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Hepatocyte growth factor at S phase induces G2 delay through sustained ERK activation

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Abstract

The effect of growth factors on the cell cycle progression, except G1/S transition, is poorly understood. Herein, we examined the effect of hepatocyte growth factor (HGF) treated at S phase on the cell cycle progression of HeLa cells. Interestingly, the treatment resulted in G2 delay, evidenced by flow cytometric and mitotic index analyses. The delay corresponded with the delay of degradation of cyclin A and cyclin B, and the delay of decrease of Cdk1/cyclin B and Cdk2/cyclin A kinase activities. As for the signaling responsible, sustained activation of ERK, but neither of p38MAPK nor of JNK, was observed after HGF treatment at S phase. Furthermore, U0126, an inhibitor of MEK1, and DN-MEK partially abrogated the G2 delay, indicating that activation of MEK-ERK pathway is involved. Taken together, HGF treatment of HeLa cells at S phase induces G2 delay partially through sustained activation of ERK signaling.

Keywords: S phase; Hepatocyte growth factor; G2 delay; ERK

Growth factors are well known to promote the transition from G1 to S phase in cell cycle progression. There is a window of time, the restriction point, in the early/mid G1 phase of the cell cycle during which mammalian cells are responsive to extracellular signals such as growth factors or nutrients. At this time period, the retinoblastoma (Rb) protein, which enables the cells to pass through the restriction point and to complete the remaining phases of the growth cycle, is phosphorylated in the presence of growth factors [1,2]. Other than G1/S transition, however, growth factors have been considered non-essential on the proliferating cell cycle [3].

Although they seem to be non-essential, it is theoretically possible that growth factors affect the progression of other phases of cell cycle, except G1/S transition. For example, when applied during late G2 phase, epidermal growth factor (EGF) has been shown to induce G2 delay in HeLa cells and A431 cells via inhibition of activating dephosphorylation of p34Cdc2 by Cdc25C [4,5]. We also observed that growth factors other than EGF, such as hepatocyte growth factor (HGF) and fibroblast growth factor (FGF) also induce G2 delay when applied during G2 phase through MEK–ERK–RSK pathway (manuscript submitted).

HGF is a pleiotropic growth factor which interacts with Met, a transmembrane receptor tyrosine kinase [6,7], and is involved in embryonic development, angiogenesis and tissue regeneration under normal conditions [8,9]. In addition to its role in normal physiology, the dysregulation of HGF and Met has been observed in many different types of carcinoma and sarcoma cells [10,11], and dysregulation and activation of HGF/Met signaling have been shown to be a crucial feature of many human malignancies [12].

In an attempt to understand the effect of growth factors on the cell cycle other than G1/S transition, we treated HeLa cells with HGF at S phase, and found that G2 delay

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was induced by the treatment and that sustained activation of ERK signaling was at least partially involved in the G2 delay.

Materials and methods

Reagents and antibodies. Antibodies against phospho-RSK, RSK, JNK, phospho-ERK and ERK were purchased from Cell Signaling Technology (Berberly, MA). Antibodies against cyclin B, cyclin A, phospho-JNK and phospho-p38MAPK were acquired from Santa Cruz laboratory (Santa Cruz, CA). Thymidine, caffeine and U0126 were purchased from Sigma Aldrich (St. Louis, MO), histone H1 was from Roche Diagnostics (Indianapolis, IN), and aceto-orcein was from Merck (West Drayton, UK).

Cell culture and synchronization by double thymidine block (DTB). HeLa cells were cultured in Dulbecco's modified Eagle's medium nutrient mixture F-12 HAM (DMEM/F12; Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco-BRL, Gaithersberg, MD) in 37 °C incubator with 5% CO_2 in air. Cells (7×10^4) were seeded into 60 mm culture plates, cultured for 1 day in DMEM containing 10% FBS and treated with 1 μM thymidine for the first synchronization. Twenty hours later, cells were washed with thymidine-free medium (1st release) and cultured in complete medium for 8 h. Then, cells were cultured again in thymidine-containing medium for 14 h for the second round of synchronization. Then, cells were washed with thymidine-free medium and cultured in complete medium (2nd or final release). In some test, synchronized cells were treated with 10 μM U0126 at 6 h after the final release.

DNA FACS and mitotic index analyses. HeLa cells released from DTB were treated with or without HGF (100 U/ml) at S phase. At the indicated time points later than HGF treatment, cells were harvested and fixed with 70% cold ethanol for 30 min. Samples were washed with phosphate-buffered saline (PBS) prior to be resuspended in a solution containing RNase A for 5 min, and then propidium iodide was added. After 5 min of incubation, samples were subjected to FACS analysis using a FACS Vantage flow cytometer (Becton–Dickinson, San Jose, CA). Mitotic index analysis was performed by staining the harvested cells with aceto-orcein, with which mitotic condensed chromatin can easily be identified. Mitotic index was determined by counting the percentage of cells with condensed chromatin.

Western blot and immunoprecipitation (IP)-Western blot analyses. Cells harvested at the indicated time points were washed twice with PBS and lysed in RIPA buffer (10 mM Tris–Cl, pH 7.5; 150 mM NaCl; 1% Triton X-100, 1 μg/ml each of aprotinin and leupeptin, 1 mM NaF and 5 mM Na_3VO_4) for 1 h on the ice. For immunoblotting, 45 μg each of centrifuged lysate was subjected to SDS–PAGE. For IP-Western blot analysis, 300 μg each of centrifuged lysate was incubated with indicated antibodies along with protein G–Sepharose beads (Invitrogen, Carlsbad, CA) for 3 h at 4 °C. Immune complexes were washed three times with RIPA buffer, and then subjected to SDS–PAGE. Western blot analysis was performed as described previously [12,13].

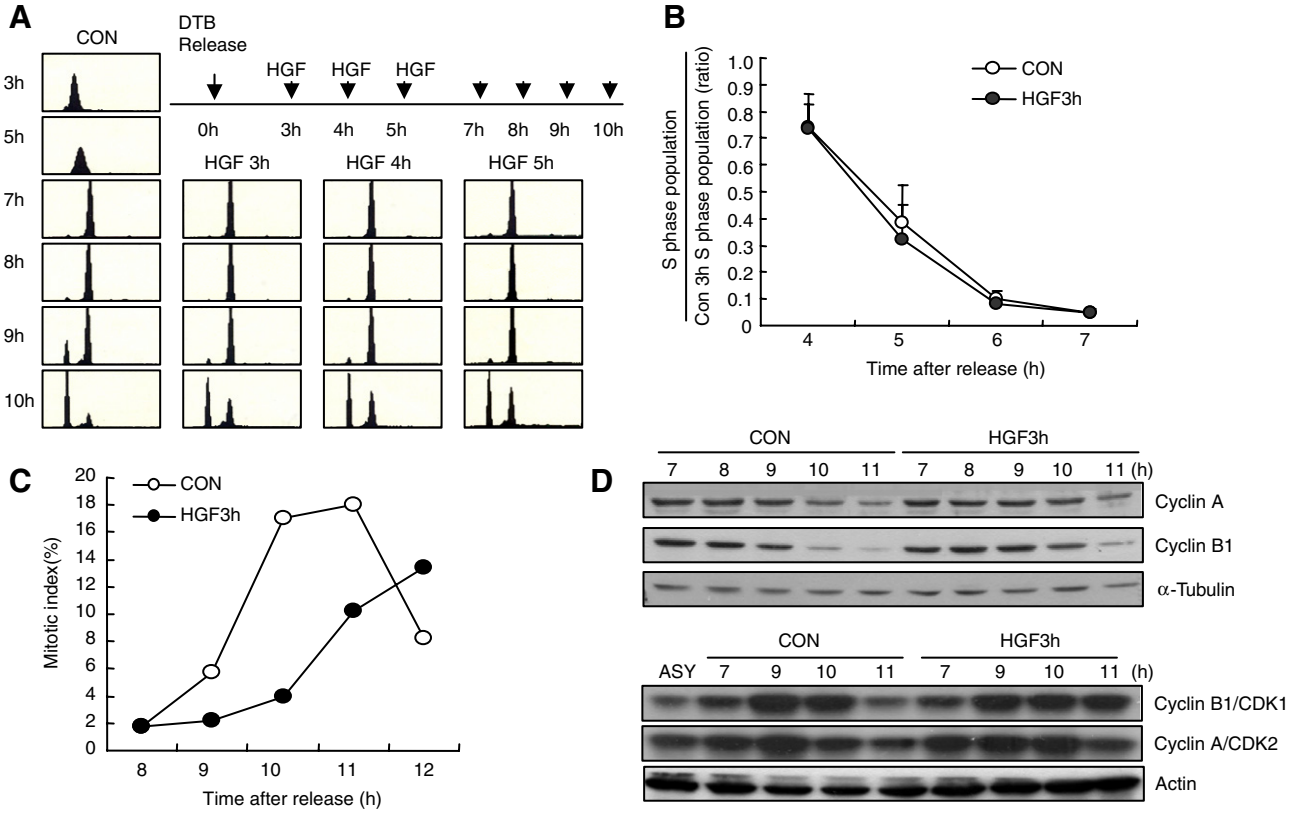

Fig. 1. G2 delay induced by HGF treatment at S phase. (A) Histograms of FACS showing DNA contents of HeLa cells in the absence or presence of HGF at the indicated time points. HGF was added at 3, 4 or 5 h after the release, respectively. (B) The ratio of each S phase population harvested at the indicated time points to that of control cells harvested at 3 h after the release from DTB. Cells harvested at indicated time points after the release were analyzed by FACS. The ratios of S phase population are averages of six independent experiments by flow cytometry. Data shown are means ± SD of three independent experiments. (C) Mitotic index of cells in the absence or presence of HGF harvested at the indicated time points after the DTB release was measured by acetio-orcein staining. (D) Protein levels of cyclin A, cyclin B and α-tubulin were examined by Western blotting. The kinase activities of Cdk2/cyclin A and Cdk1/cyclin B from cells treated with or without HGF were assessed by phosphorylation of histone H1. Each time point represents the time after the release from DTB.

CDK kinase assay. Cell lysates prepared as described above were incubated with each anti-cyclin antibody (Santa Cruz) along with protein G–Sepharose beads for 3 h at 4 °C. Immune complexes were washed three times with RIPA buffer, and then resuspended in kinase buffer (50 mM Tris, pH 7.4, 1 mM DTT, 10 mM $MgCl_2$) with the addition of 5 μg of histone H1, 4 μCi of [γ-^{32}P]ATP, 50 mM Tris, pH 7.4, 1 mM DTT, 10 mM $MgCl_2$ per reaction. Reactions were run for 30 min at 37 °C and then terminated by the addition of SDS–PAGE sample buffer. The reaction mixtures were subjected to SDS–PAGE. Gels were stained with Coomassie brilliant blue staining solution and dried. Phosphorylated substrates were detected by autoradiography.

Transfection. Cells (1×10^5) were plated out onto 60 mm dishes, and 1st thymidine block was performed. Transfection was conducted at the same time as the start of the 1st release. DN-MEK cDNA was incubated with PEI reagent (Polysciences) in the culture plate for 24 h, and cells were then washed and subjected to 2nd thymidine block and release.

Results and discussion

G2 delay induced by HGF treatment at S phase

To examine whether the treatment with HGF at S phase affects the cell cycle progression, we first checked the time points of S phase in HeLa cells by FACS analysis after the release from DTB. S phase cells were determined from 3 to 5 h after the final release (data not shown), and HGF was added at those time points, respectively. Interestingly, FACS analysis revealed about 1 h-delay of the cell cycle progression in all cases of HGF treatment at S phase; i.e., delay of cell cycle progression from G2/M to next G1 phase (Fig. 1A). However, it cannot clearly be concluded whether this delay stems from M phase or G2 phase. In addition, there is a possibility of the change in the cell cycle progression rate even during S phase.

To clarify those issues, we first compared S phase cell population of HGF-treated group to that of control group harvested at indicated time points. There was little change of S phase population between control group and HGF-treated group, indicating that the delay does not stem from S phase (Fig. 1B). On the other hand, the mitotic index analysis showed that HGF-treated group entered into mitosis 1–2 h later than control group, indicating that the cell cycle delay starts at G2 phase (Fig. 1C). Therefore, it is almost certain that the cell cycle delay induced by HGF treatment at S phase stems mainly from its G2 delaying effect.

It is well known that protein levels of cyclin A and B decrease during the progression of M phase, resulting in the decrease of respective cyclin-dependent kinase activity [14,15]. It is, therefore, likely that G2 delay should delay the decrease of the proteins if there is no considerable shortening of the duration of M phase. Indeed, the decrease in the amount of both cyclin A and B in cells treated with HGF was observed 1–2 h later than those in HGF non-treated cells (Fig. 1D, upper panel). Similarly, the

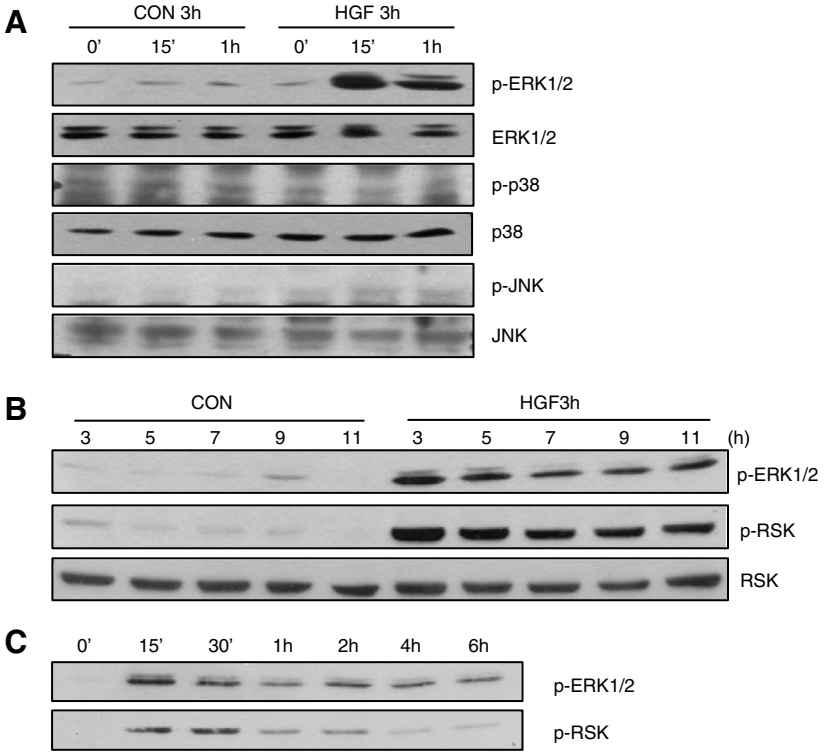

Fig. 2. Sustained activation of ERK by HGF treatment at S phase. (A) Expression levels of pERK, pJNK and pP38 were determined in cells synchronized by DTB and treated with or without HGF 3 h after the final DTB release. Each time point represents the time after treatment with or without HGF. (B) Expression levels of pERK and pRSK were determined in synchronized cells in the presence or absence of HGF treatment at 3 h after the release. Each time point represents the time after the release from DTB. (C) pERK and pRSK levels were determined in asynchronous cells after HGF treatment. Each time point represents the time after treatment with or without HGF. All the experiments were performed at least twice, and representative data are shown.

decrease of the activities of Cdk2/cyclin A and Cdk1/cyclin B in HGF-treated cells was also observed 1–2 h later than that of HGF non-treated cells (Fig. 1D, lower panel). Taken together, it is clear that G2 delay, but not S phase or M phase delay, was induced by HGF treatment at S phase.

Sustained ERK activation by HGF treatment at S phase

ATM/ATR-mediated DNA damage checkpoint signaling is one of the main checkpoint pathways that control G2/M transition [16], targeting Cdc25B and Cdc25C phosphatases and controlling the cyclin B/Cdc2 activity.

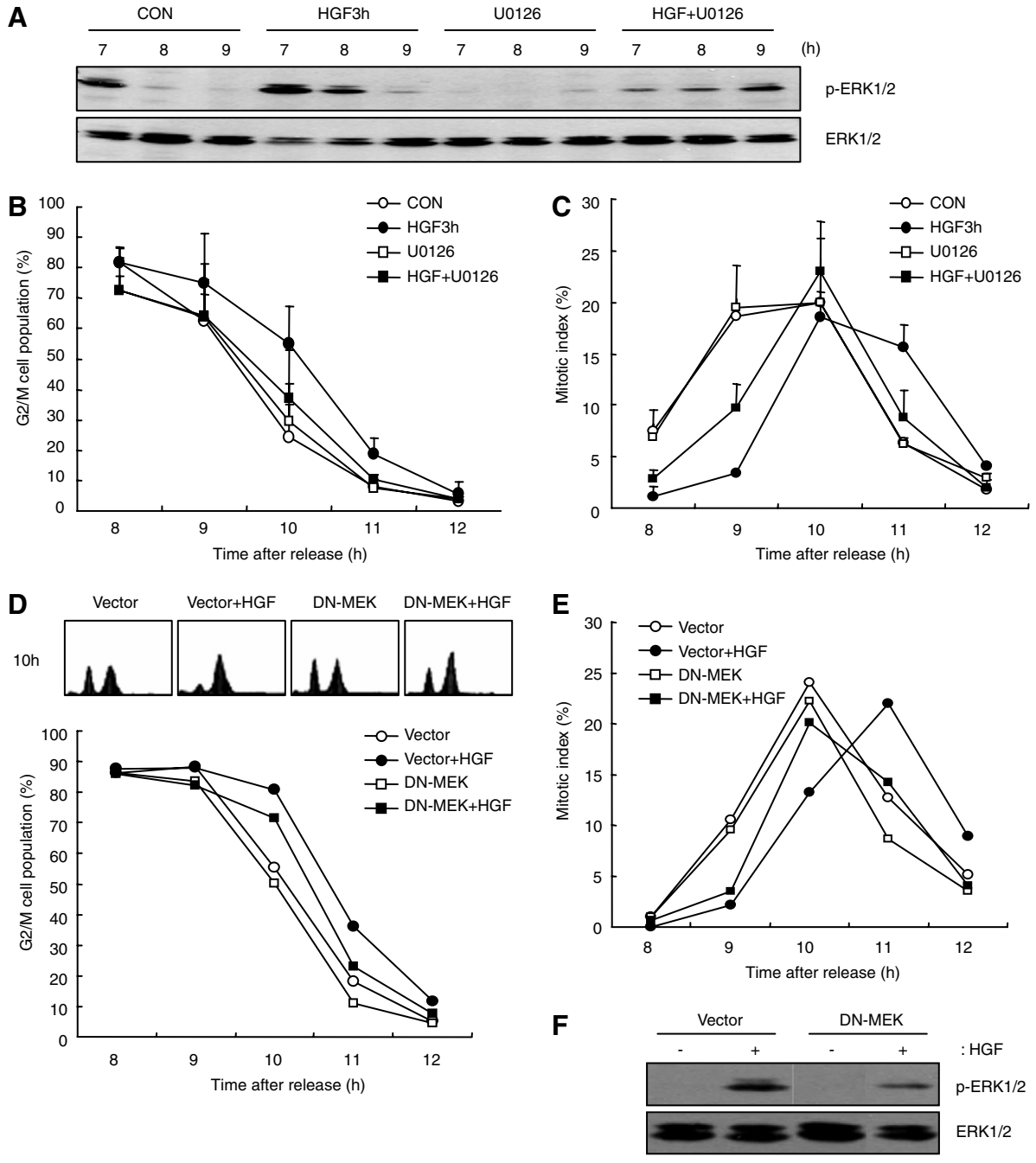

Fig. 3. Involvement of ERK signal in the G2 delay. (A–C) Cells were treated with or without HGF at 3 h after the final DTB release with or without 10 μM U0126 at 6 h after the final DTB release. Each time point represents the time after the release from DTB. (A) pERK level was determined by Western blotting. U0126 effectively inhibited HGF-induced pERK expression. (B) G2/M cell population was obtained by flow cytometry. (C) Mitotic index was measured by aceto-orcein staining. (B,C) Data shown are means ± SD of three independent experiments. (D–F) Cells were transfected with DN-MEK DNA or vector DNA. Each time point represents the time after the release from DTB. (D) G2/M cell population was obtained by flow cytometry. (E) Mitotic index was measured by aceto-orcein staining. (F) Decrease of pERK level in DN-MEK DNA transfected cells was verified by Western blotting.

Therefore, we investigated whether ATM/ATR-dependent checkpoint signaling was involved in the G2 delay induced by HGF using caffeine, an inhibitor of ATM/ATR kinase [17], and siRNAs for ATM and ATR. Both approaches failed to abrogate HGF-induced G2 delay, indicating that ATM/ATR kinases are not involved here (Supplementary Fig. 1). In addition, we found no differences in pChk1 and pChk2 levels between HGF-treated and non-treated groups suggesting no involvement of DNA damage checkpoint pathway (data not shown).

Next, we addressed the possibility of the presence of sustained signaling in our system, specifically the MAPK pathways. As shown in Fig. 2A, we first checked the activation pattern of the three representative MAPKs, such as extracellular signal-regulated kinase (ERK), p38-mitogenic activated protein kinase (p38MAPK), and c-Jun NH2-terminal kinase (JNK), by HGF treatment at 3 h after the release from DTB, and found that, although ERK activation was prominent by HGF treatment, p38MAPK and JNK activations were not observed clearly until 1 h after the treatment. Thus, we focused on the activation of ERK and its downstream substrate, RSK, in our experimental system. Indeed, the activity of both ERK and RSK in HGF-treated group was robustly increased comparing to control group and remained until when cells entered G2 phase, showing the presence of sustained MAPK signaling in our system (Fig. 2B). It is of note that the activated forms of both ERK and RSK lasted longer in synchronized cells than in asynchronous cells (Fig. 2C), suggesting that there might exist different extents of down-regulation of ERK in a cell cycle-dependent manner. In support of this possibility, basal ERK phosphorylation is known to increase along with cell cycle progression from G2 to M phase [18]. Taken together, these results suggest that, once stimulated by the addition of HGF at S phase, ERK in HeLa cells, but neither JNK nor p38MAPK, is activated and sustained until G2 phase, thus inducing the G2 delay.

ERK signal is involved in the G2 delay

The next issue that we addressed was to clarify the importance of ERK activation in the G2 delay induced by HGF treatment at S phase. To this end, we employed the MEK inhibitors, U0126 and DN-MEK. To check the necessity of the 'durability' of ERK signal in the induction of G2 delay, U0126 was added at 3 h after HGF treatment to abolish MEK activity at a late time point. Indeed, U0126 treatment induced partial, but significant, abrogation of the G2 delay, evidenced by both FACS and mitotic index analyses (Fig. 3B and C). The result indicates that ERK activation during G2 phase is necessary for the G2 delay, even though its activation starts at S phase. Related to our recent observation that HGF treatment during G2 phase induces G2 delay with the involvement of MEK-ERK signal (manuscript submitted), it is quite natural to assume that aberrant activation of ERK during G2 phase induces G2 delay irrespective of whether the ERK activation starts from S phase or G2 phase. Transfection of DN-MEK also resulted in a partial, but significant, abrogation of G2 delay (Fig. 3E and F), further strengthening the involvement of MEK-ERK pathway in this process. Taken together, under the condition of adding HGF at S phase, the sustained MAPK signaling pathway through ERK at least partially contributes to G2 delay.

Our present finding that G2 delay is induced by HGF treatment at S phase provides not only one more example that growth factor, when treated not at a specific time point in G1 phase, can also affect cell cycle progression, but also is the first demonstration of the effect of growth factor on cell cycle progression, when treated at S phase. Although we could not explain the exact biological significance of the G2 delay, the fact that a change in cell cycle, such as G2 delay, is induced by growth factor added at S phase, suggests a new aspect of effect of growth factor on the cell cycle regulation.

The activation of ERK pathways is required for mammalian cell proliferation, and their importance has best been defined in G1 phase [3,19]. Recently, the effect of ERK pathway during G2 and mitosis has also been reported. In studies using Xenopus egg extracts and fertilized eggs, the elevation of ERK activity has been shown to arrest cells in G2 prior to chromosome condensation and nuclear envelope breakdown, implying that ERK suppresses cdc2 activation and mitotic entry [20,21]. Finally, it should also be mentioned that similar to HGF, EGF treatment at S phase also induced G2 delay (Supplementary Fig. 2), indicating that the induction of G2 delay by growth factor treatment at S phase is not confined only to HGF, a specific growth factor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.123.

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