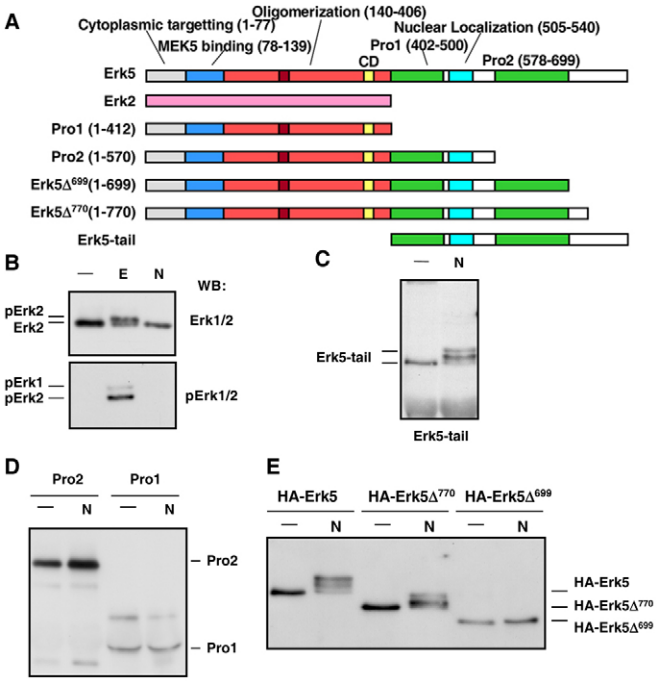

Fig. 4. Erk5 is phosphorylated at its C-terminal end. (**A**) Schematic representation of the different domains present in Erk5 (upper part) and of some mutants used to map the phosphorylation sites. (**B**) The MAPKs Erk1/2 are not phosphorylated in mitosis. 50 μg of extracts from HeLa cells treated with EGF or nocodazole were analyzed by western blot with anti-Erk1/2 or anti-Erk1/2-*P* antibodies. (**C**) Erk5 phosphorylation in mitosis occurs at its C-terminal half. The unique tail of Erk5 was subcloned in frame with an HA epitope and transfected into HeLa cells. After nocodazole treatment, the mobility of the tail was determined by immunoprecipitation with the anti-HA antibody and western blotting. (**D**,**E**) The indicated mutants lacking different regions of the unique tail were transfected and their response to nocodazole treatment was analyzed.

treatment with nocodazole caused retardation in the migration of the Erk5-tail mutant. To more precisely map the residues of Erk5 that are phosphorylated in mitosis, several deletion mutants lacking different parts of the C-terminal end of the molecule were used. The mutant form Pro1 (generated by inserting a stop codon after amino acid 412) was constructed by deleting the entire C-terminal tail of Erk5, and was created to correspond to analogous domains present in Erk1/2. This Erk5 form did not change its mobility in mitotic cells, indicating that in fact the Erk1/2-like region of Erk5 did not undergo phosphorylation during mitosis (Fig. 4D). A second deletion mutant of Erk5, created by inserting a stop codon before the second proline-rich region (Pro2, amino acid 570) was also unable to undergo a mobility shift in mitotic cells. Further deletion mutants defined the region where phosphorylation of Erk5 occurred beyond amino acid 699, and more specifically in the C-terminal 120 amino acids (Fig. 4E).

Within this region of Erk5, Morimoto and collaborators (Morimoto et al., 2007) had identified several residues phosphorylated by active Erk5 after transfection of a constitutively activated MEK5 form (MEK5DD). Even though our data excluded MEK5 or Erk5 as the C-terminal mitotic kinases, the possibility that those residues were the targets for such a phosphorylation, due perhaps to a predisposition for being phosphorylated, led us to create specific point-mutants in which those potentially phosphorylated residues were mutated to non-phosphorylatable ones (alanine residues). In addition, we created mutants targeting other potentially phosphorylatable sites (Fig. 5). Initially the Thr732 and two clusters of serines (that we termed S700s and S800s) were mutated to alanines. As deduced from the decrease in the phosphorylation of these mutants in response to nocodazole, Thr732 and at least one of the serines of each cluster of Erk5 were phosphorylated in mitotic cells (Fig. 5B). A more detailed analysis, in which each of the individual serines in each cluster was changed, allowed the identification of Ser773 and Ser706 as the residues phosphorylated in mitosis, in addition to Thr732 (Fig. 5C and data not shown). Another set of experiments identified Ser753 as another amino acid phosphorylated in mitosis (Fig. 5C). These approaches permitted the identification of four different residues in the Erk5 tail that are phosphorylated in mitosis (Fig. 5C). In agreement with this, when double (HA-Erk5$^{T732A-S753A}$ or HA-Erk5$^{T732A-S773A}$) and triple (HA-Erk5^{3XPA}) mutants were generated, the mobility shift was sequentially diminished but not completely eliminated (Fig. 5D). In fact, only when the four identified phosphorylation sites where mutated together to alanine (HA-Erk5^{4XPA}), was the bandshift induced by nocodazole treatment completely abolished (Fig. 5E), indicating that those four residues are phosphorylated in mitosis.

To investigate whether those residues were, in fact, phosphorylated in mitosis, phosphospecific antibodies against some of them (T732-*P* and S753-*P*) were generated. Immunoprecipitation of Erk5 from nocodazole-treated cells with the anti-T732-*P*–Erk5 or anti-S753-*P*–Erk5, followed by western blotting with anti-Erk5 antibodies resulted in the identification of a band that migrated with the same apparent relative molecular mass as Erk5 immunoprecipitated from HeLa cells treated with nocodazole (Fig. 6A). The anti-T732-*P*–Erk5 failed to detect Erk5 in interphase cells, whereas the anti-S753-*P*–Erk5 gave a weak signal. Analogous results were obtained when the phosphospecific antibodies were used in western blotting using immunoprecipitated Erk5 (Fig. 6B). The specificity of these phosphospecific antibodies was analyzed using single and double mutants in whom the residues of interest had been substituted by alanines. As shown in Fig. 6C, the

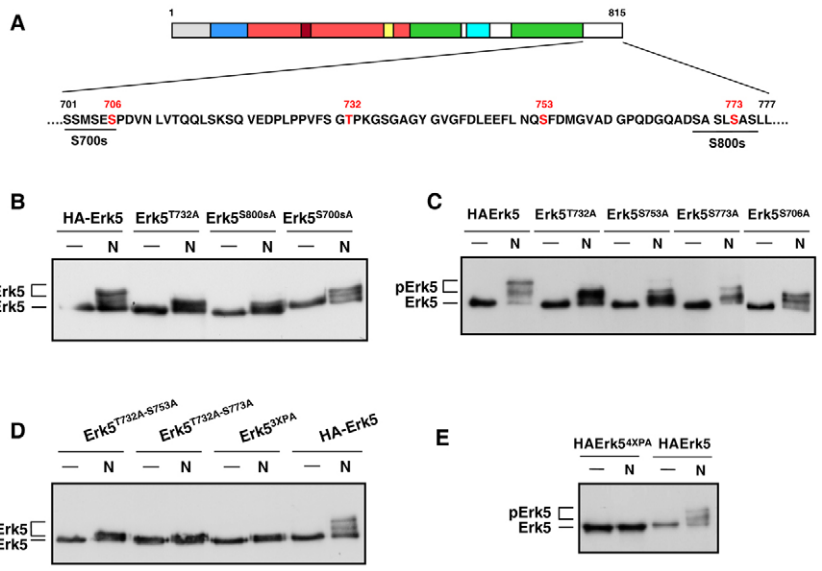

Fig. 5. Identification of the residues in the Erk5 tail phosphorylated in mitosis. (**A**) Schematic representation of the residues at the C-terminal end identified to be phosphorylated in mitosis. The identified residues are indicated in red. (**B–D**) Identification of the residues in the tail of Erk5 phosphorylated in mitosis. Different mutants in which the indicated residues had been replaced by alanine were transiently transfected and their response to nocodazole determined. (**E**) A mutant in which the four identified residues were replaced by alanines (HA-Erk5^{4XPA}) was generated, transfected and its response to nocodazole treatment analyzed.

antibodies specifically recognized those mutant Erk5 forms that contained the indicated amino acids, but were unable to interact with the mutant Erk5 forms in which the residue had been substituted for a non-phosphorylatable alanine. Further proof that the Thr732 and Ser753 were phosphorylated in mitosis came from phosphatase experiments (Fig. 6D). Both antibodies failed to recognize Erk5 from samples exposed to lambda phosphatase in an in vitro phosphatase assay. We also analyzed the kinetics of Erk5 phosphorylation at these sites as cells progress through mitosis, using the double thymidine block and release protocol described above. These experiments showed that Thr732 and Ser753 are phosphorylated, coincident with the progress through mitosis, and reach a phosphorylation peak at these residues at 10 hours after the release (Fig. 6E). Moreover, treatment with different drugs that provoke accumulation of cells in mitosis caused T732-*P* and S753-*P* Erk5 phosphorylation (Fig. 6F). Mitotic phosphorylation of Erk5 at these residues was also observed in a broad panel of cell lines, indicating that these mitotic phosphorylations are a general phenomenon (Fig. 6G).

C-terminal phosphorylation of Erk5 regulates its subcellular location

In search of the biological implications of the phosphorylation of Erk5 at those residues, we noticed that they fall within a region of Erk5 previously reported to control its subcellular location (Borges et al., 2007). This region has been shown to participate in the exit of Erk5 from the nucleus, as its absence provokes nuclear retention of Erk5 (Borges et al., 2007). Therefore, the possibility that the phosphorylation at these C-terminal residues could affect Erk5 subcellular distribution was explored in interphase cells, as the nuclear compartment is disrupted in mitosis. For these experiments, we used Erk5-deficient cells that were transfected with HA-tagged wild-type as well as Erk5 mutants in which the four sites identified in the molecular and biochemical studies were converted to Ala (Erk5^{4XPA}) or to Glu (Erk5^{4XPE}) (Fig. 7A). The HA-Erk5^{4XPA} form migrated faster than wild-type Erk5, whereas HA-Erk5^{4XPE} migrated slower. Immunofluorescence with anti-HA antibodies revealed that wild-type HA-Erk5 and HA-Erk5^{4XPA} maintained an essentially cytoplasmic distribution, according to the distribution most frequently described for Erk5 (Borges et al., 2007) (Fig. 7B). By contrast, the phosphomimetic HA-Erk5^{4XPE} had a nuclear location, supporting the idea that the residues changed are important for Erk5 subcellular location.

Another function attributed to the C-terminal region of Erk5 is the regulation of transcription of several target genes (Kasler et al., 2000). We analyzed the effect of the Erk5 mutants on the activity of a Nur77 promoter that has been used as a readout of Erk5-dependent transcriptional activity (Carvajal-Vergara et al., 2005). Expression of Erk5 increased transcriptional activity of the wild-type Nur77 reporter (Fig. 7C,D). By contrast, expression of Erk5^{4XPA} had a very small effect on the transcriptional activity of the reporter construct. The most prominent effect on the transcriptional activity was observed with the phosphomimetic Erk5^{4XPE}, which increased transcriptional activity 4-fold over that of wild-type Erk5 (Fig. 7C). As Erk5^{4XPE} was located in the nucleus, it is possible that such localization could be responsible for the increased transcriptional activity, independently of its phosphorylation at the C-terminal region. To explore this, we used a variant form of Erk5 that has previously been shown to be retained in the nucleus of HeLa cells (Borges et al., 2007). This form, termed Erk5Δ^{699}, was created by elimination of the nuclear exclusion region present in the C-terminus of Erk5, and is devoid of the C-terminal phosphorylation sites of Erk5 described here. Immunofluorescence experiments demonstrated that Erk5Δ^{699} was located in the nucleus of *Erk5*$^{-/-}$ cells, suggesting that this exclusion mechanism of Erk5 is also conserved in MEFs (Fig. 7B, lower panels). However, Erk5Δ^{699} was unable to reproduce the increase in transcriptional activity observed with Erk5^{4XPE} or even wild-type Erk5 (Fig. 7D). Moreover, the luciferase activity observed upon transfection of Erk5Δ^{699} was analogous to that obtained with the non-phosphorylatable mutant Erk5^{4XPA} (Fig. 7D). Therefore, the mere nuclear positioning of Erk5 is insufficient to confer increased transcriptional activity, which requires phosphorylation of the sites present in the C-terminal region.

Discussion

The mitogen-activated protein kinase Erk5 participates in animal development, and its deregulation has been linked to the aggressiveness of breast and prostate cancer (McCracken et al., 2008; Montero et al., 2009). Activation of Erk5 involves dual

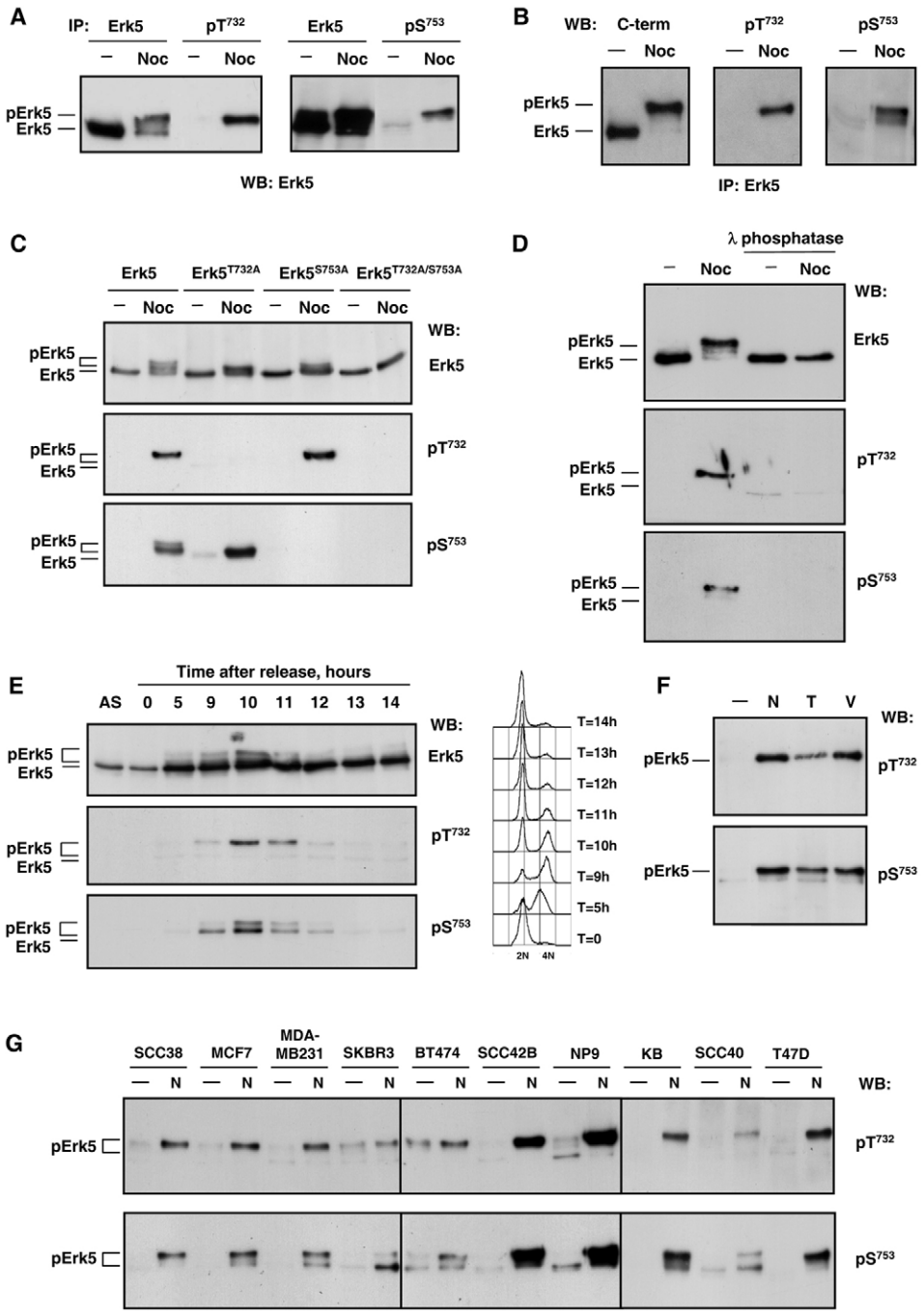

Fig. 6. Endogenous Erk5 is phosphorylated at T732 and S753. (**A**) The purified anti-T732-*P* (pT^{732}; left panel) or anti-S753-*P* (pS^{753}; right panel) antibodies were used in immunoprecipitation assays on HeLa cells after nocodazole treatment. Western blots were probed with the anti-Erk5 antibody. (**B**) Performance of the different antibodies generated in western blotting. Erk5 was immunoprecipitated with the anti-Erk5 antibody and the western blots were probed with the different antibodies generated, as indicated. (**C**) Verification of the specificity of the generated antibodies. Wild-type HA-Erk5 or the different HA-labeled mutants in which one or two of the residues identified and recognized by the generated antibodies had been mutated to alanine were transiently transfected. After nocodazole treatment, lysates were immunoprecipitated with the anti-HA antibody and western blots probed with the indicated antibodies. (**D**) HeLa cells were treated with nocodazole and in vitro phosphatase assay was performed as in Fig. 2A. Western blots were probed with the antibodies indicated on the right. (**E**) Erk5 is in vivo phosphorylated at T732 and S753 when cells transit through mitosis. G1–S-synchronized HeLa cells were released into normal medium and harvested at the indicated times after the release (in hours). Part of the sample was fixed for determination of DNA content by flow cytometry (right panel), and the other part lysed for protein preparation. Erk5 was immunoprecipitated and the western blots probed with the indicated antibodies. (**F**) HeLa cells treated with nocodazole (N), taxotere (T) or vincristine (V) were lysed and Erk5 analyzed as described above. (**G**) Erk5 is phosphorylated at T732 and S753 in many cell lines. Erk5 was immunoprecipitated from control or mitotic extracts, and the WB probed with the indicated phospho-specific antibodies.

phosphorylation at the TEY microdomain within its activation loop, a process catalyzed by the upstream activating kinase MEK5 (Kato et al., 1997; Lee et al., 1995; Zhou et al., 1995). This pathway involves growth factors or other stimuli to provoke phosphorylation-mediated activation of Erk5.

Here we describe a second pathway that controls Erk5 phosphorylation and that is active in mitotic cells. Previous reports indicated the possibility that Erk5 could be phosphorylated in mitotic cells, as its mobility, analyzed by SDS-PAGE and western blotting, was analogous to that obtained by activation of growth factor receptors (Cude et al., 2007; Girio et al., 2007). However, whether such a mobility change was due to phosphorylation was not explored. Our in vitro phosphatase experiments demonstrate that, in fact, the Erk5 mobility shift in mitosis was due to phosphorylation. However, evidence indicated that such phosphorylation did not occur at the classical consensus sites located at the TEY microactivation domain. First, immunoprecipitation of mitotic cell extracts with an antibody that recognized Erk5 phosphorylated at the TEY microdomain failed to precipitate mitotic Erk5, whereas it readily precipitated Erk5 obtained from EGF-stimulated cells. Second, an Erk5 mutant in which the TEY sequence was changed to AEF still underwent a mobility shift in mitosis. This latter finding also indicated that the phosphorylation of Erk5 in mitosis is independent of a previous phosphorylation step occurring at these sites within the microactivation domain, and suggested that MEK5 could not be involved in the novel phosphorylation events occurring in mitosis.

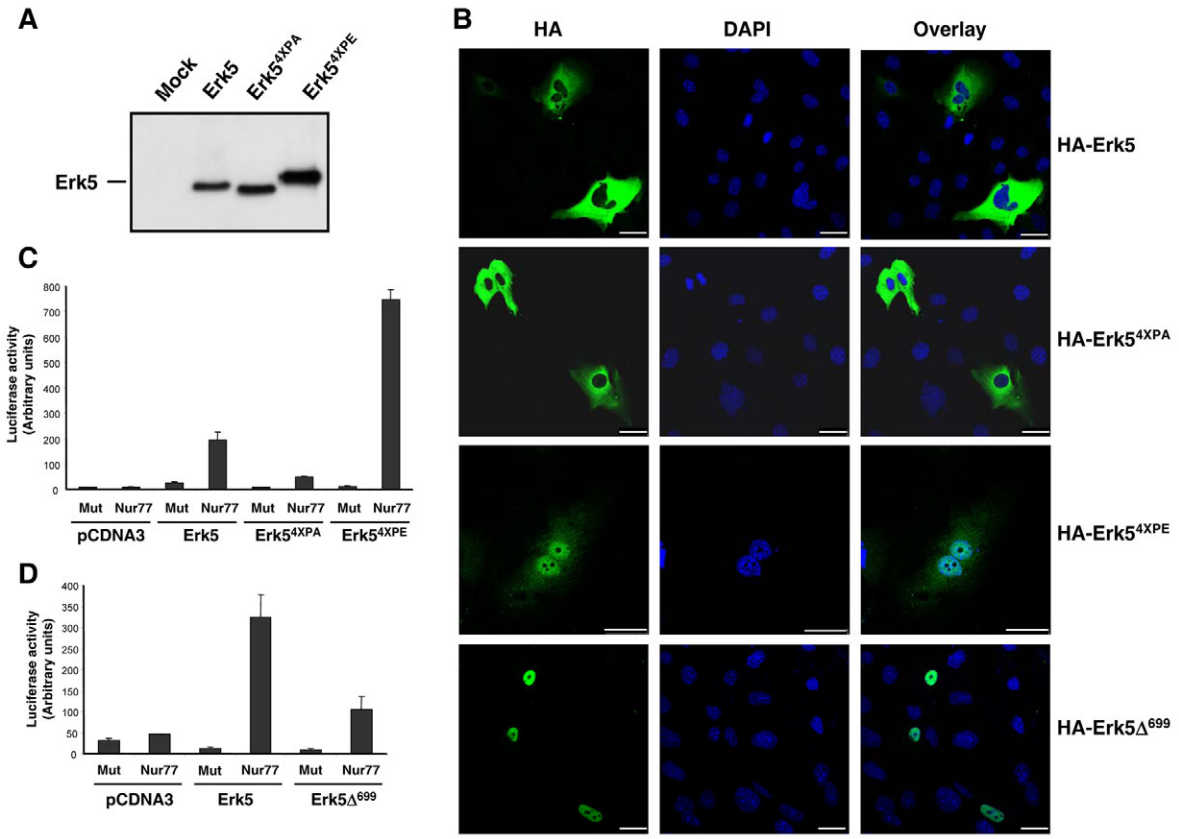

Fig. 7. The phosphomimicking mutant of Erk5 is retained in the nucleus. (**A**) Transient reconstitution of *Erk5*$^{-/-}$ MEFs with phosphomimetic and unphosphorylatable constructs. Erk5-deficient MEFs were transfected with empty vector, HA-Erk5, HA-Erk5^{4XPA} or HA-Erk5^{4XPE}. The Erk5 forms were immunoprecipitated with anti-HA antibodies, and the western blot probed with the anti-Erk5 antibody. (**B**) Subcellular localization of the different Erk5 mutants. Transfected *Erk5*$^{-/-}$ cells were grown on glass coverslips and the Erk5 mutants and wild type identified by immunofluorescence with the anti-HA antibody, followed by Alexa-488-labeled anti-rabbit antibody. Scale bars: 25 µm. (**C**) Transcriptional activity of the different Erk5 mutants. The indicated plasmids were co-transfected into Erk5-deficient MEFs together with a *Renilla* reporter, and their transcriptional levels measured as described in the Materials and Methods section. A representative experiment is shown; values are means of triplicate transfections with their standard deviations. (**D**) Erk5 nuclear location itself does not induce its transcriptional activation.

In fact, studies carried out in MEK5-deficient cells demonstrated that this kinase was required for growth factor-induced Erk5 phosphorylation at the TEY microdomain, and corroborated that the phosphorylation of Erk5 that occurs in mitosis was independent on the activity of MEK5. This finding, besides demonstrating that mitotic Erk5 phosphorylation was MEK5 independent, opened up the question of which kinase(s) is (are) involved in phosphorylating Erk5 in mitosis. So far, in addition to MEK5, Erk5 has been demonstrated to be autophosphorylated (Morimoto et al., 2007), raising the possibility that the phosphorylation event occurring in mitosis could be Erk5 dependent. However, it is unlikely that Erk5 is responsible for its autophosphorylation in mitosis, since a kinase-dead version of the molecule still undergoes mitotic phosphorylation.

The different synchronization experiments performed indicated that Erk5 is phosphorylated at the beginning of mitosis and dephosphorylated at its end, pointing to a mitotic kinase as the one responsible for Erk5 phosphorylation in mitosis. One of the main kinases orchestrating mitosis is CDK1 (Santamaria et al., 2007). Using inhibitors of this kinase, such as RO3306, we found that CDK1 activity is required to sustain Erk5 phosphorylation in mitosis. Thus, even a 10-minute treatment with this inhibitor resulted in a significant reduction of Erk5 phosphorylation. This fact may be consistent with CDK1 acting as a direct Erk5 kinase in mitosis. Moreover, two of the four phosphorylation sites of Erk5 (S706 and T732) are followed by a proline, and are CDK1 consensus target sites. However, we cannot conclude that CDK1 is the Erk5 mitotic kinase, or at least the unique kinase, as two out of the four identified phosphorylation residues are not consensus phosphorylation sites for CDK1. Furthermore, even though CDK1 co-precipitated with Erk5 in mitotic cells it was unable to efficiently phosphorylate Erk5 in vitro. Perhaps CDK1 may need a co-factor not present in the in vitro assay, or as happens with other mitotic kinases such as Plk1 (Elia et al., 2003), some priming could occur in Erk5 to be afterwards phosphorylated by CDK1. Based on these findings, we favor the hypothesis that Erk5 may be phosphorylated by various mitotic kinases, possibly including CDK1. This is compatible with the fact that RO3306 inhibits phosphorylation of Erk5 in mitosis, and can be explained by (1) a direct mechanism in which CDK1 acts as a kinase that regulates the activity of the other Erk5 mitotic kinases, or (2) an indirect mechanism in which maintenance of cells in mitosis is critical for their functioning as Erk5 mitotic kinases.

The molecular studies we performed identified S706, T732, S753 and S773 as the mitotic Erk5 phosphorylated sites. Moreover, phosphospecific antibodies raised to two of them (T732-*P* and S753-*P*) demonstrated that these residues are, in fact,

phosphorylated during mitosis, as indicated in the double thymidine block–release experiments. The phosphorylated residues of Erk5 fall within a relatively short C-terminal region. We have previously reported that deletion of this region (Erk5Δ^{699}) prevented nuclear exclusion of Erk5 (Borges et al., 2007). We therefore postulated that the phosphorylations at the mitotic sites could control the subcellular location of Erk5. In fact, substitution of the four residues with glutamic acid residues, in an attempt to mimic the charge modifications occurring upon phosphorylation, resulted in an Erk5 mutant that was retained in the nucleus of interphase cells. This is important, as some of the biological actions of Erk5, such as its transcriptional activity or the conferring of resistance to cell death, are expected to depend on its nuclear residency (Borges et al., 2007; Kasler et al., 2000). In fact, when the transcriptional activity of different mutants was analyzed, the mutant mimicking phosphorylated Erk5, that was nuclear, showed higher transcriptional activation properties than wild type Erk5. However, experiments using Erk5Δ^{699}, which is nuclear but does not include the phosphorylatable C-terminal sites, indicated that Erk5 merely being resident in the nucleus cannot explain its action on transcription. Therefore phosphorylation of these sites in interphase cells could represent a mechanism that controls the nucleocytoplasmic trafficking of Erk5 as well as its transcriptional activity. Interestingly, Morimoto and collaborators identified several residues in the Erk5 tail that are autophosphorylated in response to the activation of the canonical MEK5-dependent pathway (Morimoto et al., 2007) and some of them are coincident with those that we report here. This is important as it indicates that phosphorylation of Erk5 at its C-terminus may be involved in signaling through the canonical MEK5-dependent pathway, as well as in the novel MEK5-independent pathway described here.

In addition to the above mentioned studies that are required to gain further insights into the role of Erk5 in mitosis, our work opens several avenues of research in the Erk5 field. One important question that needs to be studied refers to the degree of crosstalk between the canonical and the non-canonical Erk5 phosphorylation pathways. Whether the novel C-terminal phosphorylations may also be triggered under other circumstances that activate the canonical MEK5-mediated pathway, such as growth factor receptor activation or stress conditions, must be analyzed. In fact, some preliminary data show that activation of the canonical pathway is able to induce Erk5 phosphorylation at least at some of the residues identified as being important in mitosis. In addition, future identification of the mechanisms involved in the phosphorylation of the C-terminal residues may allow identification of the kinase(s) responsible for Erk5 phosphorylation at these sites both in mitosis, and also in interphase cells. From the clinical point of view, and given the link found between Erk5 expression and patient prognosis in cancer, it will be interesting to evaluate to what degree phosphorylation of the Erk5 C-terminal residues is associated with clinical parameters such as overall survival, or disease progression. Also, as phosphorylation of these residues was found to occur in cells treated with agents used in the oncology clinic, analysis of Erk5 phosphorylation at these residues could serve as a biomarker to assess the action of those or other novel antitumoral compounds. Studies on some of these aspects are already underway.

Materials and Methods

Reagents and immunochemicals

Cell culture media, sera and G418 were purchased from Invitrogen, protein-A–Sepharose from GE Healthcare and Immobilon P membranes from Millipore Corp. Nocodazole, thymidine, taxotere and vincristine were from Sigma Chemical Co, EGF was from Prospec, histone H1 from Merck, active CDK1-cyclin B complex from New England Biolabs and RO-3306 from Calbiochem. Other generic chemicals were purchased from Sigma Chemical Co., Roche Biochemicals or Merck.

The anti-hemagglutinin (HA) monoclonal antibody was from Roche. The polyclonal anti-HA, anti-Erk5 (C-terminal) and anti-phospho-Erk5 (TEY) have previously been described (Esparis-Ogando, 2002). The anti-Erk1/2, anti-phospho-Erk1/2, anti-CDK1 and anti-cyclin B were from Santa Cruz Biotechnology, the anti-β-tubulin from Sigma, the anti-phospho-histone H3 was from Millipore and the anti-S-*P*–CDK specific from Cell Signaling.

Cell culture and transfections

Human cancer cell lines were derived from a cervix adenocarcinoma (HeLa), from human breast adenocarcinomas (MCF7, MDA-MB231 and SKBR3), ductal breast carcinomas (BT474, T47D), head and neck cancers (KB, SCC38, SCC40, and SCC42B) or pancreatic cancer (NP9). *Erk5*$^{-/-}$ and *Mek5*$^{-/-}$ cells are immortalized MEFs that were generated in the laboratory of Dr. C. Tournier (Wang et al., 2006; Wang et al., 2005). Cells were cultured at 37°C in a humidified atmosphere in the presence of 5% CO_2, 95% air. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing high glucose and antibiotics, and supplemented with 5% (HeLa cells) or 10% (all the others) fetal bovine serum. Mouse embryonic fibroblasts (MEFs) or HeLa cells were transfected with JetPEI™ reagent (Polyplus-transfection Inc.) following the manufacturer's instructions. Clones were obtained after selection with the appropriate antibiotics or transiently transfected cells were used to perform experiments two days after the transfection.

Cell cycle synchronization and DNA analysis by flow cytometry

Cells were arrested at the different phases of the cell cycle. For G2–M synchronization, cells were treated with 200 ng/ml nocodazole for 16 hours, and the mitotic population was obtained by shaking them off the Petri dish. In order to synchronize cells in G1–S, a double thymidine block was performed with sequential exposure to 2 mM thymidine, twice for 14 hours each and with an intermediate release of 10 hours in normal medium. After a second release and at the indicated times, samples were harvested and an aliquot was fixed and processed for flow cytometry. The rest of the sample was lysed for protein extraction and measurement. For DNA analysis by flow cytometry, ethanol-fixed cells were stained with 5 μg/ml propidium iodide (PI; Sigma) and 250 μg DNAse-free RNAse (Roche). A total of 30,000 cells were acquired in the PI gate using a FACSCalibur flow cytometer and CELLQUEST software (Becton Dickinson) and analyzed on MODFIT LT™ software (Verity software).

Protein extraction, quantification, immunoprecipitation and western blotting

Cells were washed with phosphatase-buffered saline (PBS) and lysed as described elsewhere (Esparis-Ogando et al., 2002). Protein content was determined by Bradford assay and the immunoprecipitation and western blotting performed with the indicated antibodies as described previously (Esparis-Ogando et al., 2002). For protein co-immunoprecipitation experiments, a milder buffer was used (Vander Haar et al., 2007). This buffer contained 40 mM HEPES at pH 7.4, 120 mM NaCl, 1 mM EDTA, 1 mM PMSF, 50 mM NaF, 1.5 mM Na_3VO_4, 10 mM β-glycerophosphate, 0.3% CHAPS and EDTA-free protease inhibitors (Roche). After a 2-hour immunoprecipitation with the desired antibodies, beads were washed and separated by SDS-PAGE, and the membranes blotted as usual.

Antibody generation

Anti–Erk5-*P*-specific antibodies were generated in rabbits injected with the sequences NH_2-CPPVFSGT-*P*PKGSGA-COOH (anti-T732-*P*) or NH_2-CEFLNQS-*P*FDMGV-COOH (anti-S753-*P*) corresponding to the indicated residues in the C-terminal tail of Erk5. The keyhole limpet hemocyanin-coupled peptides were injected into rabbits that were bled after the second boost. The antibodies from the sera were affinity purified as described elsewhere (Esparis-Ogando et al., 2002). Fractions with an A_{280} greater than 0.8 were pooled and characterized by IP and western blotting as shown in Fig. 6.

In vitro phosphatase and kinase assays

Erk5 was immunoprecipitated from cell lysates and the pellets washed as usual. Phosphatase treatment was carried out in 70 μl of phosphatase buffer containing 400 units of purified lambda phosphatase (New England Biolabs) for 2 hours at 30°C. Erk5 was resolved in SDS-PAGE gels and western blotting performed as described.

For the in vitro kinase assay, immunoprecipitates were washed in kinase buffer (New England Biolabs) and afterwards placed in a final volume of 30 μl of the same buffer supplemented or not with 100 μM ATP and 4 units of the active complex CKD1–cyclin B (New England Biolabs). An additional source of active CDK1–cyclin B was from mitotic HeLa cells in which the complex was immunoprecipitated with anti-cyclin B antibodies. The reaction was carried out at 30°C for 2 hours and afterwards western blotting was performed to detect the slowly migrating band. In parallel, another assay was performed on purified histone H1 (a known substrate of CDK1) and the western blot probed with the anti-Ser-*P*–CDK-specific antibody as a positive control of the reaction.

Mutant generation

Mutagenesis of Erk5 was performed using the QuikChange site-directed mutagenesis kit (Stratagene) on an Erk5 version tagged with an HA epitope at its N-terminal end that has been shown to be fully functional (Esparis-Ogando et al., 2002). The indicated amino acids were changed as follows: $Erk5^{T732A}$ (Thr732 to Ala), $Erk5^{S753A}$ (Ser753 to Ala), $Erk5^{S773A}$ (Ser773 to Ala), $Erk5^{S706A}$ (Ser706 to Ala), $Erk5^{S800sA}$ (Ser769, 771, 753 and 775 to Ala), $Erk5^{S700sA}$ (Ser701, 702, 704 and 706 to Ala), $Erk5^{T732A\text{-}S753A}$ (Thr732 and Ser753 to Ala), $Erk5^{T732A\text{-}S773A}$ (Thr732 and Ser773 to Ala), $Erk5^{3XPA}$ (Thr732, Ser753 and Ser773 to Ala), $Erk5^{4XPA}$ (Thr732, Ser706, Ser753 and Ser773 to Ala), $Erk5^{4XPE}$ (Thr732, Ser706, Ser753 and Ser773 to Glu), $Erk5^{KD}$ (Lys83 and 84 to Ser and Leu, respectively). In addition, a construct containing only the unique tail of Erk5 was generated by PCR amplification. Erk5 tail was subcloned into the pCDNA3 vector in frame with an HA epitope using conventional molecular biology techniques. The deletion mutants as well as the $Erk5^{AEF}$ mutant have previously been described (Borges et al., 2007; Esparis-Ogando et al., 2002).

Immunofluorescence

Cells cultured on glass coverslips were fixed in 2% *p*-formaldehyde for 30 minutes and permeabilized in PBS supplemented with 0.1% Triton X-100 that was afterwards quenched in 50 mM NH_4Cl. After blocking in PBS supplemented with 0.2% BSA, cells were incubated with the anti-HA antibody (Roche) for 2 hours at room temperature. After three washes of 5 minutes each, monolayers were incubated with Alexa-488-conjugated secondary antibody for 30 minutes, washed and counterstained with DAPI. Once mounted, samples were analyzed by confocal immunofluorescence microscopy using a SP5 Leica apparatus with the LAS-AF software.

Reporter gene analysis

Empty pCDNA3, pCDNA3-HAErk5, pCDNA3-HAErk5^{4XPA}, pCDNA3-HAErk5^{4XPE}, or pCDNA3-HAErk5Δ^{699} plasmids were co-transfected into Erk5-deficient MEFs together with plasmids encoding a luciferase gene driven by the wild-type Nur77 promoter or a Nur77 promoter with a mutated MEF2 binding site, which have been described previously (Woronicz et al., 1995). As a control for the transfection efficiency, a pRLSV40 *Renilla* vector was also co-transfected. The final molar ratio for the different vectors was 22:8:1 (pCDNA construct:Luciferase:*Renilla*). 24 hours after transfections, cells were harvested with Passive Lysis Buffer (Promega). The Dual Luciferase System (Promega) was used according to the manufacturer's protocol. Readings were taken in triplicates in a Synergy4 multi-mode microplate reader (Biotek), using the Gen5 1.05 software. Experiments were repeated at least twice, and results show as the mean ± s.d. of triplicates from a representative experiment.

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