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Biochemical Pharmacology 68 (2004) 2221–2227
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Induction of Cdc25B expression by epidermal growth factor and transforming growth factor-α

Tetsuya Oguri[a,b], Kaoru Nemoto[a,c], Pallavi Bansal[a], Peter Wipf[a], John S. Lazo[a,*]

[a]*Departments of Pharmacology and Chemistry, University of Pittsburgh, Pittsburgh, PA 15261, USA*
[b]*Department of Internal Medicine and Molecular Science, Nagoya City University, Graduate School of Medical Sciences, Nagoya 467-8601, Japan*
[c]*Department of Urology, Nippon Medical School, Tokyo 113-8602, Japan*

Received 6 January 2004; accepted 7 August 2004

Abstract

The dual specificity protein phosphatase Cdc25B regulates of the mitotic cell cycle checkpoint and is over expressed in human tumors. Given the importance of growth factors in initiating and sustaining cell proliferation, we examined their effects on Cdc25B protein expression in human cancer cells. Within 1 h after epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) treatment, Cdc25B protein levels increased in growth factor responsive A549 and SCC25 cells, but not in non-responsive MDA-MB-231 cells. A functional consequence of elevated Cdc25B was implied by the concomitant decrease in phosphorylated cyclin dependent kinase, a known Cdc25B substrate, after growth factor treatment of A549 and SCC25 cells. The EGF-mediated induction of Cdc25B required a functional EGF receptor (ErbB1), as mouse embryonic fibroblasts lacking ErbB1 did not have increased Cdc25B levels after EGF treatment. Moreover, the EGFR receptor-selective tyrosine kinase inhibitor AG1478 and mitogen activated kinase kinase inhibitor U0126 blocked growth factor-mediated Cdc25B induction. Thus, EGF and TGF-α appear to induce cellular Cdc25B through the mitogen-activated protein kinase pathway.

Keywords: Cdc25B; EGF; TGF-α; MAPK; Dual specificity phosphatase; Cyclin dependent kinase

1. Introduction

EGF[3]-related peptide growth factors, such as EGF and TGF-α, control cell proliferation and death in cancer cells through the EGFR/ErbB family of receptors [1]. The EGF-related ligands induce the homo- and hetero-dimerization of the four ErbB receptors, resulting in the activation of an intrinsic tyrosine kinase activity, which facilitates cell proliferation, angiogenesis and metastasis of cancers. Elevated levels of these EGF receptors and/or their ligands have been observed in many human cancers, and have been associated with a more aggressive clinical behavior and poor prognosis [2]. For this reason, the ErbB receptor family and their ligands have been the focus of considerable attention both as fundamental factors in cancer development and as attractive potential anticancer therapeutic targets [3].

The Cdc25 dual specificity phosphatases control cell cycle progression through the dephosphorylation and activation of Cdks [4]. The human Cdc25 family comprises three phosphatases: Cdc25A, Cdc25B, and Cdc25C. Cdc25A mediates the transition from G1 and S phase [5] and through mitosis [6]. Cdc25B participates in S and G_2/M transition and Cdc25C is involved in the onset of mitosis [7,8]. Linkage between the cell cycle controlling Cdc25A and Cdc25B phosphatases and mitogenic signaling pathways was first suggested when Raf-1 was found to associate with the phosphatases [9,10]. Subsequently, Cdc25A was observed to bind and dephosphorylate the EGFR [11]. Transformation of primary fibroblast with SV40 large T antigen increases Cdc25B expression [12] and a human pulmonary carcinogen induces Cdc25B in

Abbreviations: EGF, epidermal growth factor; TGF-α, transforming growth factor-α; EGFR, EGF receptor; Cdk, cyclin-dependent kinase; BME, basal medium eagle; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MAPK, mitogen activated protein kinase; MEK, MAPK/ERK kinase; ERK, extracellular signal regulated protein kinase

* Corresponding author. Tel.: +1 412 648 9319; fax: +1 412 648 2229.
E-mail address: lazo@pitt.edu (J.S. Lazo).

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doi:10.1016/j.bcp.2004.08.011

human lung cells [13]. Cdc25A and Cdc25B have been reported to transform fibroblasts lacking functional retinoblastoma protein or harboring mutated Ras protein [14]. Furthermore, over expression of Cdc25A and/or Cdc25B has been observed in a substantial number of human tumors and is correlated with poor patient survival [4,15].

Although the 5′ promoter region for some Cdc25 phosphatases have been described [16–18], very little is known about the physiological regulation of Cdc25 expression. Human Cdc25C protein levels do not markedly change during the cell cycle, although the Cdc25C promoter is regulated in G0/G1 phase by the transcriptional repressor CDF-1 [17]. EGF has been reported to cause a modest reduction in Cdc25C activity but not protein levels in G2 phase cells, possibly by affecting the phosphorylation status of Cdc25C [19]. Expression of Cdc25A fluctuates only modestly over the cell cycle and transcription can be suppressed by over-expression of catalytically active Cdc25A [18]. The murine Cdc25B promoter contains nuclear factor Y/CAAT box activating factor binding sites as well as cell cycle-regulated repressor elements that might have a role in regulating Cdc25B expression throughout the cell cycle [16]. After EGF treatment of rat ovarian surface epithelial cells, a rapid increase in Cdc25A mRNA has been observed, although only a modest increase in Cdc25A protein was detected [20]. Thus, we have investigated whether EGF or TGF-α altered Cdc25A or Cdc25B protein levels in human cancer cells to help clarify the factors that influence the expression of these oncogenic Cdc25 phosphatases.

2. Materials and methods

2.1. Cell culture and chemicals

The human lung cancer cell line A549, the human head and neck cancer cell line SCC25, and the human breast cancer cell line MDA-MB-231 were purchased from American Type Culture Collection. A549 cells were maintained in BME media supplemented with 1% FBS at 37 °C in a humidified atmosphere 5% CO_2. SCC25 and MDA-MB-231 cells were cultured in RPMI 1640 medium with 10% FBS, 100 IU penicillin and 100 mg/ml streptomycin at 37 °C in a humidified atmosphere 5% CO_2. Primary cultures, which were the generous gift of Drs. Jennifer Rubin Grandis and Jill Marie Siegfried, (University of Pittsburgh), were generated from murine embryonic fibroblasts derived from EGFR knockout mice and their corresponding wild-type littermates (CD1 background) obtained at the age of 16.5 days. The genotype and phenotype of these cells have been confirmed and previously described [21]. Cells were maintained in Dulbecco's Minimal Essential Medium containing 20% heat-inactivated FBS. Cells were passed every 1–2 week and the medium changed twice weekly. EGF and TGF-α were purchased from Sigma, and the EGFR tyrosine kinase inhibitor AG1478 and MEK inhibitor U0126 were obtained from Calbiochem.

2.2. Effect of growth factor treatment on growth of cancer cells

The effect of EGF and TGF-α on the growth of cancer cells was determined by a MTT assay. Cancer cells (1×10^3) were plated in 96-well microtiter plates and after 3 days the viability of 10 ng/ml of EGF- or TGF-α-treated and non-treated cells was assayed by determining the color development caused by the reduction of MTT (Sigma) according to the manufacturer's instructions using a microplate reader.

2.3. Western blot analysis

Cells (1×10^6) were seeded on 10-cm^2 tissue culture dishes and after a 24 h preincubation period cells were exposed to vehicle, EGF (10 ng/ml), or TGF-α (10 ng/ml) for 0–4 h for Cdc25 induction studies. These were concentrations we previously found induced Erk phosphorylation. To examine the effect of EGFR or MEK blockade by AG1478 or U0126 on Cdc25B expression, we pre-treated A549 and SCC25 cells for 30 min with pharmacologically active concentrations of AG1478 (1 μM), or U0126 (10–50 μM) before a 1 h treatment with 10 ng/ml of EGF or TGF-α. To examine the effect of protein synthesis inhibition, we pre-treated A549 and SCC25 cells for 30 min with 25 μg/ml of cyclohexamide before the 2 h treatment with 10 ng/ml of EGF or TGF-α. The growth factor treated cells were then rinsed with PBS and lysed in Triton X-100 buffer (30 mM HEPES, 1% Triton X100, 10% glycerol, 5 mM $MgCl_2$, 25 mM NaF, 1 mM EDTA (pH 8.0), 10 mM NaCl, 0.2 mM sodium vanadate, 10 μg/ml trypsin inhibitor, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml serine protease inhibitor). Protein concentration was determined by the Bradford assay (Bio Rad). Thirty micrograms of whole cell protein lysates was subjected to SDS PAGE and transferred to nitrocellulose membrane. After protein transfer, blots were incubated with the blocking solution and probed with anti-EGFR antibody (Santa Cruz Biotechnologies, Inc.), anti-Cdc25B antibody (BD Transduction Laboratories), anti-Cdc25A antibody (NeoMarkers), Cdk1 antibody (Santa Cruz Biotechnologies, Inc.), anti-tyrosine^{15} residue phosphorylated Cdk1 antibody (Santa Cruz Biotechnologies, Inc.), or anti-actin antibody (Santa Cruz Biotechnologies, Inc.) or anti-tubulin antibody (Cedarlane Laboratories Ltd.) followed by washing. The protein content was visualized using horseradish-conjugated secondary antibodies followed by enhanced chemiluminesence (PerkinElmer Life Sciences).

2.4. RT-PCR

Total cellular RNA was extracted from the cells using TRIzol reagent (Invitrogen). cDNA was synthesized using random hexamer (Amersham) with Superscript RNase H-reverse transcriptase (GIBCO-BRL). The reverse-transcribed cDNA from each sample was subjected to PCR amplification using Taq polymerase (Promega) and primers. The sequences of the Cdc25B and β-actin primers and PCR condition were as described previously [13]. Amplified products were separated by 2% agarose gel electrophoresis, and bands were visualized by staining with ethidium bromide.

To confirm our semi-quantitative PCR results, we performed quantitative RT-PCR with the LightCycler FastStart DNA SYBR Green kit (Roche Applied Science). The primer sets were designed for Cdc25B as described previously [22]. To control for specificity of the amplification products, we conducted a melting curve analysis. The number of transcripts was calculated from a standard curve obtained by plotting known input of six different concentrations to the PCR cycle number at which the detected fluorescence intensity reaches a fixed value (z). The copy number of Cdc25B was normalized by the housekeeping gene GAPDH.

2.5. Immunoprecipitation

To examine functional consequence of elevated Cdc25B, we investigated the phosphorylated cyclin dependent kinase in cells after EGF treatment with or without a previously described Cdc25B inhibitor DA-3003-2 [23]. We treated A549 cells for 1 h with 10 ng/ml of EGF in the presence or absence of DA-3003-2 (10 μM). The phosphorylation status of Cdk1 was determined using our previously described methods [13]. Briefly, cyclin B1 was immunoprecipitated from 1 mg of whole cell lysate using an overnight incubation with an anti-cyclin B1 monoclonal antibody (Santa Cruz Biotechnologies, Inc.) with protein G-agarose (Santa Cruz Biotechnologies, Inc.). The protein G-agarose pellets were washed three times with PBS, and boiled in 20 μl loading buffer for 5 min. The samples were subjected to SDS PAGE and transferred to nitrocellulose membrane. After protein transfer, blots were incubated with the blocking solution and probed for anti-Cdk1 antibody (Santa Cruz Biotechnologies, Inc.), anti-tyrosine[15] residue phosphorylated Cdk1 antibody (Santa Cruz Biotechnologies, Inc.), followed by washing. The protein content was visualized using horseradish-conjugated secondary antibodies followed by enhanced chemiluminesence (PerkinElmer Life Sciences).

3. Results

We first determined the effect of EGF or TGF-α on the growth rate of EGFR positive-cancer cells when grown in complete medium with 10% FBS using a MTT assay. All three cell lines have similar population doubling times of 20–22 h. In preliminary studies we observed substantial tyrosine phosphorylation of the immunoprecipitated EGFR and Erk phosphorylation in A549 cells after treatment with 10 ng/ml of EGF (data not shown) and this concentration of EGF was used for the subsequent cellular studies. Three days after EGF (10 ng/ml) exposure, the population cell number of EGF-treated A549 and SCC25 cells was 30% and 80% ($p < 0.05$) greater than that seen with the untreated cells (Fig. 1A). In contrast, EGF treatment did not increase the number of MDA-MB-231 cells (Fig. 1A). Three days after TGF-α (10 ng/ml) exposure, the population cell number of TGF-α-treated A549 and SCC25 cells was 40% and 30% ($p < 0.05$) greater than that seen with the untreated cells; in contrast, TGF-α treatment did not increase the number of MDA-MB-231 cells (Fig. 1B). These results confirm previous studies showing that MDA-MB-231 cells are not responsive to EGF or TGF-α, although they contain significant levels of EGFR [24].

We next examined Cdc25B and Cdc25A expression in these cancer cells after EGF or TGF-α exposure. Cdc25B expression was increased within 1 h after EGF (10 ng/ml) or TGF-α (10 ng/ml) treatment of A549 and SCC25 cells,

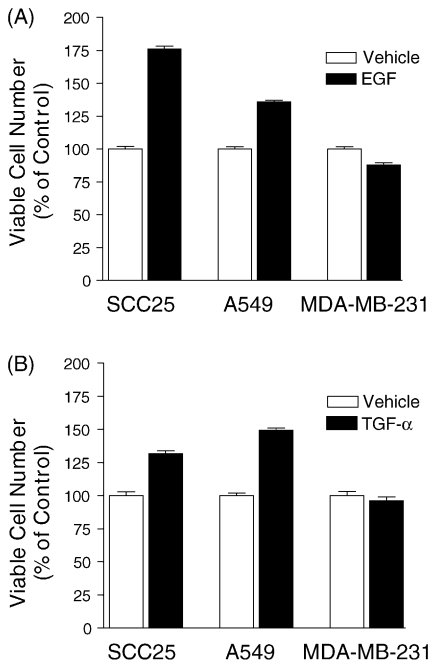

Fig. 1. EGF and TGF-α stimulated cell proliferation. Panel A. Cell growth of EGF- or vehicle-treated cells. Cells were plated in 96-well microtiter plates with RPMI 1640 and 10% FBS. After 24 h, cells were treated with 10 ng/ml EGF or vehicle control. After 72 h, cell viability was assayed using MTT. Mean value of 12 determinations and bars are S.D. All three cell lines have similar population doubling times. Panel B. Cell growth of TGF-α- or vehicle-treated cells as measured by a MTT assay. Cells were plated and treated with 10 ng/ml TGF-α as in Panel A. Mean value of 12 determinations and bars are S.D.

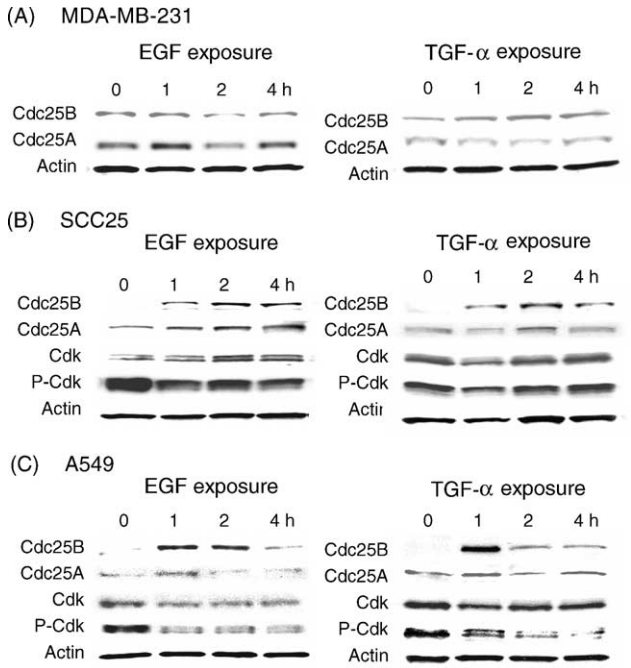

Fig. 2. The effect of EGF and TGF-α on Cdc25B phosphatase expression. Cells were treated with 10 ng/ml of EGF or 10 ng/ml of TGF-α for 0–4 h. Panel A. Western blot analysis for Cdc25A, Cdc25B and actin expression in MDA-MB-231 cells after EGF or TGF-α exposure. Panel B. Western blot analysis for Cdc25A, Cdc25B, Cdk1, phosphorylated-Cdk, and actin in SCC25 cells after EGF or TGF-α exposure. Panel C. Western blot analysis for Cdc25A, Cdc25B, Cdk1, phosphorylated-Cdk, and actin in A549 cells after EGF or TGF-α exposure. This is representative of two independent experiments.

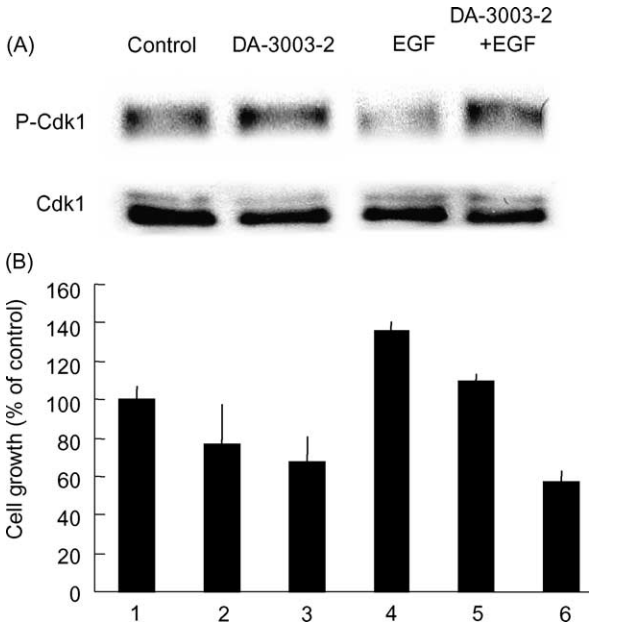

Fig. 3. Dephosphorylation of Cdk1 after EGF stimulated and inhibition by DA-3003-2. Panel A. Cdk1 phosphorylation after treatment with EGF in the presence or absence of DA-3003-2. A549 cells were treated for 1 h with vehicle control (C) or 10 ng/ml of EGF in the presence or absence of DA-3003-2 (10 μM). Protein lysates were isolated and 1 mg of whole cell lysate was immunoprecipitated with an anti-cyclin B1 monoclonal antibody. The samples were subjected to SDS PAGE and transferred to nitrocellulose membrane. After protein transfer, blots were incubated with the blocking solution and probed an anti-Cdk1 antibody and anti-tyrosine[15] residue phosphorylated Cdk1 antibody. Panel B. Cell growth of A549 cells after treatment of DA-3003-2 or EGF with DA-3003-2. A549 cells were plated in 96-well microtiter plates with RPMI 1640 and 10% FBS. After 24 h, cells were treated with vehicle control (lane 1), 3 μM DA-3003-2 (lane 2), 5 μM DA-3003-2 (lane 3), 10 ng/ml EGF (lane 4), or 10 ng/ml EGF with 3 μM DA-3003-2 (lane 5) or 5 μM DA-3003-2 (lane 6). After 72 h, cell viability was assayed using MTT. Mean value of 12 determinations and bars are S.D.

but not MDA-MB-231 cells (Fig. 2). Interestingly, MDA-MB-231 cells appeared to have higher basal Cdc25B levels than either SCC25 or A549 cells. Cdc25A levels either were transiently increased slightly or were not markedly increased in A549, SCC25 or MDA-MB-231 cells after a similar EGF or TGF-α exposure (Fig. 2). These results are in accord with the study of Abdollahi et al. [20], who observed no marked increase in Cdc25A protein levels in rat ovarian cells treated with 250 ng/ml of EGF. To assess the potential functional consequence of elevated Cdc25B levels, we examined the phosphorylation status of the known Cdc25B substrate Cdk and found decreased Cdk phosphorylation in A549 and SCC25 cells, coincident with the increased Cdc25B levels (Fig. 2B and C). Decreased Cdk1 phosphorylation was also detected after immunoprecipitation of protein lysates from A549 cells treated for 1 h with 10 ng/ml EGF (Fig. 3A) and this effect was blocked when cells were co-treated with the Cdc25B inhibitor DA-3003-2. A 3-day treatment of this same concentration of DA-3003-2 blocked the EFG-induced increase in A549 cell proliferation (Fig. 3B).

To probe the involvement of the EGFR/MAPK signaling cascade in Cdc25B induction after EGF or TGF-α treatment, we pre-treated cells with an EGFR-selective inhibitor AG1478 or the MEK-specific inhibitor U0126. We found that a 30 min pre-treatment with AG1478 (1 μM) completely inhibited the increased expression of Cdc25B in both A549 and SCC25 cells (Fig. 4A and B). In SCC25 cells, Cdc25B expression was decreased with a 10 μM U0126 pre-treatment and was completely blocked with a 50 μM U0126 pre-treatment after EGF. Cdc25B expression was not detected in SCC25 cells pre-treated with 10 μM of U0126 after TGF-α exposure (Fig. 4A). Cdc25B expression was not detected in A549 cells pre-treated with 10 μM of U0126 and treated with either EGF or TGF-α (Fig. 4B).

We found no marked changes in Cdc25B mRNA levels in A549 and SCC25 cells as measured by RT-PCR after a 1–4 h exposure to 10 ng/ml of EGF or 10 ng/ml of TGF-α exposure (Fig. 5A). We also used quantitative RT-PCR to measure mRNA expression levels and normalized them to GAPDH as an internal control and found no marked increase in Cdc25B mRNA in A549 cells (Fig. 5C). A 30 min pre-treatment of A549 and SCC25 cells with 25 μg/ml of the protein synthesis inhibitor cyclohexamide blocked the increase in Cdc25B expression levels mediated

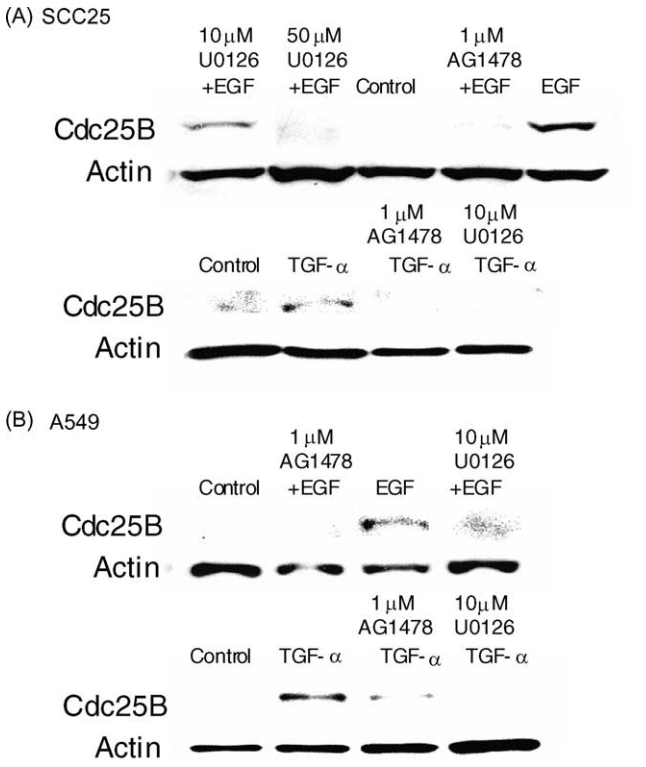

Fig. 4. Involvement of the MAPK pathway in the EGF and TGF-α-mediated Cdc25B induction. A549 and SCC25 cells were pre-treated with 1 μM AG1478 or 10 or 50 μM U0126 for 30 min before EGF treatment. Protein lysates were isolated and probed by Western blotting as described in the Section 2. This is representative of two independent experiments.

by EGF and TGF-α, indicating the need for de novo protein synthesis (Fig. 5B). These results are most consistent with the hypothesis that induction of Cdc25B protein expression was a post-transcriptional phenomenon, although we cannot exclude some level of transcriptional regulation.

Because EGFR-selective tyrosine kinase inhibitors can block ErbB2 as well as the EGFR, we repeated the experiments using embryonic fibroblasts derived from mice, in which EGFR (ErbB1) had been deleted and their wild-type littermates. As illustrated in Fig. 6, treatment of wild-type mouse embryonic fibroblasts with 10 ng/ml EGF for 1 or 2 h caused a robust induction of Cdc25B protein levels of 6.5 (±2.7, S.E.M.)- and 4.4 (±1.6)-fold, respectively, when normalized to tubulin levels; in contrast, we observed no similar induction in the EGFR knockout fibroblasts where the Cdc25B levels after 1 or 2 h of EGF treatment were 1.2 (±0.3) and 1.0 (±0.3)-fold, respectively. Interestingly, fibroblasts derived from mice lacking the EGF receptor appeared to have higher basal Cdc25B levels. We speculate that the elevated basal Cdc25B levels could be due to a founder effect or to compensatory mechanisms that emerge during the generation of the null fibroblasts but this will require additional mechanistic studies to resolve. These results suggest that a functional EGFR had an important role in regulating Cdc25B expression following EGF stimulation.

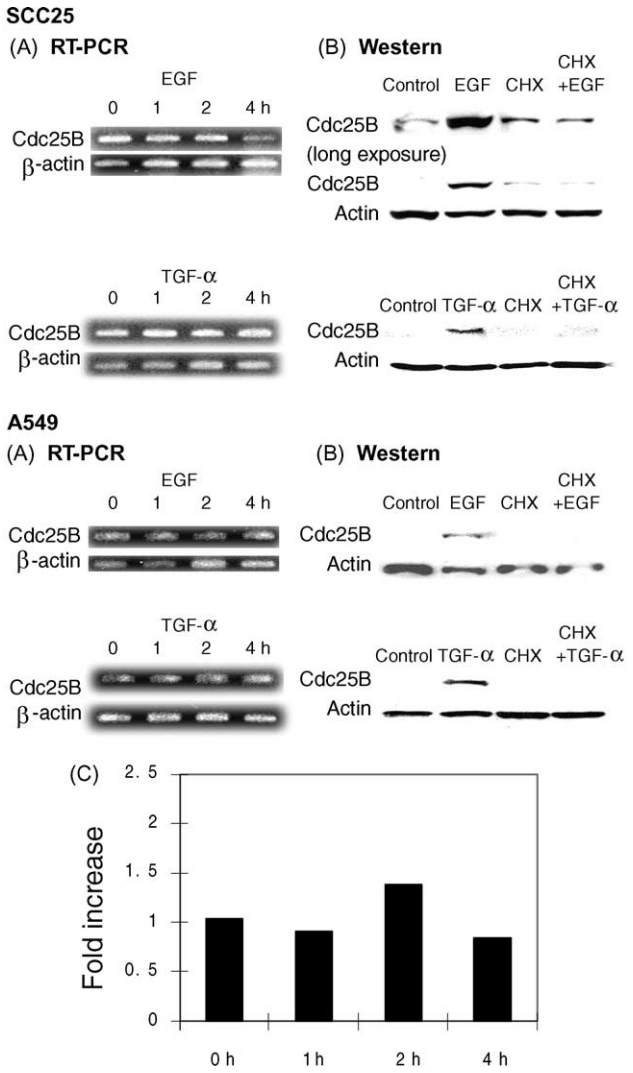

Fig. 5. Regulation of Cdc25B expression by EGF and TGF-α. Panel A. RT-PCR for Cdc25B expression in A549 and SCC25B cells after EGF or TGF-α exposure. Cells were treated with 10 ng/ml of EGF or 10 ng/ml of TGF-α for 0–4 h. Panel B. The effect of 25 μg/ml of cyclohexamide treatment on Cdc25B levels after EGF or TGF-α treatment analyzed by Western blotting as described in Section 2. This is representative of two independent experiments. Panel C. Quantitative RT-PCR for mRNA expression levels of Cdc25B in A549 cells. Cells were exposed to 10 ng/ml EGF for 0–4 h and mRNA levels determined with PCR primer sets as described in Section 2. GAPDH was used as an internal control. The ordinate indicates fold increase.

4. Discussion

Cdc25 dual specificity phosphatases control cell cycle progression through dephosphorylation and activation of Cdks [4]. Although the regulation of Cdc25B expression by growth factors has not previously been reported, TGF-β-mediated growth inhibition has been linked to Cdc25A inactivation through activation of RhoA and $p160^{ROCK}$ [25]. This inhibition of Cdc25A activity leads to Cdk2 inactivation [23]. Although there has been some speculation that Cdc25 might influence pathways important for mitogenesis [9,10], our study is the first to document

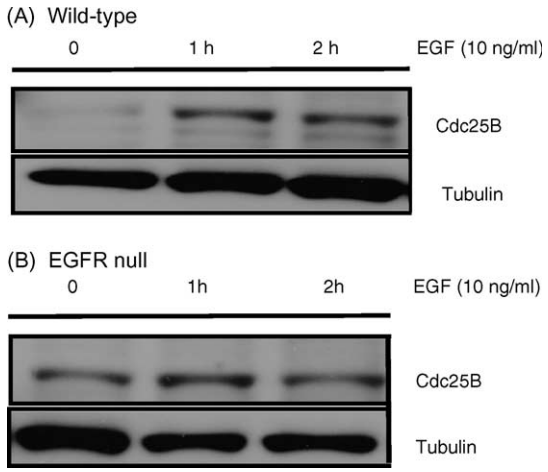

Fig. 6. Requirement for EGFR for EGFmediated Cdc25B induction. Primary mouse embryonic fibroblasts derived from EGFR knockout mice and corresponding wild-type littermates were treated with 10 ng/ml of EGF for 0–2 h. After the wild-type (Panel A) and EGFR knockout (Panel B) cells were washed, whole cell lysates were obtained and cellular proteins were subjected to SDS PAGE, transferred to nitrocellulose membrane and analyzed by Western blotting for Cdc25B and tubulin.

induction of the dual specific phosphatase Cdc25B by a growth factor in cancer cells.

EGF-related peptide growth factors and EGFR control cell proliferation and death in cancer cells [1]. Although most studies have focused on the role of EGF-related peptide growth factors and EGFR in mitogenesis, EGFR activation can control G2/M progression and prevent apoptosis [26]. Our study is of interest because we observed a rapid induction of Cdc25B, which is known to have a central role in coordinating G2/M-phase transition and possibly S-phase by regulating the phosphorylation status and activity of Cdk1 and Cdk2 [4,14]. This induction occurred with a loss of Cdk1 phosphorylation as illustrated by the immunoprecipitation results (Fig. 3A). Furthermore, the antibody used in our whole cell lysate Western blot studies was primarily directed against phospho-Cdk1, so it also may recognize phospho-Cdk2. Both Cdk1 and Cdk2 are potential substrates of Cdc25B. Thus, the decreased Cdk phosphorylation seen after exposure of EGF-related peptide growth factors could be a result of the subsequent increase in Cdc25B activity. The ability of the Cdc25B inhibitor DA-3003-2 to block the hypophosphorylation further supports this conclusion. The increase in Cdc25B expression provides an attractive alternative hypothesis for the facilitating role of EGF-related peptide growth factors may have on cell proliferation because the activation of Cdk may release cells from normal cell cycle checkpoint controls.

It is noteworthy that all of our studies have been conducted in the presence of serum. We have also examined Cdc25B expression after EGF or TGF-α treatment in serum free culture conditions, but we have not found Cdc25B induction (data not shown). Thus, the induction of Cdc25B by EGF and TGF-α may reflect the need to have additional growth factors present or require cell proliferation but this will require additional studies to clarify.

The ErbB signaling network involves a complex of interactive linear cascades including the MAPK pathway, the stress-activated pathway, protein kinase C and, the Akt pathway [27]. Our pharmacological results showing the EGFR tyrosine kinase inhibitor AG1478 and the MEK inhibitor U0126 inhibited increased expression of Cdc25B after exposure growth factors suggest that up-regulation of Cdc25B may be mediated by the MAPK pathway. The studies with murine fibroblasts suggest that a functional ErbB1 receptor may be required for the Cdc25B induction. Although we cannot exclude growth factor-mediated transcriptional activation of Cdc25B at this time, the kinetics of Cdc25B induction were rapid and we favor a post-transcriptional phenomenon. Further studies will be required to elucidate the precise mechanism of up-regulation of Cdc25B through MAPK pathway.

Acknowledgments

We thank Alexander P. Ducruet for his helpful comments and critical review of this manuscript. We are thankful for the assistance of Dr. Hiroyuki Achiwa with the quantitative RT-PCR. These studies were supported in part by NIH grant CA78039 and the Fiske Drug Discovery Fund.

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