ERK1c regulates Golgi fragmentation during mitosis

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Extracellular signal-regulated kinase 1c (ERK1c) is an alternatively spliced form of ERK1 that is regulated differently than other ERK isoforms. We studied the Golgi functions of ERK1c and found that it plays a role in MEK-induced mitotic Golgi fragmentation. Thus, in late G2 and mitosis of synchronized cells, the expression and activity of ERK1c was increased and it colocalized mainly with Golgi markers. Small interfering RNA of ERK1c significantly attenuated, whereas ERK1c overexpression facilitated, mitotic Golgi fragmentation. These effects were also reflected in mitotic progression, indicating that ERK1c is involved in cell cycle regulation via modulation of Golgi fragmentation. Although ERK1 was activated in mitosis as well, it could not replace ERK1c in regulating Golgi fragmentation. Therefore, MEKs regulate mitosis via all three ERK isoforms, where ERK1c acts specifically in the Golgi, whereas ERK1 and 2 regulate other mitosis-related processes. Thus, ERK1c extends the specificity of the Ras-MEK cascade by activating ERK1/2-independent processes.

Introduction

Extracellular signal-regulated kinases (ERKs) are members of the MAPK family of signaling proteins, which play a crucial role in the intracellular transmission of extracellular signals (Seger and Krebs, 1995; Yoon and Seger, 2006). Induction of this signaling cascade leads to phosphorylation of several target proteins that eventually regulate proliferation and other cellular processes (Yoon and Seger, 2006). The ERK-induced proliferation is regulated by a multistep mechanism that involves several cell-cycle stages (Zhang and Liu, 2002), including the regulation of G0, G1, S, and M (Tamemoto et al., 1992; Lavoie et al., 1996; Wright et al., 1999; Zhang and Liu, 2002; Edmunds and Mahadevan, 2004). Thus, aside from its role in the acute transmission of extracellular signals, the ERK cascade plays a role in the regulation of other cellular processes, which are mediated via a large set of effectors (Yoon and Seger, 2006).

One role of the ERK cascade is the regulation of G2/M and mitosis progression. Indeed, all components of the cascade were shown to undergo activation during the late G2 and M phases of the cell cycle (Tamemoto et al., 1992; Edelmann et al., 1996; Shapiro et al., 1998). In addition, inhibition of MEKs' activities by dominant-negative constructs or with pharmacological inhibitors delayed the progression of cells through the same stages (Wright et al., 1999; Roberts et al., 2002). Several molecular mechanisms have been implicated in the regulation of G2/M by

the ERK cascade, including the phosphorylation of centromere protein E (Zecevic et al., 1998), SWI–SNF (Sif et al., 1998), and polo-like kinase 3 (Plk3) (Xie et al., 2004), as well as the indirect activation of Plk1, Cdc2 (Liu et al., 2004), and Myt1 (Palmer et al., 1998). However, one of the best studied mechanisms by which the cascade can influence mitosis is the regulation of Golgi fragmentation, which is the focus of this study.

During mitosis, a mammalian cell needs to split its Golgi apparatus between two daughter cells. The mechanism that allows the proper division to occur is a massive fragmentation of the Golgi into thousands of vesicles that are later shared by the splitting cells (Shorter and Warren, 2002; Colanzi et al., 2003). This process occurs during the prophase/anaphase stages of mitosis, and is essential for the proper progression of cell division (Sutterlin et al., 2002). One of the kinases that participates in the regulation of this process is MEK1 (Acharya et al., 1998), which normally acts as an activator of ERK1 and ERK2 (ERK1/2; Yoon and Seger, 2006). Interestingly, these ERKs were not found to be associated with the fragmented Golgi. In addition, it was later shown that MEK action in the Golgi can proceed even in the absence of their NH$_2$-terminal D domain, which is essential for their activity toward ERK1/2 (Chuderland and Seger, 2005), indicating the presence of a different MEK substrate in the Golgi (Colanzi et al., 2000).

In recent years, several MEK1-induced, ERK1/2-independent proteins were proposed to play a role in mitotic Golgi fragmentation, including an ERK-like protein (Acharya et al., 1998) that appears to be mono-Tyr phosphorylated before fragmentation (Cha and Shapiro, 2001). The mono-Tyr phosphorylation of the ERK-like protein suggests that its mode of regulation

is distinct from that of ERK1/2, which are usually found either nonphosphorylated, under resting conditions, or double Thr and Tyr phosphorylated, upon activation (Yoon and Seger, 2006). The mechanism by which the ERK-like kinase executes MEKs' signals in the Golgi is not fully understood, but may involve phosphorylation of the Golgi reassembly stacking protein of 55 kD, which serves as an ERK2 substrate in the Golgi (Jesch et al., 2001), or activation of Plk3, which was recently proposed to mediate MEK1 signals in the Golgi (Xie et al., 2004). It should be noted, however, that the exact role of MEKs and their substrates in Golgi fragmentation is controversial, as it was shown that the phosphorylation of GM130, which is an important component of this process, mainly requires the activity of CDC2 without the involvement of MEKs (Lowe et al., 1998; Shorter and Warren, 2002; Uchiyama et al., 2003). Therefore, this study may provide new information on this controversial issue.

Recently, we cloned an alternatively spliced isoform of ERK1 in human cells and named it ERK1c (Aebersold et al., 2004). This ERK isoform is regulated differently from ERK1/2, mainly because of its altered cytosolic retention sequence/common docking motif. In addition, it was shown that it is localized in the Golgi of confluent cells and that its localization is regulated mainly by monoubiquitination (Aebersold et al., 2004). We extended our studies on the Golgi function of ERK1c, and found that its expression, phosphorylation, activity, and Golgi localization are increased in mitosis. Knockdown experiments revealed that ERK1c attenuates Golgi fragmentation during mitosis, and as a consequence it slows down cell cycle progression. Similar to the situation in high density cell culture, the ERK1c effect could not be substituted by ERK1 or ERK2, making it a unique MEK effector in mitosis. These results suggest that ERK1c mediates the MEK-regulated Golgi fragmentation during mitosis. In addition, this unique role of ERK1c provides a molecular mechanism by which ERK cascade may execute its multiple distinct, and even opposing, effects.

Results

ERK1c expression and activity are elevated during mitosis

Golgi fragmentation in mitosis is regulated by several protein kinases, including MEKs (Colanzi et al., 2003). Because ERK1/2, which are the known downstream targets of MEKs, are probably not involved in this Golgi fragmentation (Acharya et al., 1998), the mechanisms by which MEKs regulate this process are not clear. Recently, we identified ERK1c, which is an ERK1 isoform, and showed that it is localized in the Golgi of cells from high density cultures (Aebersold et al., 2004). Therefore, we undertook an examination of whether ERK1c plays a role in mitosis-related, MEK-dependent Golgi fragmentation. To do so, we synchronized HeLa cells using the double thymidine block procedure (Merrill, 1998), which arrests cells in early S phase and allows a synchronized cell cycle progression upon block release. Indeed, most of the cells were in S phase shortly after the release, shifted to G2 within 6–9 h, and cycled back to the next G1 within 12 h (Fig. 1 A). These results were complemented by mitotic index determination, in which

we found that the mitosis of the synchronized cells peaked 11 h after release (Fig. 1 B and Fig. S1, available at http://www. jcb.org/cgi/content/full/jcb.200509063/DC1). We then examined the behavior of ERK1c, as compared with ERK1 in the synchronously cycling cells, 6 h (G2), 9 h (late G2), and 11 h (peak mitosis) after release. The expression levels of ERK1c, which were obtained by blotting with specific ERK1c antibody, were significantly increased during mitosis (Fig. 1 C). The apparent lower levels of ERK1c in S and G2 cells, as compared with the nonsynchronized cells, were probably attributable to the observation that in the latter cells the expression of ERK1c was the mean between the low amount in S phase and the much higher amount in mitosis. On the other hand, the expression levels of ERK1/2 were unchanged during the times examined, although the regulatory phosphorylation of the ERKs, which was determined using diphospho-ERK (pERK) antibody (Yao et al., 2000), was elevated in late G2 and during mitosis, supporting previous works that demonstrated activation of MEKs and ERK1/2 during these stages of the cell cycle (Wright et al., 1999; Roberts et al., 2002).

Because ERK1c migrates together with ERK2 on an SDS-PAGE (Aebersold et al., 2004), it was not clear from the results whether the 42-kD band recognized by the pERK antibody contained any phosphorylated ERK1c or consisted only of phosphorylated ERK2, which is much more abundant. Therefore, to follow the specific activation of ERK1c and compare it to ERK1, these two proteins were immunoprecipitated with either specific ERK1c or ERK1 antibodies and subjected to an in vitro kinase assay using myelin basic protein (MBP) as a substrate. Using this protocol, nonsynchronous cells were found to contain relatively low ERK1c activity and even lower ERK1 activity (Fig. 1 D). The activity of ERK1c was lower in the S and G2 phases and significantly increased during late G2 and mitosis (sevenfold at 11 h). This rate of activation was different from that of ERK1, which constantly increased throughout the experiment, up to 12-fold higher than its activity in S phase. Because the increased activity of ERK1c could be caused by its elevated expression, we equilibrated the amount of ERK1c protein from nonsynchronized and mitotic cells to measure changes in their specific activity. Elevated levels of pERK1c were detected in the mitotic cells as compared with the nonsynchronous cells (Fig. 1 E), indicating that the elevation of ERK1c activity in mitosis is achieved by elevation in its expression, as well as in its activatory Thr and Tyr phosphorylation.

To eliminate the possibility that ERK1c activation was induced by the thymidine treatments and not by cell cycle progression, we used nocodazole, which arrests cells in mitosis via a distinct mechanism (Merrill, 1998). Indeed, application of nocodazole to HeLa cells resulted in their mitotic arrest within 24 h, as judged by FACS analysis (Fig. 2 A) and mitotic index determination (not depicted). The treated cells were then used to examine the expression and activation of ERK1c and ERK1 in a manner similar to that described for the double thymidine–based synchronization. Thus, similar to the double thymidine results, ERK1c expression, as well as its phosphorylation and activity, were elevated in the nocodazole- arrested cells (Fig. 2, B and C). On the other hand, in agreement with a previous study
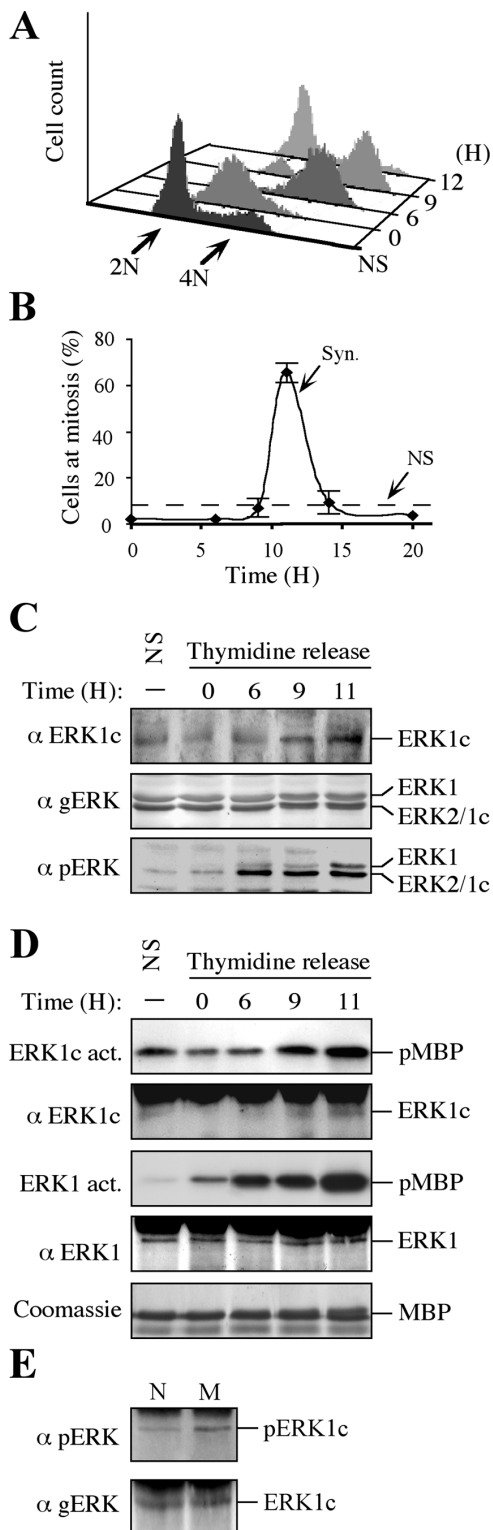
Figure 1. **Elevated ERK1c expression, phosphorylation, and activity in mitosis.** HeLa cells were either synchronized by double thymidine arrest, which was followed by the removal of the block to enable cell cycle progression, or left untreated (NS, nonsynchronized) as a control. (A) Representative flow cytometry analysis of the synchronized HeLa cells. At the indicated times after the block release, the cells were fixed, stained with propidium iodide, and analyzed by flow cytometry to determine the amount of DNA per cell (2N and 4N). (B) Mitotic index determination. The tested cells were fixed and stained with DAPI. The percentage of mitotic cells out of the total number of nuclei counted is presented as the means ± SEM of three experiments. n = 300. (C) ERK1c expression is elevated in
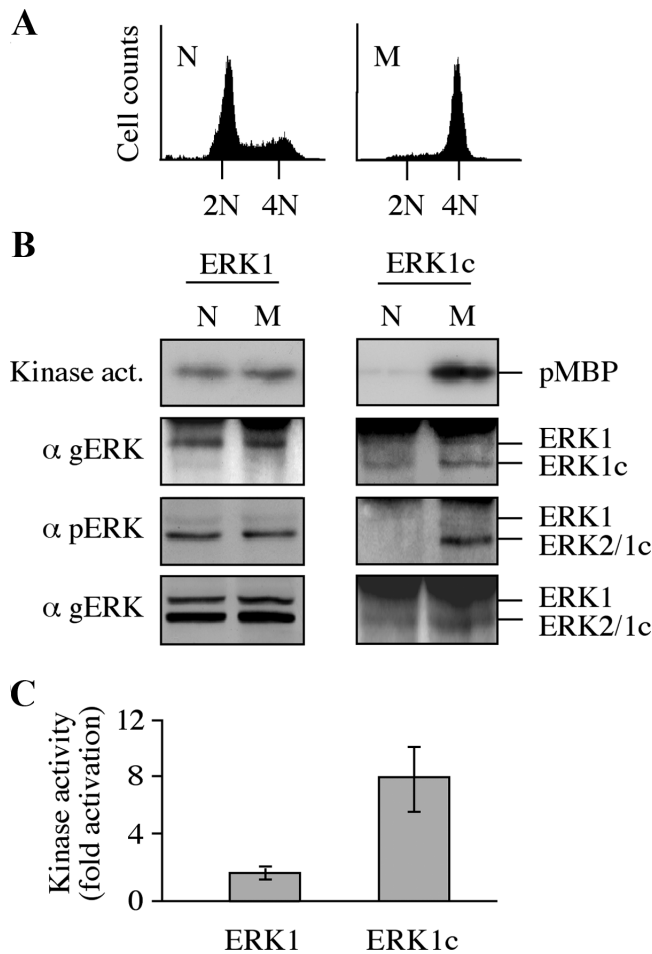
(Takenaka et al., 1998), the expression of ERK1 was not affected, and the activity of this protein was only slightly elevated by the nocodazole treatment, suggesting a specific mode of ERK1c activation in these stages of mitosis. Collectively, our results clearly show that both the expression and activity of ERK1c are elevated by cell cycle progression and not by individual chemical treatments.

ERK1c is colocalized with Golgi markers during the early stages of mitosis

In a previous study, we showed that ERK1c resides in the Golgi of confluent cells (Aebersold et al., 2004); therefore, we undertook to examine whether ERK1c can be localized in the Golgi apparatus during mitosis as well. To do so, the double thymidine–synchronized mitotic HeLa cells were costained with ERK1c, together with either the general Golgi marker GM130 or the cis-Golgi marker p58 antibodies. The exact stage of mitosis was determined by DAPI staining, which showed a typical chromosomal arrangement for each phase and confirmed that the sample depicted 11 h after the release from the thymidine block contained mainly cells ranging from late G2 to telophase. The staining of these cells with the Golgi markers (Fig. 3) revealed the expected (Colanzi et al., 2003) two-step fragmentation, first into bulbs (prophase and early prometaphase) and later into microvesicles (late prometaphase and metaphase), which were spread throughout the cell.

We then followed ERK1c localization in the various stages of mitosis (Fig. 3 A). In interphase cells, ERK1c was diffusely spread throughout the cells, without any Golgi preferences. In prophase, which is when the DNA started to condense, much of the ERK1c staining was found colocalized with the partially fragmented Golgi in the perinuclear region, although some ERK1c remained spread throughout the cells. In prometaphase, when the DNA was already condensed and on its way to the equator, ERK1c was mostly colocalized with the Golgi markers in some bulbs and microvesicles that covered a significant portion of the cytosol. In metaphase, when the condensed DNA was localized in the cell equator, both ERK1c and the Golgi markers were homogenously spread throughout the cells; similar distribution was also observed with the mitotic nocodazole-arrested cells (not depicted). Interestingly, during telophase there was still interaction of ERK1c with the recomposing Golgi, as well as with the kinetochore (Fig. 3 B), but this colocalization was rapidly changed, and in new G1 cells ERK1c was again diffusely spread. The observation that ERK1c is concentrated

the G2/M phase of the cell cycle. The tested cells were harvested and subjected to a Western blot with ERK1c, ERK1/2, and pERK antibodies. (D) ERK1c activity is enhanced in the G2/M phase of the cell cycle. The tested cells were harvested and ERK1 or ERK1c were immunoprecipitated using their respective antibodies. The immunoprecipitated proteins were either subjected to an in vitro kinase assay using MBP as a substrate (ERK1c and ERK1 act.) or detected with ERK1c and ERK1 antibodies. Coomassie blue staining of the phosphorylated MBP is shown to confirm equal amounts of substrate. (E) The diphosphorylation of ERK1c is elevated during mitosis. Synchronized HeLa at mitosis (M) or untreated cells (N) were harvested, followed by immunoprecipitation of their ERK1c using ERK1c antibody. Equal amounts of ERK1c (predetermined by Western blotting) were subjected to Western blots with pERK or ERK1c antibodies.

A

Cell counts

N   M

2N 4N   2N 4N

B

ERK1   ERK1c

N M   N M

Kinase act.   pMBP

α gERK   ERK1 / ERK1c

α pERK   ERK1 / ERK2/1c

α gERK   ERK1 / ERK2/1c

C

Kinase activity (fold activation)

ERK1   ERK1c

Figure 2. **Nocodazole treatment induces ERK1c expression and activation.** (A) Nocodazole arrests HeLa cells in the G2/M phase of the cell cycle HeLa cells were either treated with 100 ng/ml nocodazole (M) for 24 h or left untreated as control (N). The cells were then fixed, stained with propidium iodide, and analyzed by flow cytometry to determine the amount of DNA per cell (2N and 4N, as indicated). (B) Nocodazole-dependent mitotic arrest induces ERK1c activation. HeLa cells were treated with nocodazole (M) or left untreated (N). ERK1c and ERK1 were immunoprecipitated from these cells using their respective COOH-terminal antibodies and either subjected to an in vitro kinase assay (Kinase act.) or Western blot with gERK antibody. In addition, the cell extracts were either subjected to Western blot (left, bottom middle, and bottom) or immunoprecipitated with ERK1c antibody followed by Western blot (right, bottom middle, and bottom) with pERK or ERK1c antibodies (right, bottom middle, and bottom). (C) Quantification of kinase activation. Fold activation of ERK1c and ERK1 was quantified by densitometry and presented as means ± SEM. n = 3.

in the Golgi in the various stages of mitosis was also confirmed by costaining with lamin, which is disassembled together with the entire nuclear envelope after prophase (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200509063/DC1). It should also be noted that the Golgi localization was specific to ERK1c, as the more abundant ERK1 isoform was not detected in the Golgi during prophase (Fig. 3 C) or any other stage of the cell cycle. The colocalization of ERK1c with p58 or GM130 was confirmed by an electronic merge of the two stainings, showing again that the major portion of ERK1c is colocalized with the Golgi markers that are already in prophase (Fig. S3). Quantification of mitotic cells (prophase/telophase) revealed

costaining of ERK1c and the Golgi markers in ∼75% of the cells as compared with only ∼23% in interphase cells (Fig. 3 D). However, it is possible that the percentage of mitotic cells with Golgi-localized ERK1c is even higher because cells in which the Golgi or the ERK1c were not properly stained were not counted as ERK1c-Golgi–colocalized cells. These results indicate that ERK1c specifically interacts with the Golgi in mitosis and not only upon an elevation in cell density.

Monophosphorylation of ERK1c downstream of MEKs in the Golgi

We have previously shown that, similar to ERK1/2, ERK1c can be activated by the upstream MEK kinases (Aebersold et al., 2004). However, the different kinetics of ERK1c and ERK1 activation during the cell cycle (Fig. 1) challenged the involvement of MEKs in ERK1c activation; therefore, it was important to examine whether ERK1c is directly activated by MEKs in the mitotic Golgi. For this purpose we first overexpressed a constitutively active (CA) form of MEK1 (Jaaro et al., 1997) or added the MEK inhibitor PD98059 to synchronized G2/M HeLa cells. Immunoprecipitation of ERK1c from the treated cells, followed by an in vitro kinase assay, revealed that ERK1c was highly activated in the mitotic cells containing CA-MEK1 and that its activation was reduced with PD98059 (Fig. 4 A). These activations correlated with the double-Thr and -Tyr phosphorylation of ERK1c, again indicating that ERK1c is a substrate for MEK1 during mitosis. In addition, ERK1c appeared to be heavily mono-Tyr phosphorylated in the mitotic cells, and this monophosphorylation was increased in the mitotic cells expressing the CA-MEK and prevented by PD98059 (Fig. 4 A). This phosphorylation was distinct from that of ERK1/2, which were mainly diphosphorylated (not depicted). Therefore, these results indicate that although both ERK1c and ERK1/2 are activated by MEKs (Aebersold et al., 2004; Yoon and Seger, 2006) they are subjected to different modes of regulation in mitotic cells.

It was previously reported that monophosphorylated ERK–like proteins are present in the Golgi just before fragmentation (Cha and Shapiro, 2001). Indeed, staining of cells in prophase or interphase confirmed the reported appearance of monophosphorylated ERK in mitosis, without an accompanied accumulation of diphosphorylated ERK in the Golgi under any conditions examined (Fig. 4 B). This staining experiment also revealed that the monophosphorylated ERK colocalized with ERK1c but not with ERK1/2, supporting the possibility that the monophosphorylated ERK in the mitotic Golgi is ERK1c.

Because our results indicate that ERK1c is activated by MEKs, we examined whether, unlike the diphosphorylation of ERK1/2 (Yoon and Seger, 2006), MEK1 phosphorylates ERK1c preferentially on its regulatory Tyr residue. Therefore, we examined the possibility that ERK1c, which is not recognized by most general ERK (gERK) antibodies but can be identified by the monophosphorylated ERK antibody, is the monophosphorylated ERK in the mitotic Golgi. To do so, we immunoprecipitated MEK1-GFP from EGF-treated cells and, in parallel, we purified recombinant GST-ERK1c or GST-ERK1 from bacteria. Incubation of the active MEK1 with ERK1c in the presence of ATP and magnesium resulted in a fast accumulation of
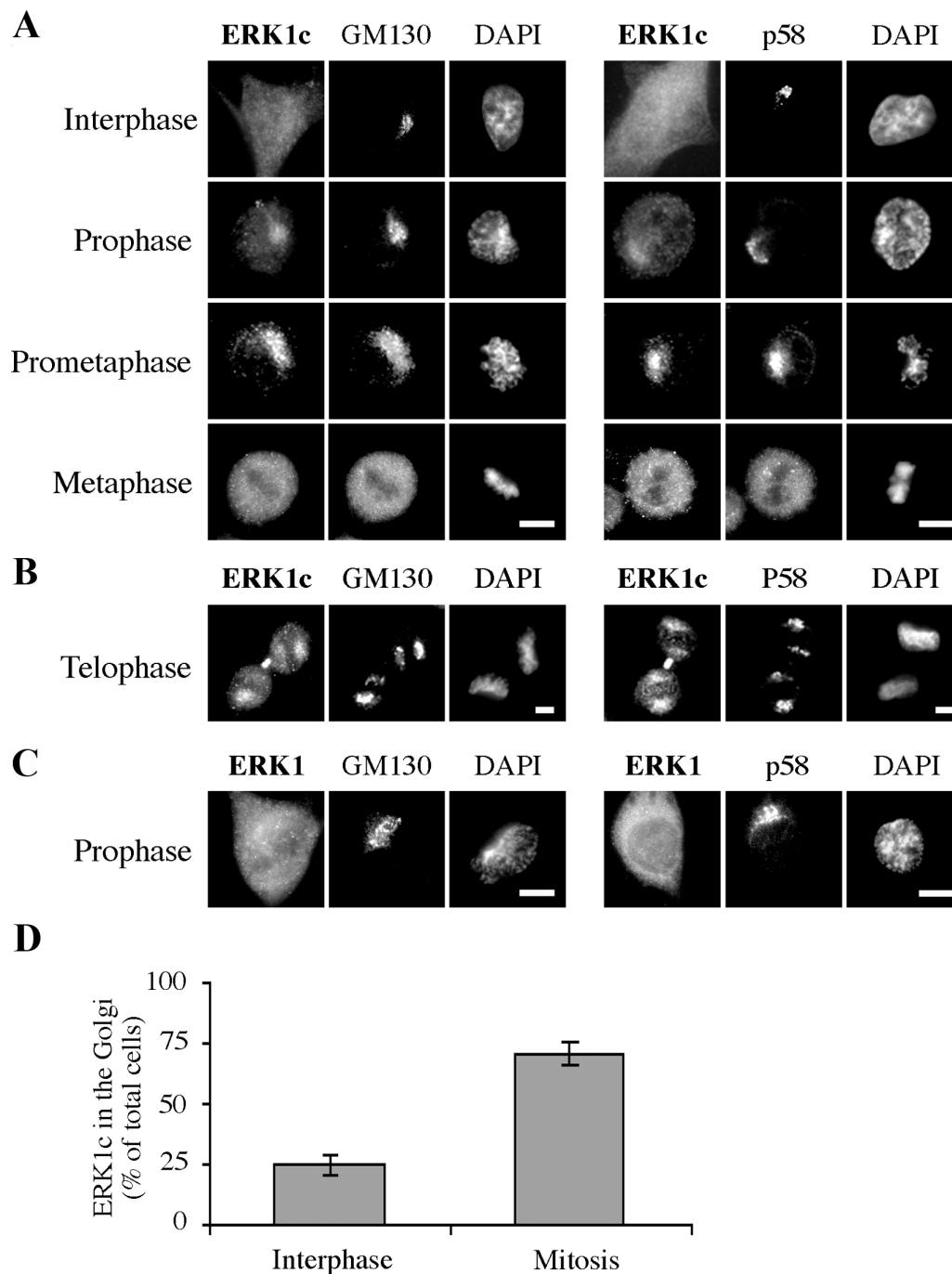
Figure 3. **The subcellular localization of ERK1c during mitosis.** Synchronized HeLa cells 11 h after the double thymidine block release were fixed and stained with ERK1c, GM130 or p58 antibodies, and DAPI. Cells from interphase, prophase, prometaphase, metaphase (A), and telophase (B) were selected by their DNA structure, which was observed using DAPI staining. Similar results were detected with at least 20 distinct cells for each condition. (C) ERK1 does not translocate to the Golgi during mitosis. HeLa cells were fixed and stained with ERK1, GM130 or p58 antibodies, and DAPI. Cells in prophase were selected by their DNA structure, which was observed using DAPI staining. Similar results were detected with at least 20 distinct cells for each condition. (D) Quantification of ERK1c colocalization in the Golgi during mitosis. The percentage of mitotic or interphase cells in which ERK1c was colocalized with the Golgi markers was determined by counting 200 cells in each slide. The results in the bar graph represent the mean ± SEM of three experiments. Bars, 10 μm.

monophosphorylated ERK1c (Fig. 4 C) that was much faster than the appearance of pERK1c that correlated with the low activity of the kinase. Under these conditions, the diphosphorylation of ERK1/2 was much faster than the mono-Tyr phosphorylation, which is in agreement with previously published studies (Yao et al., 2000; Yoon and Seger, 2006). These results indicate
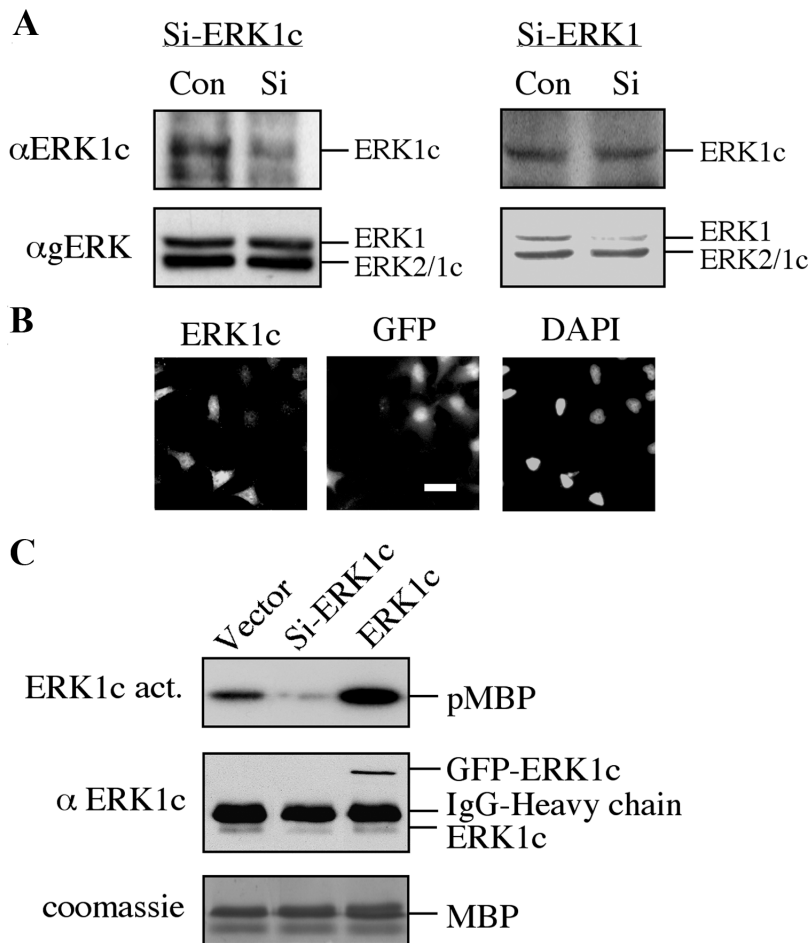
that MEKs phosphorylate ERK1c preferentially on its Tyr204 and suggest that the monophosphorylated ERK in the Golgi can be ERK1c, which is directly mono-Tyr phosphorylated by MEKs. Therefore, the presence of monophosphorylated ERK1c in the Golgi does not seem to require the activity of the Ser/ Thr phosphatase PP2A, which had previously been suggested

Figure 4. **ERK1c is activated by MEKs and is mainly mono-Tyr204 phosphorylated.** (A) Mitotic ERK1c is either mono-Tyr phosphorylated or diphosphorylated and is activated downstream of MEKs. HeLa cells were transfected with CA-MEK (ΔN-EE-MEK1, marked as MEK) or with GFP control. The cells were then synchronized by double thymidine block and released for 11 h. 25 μM PD98059 was added to one of the GFP plates (PD) 9 h after release, and the other GFP plate was left untreated (Con). After harvesting, ERK1c was immunoprecipitated and either subjected to an in vitro kinase assay (ERK1c act.) or to a Western blot with pERK, monophosphorylated ERK, and ERK1c antibodies. Coomassie blue staining of the MBP is shown as a control for equal substrate amounts. (B) ERK1c colocalizes with monophosphorylated ERK during mitosis. The synchronized HeLa cells were fixed and stained with ERK1c, ERK1, pY-ERK or pERK antibodies, and DAPI. Cells from interphase and prophase were selected by their DNA structure, which was observed using DAPI staining. Similar results were detected with at least 20 distinct cells for each condition. Bar, 5 μm. (C) ERK1c undergoes preferential phosphorylation on its Tyr204. HeLa cells were transfected with MEK1-GFP. The cells were serum starved for 16 h and then stimulated with 50 ng/ml EGF for 3 min. MEK1-GFP was immunoprecipitated and used to phosphorylate purified GST-ERK1c or GST-ERK1 for the indicated times. The amount of ERK1c and ERK1, as well as their phosphorylation, were determined by Western blotting using pY-ERK, pERK, and ERK1 antibodies. The results in the graphs represent the mean and SEM of three experiments. Squares represent diphosphorylation and circles represents monophosphorylated ERK1 or ERK1c.
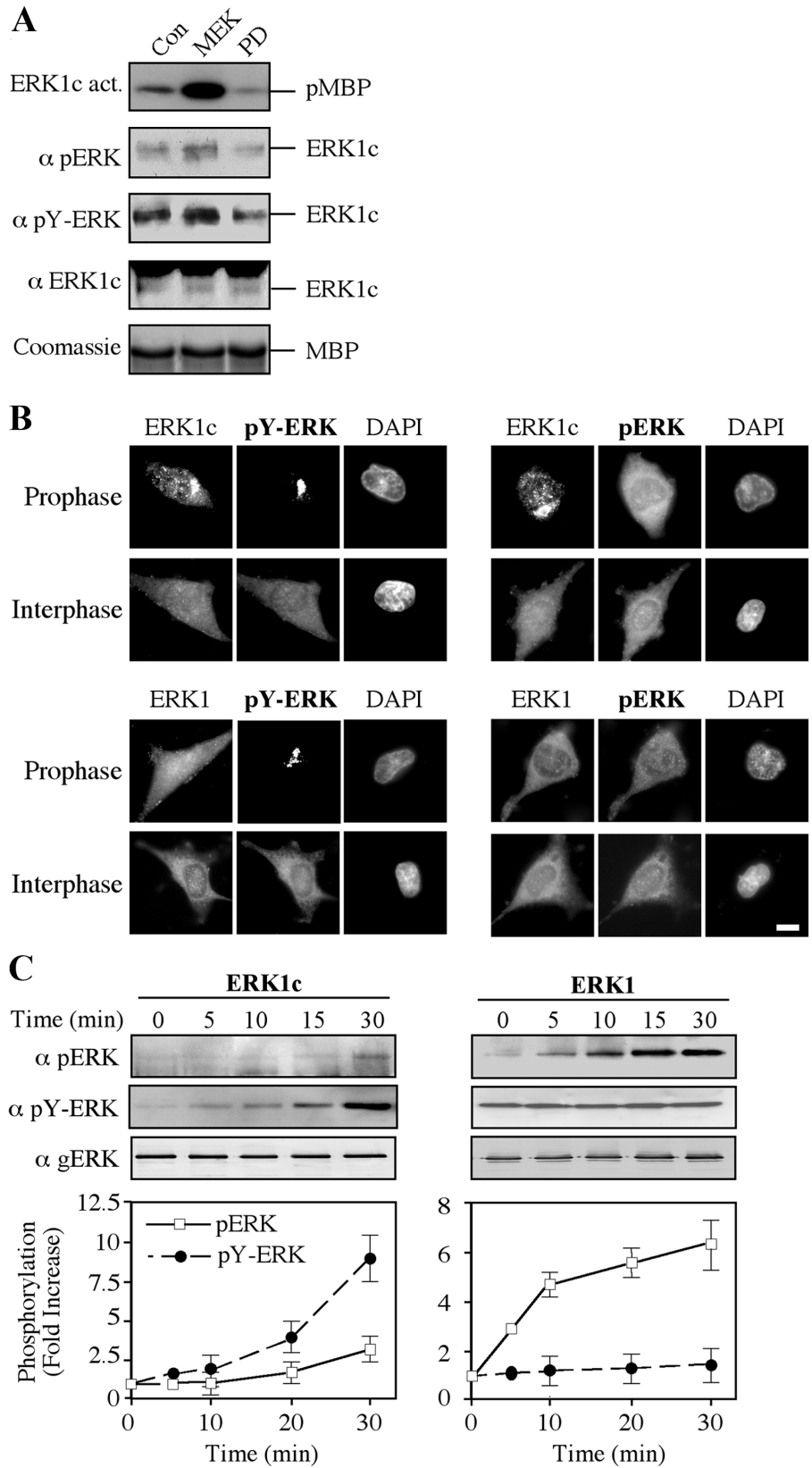
as a potential cause for the presence of monophosphorylated ERK because of its activity on the phosphorylated Thr of diphosphorylated ERK-like protein in the Golgi (Hancock et al., 2005). The observation that monophosphorylated ERK1c was identified only in the Golgi, although it was also localized to some extent in other parts of the cytosol, suggests that the regulation of this enzyme in the Golgi is distinct from its regulation in other cellular locations.

A

Si-ERK1c   Si-ERK1

Con Si   Con Si

αERK1c ⎯ ERK1c   ⎯ ERK1c

αgERK ⎯ ERK1   ⎯ ERK1
     ⎯ ERK2/1c   ⎯ ERK2/1c

B

ERK1c   GFP   DAPI

C

Vector Si-ERK1c ERK1c

ERK1c act. ⎯ pMBP

α ERK1c ⎯ GFP-ERK1c
    ⎯ IgG-Heavy chain
    ⎯ ERK1c

coomassie ⎯ MBP

Figure 5. **Modulation of ERK1c expression and activity with siRNA and GFP-ERK1c.** (A) Specific knockdown of ERK1c and ERK1 by their corresponding siRNAs. HeLa cells were transfected with GFP together with either a plasmid containing si-ERK1c, a plasmid containing si-ERK1, or additional GFP (Con). 72 h after transfection, the cells were harvested and subjected to a Western blot with ERK1c and ERK1/2 (αgERK) antibodies. (B) si-ERK1c reduces the amount of ERK1c in transfected cells. HeLa cells were cotransfected with si-ERK1c and GFP, and 72 h after transfection the cells were fixed and stained with αERK1c antibody and DAPI. Bar, 30 μM. (C) Modulation in ERK1c expression correlates with its MBP phosphorylation. HeLa cells were transfected with pEGFP (Vector), si-ERK1c, or GFP-ERK1c (ERK1c) synchronized by double thymidine block and release for 11 h. After harvesting, ERK1c was immunoprecipitated from the cells and was either subjected to an in vitro kinase assay (ERK1c act.) or Western blot using ERK1c antibody. Coomassie blue staining of the MBP confirmed equal substrate.

ERK1c regulates Golgi fragmentation

Our results clearly indicated that ERK1c is localized and active in the Golgi during mitosis, but its exact role there was not resolved. Therefore, we undertook to study whether ERK1c is the enzyme that participates in the regulation of mitotic Golgi fragmentation downstream of MEKs. To this end, we altered ERK1c (and ERK1 as control) expression in the mitotic cells by either overexpressing the GFP-conjugated wild-type constructs (68 and 70 kD of GFP-ERK1c and GFP-ERK1, respectively; Aebersold et al., 2004) or by knocking down the expression of the endogenous proteins using specific small interfering RNA (siRNA). To confirm a specific reduction of either ERK1c or ERK1, the interfering sequences were directed toward the unique COOH-terminal sequences of the ERKs. Indeed, expression of the ERK1c-interfering sequence (siRNA of ERK1c [si-ERK1c]) in HeLa cells, resulted in a reduction of 75 ± 7% in the expression of endogenous ERK1c, with no effect on the expression of ERK1/2 (Fig. 5 A). Similarly, expression of the ERK-interfering sequence (si-ERK1) specifically reduced the expression of ERK1 (80 ± 10%) without any effect on ERK1c or ERK2. The reduction in ERK1c expression by its interfering sequence was also demonstrated by immunostaining with ERK1c antibody (Fig. 5 B), which showed a marked reduction in the amount of ERK1c with no significant change of its localization in the si-ERK1c–expressing cells (Fig. 5 B and Fig. S4, available at

http://www.jcb.org/cgi/content/full/jcb.200509063). It should also be noted that this correlation between the Western blot and the immunostaining confirmed the specificity of ERK1c staining by the antibody used in this study. As expected, activity assays showed that overall ERK1c activity in mitotic cells overexpressing GFP-ERK1c was significantly higher than the activity derived from vector control cells, whereas the activity derived from the cells transfected with si-ERK1c was markedly lower (Fig. 5 C). Therefore, this system was judged suitable for the study of downstream effects that are mediated either by phosphorylation or by changes in expression and localization of ERK1c.

To determine whether ERK1c is indeed involved in the regulation of Golgi fragmentation, we used both GM130 and p58 markers to follow Golgi architecture in cells expressing GFP, GFP-ERK1c, and si-ERK1c. In these experiments, the ERK1c constructs clearly affected Golgi fragmentation during interphase and the different stages of mitosis (Fig. 6). Thus, in interphase, overexpression of ERK1c increased Golgi fragmentation as previously reported (Aebersold et al., 2004), whereas the si-ERK1c had no significant affect (Fig. 6 A). Acceleration of Golgi fragmentation by cells overexpressing ERK1c was also observed in prophase and to some extent in prometaphase, when the Golgi appeared much more broken than in cells transfected with GFP control. In these stages of mitosis, si-ERK1c expression significantly attenuated fragmentation, as only few Golgi

Figure 6. Modulation of ERK1c expression affect Golgi fragmentation during mitosis. HeLa cells were transfected with GFP, GFP-ERK1c (ERK1c), or si-ERK1c with GFP (si-ERK1c). The cells were synchronized by double thymidine block, and 11 h after the release were stained with GM130 or p58 antibodies and DAPI. (A) si-ERK1c and GFP-ERK1c affect Golgi architecture during prophase and prometaphase. Cells from interphase, prophase, and prometaphase were selected by their DNA structure. The percentage of cells with fragmented Golgi apparatus is presented as means ± SEM of three experiments. $n = 40$. (B) Knockdown of ERK1c attenuates the formation of Golgi haze in metaphase. Cells in metaphase were selected by their DNA structure. The percentage of cells with big vesicles out of total metaphase cells is presented as means ± SEM of three experiments. $n = 40$ cells. (C) Knockdown of ERK1c results in unequal Golgi division. Telophase cells were selected by their DNA structure. The percentage of cells with unequal Golgi division from total telophase cells is presented as means ± SEM of three experiments. $n = 40$. Bars: (A and C) 10 μm; (B) 5 μm.
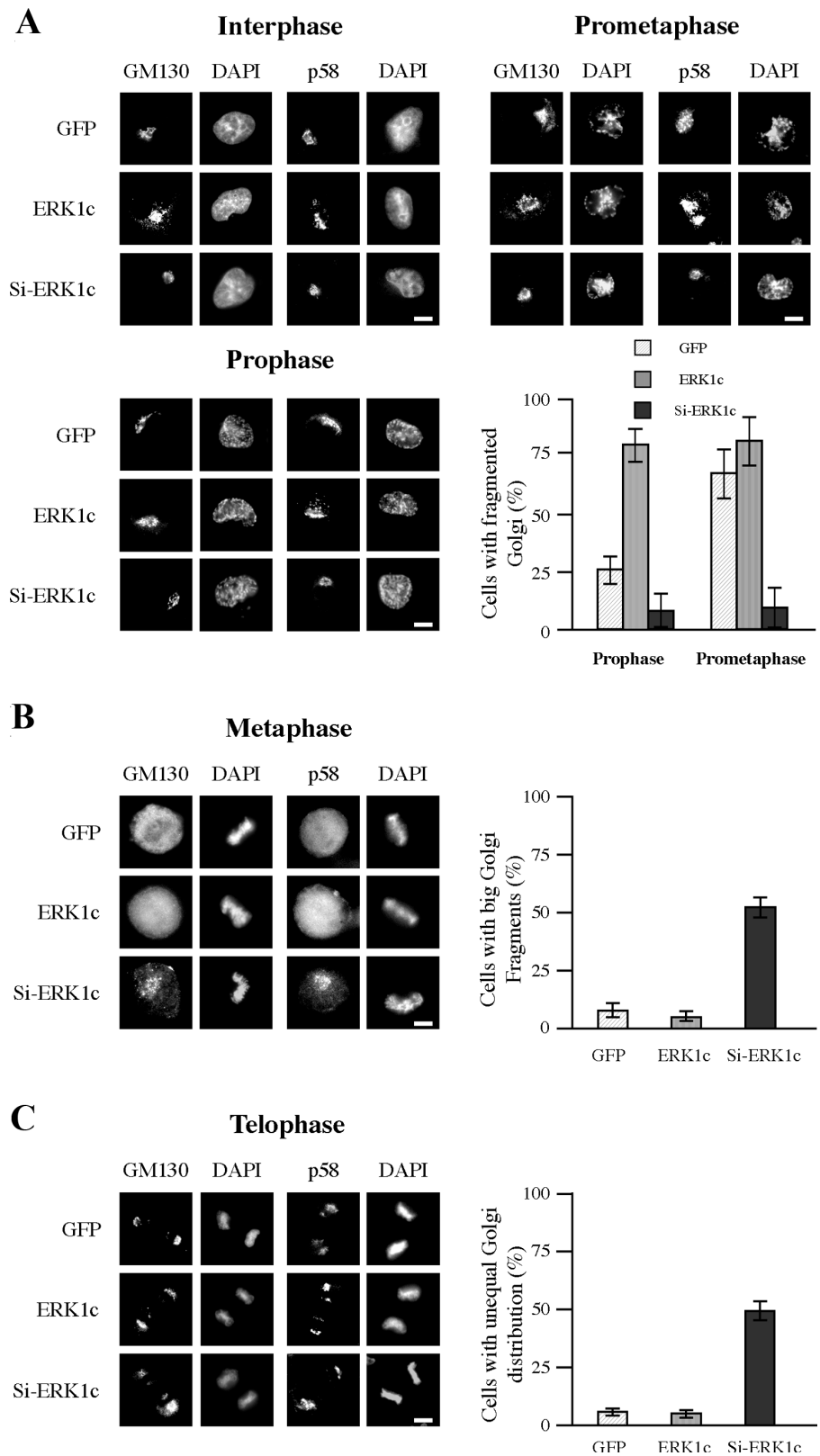

fragments could be detected in most of the cells (quantified in Fig. 6 A, right bottom).

We next followed the Golgi architecture in metaphase, when the Golgi markers appeared to be spread throughout the GFP control cells (Fig. 6 B), indicating that the Golgi was

completely fragmented, as expected (Colanzi et al., 2003). Interestingly, the overexpression of GFP-ERK1c did not change the distribution of the Golgi markers. However, in 55% of the siRNA-expressing cells the Golgi was not completely spread, but was rather broken into bulbs that were still concentrated in
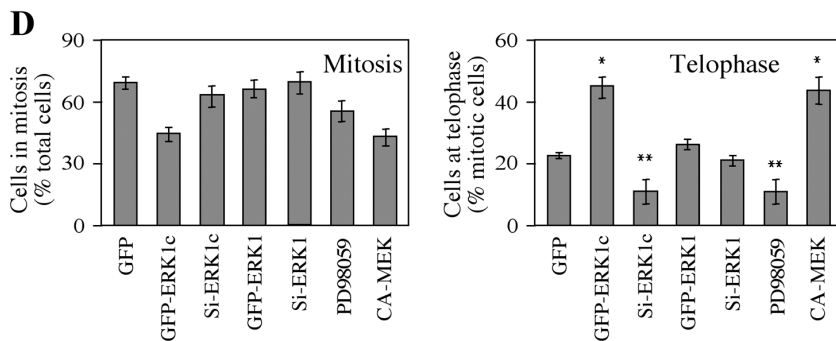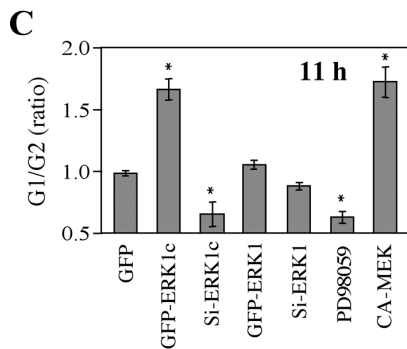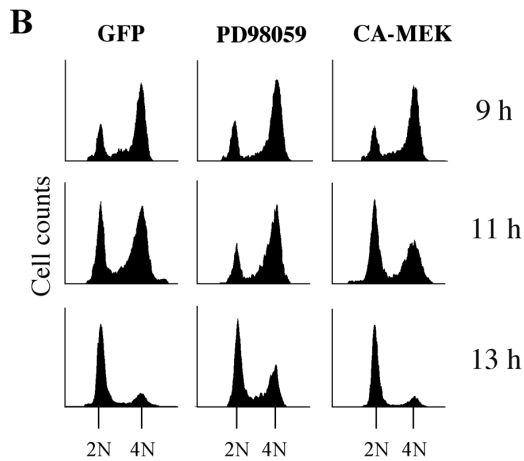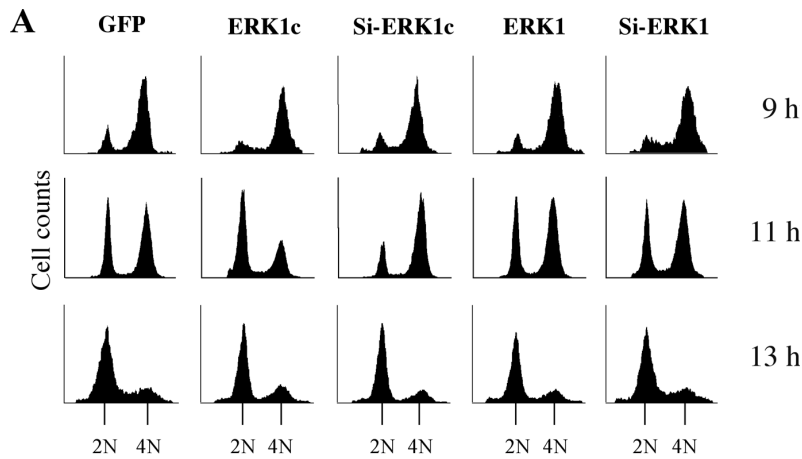
Figure 7. **Effects of ERK1c on mitotic progression.**
(A) Modulation of ERK1c expression affects cell cycle progression. HeLa cells were transfected with GFP, GFP-ERK1c, (ERK1c), si-ERK1c together with GFP (si-ERK1c), GFP-ERK1 (ERK1), and si-ERK1 together with GFP (si-ERK1). The cells were synchronized by double thymidine block as in Fig. 6. At the indicated times, the cells were fixed, stained with propidium iodide, and analyzed by flow cytometry for DNA content (2N or 4N). (B) Effect of MEK activity on cell cycle progression. HeLa cells were transfected with either GFP (GFP and PD98059) or with CA-MEK1 (CA-MEK). The cells were synchronized by double-thymidine, as in Fig. 6. 25 μM PD98059 was added to one of the GFP plates (PD98059) 2 h before fixation, whereas the other GFP plate was left untreated as control. At the indicated times, the cells were fixed, stained with propidium iodide, and analyzed by flow cytometry for their DNA content (2N or 4N). (C) Quantification of the effect of the various treatments. The ratio between G1/G2 was compared with that of the control cells. The results are means ± SEM of three experiments. A *t* test was used for statistical analysis (*, P < 0.01). (D) Determination of mitotic index and percentage of cells in telophase. HeLa cells were transfected and synchronized as in A, and stained with DAPI. The percentage of mitotic cells (left, Mitosis) and cells at telophase out of the mitotic cells (right, Telophase) is presented in the bar graph as the means ± SEM of three experiments. n = 200 cells each. A *t* test was used for analysis. *, P < 0.05; **, P < 0.01.

the cytoplasm, similar to its appearance in control prophase cells. Thus, si-ERK1c significantly inhibited Golgi fragmentation in the prophase/metaphase stages of the cell cycle. In telophase (Fig. 6 C), the Golgi was already rebuilt and equally divided between the daughter cells in the GFP- and ERK1c-expressing cells, whereas in the si-ERK1c cells the division of Golgi between the daughter cells was not equal. In these cultures, 60% of cell pairs had one cell with a bigger Golgi, whereas

the Golgi of the mate cell was much smaller. These results could be a consequence of the incomplete fragmentation of the Golgi at metaphase that allowed a distribution of some unprocessed Golgi bulbs. These results may also suggest that the pace of mitotic progression is not fully synchronized with or controlled by Golgi fragmentation, as previously suggested (Uchiyama et al., 2003). As expected from previous studies involving overexpression of ERK1 (Aebersold et al., 2004) and the absence of ERK1 from mitotic Golgi (Fig. 3), ERK1 had no significant effect on Golgi architecture under any of the conditions used (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200509063; and not depicted), again supporting the specific role of ERK1c in the regulation of Golgi fragmentation.

ERK1c regulates mitotic progression

Golgi fragmentation was found to play a critical role in the progression of mitosis (Sutterlin et al., 2002). Therefore, it was important to examine whether ERK1c regulates G2/M progression via its involvement in Golgi breakdown. To this end, HeLa cells overexpressing GFP only, GFP-ERK1c, GFP-ERK1, si-ERK1c, or si-ERK1 were synchronized using the double thymidine block, and examined by FACS for their cell cycle stage. Thus, 9 h after block release the various transfected cells were found in the G2 phase of the cell cycle, indicating that neither ERK1c nor ERK1 play a role in S/G2 progression of HeLa cells (Fig. 7 A). On the other hand, 11 h after release the GFP-ERK1c, but not GFP-ERK1, had a marked influence on the mitosis progression (Fig. 7 A). As expected, the control GFP-expressing cells were reproducibly found equally in their 2N or 4N forms, indicating that most cells are in the midst of mitosis at this stage. Overexpression of ERK1c shifted this distribution toward G1 (28% in G2 and 66% in G1; quantification in Fig. 7 C), whereas the si-ERK1c inhibited the transition (72% in G2 and 22% in G1). These effects were restricted to 10–12 h after release from the double thymidine block because when these experiments were performed 13 h after the release the cells were all found in the next G1 phase with no significant effect of any of the ERK constructs. These results indicate that the effect of ERK1c is restricted to the initial stages of mitosis and is not mimicked by ERK1. Interestingly, the effects of ERK1c during mitosis were similar to those obtained by either inhibiting or activating the upstream kinase MEK1 (Fig. 7 B, 11 h). However, unlike ERK1c, the modulation of MEKs activity still maintained a minor, but significant, effect 13 h after release. Thus, PD98059 retained 31% of the cells in G2 as compared with 12% observed in GFP control, and the CA-MEK1 reduced the percentage of cells in G2 to 7%. The longer effect of the MEKs inhibitor, as compared with si-ERK1c, may suggest that MEKs operate in part via Golgi-independent mechanisms to regulate the progression of mitosis (Roberts et al., 2002).

To further establish the mitotic role of ERK1c and MEKs, we monitored the percentage of mitotic cells 11 h after the double thymidine release, as well as the percentage of cells in telophase, out of the total mitotic cells. The percentage of mitotic cells was not affected by si-ERK1c expression (Fig. 7 D, left), but the percentage of cells in telophase was significantly inhibited (Fig. 7 D, right), indicating again that ERK1c affects cell

cycle at early stages of mitosis, without a significant effect on the G2 phase and the entrance to mitosis. The overexpression of ERK1c or CA-MEKs reduced the percentage of cells in mitosis but increased the portion of cells in telophase. This could be explained by the accelerated rate of mitotic progression in these cells, which pushed most of the cells to either telophase or to the G1 phase of the following cycle. Addition of PD98059 9 h after release from the double thymidine arrest inhibited the entrance and, even more so, the progression of cells in mitosis, indicating that unlike the si-ERK1c, it had some role at the G2/M phase, probably by its additional effect on ERK1/2. This conclusion is supported by the stronger effect of PD98059, when it was combined with the release from the double thymidine arrest (not depicted). Finally, the ERK1 constructs had no effects at these stages. These results further corroborate the specific role of ERK1c in the progression of mitosis, which is probably attributable to its effects on Golgi fragmentation at this stage.

Discussion

In this study, we show that ERK1c is an important regulator of Golgi fragmentation during mitosis and thereby plays a role in controlling mitotic progression in human cells. Mitotic Golgi fragmentation is a two-step process in which the pericentriolar Golgi stacks are converted into bulbs and then either undergo further vesicular spreading or fuse with the ER (Colanzi et al., 2003). The first part of this process seems to be regulated by phosphorylation, which is mediated by several protein kinases, including MEK1 (Acharya et al., 1998). The role of MEK1 in Golgi fragmentation was clearly demonstrated in various cell types. However, the mechanisms by which MEK1 functions during this process was not clear because the known MEK1 substrates (ERK1/2) were not found to participate in the process (Acharya et al., 1998). Rather, it was suggested that mitotic MEKs may function via an ERK-like protein (Acharya et al., 1998), which may be mono-Tyr phosphorylated (Cha and Shapiro, 2001), or via other protein kinases such as Plk3 (Xie et al., 2004).

In this study, we present data suggesting that ERK1c, but not ERK1/2, might be the mediator of the MEK-induced Golgi fragmentation during mitosis. This is strongly supported by the following observations: (a) the expression levels of ERK1c, but not ERK1/2, are elevated in late G2 and mitosis (Fig. 1); (b) the activity of ERK1c is elevated in late G2 and during mitosis (Fig. 1); (c) ERK1c, and not the other ERKs, is localized in the Golgi during the early stages of mitosis (Fig. 4); (d) si-ERK1c, but not of ERK1, reduces Golgi fragmentation during mitosis (Fig. 6), which attenuates mitotic progression (Fig. 7); and (e) ERK1c is preferentially phosphorylated on its Tyr204, which correlates with the appearance of pY-ERK in the mitotic Golgi (Fig. 3). This is unlike ERK1/2, which under most conditions are phosphorylated on both their activatory Thr and Tyr residues at a comparable rate (Fig. 4; Seger and Krebs, 1995; Yao et al., 2000). This observation, together with the mitotic Golgi distribution of ERK1c, strongly suggests that ERK1c is the mono-phosphorylated ERK that was suggested to play a role in Golgi fragmentation during mitosis (Cha and Shapiro, 2001).

It has previously been demonstrated that Golgi fragmentation is a crucial step in the progression of mitosis, and it was suggested that this fragmentation, in fact, provides a sensor for controlling entry into mitosis in mammalian cells (Sutterlin et al., 2002). Our results demonstrate that, indeed, inhibition of Golgi fragmentation achieved with the si-ERK1c caused a delay in the onset of mitosis. However, the block was never full and could not be detected 13 h after the double thymidine release. The incomplete arrest of the HeLa cells in mitosis could be caused by an incomplete inhibition of ERK1c activity because of the partial effect of the siRNA. However, it is also possible that Golgi fragmentation is not the only sensor for mitotic progression. This suggestion is supported by the observation that in cells expressing si-ERK1c, the distribution of Golgi fragments to daughter cells during telophase was not equal (Fig. 6 C). Therefore, it is possible that the progression through mitosis is governed by a multistep mechanism, which requires Golgi fragmentation at only one point during mitosis. Thus, partial Golgi breakdown (such as the one observed in si-ERK1c prometaphase in Fig. 6) is probably a sufficient signal to allow the mitotic progression. Because some of the Golgi membranes were still in bulb form and not in spread vesicle form, and the cells proceeded through telophase without waiting for the Golgi to complete its breakdown, an unequal distribution of particles resulted, and thus, an unequal Golgi size in the daughter cells. Interestingly, the cells overexpressing GFP-ERK1c contained similar amount of Golgi fragments in the daughter cells, although the progression through early mitosis was accelerated in these cells. This indicates that once the Golgi is fully degraded it can be homogenously divided into the daughter cells at the right stage of mitosis. Therefore, our results may indicate that Golgi fragmentation is an important step in the initiation of mitosis, but that once the set of events that are required for this progression have started, the synchronization of the process is probably regulated by other mechanisms.

ERK1c is an alternatively spliced form of ERK1, which is altered in the cytosolic retention sequence/common docking regulatory region; therefore, many of its regulatory aspects are distinct from those of ERK1/2. Indeed, in a previous study we showed that the kinetics of activation of this protein is distinct from its relative ERKs upon EGF or NaCl stimulation (Aebersold et al., 2004). Furthermore, the subcellular localization of ERK1c seems to be distinct from that of ERK1/2, as it was found mainly in the Golgi under various conditions (Fig. 3; Aebersold et al., 2004). In our previous work, we suggested that the Golgi translocation of ERK1c is regulated by its monoubiquitination. In mitosis, this is probably not the situation, as we failed to obtain ERK1c at any other molecular weight than the nonmodified endogenous 42-kD protein (Aebersold et al., 2004). Therefore, the mechanism that allows ERK1c to specifically associate with the Golgi has yet to be clearly identified.

Additional information on ERK1c and ERK1 during mitosis was observed using nocodazole, which arrests the HeLa cells in a prometaphase-like stage. Similar to cells synchronized by double thymidine block, nocodazole induced higher expression (Fig. 2) and Golgi localization (not depicted) of ERK1c, indicating that these effects are indeed mediated by cell cycle

arrest and not by the individual effect of the drugs. However, despite the pronounced activation of ERK1c after nocodazole treatment (Fig. 2), which was higher than that observed upon double thymidine treatment (Fig. 1), the activation of ERK1 was much smaller (Fig. 1). This result is in agreement with a previous work, which showed that nocodazole does not induce ERK1 activation (Takenaka et al., 1998). The reason for the difference is not clear and could be the outcome of some nonspecific effects of the cell cycle–arresting drugs. However, this difference could also be attributable to the homogenous arrest of cells at the prometaphase-like stage upon nocodazole treatment, unlike the wider cell cycle distribution in cells 11 h after release from double thymidine block (G2/anaphase). This may indicate that the activation of ERK1/2 occurs mainly during the G2–prophase stage and that this activity is reduced at prometaphase, whereas ERK1c is activated somewhat later than ERK1/2; its activity is maximal at prometaphase and may proceed even later in mitosis.

Importantly, the results of this study strongly suggest that ERK1c has unique functions that are not shared by ERK1/2 in regulating Golgi fragmentation. The functional difference between the isoforms was also manifested in the stronger block in cell cycle progression by the MEK inhibitor PD98059, as compared with that of the si-ERK1c (Fig. 7). Because both si-ERK1c and PD98059 reduced ERK1c activity to a similar level (Figs. 4 and 5), it is likely that MEKs have some ERK1c-unrelated effects on the progression of mitosis that are probably mediated by ERK1/2. This is supported by the fact that ERK1/2 are activated in the onset of mitosis as well (Fig. 1). In addition, ERK1/2 are not localized in the Golgi at any stage of the cell cycle, suggesting that they participate in other, Golgi-independent signaling events. The observation that reductions in ERK1 levels and activity did not exert any significant effect on the progression through mitosis (Fig. 7) may be explained by the ability of its close homologue ERK2 to compensate for its shortage. On the other hand, our results clearly indicate that ERK1c cannot overcome the effects of ERK1/2 and vice versa, which can be explained by their distinct localization and differential regulation (Aebersold et al., 2004). Thus, we propose a pathway in which activated MEKs colocalize with ERK1c in the Golgi, activate it, and thereby induces the process of Golgi remodeling during mitosis. At the same time, other MEK molecules phosphorylate ERK1/2, and those molecules participate in the regulation of distinct mitotic processes. Therefore, MEKs seem to orchestrate a large part of the mitotic events by activating three ERK isoforms that may each function in a different location and regulate distinct processes.

In summary, we characterized the function of ERK1c in the Golgi and found that it plays an important role in the regulation of Golgi fragmentation during mitosis and thereby in the regulation of cell cycle progression. The ERK1c effects could not be complemented by ERK1/2, indicating that this isoform is the unique MEK effector in the mitotic Golgi. These results shed light on the MEK-dependent regulation of Golgi fragmentation and division into the daughter cells during mitosis. In addition, this unique role of ERK1c extends the substrate specificity of the signaling by the ERK pathway and may suggest a molecular mechanism for the ability of the ERK cascade to regulate distinct, and even opposing, cellular processes.

Materials and methods

Reagents and antibodies

Thymidine, nocodazole, MBP, propidium iodide, EGF, and ATP were purchased from Sigma-Aldrich. GM130, lamin A/C antibodies, and protein A/G PLUS–agarose beads were obtained from Santa Cruz Biotechnology, Inc. Sepharose-immobilized protein A, an ECL kit, glutathione beads, and $\gamma[^{32}P]ATP$ were purchased from GE Healthcare. DAPI was purchased from Invitrogen, and PD98059 was obtained from Calbiochem. p58, pERK, monophosphorylated ERK, gERK, and ERK1 antibodies were obtained from Sigma-Aldrich. The ERK1c antibody was prepared by the Antibody Unit of the Weizmann Institute of Science, as previously described (Aebersold et al., 2004). GFP antibody was purchased from Roche. The developing substrate NBT/BCIP was obtained from Promega. FITC-, rhodamine-, alkaline phosphatase–, and horseradish peroxidase–conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories.

Buffers

Buffer A consists of 50 mM β-glycerophosphate, pH 7.3, 1.5 mM EGTA, 1 mM EDTA, 1 mM DTT, and 0.1 mM sodium vanadate. Buffer H consists of Buffer A supplemented with 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A. Buffer RM (reaction mixture at threefold concentration) consists of 30 mM $MgCl_2$, 4.5 mM DTT, 75 mM β-glycerophosphate, pH 7.3, 0.15 mM sodium vanadate, 3.75 mM EGTA, 30 μM calmidazolium, and 2.5 mg/ml bovine serum albumin. Radio-immunoprotein assay buffer consists of 137 mM NaCl, 20 mM Tris, pH 7.4, 10% (vol/vol) glycerol, 1% Triton X-100, 0.5% (wt/vol) deoxycholate, 0.1% (wt/vol) SDS, 2.0 mM EDTA, 1.0 mM PMSF, and 20 μM leupeptin. Buffer HNTG consists of 50 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol.

DNA constructs and siRNA

GFP-ERK and GFP-ERK1c were prepared in plasmid of EGFP (pEGFP) vector (Aebersold et al., 2004). CA-MEK1 (DN-EE-MEK1) was prepared in phosphorylated cDNA1 (Jaaro et al., 1997). GFP in pEGFP was purchased from CLONTECH Laboratories, Inc. GST-ERK1c was prepared by inserting ERK1c into the BamHI and EcoRI sites of pGEX-2T. The GST protein was purified according to the manufacturer's instructions and eluted from the glutathione beads using 10 mM of reduced glutathione. To generate the pSUPER-ERK1c and pSUPER-ERK1 we used the pSUPER plasmid (Brummelkamp et al., 2002). The sequence that was used (CGACGGATGAGGTGGGCCA; 1,007–1,025 bp) was derived from the unique sequence of human ERK1c. For human ERK1, the sequence that was used (AGCTGGATGACCTACCTAA; 1,052–1,070 bp) was derived from the COOH terminus of human ERK1 that is not present in ERK1c.

Cell culture and transfection

HeLa cells were grown in DME supplemented with 10% FCS (Invitrogen). Transfection into the cells was performed using the polyethylenimine method, as previously described (Boussif et al., 1995). In brief, the cells were grown to 50–70% confluence in 12-well plates. The 1.5-μg plasmid was suspended in 125 μl PBS and mixed with polyethylenimine solution (5 μl of 3 mM polyethylenimine in 125 mM NaCl). The mixture was left at 23°C for 15 min and then incubated with the cells for 90 min, after which the cells were washed and placed in DME + 10% FCS.

Immunofluorescence microscopy

Cells were fixed (20 min in 3% paraformaldehyde in PBS or 10 min in methanol at −20°C), followed by a 20 min permeabilization/blocking solution (0.1% Triton X-100/2% BSA). Antibodies of choice were added for 1 h, washed, and developed with fluorescence-tagged secondary antibodies (Cy2, FITC, or rhodamine; Jackson ImmunoResearch Laboratories) for 1 h at 23°C. Nuclei were stained with 0.1 mg/ml DAPI (Invitrogen) in PBS. The slides were visualized using a fluorescence microscope (Optiphot; Nikon) with 100×, 1.3 NA, or 40×, 0.7 NA, immersion oil objectives (Nikon). Digital images of cells were captured using a camera (DVC) and C-view V2.1 Imaging software (DVC). Images were processed using Photoshop 7.0 (Adobe).

Preparation of cell extracts and Western blotting

After treatment, the cells were rinsed twice with ice-cold PBS and once with ice-cold buffer A. Cells were scraped into buffer H (0.5 ml per plate) and disrupted by sonication (two 50-W pulses for 7 s). The extracts were centrifuged (20,000 g for 15 min at 4°C) and the supernatants were kept at 4°C. The supernatants were then separated by a 10% SDS-PAGE, transferred onto a nitrocellulose membrane, and probed with the appropriate antibodies, as previously described (Yao et al., 2000).

Immunoprecipitation

Cells extracts prepared as described in Cell culture and transfection were incubated (2 h at 4°C) with ERK1c (affinity purified), ERK1, or GFP antibodies coupled to protein A/G–Sepharose. For determination of ERK1c activity, the beads were washed once with HNTG buffer, twice with 0.5 M LiCl in 0.1 M Tris, pH 8.0, and once with 1 ml of buffer A. Immunoprecipitates were subjected either to Western blotting or subjected to an in vitro kinase assay.

In vitro kinase assay

Immunoprecipitated ERKs attached to 15-μl beads were used as kinases by mixing them with MBP (8 μg/reaction). Similarly immunoprecipitated active MEKs attached to 15-μl beads were mixed with either recombinant GST-ERK1c or GST-ERK1 (0.5 μg per reaction). Buffer RM containing 100 μM $\gamma[^{32}P]ATP$ (4,000 cpm/pmol) was added to the reaction at a final volume of 30 μl and incubated for 20 min at 30°C. The reaction was terminated by adding 10 μl of 4× sample buffer, and the phosphorylated proteins were resolved on SDS-PAGE and subjected to autoradiography and Western blot analysis with the proper antibodies.

Cell synchronization and FACS analysis

Cells were synchronized at the G1/S boundary by the double thymidine block (Merrill, 1998). In short, HeLa cells were treated with 2 mM thymidine in DMSO, washed twice with PBS, grown for 8 h in regular medium, and then treated again with 2 mM thymidine for 16 h and washed with PBS. This marks time 0, after which the cells were grown under the regular conditions for the indicated times (Merrill, 1998). HeLa cells were also synchronized at the M phase using 100 ng/ml nocodazole for 24 h (Merrill, 1998). For FACS analysis, HeLa cells were trypsinized, washed with PBS, and fixed in 70% ice-cold methanol for at least 1 h. The samples were then centrifuged (500 g for 2 min) and resuspended in 0.5 ml of staining solution (0.001% Triton X-100, 0.1 mM EDTA, 100 μg/ml RNase, and 50 μg/ml propidium iodide in PBS). The cells were analyzed by FACsort (Becton Dickinson), and the percentage of cells at different stages was calculated using the CellQuest software (BD Biosciences).

Online supplemental material

Fig. S1 shows the mitotic index of the synchronized cells. Fig. S2 shows that ERK1c translocation to the Golgi correlates with nuclear envelope breakdown. Fig. S3 shows the subcellular localization of ERK1c during mitosis (color images). Fig. S4 shows si-ERK1c in transfected cells. Fig. S5 shows that ERK1 does not affect the Golgi architecture. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200509063/DC1.

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