Glypican-1 Regulates Anaphase Promoting Complex/Cyclosome Substrates and Cell Cycle Progression in Endothelial Cells

Dianhua Qiao,* Xinhai Yang,* Kristy Meyer,* and Andreas Friedl*[†]

*Department of Pathology and Laboratory Medicine, University of Wisconsin, Madison, WI 53792; and

[†]Pathology and Laboratory Medicine Service, Department of Veterans Affairs Medical Center, Madison, WI 53705

Submitted October 10, 2007; Revised March 24, 2008; Accepted April 9, 2008

Monitoring Editor: Richard Assoian

Glypican-1 (GPC1), a member of the mammalian glypican family of heparan sulfate proteoglycans, is highly expressed in glioma blood vessel endothelial cells (ECs). In this study, we investigated the role of GPC1 in EC replication by manipulating GPC1 expression in cultured mouse brain ECs. Moderate GPC1 overexpression stimulates EC growth, but proliferation is significantly suppressed when GPC1 expression is either knocked down or the molecule is highly overexpressed. Flow cytometric and biochemical analyses show that high or low expression of GPC1 causes cell cycle arrest at mitosis or the G2 phase of the cell cycle, accompanied by endoreduplication and consequently polyploidization. We further show that GPC1 inhibits the anaphase-promoting complex/cyclosome (APC/C)-mediated degradation of mitotic cyclins and securin. High levels of GPC1 induce metaphase arrest and centrosome overproduction, alterations that are mimicked by overexpression of cyclin B1 and cyclin A, respectively. These observations suggest that GPC1 regulates EC cell cycle progression at least partially by modulating APC/C-mediated degradation of mitotic cyclins and securin.

INTRODUCTION

Glypicans constitute a family of heparan sulfate proteoglycans (HSPGs) that are attached to the extracellular surface of the plasma membrane through a glycosyl-phosphatidylinositol (GPI) anchor. Six members of mammalian glypicans (GPC1 to GPC6) and two members of *Drosophila* glypicans (*division abnormally delayed* [*dally*] and *dally-like*) have been identified. Unlike the syndecan family of HSPGs, which are modified by both heparan sulfate (HS) and chondroitin sulfate (CS) glycosaminoglycan (GAG) chains on several attachment sites spanning the extracellular domain, glypicans are decorated only by HS chains at adjacent GAG modification sites localized ~50 amino acids external to the GPI anchor (Filmus and Selleck, 2001; Song and Filmus, 2002). Glypicans, and HSPGs in general, serve as carriers and coreceptors for different HS binding growth factors and morphogens, including fibroblast growth factors (FGFs), heparin-binding epidermal growth factor-like growth factor, Wnts, transforming growth factor- β /bone morphogenetic proteins, and Hedgehog (Whitelock and Iozzo, 2005). However, the characteristic structure of glypicans endows them with several distinctive functional properties. Because of

their GPI anchorage, glypicans are actively and selectively released from the membrane by phospholipases (Song and Filmus, 2002). Moreover, the proximity of the HS chains to the cell surface may make them more accessible to integral membrane proteins (e.g., receptors) in contrast to other HSPGs (Tumova *et al.*, 2000). In addition, glypicans may preferentially localize to cholesterol-rich lipid rafts and caveolae in the membrane, leading to enhanced cell signaling, active endocytosis, and recycling (Ilanguvaran *et al.*, 2000; Cheng *et al.*, 2002). Glypican endocytosis and recycling play an important role in cellular glypican HS remodeling and polyamine and basic peptide uptake (Cheng *et al.*, 2002; Belting *et al.*, 2003).

Glypicans are expressed during normal development in a stage- and tissue-specific manner, suggesting their involvement in morphogenesis (Filmus and Selleck, 2001; Song and Filmus, 2002). This critical role is well established for *dally* in *Drosophila* and GPC3 in vertebrates, because mutations of these glypicans are directly linked to developmental defects and to abnormal overgrowth in Simpson-Golabi-Behmel syndrome, respectively (Nakato *et al.*, 1995; Jakubovic and Jothy, 2007). In adults, increasing evidence implicates glypicans in cancer development. GPC3 seems to play a dual role both as an oncofetal protein in hepatocellular carcinoma (Yamauchi *et al.*, 2005) and as a tumor suppressor in ovarian carcinoma (Lin *et al.*, 1999), malignant mesothelioma (Murthy *et al.*, 2000), and breast cancer (Xiang *et al.*, 2001). A tumor-promoting effect has been ascribed to GPC1 in several human malignancies, including pancreatic cancer (Kleeff *et al.*, 1999), glioma (Qiao *et al.*, 2003; Su *et al.*, 2006), and breast cancer (Matsuda *et al.*, 2001). One possible explanation for this dual role of GPCs in growth control and tumorigenesis would be their ability to act as coreceptors for both tumor growth-promoting and -inhibiting factors.

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E07-10-1025>) on April 30, 2008.

Address correspondence to: Andreas Friedl (afriedl@wisc.edu).

Abbreviations used: APC/C, anaphase promoting complex/cyclosome; BMVEC, brain microvascular endothelial cell; CDK, cyclin-dependent kinase; *dally*, *division abnormally delayed*; EC, endothelial cell; GPC, glypican; HS, heparan sulfate; HSPG, heparan sulfate proteoglycan; PKA, protein kinase A; SCF, Skp1-Cullin-F-box protein complex; SDC1, syndecan-1; shRNA, small hairpin RNA.

We described recently that GPC1 is overexpressed in human glioma blood vessel endothelial cells (ECs), whereas it is consistently undetectable in normal brain blood vessel ECs (Qiao *et al.*, 2003). The introduction of modest levels of exogenous GPC1 into mouse brain ECs *in vitro* by stable transfection stimulated cell growth and a mitogenic response to FGF2, a potent angiogenic factor in gliomas. The present study examines whether GPC1 is required for EC proliferation, describes the phenotype of GPC1-overexpressing cells, and explores the mechanisms of GPC1-mediated cell cycle regulation.

MATERIALS AND METHODS

Reagents and Plasmids

Dibutyl cAMP (DBcAMP) and KT5720 were from Sigma-Aldrich (St. Louis, MO). Antibodies for cyclin B1, A, and E; phospho-p38 (Tyr 182); phospho-JNK (Thr 183 and Tyr 185); Cdc2; phospho-Cdc2 (Tyr 15); Cdc20; p27; and p21 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against securin, BubR1, Cdh1, and APC2 were purchased from Abcam (Cambridge, MA). Antibodies for phospho-Src (Tyr 416) and total focal adhesion kinase (FAK) were from Upstate Biotechnology (Charlottesville, VA). Antibodies for phospho-Akt (Ser 473) and phospho-FAK (Tyr 397) were from Cell Signaling Technology (Danvers, MA) and BD Biosciences (Palo Alto, CA), respectively. Antibodies for Emi1 were from Invitrogen (Carlsbad, CA). pBS/U6, which was generated by cloning the mouse U6 RNA promoter sequence (1-315 base pairs, GenBank accession no. X06980) into pBluescript II SK (+/-) (Stratagene, La Jolla, CA) at KpnI and ApaI, was kindly provided by Dr. Yang Shi (Department of Pathology, Harvard Medical School, Boston, MA). The cDNA clones of mouse GPC1, cyclin B1, and cyclin A were purchased from Open Biosystems (Huntsville, AL).

Cell Culture, Treatment, and Transfection

Mouse microvascular endothelial cells derived from various organ sites of *H-2K^b-tsA58* ImmortoMice (Langley *et al.*, 2003) were provided by Dr. Isaiah J. Fidler (University of Texas, M. D. Anderson Cancer Center, Houston, TX), and they were grown in DMEM supplemented with 10% fetal bovine serum, 2-mM L-glutamine, 100 U/ml penicillin/streptomycin, sodium pyruvate, nonessential amino acids, and a vitamin solution (Invitrogen, Rockville, MD). Human dermal primary microvascular endothelial cells (HMVECs) were from Cambrex (East Rutherford, NJ), and they were grown in EGM-2 endothelial cell growth medium (Cambrex). Transient transfection of mouse brain microvascular endothelial cells (BMVECs) was conducted using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Generation of Adenoviruses for Gene Silencing and Overexpression

Adenoviruses containing one of two independent GPC1 small hairpin RNAs (shRNAs) (Ad-Sh-1 and Ad-Sh-2) or their scrambled controls (Ad-Sc-1 and Ad-Sc-2) were generated. Sense and antisense oligonucleotides, each containing a reverse repeat of one shRNA target sequence (21 or 22 nucleotides) interrupted by a six nucleotide spacer (AAGCTT) and tailed with a RNA polymerase III-specific terminator (TTTTT), were synthesized and annealed (Table 1). These double-strand oligonucleotides were cloned into the pBS/U6 vector at the ApaI (blunted) and EcoRI sites. A fragment containing "U6 RNA promoter-shRNA coding sequence-terminator" cassette was excised and cloned into the pDNR-1r vector (Clontech, Palo Alto, CA) at the SmaI and PstI sites, and they were subsequently inserted into the pLP-Adeno-X-PRLS adenoviral vector according to the manufacturer's instructions (Clontech). The recombinant adenoviral DNA was linearized by PacI and transfected into 293-A cells (Invitrogen) to produce adenovirus particles, which were subsequently amplified and titered according to the manufacturer's instructions

(Invitrogen). To generate GPC1 adenoviruses, a fragment of the pcDNA3.1/myc-his B vector containing the "CMV promoter-MCS-poly (A) signal" sequences (Invitrogen) was cloned into pDNR-1r at SmaI and XbaI. Then, a 2.5-kb wild-type or mutant mouse GPC1 cDNA was inserted into this modified pDNR-1r vector at EcoRI and XbaI. Adenoviral DNA recombination and subsequent adenovirus production and titration were performed as described above.

Preparation of Polyclonal Antibodies to Mouse GPC1

To prepare the immunogen, glutathione transferase (GST)-GPC1 fusion protein was expressed in *Escherichia coli* DH5 α and isolated on glutathione agarose beads. The fusion protein was released from the beads using thrombin (Sigma-Aldrich) and separated on a 7.5% SDS-polyacrylamide gel electrophoresis gel. Aliquots of gel slices containing 0.2–0.5 mg of purified GPC1 protein were injected into rabbits to raise polyclonal antiserum by using standard techniques (Harlow and Lane, 1988).

Immunoblot Analysis

Total HSPGs were extracted from cells, digested with heparitinase and chondroitinase ABC, and analyzed by immunoblotting with rabbit anti-mouse GPC1 polyclonal antiserum and antibody 3G10 (Cape Cod, East Falmouth, MA) as described previously (Su *et al.*, 2006). For analysis of total cyclin A, B1, and E, securin, Cdc2, BubR1, Emi1, Cdc20, Cdh1, APC2, p27, p21, and FAK as well as phospho-Cdc2, -p38, -c-Jun NH₂-terminal kinase (JNK), -Akt, -Src, and -FAK, cells were lysed in a phospho-protein lysis buffer, and 50 μ g of total protein was used for immunoblot analysis as described previously (Qiao *et al.*, 2000).

Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNAs were isolated using RNeasy Mini kit (QIAGEN, Valencia, CA), and first-strand cDNAs were synthesized using Omniscript reverse transcription kit (QIAGEN) according to the manufacturer's instructions. To confirm the absence of genomic DNA contamination in the RNA samples, RT reactions excluding reverse transcriptase also were conducted. The RT reactions were diluted in water to 10 ng of total RNA/ μ l, and 5 μ l of the diluted RT reactions was applied to each quantitative PCR reaction. For 18S rRNA amplification, the RT reactions were further diluted 100 times. The quantitative PCR was performed on an iCycler using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and gene-specific PCR primers at 200 nM each (Table 2). The relative mRNA levels between experimental and control samples (R) were determined using the formula $R = (1 + E)^{-\Delta C_t}$, where E is PCR efficiency, C_t is threshold cycle number, and ΔC_t is equal to [C_t of experimental sample] – [C_t of control sample]. The PCR efficiency of each primer pair was determined by serial dilution of the RT reactions. The PCR result was normalized to 18S rRNA.

Flow Cytometry

For DNA profile analysis, cells were stained with propidium iodide (PI; Sigma-Aldrich). For p-histone H3 (Ser 10) analysis, cells were immunofluorescently labeled with mouse anti-p-histone H3 (Ser 10) monoclonal antibody (mAb) (Cell Signaling Technology, Danvers, MA) (1:25) and Alexa488-conjugated goat anti-mouse IgG (Invitrogen) (1:100) according to the manufacturer's instructions. For DNA replication analysis, cells were pulse labeled with 20 μ M 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) for 30 min before harvest. The cells were immunofluorescently stained with mouse anti-BrdU mAb (Sigma-Aldrich) (1:3.5) and Alexa488-conjugated goat anti-mouse IgG (Invitrogen) (1:50) as well as PI by a method of simultaneous nucleus extraction and acid hydrolysis as described previously (Williams *et al.*, 1990). All of the samples were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Rockville, MD), and the data are processed using FlowJo 6 software (FlowJo, Ashland, OR).

Mitotic Index Assay

The mitotic index of the adenovirus-treated cells was determined by flow cytometric analysis of p-histone H3 (as described above) or by Giemsa (Sigma-

Table 1. Oligonucleotides synthesized for GPC1 shRNA adenovirus generation

Virus	Sequence
Ad-Sh-1	5'-GGTGAGCACCTGCGGATCTGAAGCTTCAGATCCGCAGGTGCTACCCCTTTTGTG-3'
Ad-Sc-1	5'-GGCCGTGCGCAGTGACTGATAAGCTTATCAGTCACTGCGCACGGCCCTTTTGTG-3'
Ad-Sh-2	5'-CAGCGAAGTCCGCCAGATCTAAAGCTTTAGATCTGGCGGACTTCGCTGCTTTTGTG-3'
Ad-Sc-2	5'-GAATCTACTCACAGCGCCAGGAAGCTTCTGGCGCTGTGAGTAGATTCTTTTGTG-3'

All antisense oligonucleotides contain a four-nucleotide 5'-end overhang (AATT) compared with the corresponding sense oligonucleotides (antisense sequences not shown). The shRNA target sequences and their reverse repeats are underlined.

Table 2. The primers used in quantitative RT-PCR

Gene	Primer
GPC1	F: 5'-AGGTGCTCACCCGAGATCTCT-3' R: 5'-CAAATTCTCTCCATCTCACT-3'
Cyclin B1	F: 5'-CTTGACAACGGTGAATGGAC-3' R: 5'-TGACAGTCATGTGCTTTGTGA-3'
Cyclin A	F: 5'-TGCATCAGGAAGACCAAGAG-3' R: 5'-CAACCCGTCGAGTCTTGAG-3'
Securin	F: 5'-GGGAGCACCAGATCTCACTT-3' R: 5'-TCTCCAGCCTCTCTCTTCA-3'
Bub1	F: 5'-GCATGAGCAGTGGGTAGTG-3' R: 5'-AAGCAGGAAGGCTTGTGT-3'
BubR1	F: 5'-ATGACAATCCAGCCTCTGTG-3' R: 5'-TTGCATGGTGTGTCATGACTGT-3'
Bub3	F: 5'-CGAACAAGCAGGGTTATGTG-3' R: 5'-CTTTAGCCTGTGGCACTGA-3'
Mad1	F: 5'-GAAATGGCAAGAGGCAAATC-3' R: 5'-TGCTCCAGGTCTTAATCTTC-3'
Mad2	F: 5'-TGGAAAGTTTCTTGTTCCTTGA-3' R: 5'-TGCTAAATGAGCGTAGACGGA-3'
Emi1	F: 5'-CGTTCAGCTCTACAGCAAA-3' R: 5'-TGCCCACAACATATCCTCTC-3'
18S rRNA	F: 5'-AGCGAAAGCATTGCGCAAGA-3' R: 5'-GGCATCGTTATGGTCGGAA-3'

F, forward primer; R, reverse primer.

Aldrich) staining as described previously (Qi and Martinez, 2003). Mitotic cells stained with Giemsa were observed on a light microscope based on their condensed chromosomes and disintegrated nuclear envelopes. At least 500 cells were randomly counted for each sample.

Immunocytochemistry

For F-actin staining, cells were cultured on poly-D-lysine/collagen-coated coverslips. After treatment, the cells were stained with Alexa488-phalloidin (Invitrogen) (5 U/ml) according to the manufacturer's instructions. For observation of mitosis, floating and rounding cells in treated cell cultures were fully recovered by pipetting and resuspended in 50% calf serum in phosphate-buffered saline. The cells were spun onto slides on Shandon Cytospin (Anatomical Pathology USA, Pittsburgh, PA) at 500 rpm, fixed in 4% paraformaldehyde, and permeabilized in 0.2% Triton X-100. Mitotic spindles and chromosomes were stained with fluorescein isothiocyanate-conjugated mouse anti- β -tubulin mAb (Sigma-Aldrich) (1:50) and PI (1 μ g/ml), respectively. All of the slides were observed on an MRC1024 confocal scanning laser microscope (Bio-Rad) by using LaserSharp 2000 software (Bio-Rad).

RESULTS

Knockdown of GPC1 Expression Suppresses Endothelial Cell Proliferation and Cell Cycle Progression

Previous work from our laboratory has shown that GPC1 is overexpressed in human glioma blood vessel endothelial cells and that moderate overexpression of GPC1 in mouse endothelial cells *in vitro* increases cell growth and response to FGF2 (Qiao *et al.*, 2003). To better understand the role of GPC1 in endothelial cell replication, we suppressed expression of this molecule by vector-based expression of double-strand shRNA. In accordance with published guidelines on siRNA quality assurance, several controls were included (Editorial 2003). To ensure specific gene silencing, two independent shRNA constructs were designed, which share sequence homology solely with mouse GPC1 mRNA and target different regions in the GPC1 transcript. To rule out possible nonspecific effects imposed by expression of exogenous shRNA, scrambled controls for each shRNA also were constructed.

In pilot experiments, transient transfection of either of the two GPC1 shRNA constructs caused significant inhibition of GPC1 expression in mouse BMVECs, whereas their scram-

bled controls had no effect (data not shown). For more efficient delivery of these constructs and to avoid clonal variation with stable transfection, we cloned these shRNA constructs into an adenoviral DNA vector (Figure 1A). Preliminary tests with adenovirus carrying empty vector DNA failed to show any noticeable effect on cell growth or morphology at doses up to 400 multiplicities of infection (MOI) (data not shown). To determine the effectiveness and the optimum dose of the GPC1 shRNA adenoviruses, BMVECs were treated with increasing doses from 100 to 400 MOI. At 200 MOI, both of the shRNA adenoviruses were able to effectively abolish GPC1 expression at both the protein and mRNA levels within 48 h, whereas the scrambled controls had no obvious effect on the expression of GPC1 or other HSPGs (Figure 1, B–F). Immunoblot analysis with antibody 3G10, which reacts with heparan sulfate stubs after heparinase digestion and therefore detects all HSPGs regardless of the core protein, demonstrated that the gene silencing effect of the shRNAs was specific to GPC1 (Figure 1, B and C). Immunoblot analysis with mouse GPC1-specific antiserum confirmed the identity of the targeted protein as GPC1 (Figure 1, D and E). Because the consensus mechanism of shRNA-mediated gene silencing consists of double-strand shRNA-guided site-specific cleavage of mRNA by the so-called RNA-induced silencing complex (Tang, 2005), quantitative RT-PCR with a primer pair whose amplicon spans the shRNA target sites inside GPC1 mRNA was performed. Approximately 95% of GPC1 mRNA disintegrated within 48 h of transduction with either GPC1 shRNA adenovirus (Figure 1F).

Based on our previous work, which demonstrated a direct relationship between GPC1 expression and cell growth, we predicted that GPC1 expression silencing would inhibit cell proliferation. Indeed, treatment with either GPC1 shRNA adenovirus resulted in cell growth retardation by ~45% after 72 h and 60% after 96 h compared with the scrambled controls (Figure 2A). No obvious cell death, morphologic change, or cell detachment was observed during at least the first 72 h of adenovirus treatment (Supplemental Figure S1A). To gain further insight into the mechanism of mitogenesis inhibition by GPC1 expression silencing, cell cycle analysis was performed by flow cytometry. Both GPC1 shRNA adenoviruses suppressed cell cycle progression, resulting in a transient increase of tetraploid (4N) cells and a progressive accumulation of polyploid (>4N) cells at the expense of the G1 cell fraction (Figure 2, B and C, and Supplemental Figure S1B). Significant apoptosis, characterized by sub-G1 DNA content was observed at 96 h (Figure 2, B and C, and Supplemental Figure S1B). These findings are consistent with a G2 and/or M phase arrest and polyploidization as well as apoptosis in GPC1-deficient ECs.

To test whether GPC1 knockdown induced a sustained mitotic arrest, we determined the mitotic index of the adenovirus-treated cells by flow cytometric analysis of p-histone H3 (Ser 10), a well characterized mitotic marker (Crosio *et al.*, 2002). Treatment with GPC1 shRNA adenovirus induced a significant decrease in the mitotic index in the presence and absence of the microtubule depolymerization agent nocodazole (Figure 2D). A similar result was seen when the mitotic cells were directly visualized by Giemsa staining of condensed chromosomes (data not shown). These observations argue against a sustained mitotic arrest and are instead consistent with a G2 arrest and/or a short mitotic delay followed by mitotic slippage.

To investigate the mechanism of polyploidy induction and to determine whether mitotic slippage, which is usually followed by endoreduplication and polyploidization, has occurred in GPC1 shRNA adenovirus-treated cells, BrdU

Figure 1. Knockdown of GPC1 expression by shRNA in mouse BMVECs. (A) Diagram of GPC1 shRNA adenoviral DNA constructs (for details, see *Materials and Methods*). "I" and "II" represent the target sequences of GPC1 shRNA adenoviruses Ad-Sh-1 and Ad-Sh-2 in mouse GPC1 mRNA, respectively. (B and C) Immunoblot analysis with anti-pan HSPG antibody 3G10. BMVECs were infected for 48 h at different doses as indicated with Ad-Sh-1 or Ad-Sh-2 or their scrambled controls, Ad-Sc-1 and Ad-Sc-2. (D and E) Immunoblot analysis with mouse GPC1-specific antiserum. The same blots as shown in B and C were probed with anti-mouse GPC1 polyclonal antiserum. (F) Quantitative RT-PCR. Total RNAs were isolated from BMVEC cells infected for 48 h with the GPC1 shRNA adenoviruses or their scrambled controls at 200 MOI. Relative GPC1 mRNA levels comparing shRNA and scrambled control-treated cells were determined by quantitative RT-PCR. Each bar represents the mean $\pm$ SE of three independent experiments.

incorporation was measured by flow cytometry after BrdU pulse labeling. Decreased DNA synthesis was seen in the cells with a 2N~4N DNA content after GPC1 silencing, consistent with suppressed proliferation and cell cycle progression (Figure 2E). Unexpectedly, DNA synthesis was significantly increased in polyploid (>4N) cells upon knockdown of GPC1 expression, suggesting tetraploid endoreduplication without subsequent mitosis and/or cytokinesis (Figure 2E). No accumulation of binucleate cells was apparent in the shRNA virus-treated cells, refuting a cytokinesis defect or cell fusion as cause of tetraploidy and polyploidy (data not shown). Together, these results suggest that knockdown of GPC1 expression results in mitotic slippage and subsequent endoreduplication, which alone or together with a G2 arrest contributes to the accumulation of tetraploid and polyploid cells.

To further ascertain that the observed cell cycle alterations were the result of GPC1 knockdown rather than due to nonspecific off-target effects of the shRNA, we attempted the rescue of the cellular phenotype by GPC1 reexpression. Mouse GPC1 cDNA carrying silent mutations in the shRNA target sequence was coexpressed with GPC1 shRNA by adenoviral transduction. The principal cell cycle changes following GPC1 silencing, i.e., the increase in tetra- and polyploid and decrease in G1 cells, were dose-dependently

reversed by the introduction of exogenous GPC1 (Supplemental Figure S2). These results indicate that the cell cycle alterations after GPC1 shRNA treatment could be attributed to GPC1 expression silencing. Interestingly, at the highest GPC1 dose (30 $\times$), the 4N fraction increased again at the expense of G1, supporting the notion that the GPC1 dose is critical for cell cycle regulation (see below).

Flow cytometric cell cycle analysis was repeated in two additional mouse microvascular endothelial cell lines derived from the lung and bone of *H-2K^b-tsA58* mice to determine whether the effects on the cell cycle are cell line specific or whether they represent a more general regulatory mechanism. Primary HMVECs also were included in the analysis to rule out the possibility that the observed cell cycle disturbance was related to the p53-inactivating simian virus 40 large T antigen in cells from *H-2K^b-tsA58* mice. This is unlikely because the experiments were performed at the nonpermissive temperature of 37°C, where the large T antigen is rapidly inactivated.

The cell cycle change induced by silencing of GPC1 expression followed the same trend in the other three cell types, although some quantitative differences were apparent (Supplemental Figures S3 and S4). Specifically, mouse endothelial cells from the lung exhibited a cell cycle change as dramatic as in BMVECs, whereas mouse endothelial cells

Figure 2. Knockdown of GPC1 expression suppresses cell proliferation by inhibiting cell cycle progression. (A) BMVEC cells in six-well plate were treated with different adenoviruses at 200 MOI as indicated. The cells were counted at different times. (B and C) BMVECs were treated with GPC1 shRNA or scrambled control adenovirus at 200 MOI for different times as indicated. The DNA content of the cells was measured by flow cytometry. (D) BMVECs were treated with the adenoviruses at 200 MOI in the absence or presence of 200 ng/ml nocodazole for different times as indicated. The cells were immunofluorescently stained for p-histone H3 (Ser 10) and analyzed by flow cytometry. Nocodazole was added at 24 h after infection. (E) BMVECs were infected with the adenoviruses at 200 MOI for 48 h and pulse labeled with BrdU for 30 min before harvest. BrdU incorporation was analyzed by flow cytometry after immunofluorescence staining for BrdU. Each bar represents the mean $\pm$ SE of three independent experiments. * $p < 0.05$ versus scrambled control.

from bone showed a relatively muted cell cycle change (Supplemental Figure S3). The variability is not unexpected, considering the well-documented heterogeneity among endothelial cells from different organ sites. A considerable increase in tetraploidy was observed in HMVECs after GPC1 shRNA adenovirus transduction at later time points (e.g., at 96 and 120 h) (Supplemental Figure S4). The delayed time course is likely at least in part due to the slow growth rate of these primary cells, although it cannot be ruled out entirely that a more intact p53 pathway and therefore a tighter G1 tetraploidy checkpoint (Andreassen *et al.*, 2003)

may have contributed to reduced polyploidization. In summary, these results indicate that the inhibition of cell cycle progression by GPC1 knockdown is not a cell line-specific effect and suggest that GPC1 is required in endothelial cells for supporting normal cell replication.

Knockdown of GPC1 Expression Down-Regulates Mitotic Cyclins and Securin

The data presented so far are consistent with a G2 arrest and/or mitotic slippage induced by the knockdown of GPC1

expression, which lead to tetraploidy and polyploidy. Both G2/M transition and exit from mitosis are tightly regulated by mitotic cyclins, including cyclins A (A2) and cyclin B1, and the cyclin-dependent kinases CDK2 and Cdc2 (CDK1) (Fung and Poon, 2005). Cyclin A, expressed from late G1 to prometaphase, can activate both CDK2 and Cdc2. The cyclin A/CDK complexes are considered essential for the cell cycle progression in both S and G2 phases of the cell cycle (Yam *et al.*, 2002). Cyclin B1, expressed from mid-S to metaphase, can only activate Cdc2, and the cyclin B1/Cdc2 complex (also called M phase-promoting factor [MPF]) dominantly controls G2/M transition by phosphorylating substrates that are critical for entry into mitosis (Porter and Donoghue, 2003). Additionally, anaphase-promoting complex/cyclosome (APC/C) mediated ubiquitin-proteosomal degradation of both cyclin A and cyclin B1 is a prerequisite for normal exit from mitosis (Peters, 2006). APC/C-mediated degradation of securin, a small protein which inhibits the protease separase and thus stabilizes cohesion and inhibits sister chromatid segregation, also is required (Peters, 2006).

In addition to the dynamic regulation of mitotic cyclins, the activity of the mitotic CDKs, especially the cyclin B1/Cdc2 complex, is frequently regulated by altering the phosphorylation status of the CDK kinase proteins. Phosphorylation of Cdc2 at Thr 14 and Tyr 15 by Wee1 and Myt1 maintains the kinase in an inactive state before mitosis. The removal of these phosphate substitutions by Cdc25 phosphatases has been shown to be a critical step in cyclin B1/Cdc2 activation and entry into mitosis (Porter and Donoghue, 2003). To determine whether a G2 arrest might be a plausible explanation for the observed cell cycle inhibition, we investigated the effect of GPC1 knockdown on mitotic cyclins and Cdc2. Neither the protein level of Cdc2 nor its phosphorylation at Tyr 15 was affected by GPC1 knockdown (Figure 3A). In contrast, the cyclin B1 protein level was dramatically reduced as early as 36 h after infection with GPC1 shRNA virus, whereas a significant decrease in securin protein level was noticed after 48 h (Figure 3B). A moderate decline in cyclin A protein level was also observed after 48 h in GPC1 shRNA adenovirus-treated cells compared with control virus infection (Figure 3B). Conversely, the protein level of cyclin E, which is normally highly expressed at the G1/S boundary and degraded shortly afterward by non-APC/C ubiquitin ligase-mediated proteolysis (Welcker and Clurman, 2005), was unaltered (Figure 3B). A similar change in the mitotic cyclins was also observed in primary human dermal microvascular endothelial cells (Supplemental Figure S4D).

Because transcriptional regulation also plays a role in the control of the protein levels of mitotic cyclins and securin (Fung and Poon, 2005), the mRNA levels of cyclin A, cyclin B1 and securin in adenovirus treated cells were examined by quantitative RT-PCR. Treatment with GPC1 shRNA virus did not lead to a significant change in the mRNA levels (Figure 3C), suggesting that the transcription of these genes was not affected. Thus, the inhibition of GPC1 expression apparently accelerates APC/C-mediated proteolytic degradation of cyclin B1, cyclin A, and securin.

p27^{Kip1} and p21^{Cip1}, the two prominent CDK inhibitors for both CDK2 and Cdc2 during interphase, are also regulated by APC/C activity and could induce G1 or G2 arrest dependent on the timing of their accumulation. These molecules are targeted by the Skp1-Cullin-F-box protein (SCF) E3 ubiquitin ligase complex SCF^{Skp2} for ubiquitin-proteosomal degradation. SCF^{Skp2} activity is further regulated by

Figure 3. Knockdown of GPC1 expression down-regulates mitotic cyclins and securin but up-regulates p27 and p21. BMVECs were treated with GPC1 shRNA or scrambled control adenovirus at 200 MOI for different times as indicated. (A, B, and D) The protein levels of p-cdc2 and cdc2 (A); cyclins B1, A, and E, and securin (B); and p27 and p21 (D) in the cells were determined by immunoblot analysis, with β -actin as loading control. (C) Total RNAs were isolated from the cells at 48 h after infection, and quantitative RT-PCR was carried out for cyclin B1, cyclin A, and securin. The relative mRNA levels of these genes comparing shRNA and control virus treatments were determined. Each bar represents the mean $\pm$ SE of three independent experiments.

APC/C^{Cdh1} through ubiquitinating the F-box protein Skp2 (Bornstein *et al.*, 2003; Bashir *et al.*, 2004; Nakayama *et al.*, 2004). Thus, an increased APC/C^{Cdh1} activity induced by knockdown of GPC1 could lead to stabilization of p27 and p21 proteins. A significant increase in the protein levels of these CDK inhibitors was seen in GPC1 shRNA adenovirus-treated cells (Figure 3D), consistent with the reduction in mitotic cyclins and securin (Figure 3B) as well as an elevated APC/C activity.

Overexpression of GPC1 Affects Cell Growth and Cell Cycle Progression

The GPC1 knockdown experiments indicate an unexpected requirement of this molecule for proper cell cycle progres-

sion. To determine whether GPC1 regulates the cell cycle in a dose-dependent manner and to gain further insight into potential mechanisms of action, we examined the effect of forced overexpression of GPC1 on proliferating ECs. GPC1

Figure 4. Overexpression of GPC1 affects cell proliferation and cell cycle progression. BMVECs were treated with GPC1 or control adenovirus for 48 or 72 h at different MOI as indicated, and the alterations in cell proliferation and cell cycle progression were analyzed. (A) HSPG expression in the cells was measured by immunoblot analysis, with antibody 3G10 at 48 h after infection. (B) Phase contrast images of the cells at 48 h after infection. Magnification, 40 $\times$. (C) Cell proliferation. The cells were infected with the adenoviruses for 48 or 72 h as indicated, and the cells were counted. (D) The DNA profile of the cells was analyzed by flow cytometry at 48 h after infection. (E) The cells were treated with the adenoviruses for 48 h, and then immunofluorescence staining for p-histone H3 (Ser 10), and flow cytometry were performed. (F) The cells were treated with the adenoviruses for 48 h and pulse labeled with BrdU for 30 min before harvest. BrdU incorporation was analyzed by flow cytometry after immunofluorescence staining against BrdU. Each bar represents the mean $\pm$ SE of three independent experiments. * $p < 0.05$ versus control virus.

expression levels were titrated by treating BMVECs with different doses of mouse GPC1 adenovirus or empty adenovirus control (Figure 4A). Forced expression of GPC1 led to dose-dependent effects on proliferation and cell cycle progression.

Infection with 5 MOI of GPC1 adenovirus slightly stimulated cell proliferation, whereas treatment with >10 MOI of GPC1 adenovirus led to cell growth inhibition accompanied by cell rounding and detachment from the tissue culture surface (Figure 4, B and C). Further analysis by flow cytometry demonstrated a perturbed DNA profile in GPC1-overexpressing cells (Figure 4D). The cells treated with 5 MOI of GPC1 adenovirus had a relatively normal DNA profile with only a slight reduction in G1 phase cells. However, treatment with higher doses of GPC1 adenovirus induced progressive accumulation of an S phase-like subpopulation and aneuploidy as well as apoptosis, accompanied by a reduction in G1 phase cells. As a control, the cells were also transduced with increasing doses of an adenovirus containing the cell surface HSPG mouse syndecan-1 (SDC1). Importantly, even high doses of SDC1 (up to 100 MOI) had no effect on cell growth and cell cycle progression (data not shown). The mitotic index assay using flow cytometric detection of p-histone H3 (Ser 10) demonstrated an increase in the fraction of mitotic cells in GPC1-overexpressing cells at all dose levels (Figure 4E). Together with our prior observations, this result suggests accelerated cell proliferation at low GPC1 dose and mitotic arrest at higher GPC1 dose.

DNA synthesis was examined by flow cytometry following BrdU pulse labeling in cells with different DNA content to better characterize the cell cycle alterations (Figure 4F). In cells with a DNA content of 2N~4N, low levels of GPC1 stimulated DNA synthesis, whereas higher GPC1 levels (doses of 25 or 50 MOI) suppressed DNA synthesis. In cells with a DNA content of >4N, DNA synthesis was stimulated at all GPC1 doses, suggesting tetraploid endoreduplication without subsequent mitosis, cytokinesis, or both. The decrease in DNA synthesis in the cells with 2N~4N DNA content upon high level GPC1 overexpression, in contrast, suggests that the increased S phase-like subpopulation shown in Figure 4D was possibly the result of either apoptosis of higher-DNA content cells ($\geq 4N$) or due to completed abnormal mitosis with unequal chromosome distribution between daughter cells. Together, these results suggest that low-level overexpression of GPC1 leads to increased cell proliferation, whereas high-level overexpression of GPC1 causes mitotic arrest, which subsequently progresses to aneuploidy and apoptosis.

Overexpression of GPC1 Up-Regulates Mitotic Cyclins and Securin

Since the degradation of mitotic cyclins and securin was stimulated by knockdown of GPC1 expression (Figure 3), we analyzed the effect of GPC1 overexpression on the protein levels of mitotic cyclins and securin as well as Cdc2. As in the GPC1 knockdown experiments, neither total protein level of Cdc2 nor its phosphorylation at Tyr 15 were affected by overexpression of GPC1 (Figure 5A). However, consistent with the GPC1 knockdown experiments, the protein levels of cyclin B1, cyclin A, and securin were significantly increased following GPC1 overexpression in a dose-dependent manner (Figure 5B). The protein level of APC-independent cyclin E was not affected. Quantitative RT-PCR failed to reveal a change in cyclin B1, cyclin A and securin mRNA levels, indicating that the increase of these proteins by GPC1 is not caused by enhanced transcription (Figure

Figure 5. Overexpression of GPC1 up-regulates mitotic cyclins and securin. BMVECs were treated with GPC1 or control adenovirus for 48 h at different MOI as indicated. (A and B) The levels of p-cdc2 and cdc2 (A) and cyclins B1, A, and E, and securin (B) in the cells were examined by immunoblot analysis, with β -actin as loading control. (C) Total RNAs were isolated from the cells treated with the adenoviruses at 25 MOI, and quantitative RT-PCR was performed for cyclin B1, cyclin A, and securin. The relative mRNA levels of these genes comparing GPC1 and control virus treatments were determined. Each bar represents the mean $\pm$ SE of three independent experiments.

5C). These results suggest that GPC1 overexpression suppressed APC/C-mediated proteolytic degradation of cyclin B1, cyclin A, and securin.

GPC1 Overexpression Induces Multipolar Mitotic Spindles

To better characterize the mitotic defects, we visualized mitotic spindles and chromosomes with β -tubulin/PI immunofluorescence microscopy. Normal mitotic cells in prometaphase, metaphase, anaphase, and telophase and abnormal mitotic cells with either multipolar or monopolar spindles were differentiated and counted (Figure 6A). Compared with scrambled control virus, GPC1 shRNA adenovirus treatment reduced the fraction of telophase and multi-

Figure 6. Effect of GPC1 and mitotic cyclin expression on mitosis. (A) The mitotic spindles of different mitotic states in BMVECs. Mitotic spindle and chromosomal DNA were stained with FITC-conjugated β -tubulin antibody and PI, respectively, and visualized by confocal microscopy. Magnification, 600 $\times$. (B) BMVECs were treated with different adenoviruses as indicated for 48 h. Mitotic spindles and chromosomes were stained and analyzed by confocal microscopy. The percentage of the different mitotic states within the total population of mitotic cells was determined. At least 200 mitotic cells, which were pooled from at least three independent cultures, were viewed for each treatment. The following adenovirus doses were used: Ad-Sh-1 and Ad-Sc-1, 200 MOI; Ad, 50 MOI; and Ad-GPC1, 5, 10, 25, or 50 MOI. (C and D) BMVECs were transiently transfected with mouse cyclin B1, cyclin A, or control vectors. The expression of the cyclins was measured by immunoblot analysis (C). The mitoses were analyzed as described in B at 48 h after transfection (D).

polar spindle cells among mitotic cells and caused a decline of total mitotic events as described above (Figures 2D and 6B). The proportion of anaphase cells increased after GPC1 knockdown (Figure 6B). The relative increase in anaphase cells and decrease in telophase cells suggest either accelerated mitotic exit from metaphase or mitosis delay in anaphase. Overexpression of GPC1 with higher doses of virus caused a dramatic increase in the proportion of mitotic cells with multipolar spindles in a dose-dependent manner, and it was accompanied by a relative increase in metaphase cells and a decrease in both anaphase and telophase cells (Figure 6B). The significant increase in multipolar spindle cells and the change in the fraction of metaphase, anaphase, and telophase cells suggest that overexpression of GPC1 induces centrosome overproduction and mitotic arrest or delay at both prometaphase and metaphase. Monopolar spindle cells

constituted a relatively small proportion within the mitotic cells under all adenovirus treatment conditions (Figure 6B).

Cyclin B1 and Cyclin A Overexpression Mimics the Effects of GPC1 - Cyclin A Overexpression Is Sufficient to Induce Multipolar Mitotic Spindles

It is well documented that cell cycle-dependent expression and degradation of mitotic cyclins and securin play an essential role in the proper progression through mitosis in mammalian cells (Pines, 2006), and that Cdk2, paired with either cyclin A or cyclin E, acts as a licensing factor for centrosome duplication (Tsou and Stearns, 2006). We found in this study that the protein levels of mitotic cyclins and securin were regulated by GPC1 expression, i.e., up-regulated by GPC1 overexpression and down-regulated by knockdown of GPC1 expression (Figures 3B and 5B). To

Figure 7. GPC1 regulates BubR1 and Emi1 gene expression. (A and B) BMVECs were treated with 200 MOI of GPC1 shRNA adenovirus or 25 MOI of GPC1 adenovirus or their control adenoviruses as indicated for 48 h. Total RNAs were isolated, and quantitative RT-PCR was performed for Mad1, Mad2, BubR1, Bub1, and Bub3 (A) as well as Emi1 (B). The relative mRNA levels of these genes comparing GPC1 shRNA or GPC1 adenovirus and their control virus treatments were determined. Each bar represents the mean $\pm$ SE of three independent experiments. * $p < 0.05$ versus control viruses. (C and D) BMVECs were treated with 200 MOI of GPC1 shRNA or control adenovirus for different times (C), or treated with different doses of GPC1 or control adenovirus for 48 h (D) as indicated. Immunoblot analysis was performed for BubR1, Emi1, Cdc20, Cdh1, and APC2, with β -actin as loading control.

determine whether forced expression of mitotic cyclins is sufficient to replicate the mitotic alterations observed during GPC1 overexpression, cells were transiently transfected with wild-type mouse cyclin B1, cyclin A, or control vectors. Immunoblot analysis confirmed the overexpression of the cyclins following their transfection in comparison with control vectors (Figure 6C). Cyclin B1 overexpression induced a relative increase in metaphase cells and a decrease in anaphase cells, but it had no effect on the percentage of mitotic cells with multipolar spindles (Figure 6D). Conversely, overexpression of cyclin A led to a dramatic increase in multipolar spindle cells in addition to a disappearance of monopolar spindle cells (Figure 6D). Thus, forced expression of the mitotic cyclins is sufficient to induce mitotic alterations similar to those seen during GPC1 overexpression, and it suggests that these cyclins mediate the GPC1 effects on mitosis.

GPC1 Regulates BubR1 and Emi1 Gene Expression

Our findings point toward the regulation of APC/C by GPC1. APC/C specifically ubiquitinates destruction box (D-box)-containing cell cycle proteins, such as mitotic cyclins and securin, and its activity is tightly regulated by different protein kinases and protein inhibitors (Baker *et al.*, 2007). Protein kinase A (PKA) is a well-characterized negative regulator of APC/C. Therefore, we examined whether the PKA agonist DBcAMP reverses the effect of GPC1 knock-down and whether the PKA antagonist KT5720 abolishes the effect of GPC1 overexpression on the cells cycle. Even high concentrations of DBcAMP or KT5720 failed to reverse the cell cycle alterations caused by low or high GPC1 levels, respectively (Supplemental Figure S5), effectively ruling out PKA as a critical mediator of APC/C regulation by GPC1. The spindle assembly checkpoint (SAC) proteins, Bub1,

BubR1, Bub3, Mad1, Mad2, and Emi1 represent another group of APC/C inhibitors (Baker *et al.*, 2007). Quantitative RT-PCR analysis of the SAC protein and Emi1 mRNAs demonstrates that the manipulation of GPC1 expression levels regulates the mRNA levels of both BubR1 and Emi1 (Figure 7, A and B). Specifically, both BubR1 and Emi1 transcripts were significantly lowered during GPC1 knock-down and significantly elevated when GPC1 was overexpressed. BubR1 and Emi1 protein levels were consistent with these results (Figure 7, C and D). To investigate whether APC/C components could be affected by altered GPC1 expression, immunoblot analysis was also performed for the APC/C coactivators Cdc20 and Cdh1, and the APC/C subunit APC2. Cdc20 and APC2 levels were unaltered in both experimental conditions, whereas Cdh1 was significantly suppressed in GPC1-overexpressing cells (Figure 7, C and D). Overall, these observations are consistent with a positive regulation of Emi1 and BubR1 transcription by GPC1. Consequently, reduced APC/C activity upon GPC1 overexpression results in elevated levels of mitotic cyclins and securin and either accelerated cell cycle progression or a failure to normally exit from mitosis (Figure 8).

Multiple intracellular signaling pathways, including the p38 and JNK mitogen-activated protein kinase, Src/Sam68, PI3K/Akt, and FAK pathways, have been reported to participate in the regulation of mitosis in addition to PKA (Shalloway *et al.*, 1992; Yamakita *et al.*, 1999; Dangi *et al.*, 2003; Mikhailov *et al.*, 2005; Jordan *et al.*, 2006). In an effort to investigate whether these signaling pathways are involved in GPC1-mediated regulation of mitosis either upstream of or parallel to BubR1 and Emi1, the effect of changing GPC1 expression levels on these signaling pathways was also examined (Supplemental Figures S6 and S7). A forced decrease or increase in GPC1 expression had no effect on the activat-

Figure 8. Model for GPC1 regulation of cell cycle progression. GPC1 signals to up-regulate Emi1 and BubR1 expression, which could suppress the ubiquitin ligase activity of APC/C^{Cdh1} during interphase and of APC/C^{Cdc20} during mitosis. Mitotic cyclins and securin are the primary targets of APC/C for ubiquitin-proteasomal degradation. During the normal cell cycle, they must be accumulated during interphase for entry into mitosis, and they have to be destroyed around mid-M phase for the exit from mitosis. SCF^{Skp2} targets p27 and p21, which could inhibit both CDK2 and Cdc2 activity, and itself is suppressed by APC/C^{Cdh1} through ubiquitination of the Skp2 subunit. During GPC1 silencing, down-regulation of Cdc2 activity by reducing cyclin B1 and increasing p27/p21 protein levels may play a key role in the cell cycle abnormality, including G2 arrest and endoreduplication. In GPC1-overexpressing cells, dramatically increased levels of mitotic cyclins and securin may account for the development of mitotic arrest and subsequent aneuploidization. A moderate increase in mitotic cyclins could accelerate cell cycle progression without significant disturbance.

ing phosphorylation status of these molecules, arguing against their involvement in the GPC1 regulation of mitosis.

DISCUSSION

Here, we report the novel finding that GPC1 regulates proliferation and normal cell cycle progression of proliferating ECs. In agreement with our previous work, the forced expression of GPC1 to moderate levels stimulates EC proliferation without apparent disturbance of the cell cycle. In GPC1-deficient ECs, proliferation is almost abolished, accompanied by the accumulation of tetra- and polyploid cells. Robust overexpression of GPC1 disrupts the cell cycle progression apparently by mitotic arrest in addition to an induction of aneuploidy and apoptosis. These findings indicate that EC proliferation requires an optimal GPC1 concentration and that a deviation from this optimal level in either direction disrupts the cell cycle.

The knockdown of GPC1 expression induces tetraploidy, polyploidy, and apoptosis (Figure 2). Potential causes for this effect include a G2 arrest, a mitotic arrest or a premature mitotic slippage, and subsequent endoreduplication. The possibility of a mitotic arrest can be ruled out based on the observation that the mitotic index is reduced following GPC1 knockdown. Several lines of evidence point toward a G2 arrest during GPC1 expression silencing. GPC1 knockdown significantly suppresses cyclin B1 levels without affecting Cdc2 and induces the CDK inhibitors p27 and p21, which altogether should result in a significant inhibition of cyclin B1/Cdc2 kinase activity. p27 and p21 may inhibit CDK2 kinase activity in G1 phase to induce a G1 arrest as reported previously (Coqueret, 2003). However, our data suggest an elevated APC/C activity in GPC1-silenced cells,

and several studies indicate that SCF^{Skp2}, the downstream target of APC/C^{Cdh1}, targets p21 in S phase and p27 in S-G2 phase, regulating Cdc2 kinase activity and entry into mitosis (Bornstein *et al.*, 2003; Nakayama *et al.*, 2004). Cyclin B1/Cdc2, also called MPF, is a key effector for entry into mitosis (Stark and Taylor, 2006), and a threshold level of cyclin B1 is required for G2/M transition during the cell cycle (Porter and Donoghue, 2003). Given the consistent induction of G2 arrest by suppression of cyclin B1/Cdc2 in various cell types (Stark and Taylor, 2006) and the fact that Emi1, an APC/C inhibitor functioning through late G1 to early M phase (Reimann *et al.*, 2001; Hsu *et al.*, 2002), is reduced by inhibiting GPC1 expression, a G2 arrest seems likely. However, the cells are not arrested in G2 phase permanently since tetraploid endoreduplication and consequent polyploidization occur subsequently. There is experimental precedent that cells arrested at the G2 phase can adapt and enter M phase aberrantly (Andreassen *et al.*, 2001). Mitotic cells meeting obstacles in mitosis progression or undergoing mitosis errors may exit mitosis prematurely and return to interphase (G1) in a tetraploid state by so-called mitotic slippage (Rieder and Maiato, 2004). These cells face a p53-dependent G1 tetraploidy checkpoint and frequently proceed to endoreduplication during which DNA is replicated without subsequent mitosis or cytokinesis, resulting in poly- or aneuploidy (Andreassen *et al.*, 2003). Our observations support such cellular transition after knockdown of GPC1 expression, although the relative contribution of G2 arrest and mitotic slippage to the accumulation of tetraploid cells is unclear. Increasing evidence associates endoreduplication to inhibition of Cdc2 kinase activity in different organisms, including budding yeast, fission yeast, *Drosophila*, and mammalian cells (Edgar and Orr-Weaver, 2001; Nakayama *et al.*, 2004). It has been reported that premature degradation of cyclin B1 mediates mitotic slippage from a normal mitotic checkpoint in nocodazole treated cells (Brito and Rieder, 2006), and that cyclin B1 degradation is accelerated and contributes to the polyploidization in megakaryocytes (Zhang *et al.*, 1998). Depletion of securin has been found to increase arsenite-induced mitotic arrest and aberrant chromosomal segregation in human cells (Chao *et al.*, 2006). Moreover, immunodepletion of Emi1 in *Xenopus* strongly delays cyclin B1 accumulation and mitosis entry (Reimann *et al.*, 2001). A role of the APC/C complex is further supported by the observation that mice haploinsufficient for the APC/C regulator BubR1 are characterized by mitotic slippage and enhanced tumor development (Dai *et al.*, 2004).

Cellular changes induced by GPC1 overexpression are dose-dependent. Low-level overexpression of GPC1, e.g., with 5 MOI of GPC1 adenovirus, stimulates cell growth without a significant disturbance to the cell cycle. This result is consistent with a stimulation of DNA synthesis and accelerated mitotic entry and the observation that cyclin A and cyclin B1 are induced to a moderate level. Dependent on the cell cycle stage, cyclin A and cyclin B1 act synergistically to stimulate cell cycle progression from G1 phase to mid-mitosis (Fung and Poon, 2005).

In contrast, high-level overexpression of GPC1 disrupts normal cell cycle progression with an induction of mitotic arrest and aneuploidy as well as apoptosis. The stabilization of the mitotic cyclins induced by elevated GPC1 levels is a plausible mechanism for the observed mitotic arrest. Specifically, stabilized cyclin A induces centrosome overproduction, leading to multipolar mitotic spindles and mitotic arrest at prometaphase (Balczon, 2001; Faivre *et al.*, 2002; Kronenwett *et al.*, 2003). Stabilized cyclin B1 induces mitotic arrest at metaphase, consistent with a report that pro-

nounced overexpression of nondestructible cyclin B1 causes metaphase arrest without detectable sister chromatid separation in human cells (Wolf *et al.*, 2006). A large proportion of mitotic cells contain multipolar mitotic spindles both during GPC1 or cyclin A overexpression (Figure 6), implicating cyclin A as a down-stream effector of GPC1. Securin is also significantly stabilized by high-level overexpression of GPC1, which may additionally contribute to the mitotic arrest by inhibiting separate and therefore chromatid segregation (Christopoulou *et al.*, 2003). Similar to GPC1 expression silencing, the cells arrested in mitosis acquire aneuploidy, likely by mitotic slippage and subsequent endoreduplication. The tetra- and aneuploid cells ultimately undergo apoptosis as revealed by the observation that sub-G1 cells, tetra- and aneuploid and S-phase like cells constitute the detached and rounding cell population (data not shown).

The synchronized regulation of cyclin A, cyclin B1, and securin dependent on GPC1 levels points toward the APC/C complex as a critical player. GPC1 up-regulation induces gene expression of Emi1 and BubR1, both of which are known to suppress the activity of APC/C (Pines, 2006). APC/C is comprised of APC/C^{Cdc20} and APC/C^{Cdh1}, which differ in their activation subunits (i.e., Cdc20 or Cdh1). Emi1 and BubR1 have differential effects as Emi1 inhibits both APC/C^{Cdh1} and APC/C^{Cdc20} before prometaphase, whereas BubR1 inhibits APC/C^{Cdc20} only during prometaphase (Peters, 2006). The up-regulation of p27 and p21 protein levels in GPC1 silenced cells is consistent with such modulation of APC/C activity by GPC1 according to the well-characterized APC/C^{Cdh1}-SCF^{Skp2}-p27 ubiquitination pathway (Bashir *et al.*, 2004). Studies in Skp2^{-/-} and p27^{-/-} single and double mutant mice have demonstrated that stabilization of p27 by eliminating SCF^{Skp2}, which occurs in S-G2 phase to suppress Cdc2 kinase activity, is sufficient to induce a cellular phenotype, which is characterized by enlarged, polyploid nuclei and endoreduplication as well as a reduced mitotic index. These cellular events can be induced in cultured cells by an experimental reduction of Cdc2 activity (Nakayama *et al.*, 2004), and they are observed in the present study when GPC1 expression is silenced (Figure 2). Thus, it seems that reduced Cdc2 activity caused by a decrease in cyclin B1 and an increase in p27/p21 may play a key role in the cell cycle dysregulation induced by GPC1 silencing (Figure 8). The reduction of Cdh1 may contribute to the down-regulation of APC/C activity when GPC1 is highly overexpressed (Figure 7D).

How GPC1 regulates Emi1 and BubR1 expression is presently unclear. Via its HS chains, GPC1 functions as coreceptor for morphogens, growth factors, and antiangiogenic effectors (Whitelock and Iozzo, 2005). Whether this activity is responsible for the observed effects on cell cycle and mitosis is uncertain. Clearly, HS is insufficient because SDC1 overexpression does not reproduce the GPC1 overexpression phenotype. Alternative, intracellular activities of GPC1 also must be considered. GPC1 can transfer to the cytosol and translocates into the nucleus (Liang *et al.*, 1997). Interestingly, the nuclear localization pattern of GPC1 fluctuates during cell division and cell cycle, suggesting that the molecule may be directly involved in these events (Liang *et al.*, 1997).

The link between glypican and cell cycle regulation as described here is not without precedent. A genetic screen to identify mutants of cell division patterning in the developing *Drosophila* CNS led to the discovery of *dally*, one of two glypican homologues in this organism (Nakato *et al.*, 1995; Jackson *et al.*, 1997). Loss of *dally* function delays entry into mitosis in a specific set of retinal neurons; however, other tissues and overall viability are also affected. *Dally* mutants are characterized by a delay in the loss of mitotic cyclins and

in the exit from mitosis in two groups of dividing cells in the developing eye disk and larval brain (Penton *et al.*, 1997; Nakato *et al.*, 2002). The function of *dally* at least in part seems to be mediated by the core protein rather than the HS chains (Kirkpatrick *et al.*, 2006). Reduction of cyclin A but not cyclin B effectively rescues the mitotic defect (Nakato *et al.*, 2002). The effect of *dally* on mitotic cyclins is opposite to that of GPC1, and deregulation of cyclin A and cyclin B contribute differently to the mitotic defect caused by the *dally* mutation. This inconsistency may reflect differences between vertebrates and *Drosophila* in the regulation of mitotic cyclins and mitosis or indicate a completely different signaling mechanism. Nevertheless, both GPC1 and *dally* signaling seems to converge upon the proteolytic degradation of mitotic cyclins by APC/C. In summary, our findings suggest a fundamental role for GPC1 in the regulation of cell cycle progression and mitosis.

ACKNOWLEDGMENTS

We thank Korise Rasmusson for help with the manuscript. This work was supported by National Institutes of Health grant R01 NS-048921-01.

REFERENCES

- Editorial. (2003). Whither RNAi? *Nat. Cell Biol.* 5, 489–490.
- Andreassen, P. R., Lacroix, F. B., Lohez, O. D., and Margolis, R. L. (2001). Neither p21WAF1 nor 14–3-3sigma prevents G2 progression to mitotic catastrophe in human colon carcinoma cells after DNA damage, but p21WAF1 induces stable G1 arrest in resulting tetraploid cells. *Cancer Res.* 61, 7660–7668.
- Andreassen, P. R., Lohez, O. D., and Margolis, R. L. (2003). G2 and spindle assembly checkpoint adaptation, and tetraploidy arrest: implications for intrinsic and chemically induced genomic instability. *Mutat. Res.* 532, 245–253.
- Baker, D. J., Dawlaty, M. M., Galaray, P., and van Deursen, J. M. (2007). Mitotic regulation of the anaphase-promoting complex. *Cell Mol. Life Sci.* 64, 589–600.
- Balczon, R. C. (2001). Overexpression of cyclin A in human HeLa cells induces detachment of kinetochores and spindle pole/centrosome overproduction. *Chromosoma* 110, 381–392.
- Bashir, T., Dorrello, N. V., Amador, V., Guardavaccaro, D., and Pagano, M. (2004). Control of the SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase. *Nature* 428, 190–193.
- Belting, M., Mani, K., Jonsson, M., Cheng, F., Sandgren, S., Jonsson, S., Ding, K., Delcros, J. G., and Fransson, L. A. (2003). Glypican-1 is a vehicle for polyamine uptake in mammalian cells: a pivotal role for nitrosothiol-derived nitric oxide. *J. Biol. Chem.* 278, 47181–47189.
- Bornstein, G., Bloom, J., Sitry-Shevah, D., Nakayama, K., Pagano, M., and Herskho, A. (2003). Role of the SCF(Skp2) ubiquitin ligase in the degradation of p21Cip1 in S phase. *J. Biol. Chem.* 278, 25752–25757.
- Brito, D. A., and Rieder, C. L. (2006). Mitotic checkpoint slippage in humans occurs via cyclin B destruction in the presence of an active checkpoint. *Curr. Biol.* 16, 1194–1200.
- Chao, J. I., Hsu, S. H., and Tsou, T. C. (2006). Depletion of securin increases arsenite-induced chromosome instability and apoptosis via a p53-independent pathway. *Toxicol. Sci.* 90, 73–86.
- Cheng, F., Mani, K., van den Born, J., Ding, K., Belting, M., and Fransson, L. A. (2002). Nitric oxide-dependent processing of heparan sulfate in recycling S-nitrosylated glypican-1 takes place in caveolin-1-containing endosomes. *J. Biol. Chem.* 277, 44431–44439.
- Christopoulou, L., Moore, J. D., and Tyler-Smith, C. (2003). Over-expression of wild-type Securin leads to aneuploidy in human cells. *Cancer Lett.* 202, 213–218.
- Coqueret, O. (2003). New roles for p21 and p27 cell-cycle inhibitors: a function for each cell compartment? *Trends Cell Biol.* 13, 65–70.
- Crosio, C., Fimia, G. M., Loury, R., Kimura, M., Okano, Y., Zhou, H., Sen, S., Allis, C. D., and Sassone-Corsi, P. (2002). Mitotic phosphorylation of histone H3, spatio-temporal regulation by mammalian Aurora kinases. *Mol. Cell Biol.* 22, 874–885.
- Dai, W., Wang, Q., Liu, T., Swamy, M., Fang, Y., Xie, S., Mahmood, R., Yang, Y. M., Xu, M., and Rao, C. V. (2004). Slippage of mitotic arrest and enhanced tumor development in mice with BubR1 haploinsufficiency. *Cancer Res.* 64, 440–445.

- Dangi, S., Cha, H., and Shapiro, P. (2003). Requirement for phosphatidylinositol-3 kinase activity during progression through S-phase and entry into mitosis. *Cell Signal* 15, 667–675.
- Edgar, B. A., and Orr-Weaver, T. L. (2001). Endoreplication cell cycles: more for less. *Cell* 105, 297–306.
- Faivre, J., Frank-Vaillant, M., Poulhe, R., Mouly, H., Jessus, C., Brechot, C., and Sobczak-Thépot, J. (2002). Centrosome overduplication, increased ploidy and transformation in cells expressing endoplasmic reticulum-associated cyclin A2. *Oncogene* 21, 1493–1500.
- Filmus, J., and Selleck, S. B. (2001). Glypicans: proteoglycans with a surprise. *J. Clin. Invest* 108, 497–501.
- Fung, T. K., and Poon, R. Y. (2005). A roller coaster ride with the mitotic cyclins. *Semin. Cell Dev. Biol.* 16, 335–342.
- Harlow, E., and Lane, D. (1988). *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory: Cold Spring Harbor, NY.
- Hsu, J. Y., Reimann, J. D., Sorensen, C. S., Lukas, J., and Jackson, P. K. (2002). E2F-dependent accumulation of hEmi1 regulates S phase entry by inhibiting APC(Cdh1). *Nat. Cell Biol.* 4, 358–366.
- Ilangumaran, S., He, H. T., and Hoessli, D. C. (2000). Microdomains in lymphocyte signalling: beyond GPI-anchored proteins. *Immunol. Today* 21, 2–7.
- Jackson, S. M., Nakato, H., Sugiura, M., Jannuzzi, A., Oakes, R., Kaluza, V., Golden, C., and Selleck, S. B. (1997). dally, a *Drosophila* glypican, controls cellular responses to the TGF-beta-related morphogen, Dpp. *Development* 124, 4113–4120.
- Jakubovic, B. D., and Jothy, S. (2007). Glypican-3, from the mutations of Simpson-Golabi-Beckwith genetic syndrome to a tumor marker for hepatocellular carcinoma. *Exp. Mol. Pathol.* 82, 184–189.
- Jordan, K. C., Schaeffer, V., Fischer, K. A., Gray, E. E., and Ruohola-Baker, H. (2006). Notch signaling through tramtrack bypasses the mitosis promoting activity of the JNK pathway in the mitotic-to-endocycle transition of *Drosophila* follicle cells. *BMC Dev. Biol.* 6, 16.
- Kirkpatrick, C. A., Knox, S. M., Staats, W. D., Fox, B., Lercher, D. M., and Selleck, S. B. (2006). The function of a *Drosophila* glypican does not depend entirely on heparan sulfate modification. *Dev. Biol.* 300, 570–582.
- Kleeff, J., Wildi, S., Kumbasar, A., Friess, H., Lander, A. D., and Korc, M. (1999). Stable transfection of a glypican-1 antisense construct decreases tumorigenicity in PANC-1 pancreatic carcinoma cells. *Pancreas* 19, 281–288.
- Kronenwett, U., Castro, J., Roblick, U. J., Fujioka, K., Ostring, C., Faridmoghaddam, F., Laytragoon-Lewin, N., Tribukait, B., and Auer, G. (2003). Expression of cyclins A, E and topoisomerase II alpha correlates with centrosome amplification and genomic instability and influences the reliability of cytometric S-phase determination. *BMC Cell Biol.* 4, 8.
- Langley, R. R., Ramirez, K. M., Tsan, R. Z., Van Arsdall, M., Nilsson, M. B., and Fidler, I. J. (2003). Tissue-specific microvascular endothelial cell lines from H-2K(b)-tsA58 mice for studies of angiogenesis and metastasis. *Cancer Res.* 63, 2971–2976.
- Liang, Y., Haring, M., Roughley, P. J., Margolis, R. K., and Margolis, R. U. (1997). Glypican and biglycan in the nuclei of neurons and glioma cells: presence of functional nuclear localization signals and dynamic changes in glypican during the cell cycle. *J. Cell Biol.* 139, 851–864.
- Lin, H., Huber, R., Schlessinger, D., and Morin, P. J. (1999). Frequent silencing of the GPC3 gene in ovarian cancer cell lines. *Cancer Res.* 59, 807–810.
- Matsuda, K., Maruyama, H., Guo, F., Kleeff, J., Itakura, J., Matsumoto, Y., Lander, A. D., and Korc, M. (2001). Glypican-1 is overexpressed in human breast cancer and modulates the mitogenic effects of multiple heparin-binding growth factors in breast cancer cells. *Cancer Res.* 61, 5562–5569.
- Mikhailov, A., Shinohara, M., and Rieder, C. L. (2005). The p38-mediated stress-activated checkpoint. A rapid response system for delaying progression through antephase and entry into mitosis. *Cell Cycle* 4, 57–62.
- Murthy, S. S., Shen, T., De Rienzo, A., Lee, W. C., Ferriola, P. C., Jhanwar, S. C., Mossman, B. T., Filmus, J., and Testa, J. R. (2000). Expression of GPC3, an X-linked recessive overgrowth gene, is silenced in malignant mesothelioma. *Oncogene* 19, 410–416.
- Nakato, H., Fox, B., and Selleck, S. B. (2002). dally, a *Drosophila* member of the glypican family of integral membrane proteoglycans, affects cell cycle progression and morphogenesis via a cyclin A-mediated process. *J. Cell Sci.* 115, 123–130.
- Nakato, H., Futch, T. A., and Selleck, S. B. (1995). The division abnormally delayed (dally) gene: a putative integral membrane proteoglycan required for cell division patterning during postembryonic development of the nervous system in *Drosophila*. *Development* 121, 3687–3702.
- Nakayama, K., Nagahama, H., Minamishima, Y. A., Miyake, S., Ishida, N., Hatakeyama, S., Kitagawa, M., Iemura, S., Natsume, T., and Nakayama, K. I. (2004). Skp2-mediated degradation of p27 regulates progression into mitosis. *Dev. Cell* 6, 661–672.
- Penton, A., Selleck, S. B., and Hoffmann, F. M. (1997). Regulation of cell cycle synchronization by decapentaplegic during *Drosophila* eye development. *Science* 275, 203–206.
- Peters, J. M. (2006). The anaphase promoting complex/cyclosome: a machine designed to destroy. *Nat. Rev. Mol. Cell Biol.* 7, 644–656.
- Pines, J. (2006). Mitosis: a matter of getting rid of the right protein at the right time. *Trends Cell Biol.* 16, 55–63.
- Porter, L. A., and Donoghue, D. J. (2003). Cyclin B1 and CDK 1, nuclear localization and upstream regulators. *Prog. Cell Cycle Res.* 5, 335–347.
- Qi, W., and Martinez, J. D. (2003). Reduction of 14–3–3 proteins correlates with increased sensitivity to killing of human lung cancer cells by ionizing radiation. *Radiat. Res.* 160, 217–223.
- Qiao, D., Chen, W., Stratagoules, E. D., and Martinez, J. D. (2000). Bile acid-induced activation of activator protein-1 requires both extracellular signal-regulated kinase and protein kinase C signaling. *J. Biol. Chem.* 275, 15090–15098.
- Qiao, D., Meyer, K., Mundhenke, C., Drew, S. A., and Friedl, A. (2003). Heparan sulfate proteoglycans as regulators of fibroblast growth factor-2 signaling in brain endothelial cells. Specific role for glypican-1 in glioma angiogenesis. *J. Biol. Chem.* 278, 16045–16053.
- Reimann, J. D., Freed, E., Hsu, J. Y., Kramer, E. R., Peters, J. M., and Jackson, P. K. (2001). Emi1 is a mitotic regulator that interacts with Cdc20 and inhibits the anaphase promoting complex. *Cell* 105, 645–655.
- Rieder, C. L., and Maiato, H. (2004). Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint. *Dev. Cell* 7, 637–651.
- Shalloway, D., Bagrodia, S., Chackalaparampil, I., Shenoy, S., Lin, P. H., and Taylor, S. J. c-Src and mitosis. *Ciba Found Symp.* 170, 248–265, 1992; discussion 265–275.
- Song, H. H., and Filmus, J. (2002). The role of glypicans in mammalian development. *Biochim. Biophys. Acta* 1573, 241–246.
- Stark, G. R., and Taylor, W. R. (2006). Control of the G2/M transition. *Mol. Biotechnol.* 32, 227–248.
- Su, G., Meyer, K., Nandini, C. D., Qiao, D., Salamat, S., and Friedl, A. (2006). Glypican-1 is frequently overexpressed in human gliomas and enhances FGF-2 signaling in glioma cells. *Am. J. Pathol.* 168, 2014–2026.
- Tang, G. (2005). siRNA and miRNA: an insight into RISCs. *Trends Biochem. Sci.* 30, 106–114.
- Tsou, M. F., and Stearns, T. (2006). Controlling centrosome number: licenses and blocks. *Curr. Opin. Cell Biol.* 18, 74–78.
- Tumova, S., Woods, A., and Couchman, J. R. (2000). Heparan sulfate proteoglycans on the cell surface: versatile coordinators of cellular functions. *Int. J. Biochem. Cell Biol.* 32, 269–288.
- Welcker, M., and Clurman, B. (2005). Cell cycle: how cyclin E got its groove back. *Curr. Biol.* 15, R810–R812.
- Whitelock, J. M., and Iozzo, R. V. (2005). Heparan sulfate: a complex polymer charged with biological activity. *Chem. Rev.* 105, 2745–2764.
- Williams, L. S., Nordberg, J. A., and Chau, P. C. (1990). Evaluation of immunocytochemical detection methods of incorporated bromodeoxyuridine in hybridomas. *Cytometry* 11, 490–497.
- Wolf, F., Wandke, C., Isenberg, N., and Geley, S. (2006). Dose-dependent effects of stable cyclin B1 on progression through mitosis in human cells. *EMBO J.* 25, 2802–2813.
- Xiang, Y. Y., Ladedá, V., and Filmus, J. (2001). Glypican-3 expression is silenced in human breast cancer. *Oncogene* 20, 7408–7412.
- Yam, C. H., Fung, T. K., and Poon, R. Y. (2002). Cyclin A in cell cycle control and cancer. *Cell Mol. Life Sci.* 59, 1317–1326.
- Yamakita, Y., Totsukawa, G., Yamashiro, S., Fry, D., Zhang, X., Hanks, S. K., and Matsumura, F. (1999). Dissociation of FAK/p130(CAS)/c-Src complex during mitosis: role of mitosis-specific serine phosphorylation of FAK. *J. Cell Biol.* 144, 315–324.
- Yamauchi, N., et al. (2005). The glypican 3 oncofetal protein is a promising diagnostic marker for hepatocellular carcinoma. *Mod. Pathol.* 18, 1591–1598.
- Zhang, Y., Wang, Z., Liu, D. X., Pagano, M., and Ravid, K. (1998). Ubiquitin-dependent degradation of cyclin B is accelerated in polyploid megakaryocytes. *J. Biol. Chem.* 273, 1387–1392.