Negative Growth Regulation of SK-N-MC Cells by bFGF Defines a Growth Factor-sensitive Point in G2*

Received for publication, March 1, 2000, and in revised form, April 13, 2000
Published, JBC Papers in Press, April 17, 2000, DOI 10.1074/jbc.M001764200

Veronique A. J. Smits, Maartje A. van Peer, Marieke A. G. Essers, Rob Klompmaker,
Gert Rijksen, and René H. Medema‡

From the Jordan Laboratory, Department of Hematology, University Medical Center Utrecht G03.647, P. O. Box 85500,
3508 GA Utrecht, The Netherlands

Basic fibroblast growth factor (bFGF) has been shown to induce growth inhibition of the neuroepithelioma cell line SK-N-MC. Here we show that this growth inhibition occurs in G2. We show that bFGF is active on these cells during S and early G2 phase. Therefore, this constitutes a rather unusual mechanism of growth inhibition, because it is generally believed that cells become refractory to extracellular signals after passage through the restriction point. We show that bFGF treatment inhibits Tyr-15 dephosphorylation of cdc2 and prevents activation of Cdc25C, similar to what is seen upon activation of the G2 DNA damage checkpoint. Interestingly, both DNA damage- and bFGF-induced effects on cdc2 phosphorylation are reverted by caffeine. To confirm the involvement of similar pathways induced by bFGF and DNA damage, we generated tetracycline-regulatable SK-N-MC clones expressing Cdc25C-S216A. Expression of this Cdc25C mutant can revert the bFGF-induced effects on cdc2 phosphorylation and can rescue cells from the block in G2 imposed by bFGF. Taken together, these data define a growth factor-sensitive point in G2 that most likely involves regulation of Cdc25C phosphorylation.

Cells can respond to a variety of extracellular signals, which together dictate cellular behavior including the decision to proliferate, differentiate, or undergo apoptosis (1). Cell proliferation is controlled by multiple growth-regulatory pathways that act together to ensure proper cell division. At the late G1 restriction point the cell weighs the activity of positive and negative regulatory signals. After passage through the restriction point, mitogenic growth factors are no longer required for cells to complete division, and cells become refractory to growth-inhibitory signals (2–4). Instead, cells come to rely upon the intrinsic regulators of the cell cycle machinery for orderly progression through the remainder of the cell cycle (2).

Orderly progression through the mammalian cell cycle is dependent on the timed activation of cyclin-dependent kinases (5). Each cell cycle phase is characterized by the presence of distinct cyclin-cyclin-dependent kinase complexes (6). Cell cycle control by checkpoints functions through interference with activation of these complexes. For example, for the onset of mitosis the activation of cyclin B-cdc2 complexes is required (7), whereas activation of the G2 DNA damage checkpoint results in inhibition of these complexes, leading to an arrest in G2 phase progression (8).

Cyclin B is first synthesized during S phase, and cyclin B-cdc2 complexes continue to accumulate throughout G2. These complexes are held in an inactive state by phosphorylation of cdc2 at Thr-14 and Tyr-15, which is mediated by the Wee1 protein kinase (7, 9). At the end of G2, abrupt dephosphorylation of this site by the phosphatase Cdc25C triggers cdc2 activation (9). Cyclin B-cdc2 then phosphorylates and thereby further activates Cdc25C, which induces the full activation of cdc2 by forming a positive feedback loop by mutual activation (10, 11). At the same time, certain sites within the cytoplasmic retention signal in the N terminus of cyclin B are phosphorylated, which allows translocation to the nucleus (12, 13). Upon nuclear translocation, the cyclin B-cdc2 complex can phosphorylate critical substrates required for the initiation of mitosis. Completion of mitosis in turn depends on the ubiquitin-mediated degradation of cyclin B at the metaphase/anaphase transition (14).

We have investigated the mechanism of growth inhibition by bFGF1 in SK-N-MC cells, neuroepithelioma cells of embryonic neuroectodermal origin. We find that addition of bFGF delays cell cycle progression through the G2 phase of the cell cycle rather than during G1. This bFGF-induced delay in G2 progression resembles the G2 arrest seen after DNA damage, indicating the existence of a growth factor-sensitive point in G2.

EXPERIMENTAL PROCEDURES

Cell Culture, Transfections, and Synchronization—The human neuroepithelioma SK-N-MC cell line was cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium (DF12 medium, Life Technologies, Inc.). The mouse fibroblast NIH 3T3-derived cell line A14 (15) was cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.). Culture media were supplemented with 10% fetal calf serum (Life Technologies, Inc.), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin.

SK-N-MC cells were transfected using the standard calcium phosphate technique (16) with pUHD10–3 encoding the βtA hybrid protein (17). 24 h after transfection the medium was replaced with DF12 medium containing 500 μg/ml G418 (Calbiochem) and 1 μg/ml tetracycline (Roche Molecular Biochemicals). Two weeks later individual colonies were picked. Induction was tested using transient transfection with pUHD10–3 and analyzed using a luciferase assay as described (17). One clone, SKTA9 (5200-fold induction) was selected and routinely cultured in DF12 medium supplemented with 10% fetal calf serum, 500 μg/ml G418, and 1 μg/ml tetracycline. Subsequently, SKTA9 cells were transfected with 10 μg of pUHD10–3, Cdc25C-myc or pUHD10–3,Cdc25C-S216A-myc (kindly provided by Dr. H. Piwnica-Worms, Washington University School of Medicine, St. Louis, Missouri) in combination with 2 μg of pBabe.puro. 24 h after transfection the medium

* This work was supported by Grant UU96-1176 from the Dutch Cancer Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 31-30-2506515; Fax: 31-30-2511893; E-mail: R.H.Medema@lab.azu.nl.

1 The abbreviations used are: bFGF, basic fibroblast growth factor; PAA, polyacrylamide; BrdUrd, 5-bromo-2′-deoxyuridine; MAPK, mitogen-activated protein kinase.
was replaced with DF12 medium containing 0.25 μg/ml puromycin (Sigma) and 1 μg/ml tetracycline. Two weeks later individual colonies were picked and analyzed for inducible expression of Cdc25C(-S216A)-myc. Cells were maintained in DF12 medium containing 10% fetal calf serum, 0.25 μg/ml puromycin, and 1 μg/ml tetracycline. Prior to trypsinization, cells were treated with thymidine (2.5 mM) for 24 h. Cells were released from the thymidine block by washing twice with prewarmed DF12 medium for 15 min.

**Antibodies and Reagents**—Protein A/G plus agarose, the mouse monoclonal antibodies against cyclin B1 (GNS1) and cdc2 (clone 17), and the rabbit polyclonal anti-Cdc25C (C-20) were purchased from Santa Cruz Biotechnology. The polyclonal phosphospecific anti-phospho-Tyr-15-cdc2 antibody was from Biosands. Fluorescein isothiocyanate-conjugated anti-BrdUrd antibody was obtained from Becton Dickinson. The mouse monoclonal anti-e-myc (9E10) was kindly provided by Dr. H. Bos (Laboratory of Physiological Chemistry, Utrecht, The Netherlands). Recombinant bovine bFGF and histone H1 were purchased from Roche Molecular Biochemicals. Adrarnycin, caffeine, 4',6-diamidino-2-phenylindole (DAPI), and propidium iodide were obtained from Molecular Probes.

**Cell Cycle Analysis Using Flow Cytometry**—Repliecate DNA synthesis and DNA content were analyzed using bivariate flow cytometry. Cells were pulsed with 1 μM BrdUrd for 10 min at 37 °C, after which the cells were harvested by trypsinization and fixed overnight in 70% ethanol at 4 °C. After the ethanol was washed away, the cells were treated with 0.1 N HCl containing 0.5 mg/ml pepsin for 20 min at room temperature. Next, cells were treated with 2 N HCl for 12 min at 37 °C followed by the addition of 0.05 N borate buffer (pH 8.5). The cells were washed and incubated with fluorescein isothiocyanate-conjugated anti-BrdUrd antibody for 1 h at 4 °C. Finally, the cells were counterstained with propidium iodide in a solution containing 10 μg/ml propidium iodide and 10 μg/ml DNase-free RNase. The stained cells were analyzed on a fluorescence-activated cell sorter using Lysis II software flow cytometry analysis (Becton Dickinson).

For analysis of the progression of cells through S phase, the cells were pulsed with 1 μM BrdUrd for 10 min at 37 °C, after which BrdUrd was washed away from the cells and fresh medium with or without bFGF (20 ng/ml) was added to the cells. At different time points after the BrdUrd pulse, the cells were harvested and prepared for cell cycle analysis as described above.

**DNA Staining Using Immunofluorescence**—Cells were harvested, fixed in 70% ethanol, and centrifuged on slides. DNA was stained using 4',6-diamidino-2-phenylindole (20 μg/ml). The percentage of mitotic figures was scored using fluorescence microscopy.

**Western Blotting**—Cells were lysed directly in Laemmli sample buffer without β-mercaptoethanol or bromphenol blue. Protein concentration was determined using the Lowry protein assay. Subsequently, β-mercaptoethanol and bromphenol blue were added, and the samples were boiled for 5 min. Proteins were separated on a polyacrylamide (PAA) gel and blotted to a nitrocellulose membrane. Proteins were detected with mouse monoclonal or rabbit polyclonal antibodies during overnight incubation at 4 °C, followed by the secondary antibody for 1 h at room temperature. The blots were developed using enhanced chemiluminescence.

**Immunoprecipitation and In Vitro Kinase Reactions**—Cells were washed with ice-cold phosphate-buffered saline and lysed in NETN (400 mM NaCl, 20 mM Tris (pH 8.0), 1 mM EDTA, 0.5% Nonidet P-40) or EIA lysis buffer (150 mM NaCl, 50 mM Hepes (pH 7.5), 5 mM EDTA, 0.1% Nonidet P-40) for 30 min at 4 °C for cyclin B1- or myc-immunoprecipitation, respectively. Both lysis buffers were supplemented with 10 mM β-glycerophosphate, 10 mM NaF, 1 mM NaVO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml trypsin inhibitor. Lysates were centrifuged at 15,000 rpm at 4 °C. Cyclin B1 was immunoprecipitated with 0.3 μg of anti-cyclin B1 antibody, and myc-tagged proteins were immunoprecipitated with anti-myc antibody, both with protein A/G plus agarose for 4 h overnight at 4 °C. Subsequently, immunoprecipitates were washed twice with lysis buffer. For immunoblotting, precipitates were washed once again with lysis buffer, whereafter sample buffer was added. Then the samples were boiled for 5 min, the proteins were separated on a PAA gel, and proteins were detected by Western blotting.

For in vitro kinase reactions, cyclin B1 precipitates were washed with kinase buffer (20 mM Tris (pH 7.5), 5 mM MgCl2, 2.5 mM MnCl2, 1 mM dithiothreitol). Immunocomplexes were incubated in kinase buffer supplemented with 50 μM ATP, 2.5 μCi [γ-32P]ATP (Du Pont), and 10 μg of histone H1 for 30 min at 30 °C. Kinase reactions were stopped by adding sample buffer and boiling the samples for 5 min. Samples were separated on a 12% PAA gel, and the phosphorylated histone H1 was visualized by autoradiography. The amount of incorporated [32P] was determined on a PhosphorImager (Molecular Dynamics).

**RESULTS**

**bFGF Delays Progression through the G2/M Phases of the Cell Cycle**—bFGF treatment of human neuroepithelioma SK-N-MC cells has been reported to result in growth inhibition (18), but the mechanism by which bFGF mediates this growth inhibition is unknown. Therefore, we decided to study this bFGF-induced growth inhibition in more detail. We first determined the extent of growth inhibition by bFGF by analyzing the doubling time of SK-N-MC cells grown in the presence or absence of bFGF. As shown in Fig. 1A, in the absence of bFGF the doubling time of SK-N-MC cells is ~20 h, whereas the doubling time rises to ~30 h in the presence of bFGF.

To determine in which phase of the cell cycle bFGF exerts its growth-inhibitory effect, we analyzed cell cycle profiles of asynchronously growing cells treated with bFGF for 24 or 48 h. Unexpectedly, treatment of SK-N-MC cells with bFGF seemed to arrest cells with a 4N DNA content, indicating that bFGF affects progression through the G2/M, and not G1/S, phases of the cell cycle (Fig. 1B). After a culture period of 24 h we noticed an increase in the percentage of cells in the G2/M phases from 11% in the untreated population to ~29% in the bFGF-treated cultures. 48 h after the initial addition of bFGF, ~36% of the cells had a 4N DNA content. These results indicate that bFGF affects progression through the G2/M phases in these cells.

Next, we performed a BrdUrd pulse-chase experiment in SK-N-MC cells. To this end, cells were pulsed with BrdUrd for 10 min, after which the BrdUrd was washed away and the cells were allowed to grow in the presence or absence of bFGF for different time periods. In this way, we were able to follow the BrdUrd-positive cells as they passed through S, G2, and M, into the next G1 phase. As expected, BrdUrd-positive cells were equally distributed throughout S phase when harvested immediately after the BrdUrd pulse (t = 0) (Fig. 1C). After a 4-h chase, most of the untreated and bFGF-treated cells had left S phase and had entered G2/M, indicating that bFGF does not affect progression through S phase. A fraction of the cells in untreated cultures were reentering the next G1 phase after 8 h, whereas all cells treated with bFGF were still in G2/M. After 12 h of culture, when ~65% of the untreated cells had reentered the next G1 phase, only ~10% of cells in the bFGF-treated cultures had left the G2/M phases (Fig. 1C). It took up to 20 h before the bulk of cells treated with bFGF had entered the next G1 phase (data not shown), indicating that treatment with bFGF results in delay in cell cycle progression somewhere in G2/M of at least 8 h. This is consistent with the experiments performed in asynchronous cultures, where bFGF treatment induced an increase of cells with a 4N DNA content and an increased doubling time instead of blocking all cells in G2/M (Fig. 1B). The results obtained from the BrdUrd pulse-chase experiment also clearly demonstrate that bFGF affects cells after they have passed the restriction point, since we studied the effect of bFGF on cells in S phase.

**bFGF Inhibits Cell Cycle Progression at Some Point in G2** —The experiments described above do not allow discrimination between cells in G1, and those in mitosis. To further characterize the cell cycle status of bFGF-treated cells, SK-N-MC cells were synchronized at the G1/S transition using thymidine, whereafter the cells were released in the presence or absence of bFGF. Nocodazole was added after 16 h of release to ensure...
that the synchronized cells eventually arrest in the M phase. Cells were harvested at various time points, and the percentage of mitotic cells was determined by scoring for cells with condensed chromosomes using immunofluorescence microscopy. Error bars represent the S.E. of three microscopy fields.

in the M phase (~60%). However, cells treated with bFGF did not enter mitosis until 24 h after release, and after 30 h only ~30% of the cells were in M phase. These findings show that bFGF treatment of SK-N-MC cells results in an obstruction in the G2 phase rather than during mitosis. bFGF-treated cells eventually enter the M phase, again indicating that cell cycle progression through G2 is delayed instead of totally blocked.

Cyclin B-cdc2 Activation Is Prevented by bFGF—To enter M phase, kinase activity of the mitotic cyclin B-cdc2 complex is required (7). We therefore tested whether bFGF inhibits mitotic entry by affecting activation of the cyclin B-cdc2 kinase. SK-N-MC cells were harvested at different time points after release from a thymidine block, and the cyclin B-associated kinase activity was determined using an in vitro kinase reaction. As shown in Fig. 3A, cyclin B-associated kinase activity started to rise 20 h after the release in the absence of bFGF and continued to increase in time up to 30 h. In contrast, releasing cells from the thymidine block in the presence of bFGF delayed activation of cyclin B-cdc2 kinase activity. Consistent with the timing of the appearance of mitotic figures, as shown in Fig. 2, the cyclin B-associated kinase activity increased after a 24-h release in the presence of bFGF. The reduction in cyclin B-cdc2 kinase activity was not because of inhibition of cyclin B expression, because cyclin B protein levels were not influenced by bFGF treatment (data not shown). These results clearly indicate that bFGF delays cell cycle progression by interference with some event in G2, possibly the activation of the mitotic cyclin B-cdc2.

Several phosphorylation and dephosphorylation steps lead to the activation of a cyclin-cyclin-dependent kinase complex. Dephosphorylation of cdc2-Tyr-15 by Cdc25C leads to the activation of cdc2 at the G2/M transition, which results in a mobility shift on PAA gels (19). To investigate cyclin B-cdc2 activation in bFGF-treated cells, SK-N-MC cells were synchronized at the G1/S transition and released as described for determination of cyclin B-associated kinase activity (Fig. 3A). At various time points after the release, cells were lysed, cyclin B was immunoprecipitated, and cdc2 bound to cyclin B was detected by immunoblotting. As shown in Fig. 3B, activated cdc2 was present after a 22-h release in the absence of bFGF, and the amount of cdc2-Tyr-15-dephosphorylated protein increased in time. Releasing the cells in the presence of bFGF did not interfere with binding of cdc2 to cyclin B, but delayed cdc2-Tyr-15 dephosphorylation. These results indicate that the delay in activation of
immunoprecipitation or loaded in each lane. The slower migrating form is the active, phosphorylated form of cdc2. After 16 h, cells were lysed, and the phosphorylation status of cdc2-Tyr-15 was analyzed using a polyclonal phosphospecific anti-Tyr-15 antibody on Western blot. As shown in Fig. 3D, this latter result was confirmed using a phosphospecific antibody recognizing Tyr-15-phosphorylated cdc2 (Fig. 3D). To demonstrate that the bFGF-induced effect on cdc2 is associated with the growth inhibitory property of bFGF, the same experiment was done using NIH 3T3-derived A14 cells, which are growth stimulated by bFGF (20) (data not shown). Synchronization of A14 cells with nocodazole resulted in the activation of cyclin B-cdc2 kinase activity (Fig. 3C), and this activation could not be prevented by bFGF treatment.

Taken together, our data suggest that bFGF exerts its growth inhibitory effect through interference with the Tyr-15 dephosphorylation of cdc2. Dephosphorylation of cdc2-Tyr-15 is mediated by the phosphatase Cdc25C (21). Cdc25C itself is activated by phosphorylation, and this was shown to result in a mobility shift on PAA gels (11). Indeed, synchronizing cells in mitosis by nocodazole treatment resulted in the appearance of a slower migrating form of Cdc25C (Fig. 3E). However, treating cells with nocodazole in the presence of bFGF prevented the phosphorylation of Cdc25C, suggesting that bFGF might inhibit activation of Cdc25C and thereby interfere with cdc2 activation.

**Caffeine Can Rescue the bFGF-induced Inhibition of cdc2-Tyr-15 Dephosphorylation**—The data described above demonstrated that bFGF induced a G2 arrest in SK-N-MC cells, which is associated with inhibition of cdc2-Tyr-15 dephosphorylation by Cdc25C. Similar effects on cdc2 activation are seen when the G2 DNA damage checkpoint is activated (8). DNA damage-induced retention of cdc2-Tyr-15 phosphorylation can be diminished by caffeine treatment (22). We therefore examined the effect of caffeine on the bFGF-induced inhibition of cdc2-Tyr-15 dephosphorylation using the phosphospecific anti-Tyr-15 antibody on Western blot. As shown in Fig. 4, after treatment with bFGF or the DNA damaging agent adriamycin, cdc2-Tyr-15 remains phosphorylated in cells blocked with nocodazole. Treating the cells with bFGF or adriamycin in the presence of caffeine restored cdc2-Tyr-15 dephosphorylation, indicating that caffeine can overcome the bFGF-induced inhibition of cdc2-Tyr-15 dephosphorylation as well as the DNA damage-induced effect on cdc2. Taken together, these results suggest that bFGF induces a G2 arrest in SK-N-MC cells that might act in a similar fashion as the G2 DNA damage checkpoint.

Expression of Cdc25C-S216A Can Rescue the bFGF-induced G2 Delay—DNA damage results in activation of Chk1, which phosphorylates Cdc25C on Ser-216 thereby creating binding sites for 14–3-3 proteins (23–25). Binding to 14–3-3 blocks the access of Cdc25C to cyclin B, resulting in a G2 arrest (26). To study further similarities between the G2 arrest induced by bFGF, the same experiment was done using NIH 3T3-derived A14 cells, which are growth stimulated by bFGF (20) (data not shown). Synchronization of A14 cells with nocodazole resulted in the activation of cyclin B-cdc2 kinase activity (Fig. 3C), and this activation could not be prevented by bFGF treatment.

Taken together, our data suggest that bFGF exerts its growth inhibitory effect through interference with the Tyr-15 dephosphorylation of cdc2. Dephosphorylation of cdc2-Tyr-15 is mediated by the phosphatase Cdc25C (21). Cdc25C itself is activated by phosphorylation, and this was shown to result in a mobility shift on PAA gels (11). Indeed, synchronizing cells in mitosis by nocodazole treatment resulted in the appearance of a slower migrating form of Cdc25C (Fig. 3E). However, treating cells with nocodazole in the presence of bFGF prevented the phosphorylation of Cdc25C, suggesting that bFGF might inhibit activation of Cdc25C and thereby interfere with cdc2 activation.

**Caffeine Can Rescue the bFGF-induced Inhibition of cdc2-Tyr-15 Dephosphorylation**—The data described above demonstrated that bFGF induced a G2 arrest in SK-N-MC cells, which is associated with inhibition of cdc2-Tyr-15 dephosphorylation by Cdc25C. Similar effects on cdc2 activation are seen when the G2 DNA damage checkpoint is activated (8). DNA damage-induced retention of cdc2-Tyr-15 phosphorylation can be diminished by caffeine treatment (22). We therefore examined the effect of caffeine on the bFGF-induced inhibition of cdc2-Tyr-15 dephosphorylation using the phosphospecific anti-Tyr-15 antibody on Western blot. As shown in Fig. 4, after treatment with bFGF or the DNA damaging agent adriamycin, cdc2-Tyr-15 remains phosphorylated in cells blocked with nocodazole. Treating the cells with bFGF or adriamycin in the presence of caffeine restored cdc2-Tyr-15 dephosphorylation, indicating that caffeine can overcome the bFGF-induced inhibition of cdc2-Tyr-15 dephosphorylation as well as the DNA damage-induced effect on cdc2. Taken together, these results suggest that bFGF induces a G2 arrest in SK-N-MC cells that might act in a similar fashion as the G2 DNA damage checkpoint.

Expression of Cdc25C-S216A Can Rescue the bFGF-induced G2 Delay—DNA damage results in activation of Chk1, which phosphorylates Cdc25C on Ser-216 thereby creating binding sites for 14–3-3 proteins (23–25). Binding to 14–3-3 blocks the access of Cdc25C to cyclin B, resulting in a G2 arrest (26). To study further similarities between the G2 arrest induced by
bFGF and the G2 DNA damage checkpoint, we established cell lines expressing wild type Cdc25C or the Cdc25C-S216A mutant previously shown to partially override the G2 DNA damage checkpoint (24). We made use of the tetracycline-repressible system to drive expression of Cdc25C and Cdc25C-S216A (17).

A SK-N-MC-derived cell line was obtained in which tTA is stably expressed (SKTA9). SKTA9 cells were subsequently transfected with an expression plasmid containing myc-tagged human Cdc25C or Cdc25C-S216A mutant cDNA under the control of a minimal cytomegalo virus promoter fused to tetracycline operator sequences. Colonies were picked and analyzed by bivariate flow cytometry using anti-BrdUrd antibody and added. After 12 h, cells were harvested, and cell cycle profiles were washed away and fresh medium with or without bFGF (20 ng/ml) was added. After 12 h, cells were harvested, and cell cycle profiles were obtained by bivariate flow cytometry using anti-BrdUrd antibody and propidium iodide. Shown are the histograms of the DNA content of BrdUrd-positive cells.

9C11 (wild type Cdc25C), 9S11 (Cdc25C-S216A mutant), and 9S19 (Cdc25C-S216A mutant) expressed no exogenous Cdc25C when cultured in the presence of tetracycline, whereas exogenous Cdc25C expression was induced after the removal of tetracycline from the culture medium.

We next investigated whether expression of wild type Cdc25C or Cdc25C-S216A mutant can rescue the bFGF-induced retention of cdc2-Tyr-15 phosphorylation using the phosphospecific anti-Tyr-15 antibody on Western blot. As shown in Fig. 5B, bFGF or adriamycin blocked cdc2-Tyr-15 dephosphorylation in uninduced 9S19 cells. However, in 9S19 cells induced to express Cdc25C-S216A, the cdc2-Tyr-15 dephosphorylation was not efficiently blocked by bFGF treatment, indicating that expression of Cdc25C-S216A can overcome the bFGF-induced inhibition of cdc2-Tyr-15 dephosphorylation. This effect was also seen in the 9S11 clone (Cdc25C-S216A mutant) (data not shown) but not seen in 9C11 cells (wild type Cdc25C) (Fig. 5B). Treating 9S19 cells induced to express Cdc25C-S216A with adriamycin still resulted in a retention of cdc2-Tyr-15 phosphorylation. This latter observation is consistent with the notion that multiple pathways mediate the G2 arrest after DNA damage (27, 28), so that expression of Cdc25C-S216A alone is unlikely to fully override DNA damage effects on cdc2-Tyr-15 phosphorylation.

Because expression of Cdc25C-S216A could override the bFGF-induced effects on cdc2, we next tested whether the inhibition of G2 phase progression induced by bFGF can also be diminished by expression of this Cdc25C mutant. We again performed a BrdUrd pulse-chase experiment as described above and analyzed the percentage of BrdUrd-positive cells that could complete the cell cycle and enter the next G1 phase in a 12-h time course following the BrdUrd pulse. In uninduced 9S19 cells, bFGF treatment resulted in an inhibition of ~70% in entry in G1 phase, a result similar to that seen in the parental SK-N-MC cells (Figs. 5C and 1, respectively). In addition, bFGF treatment of 9C11 cells, either uninduced or induced to express wild type Cdc25C, caused a very significant inhibition of G1 entry (Fig. 5C), consistent with the notion that overexpression of wild type Cdc25C was unable to revert the inhibitory effects of bFGF on cdc2 activation (Fig. 5B). However, when 9S19 cells were induced to express Cdc25C-S216A, inhibition of G1 entry by bFGF was reduced to only ~30%, whereas cell cycle progression in the absence of bFGF was not affected (Fig. 5C). This indicates that expression of a Cdc25C-S216A mutant does not affect cell cycle kinetics of SK-N-MC cells under normal growth conditions, but that this mutant specifically overrides the negative effect of bFGF on cell growth.

**DISCUSSION**

In this study we demonstrate that bFGF can induce growth inhibition of SK-N-MC cells by a delay in G2 phase progression, a mechanism that appears similar to the G2 DNA damage checkpoint. We showed that treatment with bFGF increased the doubling time of SK-N-MC cells from ~20 to ~30 h, and we demonstrated that cells are delayed in G2/M by about 8 h. This indicates that the major, if not only, growth inhibitory effect of bFGF on SK-N-MC cells is exerted in the G2 phase and makes it unlikely that bFGF affects other cell cycle phases. This is remarkable because it is generally believed that extracellular factors can influence cell cycle progression during G1 and that cells become refractory to growth inhibitory signals after passage through the restriction point (2). Our results clearly indicate that cells can be inhibited by growth inhibitory signals after passage through the restriction point, because the addition of bFGF to cells that are in S phase still results in a delay in G2/M progression of at least 8 h. Indeed, the addition of
bFGF up to a point late in G₂ still efficiently inhibited mitotic entry (data not shown).

In addition to the results obtained with bFGF, stimulation of the Ret signaling pathway using a chimeric human epidermal growth factor receptor-Ret chimera receptor in SK-N-MC cells resulted in growth inhibition that was associated with a delay in G₂ phase progression, similar to what is seen after bFGF treatment. These results indicate that this response is not restricted to a single growth factor receptor. Indeed, others have reported minor effects on G₂ progression by epidermal growth factor. Treatment of Hela and A431 cells with epidermal growth factor resulted in a short delay (1–2 h) in mitotic entry coupled with a delay in activation of cyclin B-cdc2 complexes (29). However, in these cells no overall growth inhibition was observed with epidermal growth factor, in contrast to what we see with bFGF in SK-N-MC cells. Therefore, this is the first example of overall growth inhibition by an extracellular factor that appears to be confined to regulation of the G₂/M transition. Nevertheless, the data obtained with epidermal growth factor in Hela and A431 cells suggest that negative growth regulation in G₂ by extracellular factors might be a more general phenomenon. In addition to the extensively studied G₁ restriction point, growth regulation in G₂ may therefore play an important role in the proliferation of some cells.

It will be of interest to study the signaling molecules in the pathway by which bFGF and Ret induce growth inhibition of SK-N-MC cells. It should be noted that van Puijenbroek et al. (18) showed that growth inhibition by bFGF and Ret in these SK-N-MC cells is associated with sustained MAPK activation, whereas platelet-derived growth factor, which does not induce growth inhibition, also activates MAPK but gives rise to only a very transient activation of MAPK in these cells. Several studies support a role for MAPK in regulating progression through G₂/M (30, 31), but other studies also suggest the involvement of other signaling molecules, for example, protein kinase C (32–35). However, using pharmacological inhibitors of MAPK, protein kinase C, phosphatidylinositol 3-kinase, and p38 MAPK we have been unable to obtain evidence for a possible involvement of any of these signaling molecules in the observed bFGF-induced growth inhibition (data not shown).

A possible mediator of the G₂ arrest induced by bFGF is the cyclin-dependent kinase inhibitor p21
\(^{\text{CIP1}}\). This p53-regulated protein has been described as inhibiting kinase activity of the cyclins E, A, and B, resulting in an arrest in both the G₁ and G₂ phases of the cell cycle (36). SK-N-MC cells do not express functional p53 (37), but activation of p21
\(^{\text{CIP1}}\) expression can also occur independently of p53 (38, 39). However, up-regulation of p21
\(^{\text{CIP1}}\) could not be observed after bFGF treatment (data not shown), indicating that the inhibition of cyclin B kinase activity by bFGF must be due to another mechanism.

Because Tyr-15 dephosphorylation is a critical step in the activation of the cyclin B-cdc2 complex, we investigated the effect of bFGF on this dephosphorylation event. We found that cdc2-Tyr-15 dephosphorylation, as well as activation of the Cdc25C phosphatase responsible for this dephosphorylation, was inhibited by bFGF. DNA damage also results in the inhibition of cdc2-Tyr-15 dephosphorylation by blocking activation of Cdc25C, thereby leading to an arrest in G₂ (8). Interestingly, caffeine could revert both DNA damage- and bFGF-induced inhibition of cdc2-Tyr-15 dephosphorylation, indicating once more that these two pathways are remarkably similar. Moreover, our finding that caffeine can revert the effect of bFGF on cdc2 dephosphorylation makes it unlikely that the inhibition of cdc2 activation by bFGF is due to an arrest of cell cycle progression at an early point in G₂, prior to cdc2 activation. Thus, our data indicate that bFGF directly interferes with the activation of cyclin B-cdc2 to inhibit cell proliferation.

Our results suggested that bFGF would interfere with cdc2-Tyr-15 dephosphorylation via inhibition of Cdc25C. The inhibition of cdc2 activation induced by DNA damage results from Chk1-mediated phosphorylation of the Ser-216 site of Cdc25C, leading to the inability of Cdc25C to activate cyclin B-cdc2 complexes (23–26). We showed that expression of Cdc25C mutated on Ser-216 was able to rescue the bFGF-induced inhibition of cdc2-Tyr-15 dephosphorylation (Fig. 5) and restored the cyclin B-cdc2 kinase activity (data not shown). Expression of this mutant, in contrast to wild type Cdc25C, resulted in a rescue of the bFGF-induced G₂ delay as demonstrated by a BrdUrd pulse-chase experiment (Fig. 5). This demonstrates that regulation of Cdc25C phosphorylation is a critical factor in the bFGF-induced growth inhibition of SK-N-MC cells. Because we find a partial reversal, we cannot rule out other effects of bFGF during G₂/M. Indeed, using a BrdUrd pulse-chase assay we showed that bFGF induced an ~8 h delay in G₂ progression (Fig. 1C), whereas the mitotic entry was only inhibited for 4–6 h (Fig. 2), which could indicate that bFGF has additional effects on events later in mitosis.

Besides Chk1-mediated inhibition of Cdc25C, other pathways are activated after DNA damage to enforce a delay in G₂. For example, p53-regulated p21 expression results in a sustained G₂ arrest in response to DNA damage (27), whereas inhibition of the nuclear translocation of cyclin B by DNA damage blocks the access of cyclin B to its mitotic substrates (28). We found that expression of Cdc25C-S216A was unable to revert the inhibition of cdc2-Tyr-15 dephosphorylation that occurs in response to the DNA-damaging agent adriamycin (Fig. 5B), consistent with the existence of parallel pathways. Thus, although certain similarities were observed between the DNA damage response and the effects of bFGF described here, the efficient rescue of bFGF-induced G₂ arrest seen in cells expressing Cdc25C-S216A mutant indicates that the pathways affected by bFGF are not as diverse as those activated after DNA damage.

A potentially interesting link between bFGF-induced growth inhibition in G₂ and the DNA damage checkpoint is suggested by studies on the radioprotective effect of bFGF. It has been reported that exogenous bFGF can protect cells from the lethal effects of ionizing radiation (40, 41). This radioprotective effect of bFGF is correlated with a pronounced increase in the duration of the G₂ arrest after irradiation (42). Interestingly, bFGF expression has been reported to be present in abundant amounts in primary brain tumors, which are known for their poor responsiveness to radiation therapy (43). Given the effects of bFGF that we describe here, it is a distinct possibility that tumors producing high levels of bFGF respond so poorly to radiotherapy because of the suggested resemblance between DNA damage and bFGF in regulating cdc2 activity.

Acknowledgments—We thank Dr. Piwnica-Worms for providing the Cdc25C plasmids. We thank the members of the Bos laboratory for helpful discussions, technical assistance, and reagents. We also thank the other members of the Jordan laboratory for critical discussions.

REFERENCES
1. Beijersbergen, R. L., and Bernards, R. (1996) Biochem. Biophys. Acta 1287, 123–120
2. Pardee, A. B. (1989) Science 246, 603–608
3. Laiho, M., DeCaprio, J. A., Ludlow, J. W., Livingston, D. M., and Massague, J. (1990) Cell 62, 175–185
4. Albers, M. W., Williams, R. T., Brown, E. J., Tanaka, A., Hall, F. L., and Schreiber, S. L. (1993) J. Biol. Chem. 268, 22925–22939
5. Sherr, C. J. (1993) Cell 73, 1069–1065
6. Nurse, P. (1994) Cell 79, 547–550
7. Dunphy, W. G. (1994) Trends Cell Biol. 4, 202–207

---

\(^{2}\) V. A. J. Smits, M. A. van Peer, M. A. G. Essers, R. Klompmaker, G. Rijksen, and R. H. Medema, manuscript in preparation.
Growth Inhibition by bFGF in G2

8. Kharbanda, S., Saleem, A., Datta, R., Yuan, Z. M., Weichselbaum, R., and Kufe, D. (1994) Cancer Res. 54, 1412–1414
9. King, R. W., Jackson, P. K., and Kirschner, M. W. (1994) Cell 79, 563–571
10. Hoffman, I., Clarke, P. R., Marote, M. J., Karsenti, E., and Draetta, G. (1994) EMBO J. 13, 53–63
11. Izumi, T., and Maller, J. L. (1993) Mol. Biol. Cell 4, 1337–1350
12. Pines, J., and Hunter, T. (1994) EMBO J. 13, 3772–3781
13. Li, J., Meyer, A. N., and Donoghue, D. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 502–507
14. Pines, J. (1995) Biochem. J. 308, 697–711
15. Burgering, B. M., Medema, R. H., Maassen, J. A., van der Eb, A. J., McCormick, F., and Bos, J. L. (1991) EMBO J. 10, 1103–1109
16. Danos, O., and Mulligan, R. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 89, 5547–5551
17. van Puijenbroek, A. A. F. L., van Weering, D. H. L., van den Brink, C. E., Bos, J. L., van der Saag, P. T., de Laat, S. W., and den Hertog, J. (1997) Oncogene 14, 1147–1157
18. Nishijima, H., Nishitani, H., Seki, T., and Nishimoto, T. (1997) J. Cell Biol. 138, 1105–1116
19. Gospodarowicz, D. (1974) Nature 249, 123–127
20. Haimovitz-Friedman, A., Vlodavsky, I., Chaudhuri, A., Witte, L., and Fuks, Z. (1991) Cancer Res. 51, 2552–2558
21. Fuks, Z., Persaud, R. S., Aliferi, A., McLoughlin, M., Ehleiter, D., Schwartz, J. L., Seddon, A. P., and Haimovitz-Friedman, A. (1994) Cancer Res. 54, 2582–2590
22. Jung, M., Kern, F. G., Jorgensen, T. J., McLeskey, S. W., Blair, O. C., and Dritschilo, A. (1994) Cancer Res. 54, 5194–5197
23. Takahashi, J. A., Mori, H., Fukumoto, M., Igarashi, K., Jaye, M., Oda, Y., Kikuchi, H., and Hatanaka, M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 5710–5714
24. Jin, P., Hardy, S., and Morgan, D. O. (1998) J. Cell Biol. 141, 875–885
25. Kinzel, V., Raschkin, M., Blume, A., and Richards, J. (1990) Cancer Res. 50, 7932–7936
26. Abrieu, A., Fisher, D., Simon, M. N., Dereu, M., and Picard, A. (1997) EMBO J. 16, 6407–6413
27. Tamemoto, H., Kadowaki, T., Tohe, K., Ueki, K., Iizumi, T., Chatani, Y., Kohn, M., Kasuga, M., Yazaki, Y., and Akanuma, Y. (1992) J. Biol. Chem. 267, 20293–20297
28. Barth, H., and Kinzel, V. (1994) Exp. Cell Res. 212, 383–388
29. Hofmann, J., O'Connor, P. M., Jackman, J., Schubert, C., Ueberall, F., Kohn, K. W., and Grunicke, H. (1994) Biochem. Biophys. Res. Commun. 199, 937–943
30. Livneh, E., and Fishman, D. D. (1997) Eur. J. Biochem. 248, 1–9
31. Thompson, L. J., and Fields, A. P. (1996) J. Biol. Chem. 271, 15045–15053
32. Medema, R. H., Klompmaker, R., Smits, V. A. J., and Rijksen, G. (1998) Oncogene 16, 431–441
33. Danos, O., and Mulligan, R. C. (1998) Proc. Natl. Acad. Sci. U. S. A. 89, 5547–5551
34. van Puijenbroek, A. A. F. L., van Weering, D. H. L., van den Brink, C. E., Bos, J. L., van der Saag, P. T., de Laat, S. W., and den Hertog, J. (1997) Oncogene 14, 1147–1157
35. Moll, U. M., Ostermeyer, A. G., Haladay, R., Winkfield, B., Frazier, M., and Zambetti, G. (1996) Mol. Cell. Biol. 16, 1126–1137
36. Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y., and Wang, X. F. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 5545–5549
37. Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. (1995) Genes Dev. 9, 1831–1845
Negative Growth Regulation of SK-N-MC Cells by bFGF Defines a Growth Factor-sensitive Point in G²
Veronique A. J. Smits, Maartje A. van Peer, Marieke A. G. Essers, Rob Klompmaker, Gert Rijksen and René H. Medema

J. Biol. Chem. 2000, 275:19375-19381.
doi: 10.1074/jbc.M001764200 originally published online April 17, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M001764200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 24 of which can be accessed free at
http://www.jbc.org/content/275/25/19375.full.html#ref-list-1