ACTIVATION OF CDK2 STIMULATES PROTEASOME-DEPENDENT TRUNCATION OF TYROSINE PHOSPHATASE SHP-1 IN HUMAN PROLIFERATING INTESTINAL EPITHELIAL CELLS.

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Running head: Cyclin-dependent kinase-2-dependent SHP-1 degradation

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SHP-1 is expressed in the nuclei of intestinal epithelial cells (IECs). Increased SHP-1 expression and phosphatase activity coincide with cell cycle arrest and differentiation in these cells. Suspecting the tumor suppressive properties of SHP-1, a yeast two-hybrid screen of an IEC cDNA library was conducted using the full-length SHP-1 as bait. Characterization of many positive clones revealed sequences identical to a segment of the Cdk2 cDNA sequence. Interaction between SHP-1 and Cdk2 was confirmed by co-immunoprecipitations whereby co-precipitated Cdk2 phosphorylated SHP-1 protein. Inhibition of Cdk2 (roscovitine) or proteasome (MG132) was associated with an enhanced nuclear punctuate distribution of SHP-1. Double labeling localization studies with signature proteins of subnuclear domains revealed a co-localization between the splicing factor SC35 and SHP-1 in bright nucleoplasmic foci. Using Western blot analyses with the anti-SHP-1 antibody recognizing the C-terminus, a lower molecular weight species of 45-kDa was observed in addition to the full-length 64-65-kDa SHP-1 protein. Treatment with MG132 led to an increase in expression of the full-length SHP-1 protein while concomitantly leading to a decrease in the levels of the lower 45-kDa molecular species. Further Western blots revealed that the 45-kDa protein corresponds to the C-terminal portion of SHP-1 generated from proteasome activity. Mutational analysis of Y208 and S591 (a Cdk2 phosphorylation site) residues on SHP-1 abolished the expression of the amino-truncated 45-kDa SHP-1 protein. In conclusion, our results indicate that Cdk2-associated complexes, by targeting SHP-1 for proteolysis, likely counteract the ability of SHP-1 to block cell cycle progression of IECs.

SHP-1, an SH2 domain-containing protein tyrosine phosphatase, is a key regulator in the control of intracellular levels of phosphotyrosine. It is predominantly expressed in hematopoietic cells and epithelial cells (1-3). SHP-1 contains two Src homology (SH2) domains, a neighboring catalytic domain and a C-terminal tail. Its phosphatase activity is inhibited by the interaction between the N-terminal SH2 domain and the catalytic domain (4-7). SHP-1 acts as a negative regulator of intracellular signaling by three families of transmembrane receptors: growth factors receptors with an intrinsic tyrosine kinase activity e.g., c-kit, CSF-1, TrkA and EGF (8-12), cytokine receptors e.g., Epo-R, IFNα/β-R, IL-3R and IL-2R (9-17) and receptors involved in immune responses such as the T-cell receptor complex (TCR), CD5 and death receptor (18-21). SHP-1 binds the immunoreceptor tyrosine-based inhibition motif (ITIM) of these receptors through its SH2 domains and dephosphorylates downstream proteins. Its effect is to terminate the signal of the activated receptor or to activate other terminating pathways such as apoptosis (22).

On the other hand, very little is known as to the biological roles of SHP-1 in epithelial cells, although the existence of an epithelium-specific isoform of SHP-1 is suggestive of specific function(s) in these cells (23). Keilhack et al. (24) have previously shown that SHP-1 is an important downstream regulator of ROS signaling in epididymal epithelium. Furthermore, previous evidence indicates that SHP-1 associates with and dephosphorylates p120 catenin in EGF-stimulated A431 cells (25), suggesting a role for this PTP in...
the regulation of catenin function and cadherin-mediated epithelial cell-cell adhesion. Furthermore, SHP-1 localization differs between non-hematopoietic and hematopoietic cells, with SHP-1 protein being virtually exclusively cytoplasmic in hematopoietic cells and nuclear in non-hematopoietic cells (26). These results have implications regarding the nuclear function of SHP-1 in non-hematopoietic cells. Our recent data indicate that increased SHP-1 expression and activity coincide with cell cycle arrest and induction of differentiation of intestinal epithelial cells. Results show that overexpression of SHP-1 in intestinal epithelial crypt cells significantly inhibited dhfr, c-myc and cyclin D1 gene expression and decreased β-catenin/TCF-dependent transcription (27). Interestingly, extensive studies of SHP-1 protein and mRNA in cancer cell lines have revealed that the expression of SHP-1 protein is diminished or abolished not only in most leukemia and lymphoma cell lines and tissues but also in some non-hematopoietic cancer cell lines, such as estrogen receptor (ER) negative breast cancer cell lines as well as certain colorectal cancer cell lines (28-32). To gain insight into the molecular roles of SHP-1 in the nucleus and into its suspected tumor suppressive properties in epithelial cells, we conducted a yeast two-hybrid screen of an intestinal epithelial cell cDNA library using the full-length SHP-1 as bait. Results show that cyclin-dependent kinase-2 (Cdk2) interacts with SHP-1 and promotes its proteasome-dependent truncation in human proliferating intestinal epithelial cells.

**EXPERIMENTAL PROCEDURES**

*Materials.* [γ-32P]ATP and [γ-32P]orthophosphate were obtained from Perkin Elmer (Shelton, CT, USA). The antibodies against Cdk2 (M2), SHP-1 (C-19), PML (PG-M3), B-23 (FC-8791), lamin B (M-20), calpain (H-240), Na+/K+ATPase α (H-300) and a peptide from hemagglutinin HA1 protein were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Polyclonal antibody recognizing SHP-1 was a kind gift from Dr C. Nahmias (Dept. Cell Biology, Institut Cochin, France) and was previously described (27). Antibody recognizing the SHP-1 phosphorylated on S591 was from ECM Biosciences (Versailles, KY, USA). Monoclonal antibodies against pRb and SC35 were from Pharmingen (Mississauga, ON, Canada). The actin-recognizing antibody was purchased from Chemicon International (Billerica, MA, USA). Recombinant active cyclin E/Cdk2 complex was from Upstate Biotechnologies Inc. (Lake Placid, NY, USA) and cyclin A/Cdk2 complex was from Invitrogen. Antibodies recognizing the FLAG tag (F-3165) were from Sigma-Aldrich (Oakville, ON, Canada). Cycloheximide was purchased from Calbiochem (Mississauga, ON, Canada). The secondary antibody AlexaFluro568 rhodamine-conjugated goat anti-mouse IgG and AlexaFluro488 FITC-labelled goat anti-rabbit were from Molecular Probes (Invitrogen, Burlington, ON, Canada). The GST-FER tyrosine kinase used for in gel phosphatase assays was purchased from Invitrogen. All other materials were obtained from Sigma-Aldrich unless stated otherwise.

*Expression vectors.* The full-length mouse SHP-1 cDNA (M. Thomas, Howard Hughes Medical Institute, St. Louis, MO) was subcloned into the expression vector pGBKT7 in frame with the GAL4 DNA Binding domain (Clontech, Palo Alto, CA). The GAL4-DNA binding domain-SHP-1 construct was generated using pcDNAneoI-SHP-1 (33) as a template and the oligos: sense 5’-GGATTCCATATGATGGTGAGGTGGTTTCAC-3’ and antisense 5’-GGTTCTCTCAAGAGGAAGTGAGAATTCTT-3’. The resulting DNA fragment was subcloned into the Nde-EcoRI sites of the pGBKT7 vector. Mutation of the critical cysteine 453 of the catalytic site of the molecule for serine (SHP-1C453S) was previously described (33).

Expression vectors for HA-tagged wild-type Cdk2 (HA-Cdk2) and a dominant-negative form of Cdk2 (HA-Cdk2DN) were obtained from Dr James M. Roberts (Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington). The full-length SHP-1 cDNA was subcloned into the expression vector pcDNA3 (Invitrogen) in frame with the HA epitope. The HA-SHP-1 was constructed by adding the HA epitope to the N-terminal of SHP-1 by PCR using an oligonucleotide containing the sequence encoding the HA epitope and Kozak sequence. Primer sense: 5’ ATT CCG GAA TTC GCG CAT GTA CCC ATA CGA TGT TCC AGA TTA CGC
TCT TGT GAG GTG GTT TCA CCG G 3'.

Primer antisense: 5' ATT CCG GAA TTC TCA CTT CCT CTT GAG AG 3'. The PCR product was inserted into the EcoRI sites. The SHP-1-FLAG was constructed by adding the FLAG epitope at the C-terminus by PCR. Primer sense 3' AGGAAAGCTTATGGTAGGTTGTTCACC-3'. Primer antisense: 5'-AGGAGGCGCGCTTACTTGTACCTGTCGTCCTTGTAGTCTCTCTTTGTGAGACCTTTTTGTCTTCTCC-3' and finally the inner primers which changed tyrosine 208 for phenylalanine (underlined): 5'-GGCTTTGTCTCTCTGCGAG-3' and 5'-CTGCGCAAGAAAGAGGACCTTTGTTCTTCTCC-3'.

The PCR product was inserted into the pcDNA3 vector into the HindIII/NotI sites. SHP-1 wild-type ligated downstream of the glutathione S-transferase sequence in a pGEX-2T plasmid were a kind gift from Dr Robert Faure (Laval University, Québec, QC, Canada). The SHP-1(S160A), SHP-1(S591A), SHP-1(Y208F) and SHP-1(208-595) mutants were generated by site-directed mutagenesis. For SHP-1(S160A) mutant, the forward primer included a BamHI enzyme site:

CAACTAGGATCCGTGAGGTGGTTTCACCGGGACC-3' and the reverse primer contained a EcoRI and an HindIII enzyme sites: 5'-CGGAATGAATTCAAGCTTTTACTTCCTCTTGTACCTGTCGTCCTTCTCTTGAGAGAACCTTTGTTCTTCTCC-3'.

For SHP-1(S591A) in pGex-2T, we used the forward primer corresponding to positions 318-332 of mouse SHP-1 cDNA: 5'-AGTGAGAGGTTGACCCACCC-3'. This primer contained a KpnI enzyme site. The reverse primer included an EcoRI and HindIII enzyme sites and changed serine 591 for alanine (underlined):

GCGAAGCGCCGCTTACTTGTACCTGTCGTCCTTGTAGTCTCTCTTTGTGAGACCTTTCTTC-3'. For the Y208F mutant, a forward primer was used which included an EcoRI site enzyme and a Kozak sequence:

ATTCCGGAATTCCGCTATGGTGAGGAGTGGTTTCACC-3' and a reverse primer with NotI enzyme site and FLAG tag: 5'-AGGAGGCGCGCTTACTTGTACCTGTCGTCCTTGTAGTCTCTCTTTGTGAGACCTTTTTGTCTTCTCC-3' and finally the inner primers which changed tyrosine 208 for phenylalanine (underlined):

GGCTTTGTCTCTCTGCGAG-3' and 5'-CTGCGCAAGAAAGAGGACCTTTGTTCTTCTCC-3'.

Yeast two-hybrid screen and assay. A yeast two-hybrid screen was performed according to the Matchmaker Two-Hybrid system 3 protocol (Clontech) using pGBKT7-SHP1 as bait. Briefly, yeast strain Y187 (Saccharomyces cerevisiae) was transformed with pGBKT7-SHP1 plasmid. Transformants were selected on medium lacking tryptophan. GAL4 DNA binding domain-SHP1 constructs (in pGex-4T-2 vector) were gifts from Dr A.W. Poole (Dept. Pharmacology and Physiology, School of Medical Sciences, University Walk, Bristol) and were previously described (34).
Sequence identification and comparison were performed using the National Center for Biotechnology Information (NCBI) online service. Specific interactions were confirmed by direct cotransformation of GAL4 DNA binding domain-SHP1 and the positive clones (fused to the GAL4 activating domain) into the Saccharomyces cerevisiae Y187. Colony selection and analysis were performed as described above.

**Cell culture.** Human intestinal epithelial cells (HIEC) were cultured as described previously (35) in Dulbecco’s modified Eagle’s medium supplemented with 4 mM glutamine, 20 mM HEPES, 50 U/ml penicillin and 50 µg/ml streptomycin (all obtained from Invitrogen), 0.2 IU/ml insulin (Connaught Novo Laboratories, Willowdale, ON, Canada), and 5% fetal bovine serum (FBS). The Caco-2/15 cell line was obtained from A. Quaroni (Cornell University, Ithaca, NY, USA) and cultured in DMEM containing 10% FBS, as described previously (27). Human Embryonic Kidney 293T cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM containing 10% FBS. The LoVo cell line was obtained from ATCC and cultured in Ham’s F12 (Invitrogen) containing 10% FBS. The DLD-1 cell line was obtained from ATCC and cultured in RPMI 1640 (Invitrogen) containing 10% FBS. The HCT116 and HT-29 cell lines were obtained from ATCC and cultured in McCoy 5A (Invitrogen) containing 10% FBS.

**Protein expression and immunoblotting.** Cells were lysed in chilled lysis buffer (100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1 mM PMSF, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin, 0.5 µg/ml aprotinin, 0.2 mM orthovanadate, and 40 mM β-glycerophosphate) and lysates cleared of cellular debris by centrifugation. Proteins (50 µg) from cell lysates were separated by SDS-PAGE (polyacrylamide gel electrophoresis) and detected immunologically following electrophoresis onto nitrocellulose membranes (Amersham Biosciences Corp., Baie d’Urfé, QC, Canada). Protein and molecular weight markers (Bio-Rad, Mississauga, ON, Canada) were localized with Ponceau red. After blocking for 1 h at 25°C in PBS-0.05% Tween containing 5% powdered milk, membranes were first incubated for 2-4 h at 25°C or overnight at 4°C with primary antibodies in blocking solution, followed by a second incubation with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit (1:10000) IgG in blocking solution for 1 h. The blots were visualized by the Amersham ECL system. Protein concentrations were measured using a modified Lowry procedure with bovine serum albumin (BSA) as standard (36).

**Transient transfections.** 293 cells were transfected by lipofectamin (Invitrogen) with expression vectors. Thirty-six hours after transfection, cells were lysed in Triton X-100 lysis supplemented buffer and lysates were cleared of cellular debris by centrifugation (16 000 g, 10 min, 4°C).

**Co-immunoprecipitation experiments and Cdk2 kinase assays.** Cells were washed twice with ice-cold PBS, lysed in chilled lysis buffer and cleared of cellular debris by centrifugation. Primary antibodies were added to 500-800 µg of each cell lysate and incubated overnight at 4°C under agitation. Four µg of protein A-Sepharose (Amersham-Biosciences Corp.) were subsequently added for 1 h (4°C under agitation). Immunocomplexes were harvested by centrifugation and washed three times with ice-cold lysis buffer. Proteins were solubilized in Laemmli’s buffer and separated by SDS-PAGE. In other experiments, the beads were washed three times with lysis buffer followed by two rinses in ice-cold kinase buffer (40 mM Hepes pH 7.4, 20 mM MgCl2, 1 mM dithiothreitol, 10 mM pNpp) before performing the Cdk2 kinase assay with histone H1 as substrate.

**Immunofluorescence microscopy of cultured cells.** Caco-2/15 or HIEC cells grown on sterile glass coverslips were washed twice with ice-cold PBS. Cultures were then fixed with 3% paraformaldehyde for 20 min at 4°C, permeabilized with a 0.1% Triton X-100 solution in PBS for 10 min, and blocked with PBS and 3% bovine serum albumin for 30 min at room temperature. Cells were subsequently immunostained for 1.5 h with the primary antibody (Cdk2, SHP-1, SC35 or PML) and for 30 min with the secondary antibody (FITC-labelled goat anti-rabbit or Rhodamine-labelled goat anti-
mouse). In some experiments, nuclei were stained with DAPI. Negative controls (no primary antibody) were included in all experiments.

**GST fusion protein purification.** Recombinant plasmids was introduced into *Escherichia coli* BL21 DE3, and the fusion protein was produced by growing 500 ml of bacterial culture to an optical density between 0.4 and 0.6 and subsequently treating the culture with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. Cells were recovered and resuspended in buffer (PBS1X, pH 7.5, 10 mM EDTA, 1 mM PMSF, 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin, 0.7 µg/ml pepstatin) and sonicated. Triton X-100 was added to the lysates at a final concentration of 1%. The bacterial lysates were incubated on ice for 10 min and centrifuged at 16 000 g for 15 min. The supernatants were recovered and mixed with 1.2 ml of glutathione-Sepharose 4B beads (Amersham Biosciences, Inc.) and the resulting mixture rotated at 4°C for 1 h. The beads were then washed extensively in lysis buffer with Triton 1% and used for *in vitro* binding assays as described previously (27, 37).

**SHP-1 phosphorylation and phosphatase assays.** Kinase assays were performed using cyclin E/Cdk2 kinase or cyclin A/Cdk2 buffer supplied by the manufacturer. Briefly, the buffer was supplemented with 1 mM ATP, 2 µCi [γ-32P]ATP, 4-5 µg of GST-SHP-1 or GST-SHP-1S160A or GST-SHP-1S591A (human) or 1.5 µg GST-SHP-1S591A (mouse) with 0-1 µg of activated cyclin E/Cdk2 or activated cyclin A/Cdk2 complexes and incubated at 30°C for 5-30 min. Reactions were stopped by addition of Laemmli’s buffer. Radiolabeled GST-SHP-1 was separated by SDS-PAGE and processed for autoradiography. In some experiments, phosphatase activity was assayed by washing 3 times the other half of the reaction mixture with glutathione-Sepharose 4B beads and finally resuspending in a total volume of 80 µl of phosphatase buffer (50 mM HEPES pH 7.0, 60 mM NaCl, 60 mM KCl, 0.1 mM PMSF, 0.7 µg/ml pepstatin, 0.5 µg/ml aprotinin, 0.5 µg/ml leupeptin) supplemented with 0.1 mg/ml bovine serum albumin, 50 µM EDTA, 100 µM dithiothreitol (33). The reaction was initiated by the addition of para-nitrophenyl phosphate (pNPP) (10 mM, final concentration) for 30 min at 30°C. The reaction was stopped by the addition of 0.9 ml of 1 N NaOH, after which sample absorbencies were measured at 410 nm.

**In vivo phospholabeling.** Cdk2 and SHP-1 or their relevant expression vectors were transfected in 293 cells. Twenty-four hours after transfection, cells were labelled for 2 h in phosphate-depleted medium with 100 µCi/ml of 32P-orthophosphate. After labelling, the cells were lysed in lysis buffer and 32P-phosphate-labeled SHP-1 was isolated by immunoprecipitation with SHP-1 antibody. Incorporation of 32P-orthophosphate was detected by autoradiography.

**RNA extraction and gene expression analysis.** Total RNA was isolated and processed using the RNA easy Plus Mini Kit (QIAGEN, Mississauga, ON, Canada). Reverse-transcription PCR (RT-PCR) analysis was performed using AMV-RT (Roche Diagnostics, QC, Canada) according to the manufacturer's instructions. Human SHP-1 transcripts were amplified with a forward primer: 5’-GTTTCTTCTAGTTTCTGAC-3’ and a reverse primer: 5’-CCATCGGATGGTCCTTCTGG. hTBP was used as internal control. The forward primer was: 5’-TGAGGATAAGAGAGCCACGAA-3’ and the reverse primer was: 5’-GAGCACAAGGGCCTTAACTC-3’. PCR conditions included 1 min at 94°C, 30s at 94°C, 30s at 94°C, 30s at 94°C, 30s at 94°C, 30s at 94°C, and 30s at 94°C.

**Isolation of nuclear proteins.** Cells were washed twice with ice-cold PBS and resuspended in 100 µl of lysis buffer (10 mM HEPES pH 7.9, 60 mM KCl, 1 mM EDTA, 1mM DTT and 0.5%NP40 with 1× cocktail protein inhibitor) and kept on ice for 5 min. The nuclei were pelleted at 12 000g for 20s and the supernatant (cytoplasmic proteins) transferred into a new tube. The nuclei were resuspended in 200 µl of buffer (250 mM Tris pH 7.8, 80 mM KCl, 1 mM DTT and 1× cocktail protein inhibitor) and kept on ice for 5 min. The nuclei were pelleted at 12 000g for 20s and the supernatant (cytoplasmic proteins) transferred into a new tube. The nuclei were resuspended in 200 µl of buffer (250 mM Tris pH 7.8, 80 mM KCl, 1 mM DTT and 1× cocktail protein inhibitor). The lysates were incubated in liquid nitrogen 2 min, subsequently incubated at 37°C, 2 min and then vortexed during 5s. This last step was repeated 3 times. Thereafter, lysates were centrifuged 10 min at 12 000g at 4°C after which the nuclear proteins were stored at -80°C.

**In gel phosphatase assays.** These assays were performed as previously reported by Burridge and Nelson with minor modifications (38).
**Results**

*Interaction of SHP-1 with Cdk2 in human epithelial cells.*

To identify possible partner protein(s) that interact with SHP-1, a yeast two-hybrid screen was performed using SHP-1 as bait to search a Caco-2/15 cDNA library constructed from a mixture of mRNAs from confluent Caco-2/15 cells. Prior to screening the library, the recombinant vector pGBK7-SHP-1 was co-transformed with blank vector pGADT7 into AH109 and no positive clones appeared on selective plates. It indicated that SHP-1 itself had no transcriptional activity on the GAL4 promoter. 1 out of 12 clones were positive in the screening and all clones contained a 1100 bp insert whose sequence was identical to a segment of the cyclin-dependent kinase-2 (Cdk2) cDNA sequence (data not shown). Yeast two-hybrid interaction experiments were performed to re-test the interaction of SHP-1 with Cdk2 identified in the original screen. Survival tests on appropriate SD plate’s deficient in leucine, tryptophane, histidine and adenine were done (data not shown). When pGADT7-Cdk2 or pGBK7-SHP-1 was transformed into yeast strain AH109 separately, the cells did not grow on selective plates. However, when BD-SHP-1 and AD-Cdk2 were co-transformed into yeast, colonies could be recovered on appropriate QDO plates (data not shown) indicating a specific interaction between the two proteins.

To validate the interaction between SHP-1 and Cdk2 *in vivo*, the expression vector encoding wild-type SHP-1 or the catalytically inactive SHP-1 C453S was co-transfected with a full-length Cdk2 expression vector with a C-terminal HA epitope tag into 293 cells, followed by co-immunoprecipitation/Western blot analysis. Immunoprecipitations demonstrated the association of SHP-1 with Cdk2 (Fig. 1A, left panel, lanes 3 and 4). No interaction was present in untransfected cells (Fig. 1A, lane 1) and no significant interaction was detected in cells transfected with Cdk2 only (Fig. 1A, lane 2). Interestingly, the catalytically inactive mutant of SHP-1 (C453S) which has been described as a substrate-trapping mutant (39), exhibited strong elevated binding (Fig. 1A, lane 4). In addition, the interaction between endogenous SHP-1 and Cdk2 was also analyzed in the intestinal epithelial cell line, Caco-2/15, which spontaneously differentiates into an enterocyte phenotype after confluence (40). Immunoprecipitations confirmed the interaction between endogenous SHP-1 and Cdk2 proteins in subconfluent (sc) proliferating cells (Fig. 1A, middle panel). The interaction between SHP-1 and Cdk2 was lost upon cell confluence and differentiation. Since Caco-2/15 cells are derived from a human colonic adenocarcinoma (40), it was deemed important to validate the above results in normal human intestinal-derived cells. Hence, the interaction of SHP-1 with Cdk2 was tested in crypt-like undifferentiated HIEC cells (35). Since interaction between SHP-1 and Cdk2 was mostly detected in subconfluent growing Caco-2/15 (middle panel), it was verified whether this interaction fluctuates in a cell cycle-dependent manner in normal cells. The interaction between Cdk2 and SHP-1 was therefore analyzed in serum-deprived and serum-stimulated HIEC. Treatment of quiescent HIEC cells with serum growth factors stimulated S phase entry as previously reported by our group (41). Interaction between SHP-1 and Cdk2 became apparent at 8h and became maximal at 16h after serum addition. Moreover, this interaction was concomitant with the phosphorylation and activation of Cdk2 (right panel), thereby suggesting that serum growth factors promote the interaction between Cdk2 and SHP-1 during G1/S phase progression in HIEC cells. Localization of SHP-1 and Cdk2 proteins was further analyzed in asynchronously-growing subconfluent Caco-2/15 cells and 3-days post-confluent differentiating Caco-2/15 cells. As illustrated in Fig. 1B, bright nuclear SHP-1 staining was evident in proliferative subconfluent cells (panel 1, see...
arrows), although some SHP-1 staining was also observed in the cytoplasm (panel 1, see asterisk). Nuclear localization of SHP-1 was completely altered upon confluency, as shown by the accumulation of SHP-1 staining in the cytoplasm and at sites of cell-cell contact in post-confluent Caco-2/15 cells (panel 2). In subconfluent growing Caco-2/15 cells, Cdk2 protein staining was mostly localized in the nucleus (panel 5, see arrows), but was also visible in the cytoplasm of some cells (panel 5, see asterisks). In confluent cells, Cdk2 staining was found mostly in the cytoplasm (panel 6). Overall, these data indicate that SHP-1 interacts with Cdk2 in proliferating intestinal epithelial cells.

Cdk2 phosphorylates SHP-1 and slightly increases its phosphatase activity in vitro.

The observed interaction between SHP-1 and Cdk2 prompted us to investigate whether the association of Cdk2 with SHP-1 could modulate Cdk2 kinase activity. SHP-1 or the catalytically inactive SHP-1 C453S mutant was first co-overexpressed with Cdk2 or the catalytically inactive Cdk2 (Cdk2DN) in 293 cells. SHP-1 was immunoprecipitated and Cdk2 activity in the SHP-1 immunoprecipitates was analyzed by using histone H1 as substrate. As shown in Fig. 2A (lanes 4 and 6), SHP-1 immunoprecipitates isolated from 293 cells overexpressing either SHP-1 or SHP-1 C453S with wtCdk2 efficiently phosphorylated histone H1 protein. However, this phosphorylation was not observed when the dominant-negative mutant of Cdk2 was co-expressed (Fig. 2A, lanes 3 and 5). In addition, no difference in tyrosine phosphorylation level of Cdk2 was observed in SHP-1 immunoprecipitates (data not shown). These results indicate that histone H1 kinase found in SHP-1 immunoprecipitates is indeed Cdk2 and that SHP-1 phosphatase activity does not influence kinase activity and tyrosine phosphorylation of Cdk2.

To determine whether SHP-1-Cdk2 association may have some functional relevance on SHP-1 activity, the capacity of Cdk2 to phosphorylate and activate the SHP-1 protein was evaluated in an additional series of experiments. We first verified whether SHP-1 is phosphorylated by Cdk2 in vivo. 293 cells were transfected with either Cdk2-HA, SHP-1 or their respective empty vectors and subjected to 32P-phospholabeling. 32P-phospholabeling of immunoprecipitated SHP-1 protein showed increased incorporation of 32P into SHP-1 in cells transfected with Cdk2-HA (Fig. 2B). The phosphorylation of GST-SHP-1 by active cyclin E/Cdk2 and cyclin A/Cdk2 complexes is shown in Fig. 2C. Both Cdk2-associated complexes efficiently phosphorylated the GST-SHP-1 protein. Phosphatase assays reveal a modest increase in SHP-1 activity following Cdk2 phosphorylation (data not shown). The canonical amino-acid sequence in substrates that is recognized by Cdk2 is S/TPXK/R, where S is the phosphorylatable serine and X is any amino acid (42). We previously found such canonical amino-acid sequence in substrates that is recognized by Cdk2 is S/TPXK/R, where S is the phosphorylatable serine and X is any amino acid (42). We previously found such canonical amino-acid sequence in substrates that is recognized by Cdk2 is S/TPXK/R, where S is the phosphorylatable serine and X is any amino acid (42). We previously found such canonical amino-acid sequence in substrates that is recognized by Cdk2 is S/TPXK/R, where S is the phosphorylatable serine and X is any amino acid (42). We previously found such canonical amino-acid sequence in substrates that is recognized by Cdk2 is S/TPXK/R, where S is the phosphorylatable serine and X is any amino acid (42).
**SHP-1 protein expression inversely correlates with Cdk2 activity.**

Since both SHP-1 expression and phosphatase activity levels of SHP-1 were much more elevated in confluent growth-arrested intestinal epithelial cells as well as in differentiated enterocytes (27), it was verified whether the abundance of SHP-1 mRNA and protein fluctuates in a cell cycle-dependent manner. The protein expression of SHP-1 was therefore further analyzed in serum-deprived and serum-stimulated HIEC. Treatment of quiescent HIEC cells with serum growth factors stimulated S phase entry as monitored by Western blot analysis of pRb and Cdk2 phosphorylation (Fig. 3A, upper panel). The appearance of a faster migrating species of Cdk2 is known to be indicative of phosphorylation of the enzyme on threonine 160 by Cdk-activating kinase (44). As illustrated in Fig. 3A, in serum-deprived HIEC cells, pRb was exclusively found in its hypophosphorylated activated state. Hyperphosphorylation of pRb protein became apparent at 16h after serum stimulation, concurrent with the phosphorylation and activation of Cdk2. Interestingly, decreased expression of SHP-1 became apparent and significant 16h after serum addition concomitant with the induction of pRb hyperphosphorylation and Cdk2 activation. In Caco-2/15 cells, and consistent with our previous observations (27), decreased activity of Cdk2-associated complexes (phosphorylated histone H1) became apparent and significant after confluence, concomitantly with the induction of SHP-1 protein (3-fold) at day 5 post-confluency (Fig. 3B, upper panel). By contrast, RT-PCR analysis revealed that SHP-1 mRNA levels did not differ between subconfluent and post-confluent differentiating Caco-2/15 cells and between serum-deprived and serum-stimulated HIEC. (Fig. 3A and B, lower panels). Hence, these data indicate an inverse correlation between expression levels of SHP-1 protein and the activity of Cdk2-associated complexes.

**Inhibition of Cdk2 or proteasome increases nuclear SHP-1 expression levels in cycling intestinal epithelial cells.**

Cdk2-mediated phosphorylation is often involved in proteolytic degradation of other cell cycle and differentiation regulators such as p27<sup>kip1</sup> (45) and MyoD (46). In Fig. 2, we demonstrated that SHP-1 is a substrate for phosphorylation by Cdk2. To test the hypothesis that Cdk2-dependent phosphorylation of SHP-1 promotes its degradation, the effects of the Cdk2 inhibitor roscovitine and of the potent inhibitor of the 20S proteasome, MG132, were examined on SHP-1 expression levels in subconfluent growing Caco-2/15 cells, in which cyclin E/Cdk2 and cyclin A/Cdk2 complexes activities are elevated (41). Treatment with roscovitine or MG132 led to a significant increase in expression levels of SHP-1 protein comparatively to DMSO-treated Caco-2/15 cells at the same time points (Fig. 4A). Interestingly, treatment of serum-stimulated HIEC cells with roscovitine or MG132 also prevented serum-induced decrease expression of SHP-1 comparatively to non-treated serum-stimulated HIEC cells at the same time points (data not shown). Next, the influence of ectopic expression of Cdk2 on the levels of co-expressed SHP-1 was examined in transiently transfected 293 cells treated with cycloheximide. As shown in Fig. 4B, SHP-1 exhibited a significant shortened half-life when co-overexpressed with Cdk2. This suggests that the increased levels of SHP-1 observed following roscovitine treatment is due to a stabilization of the protein.

In addition, the treatment with roscovitine or MG132 was associated with an enhanced nuclear distribution of SHP-1 now forming a punctuate pattern (Fig. 4C, panel 5-8, see arrows in inserts), suggesting that the nuclear proteasome system degrades SHP-1 in intestinal epithelial cells.

**The nuclear proteasome degrades SHP-1 in intestinal epithelial cells.**

Nuclear proteasomes have previously been localized to PML (promyelocytic leukemia) nuclear bodies (47, 48), nucleoplasmic speckles (49) and focal clusters throughout the nucleoplasm (50). To identify the subnuclear distribution of SHP-1 protein in cells treated with roscovitine or MG132, double labeling localization studies were performed with signature proteins of subnuclear domains. As shown in Fig. 5 (panel 1), the clumpy localization of B23, a nucleolar protein (51), did not co-localize with SHP-1. In addition, PML nuclear bodies, visualized by antibodies to PML, did not contain SHP-1 (panel 8). By contrast, significant co-localization was observed between splicing factor SC35, a signature protein of
splicing speckles (52), and SHP-1 in bright nucleoplasmic foci (panel 12). Similar results were obtained in cells treated with MG132 (data not shown). These results suggest that Cdk2-dependent SHP-1 degradation occurs in splicing speckles that contain proteasome.

An amino-truncated 45-kDa SHP-1 protein is detected in cells expressing full-length SHP-1.

In Western blot analyses with the anti-SHP-1 antibody recognizing the C-terminus, several low molecular weight species were observed in addition to the full-length 64-65-kDa SHP-1 protein or the SHP-1 C/S mutant (Fig. 6A, first two panels). Of interest, the major lower molecular species detected with the C-terminus antibody, the 45-kDa protein, was also present in many untransfected colon cancer cell lines, indicating that this smaller protein is normally expressed in untransfected human cells (Fig. 6A, right panels). Western blot analysis of nuclear and cytoplasmic extracts also revealed that the 45-kDa protein accumulated into the cytoplasm (Fig. 6A, right panel). In addition, this 45-kDa SHP-1 protein was immunoprecipitated by the C-terminus SHP-1 antibody (Fig. 6B). Treatment of 293 cells with the proteasome inhibitor MG132 led to an increase in expression levels of full-length SHP-1 protein while concomitantly decreasing the expression levels of the 45-kDa SHP-1 protein (Fig. 6C). Similar results were obtained when the cells were treated with roscovitine (data not shown). Intriguingly, when anti-HA antibody was used to detect the N-terminal HA tag of SHP-1 in Western blot analyses, no lower molecular species could be detected (data not shown). To confirm that this 45-kDa fragment originated from the SHP-1 protein, we fused the FLAG tag at the C-terminus of the full-length SHP-1. As shown in Fig. 6D, the 45-kDa molecular species was now well detected with the FLAG antibody, as well as by two other different SHP-1 antibodies known to recognize the C-terminal portion of SHP-1; by contrast, when we used an antibody recognizing the N-terminal portion of SHP-1, we could not detect the 45-kDa protein. Hence, this confirms that the 45-kDa protein is a peptide fragment corresponding to the C-terminal portion of SHP-1 generated from proteasome activity.

\[ Y^{208} \] and \[ S^{591} \] residues are both important for SHP-1 proteolysis.

By using Pcleavage, a support vector machine-based method predicting constitutive proteasome cleavage sites (3; www.imtech.res.in/raghava/pcleavage/), Y208 was found to be a putative proteasome cleavage site in SHP-1 coding sequence. Therefore, this tyrosine was mutated to phenylalanine and the expression of the mutant subsequently analyzed by Western blotting. As shown in Fig. 7A, the 45-kDa SHP-1 protein was not detected in cells expressing SHP-1(Y208F) mutant (lane 4), in contrast to cells expressing full-length SHP-1 (lanes 2 and 5). Hence, our data suggest that cleavage of SHP-1 at Y208 results in a 45-kDa SHP-1 protein corresponding to the C-terminal portion of full-length SHP-1 excluding SH2 domains but including the catalytic phosphatase domain. We next generated the truncated SHP-1 mutant (Y208-595) and analyzed its expression and phosphatase activity. Following transfection of this mutant and Western blot analyses with total cell lysates, the Y208-595 SHP-1 mutant (lane 3) did not exhibit the exact same electrophoretic mobility as that observed with the 45-kDa SHP-1 peptide generated from endogenous proteasome activity (lanes 2 and 5) as seen in Fig. 7A. We next investigated whether SHP-1 phosphorylation on S591 could be responsible for this difference. As shown in Fig. 7B, wtSHP-1 exhibited two bands when overexpressed in 293 cells. Both forms of SHP-1 are phosphorylated on S591. Interestingly, the 45-kDa SHP-1 peptide generated from endogenous proteasome activity was much more phosphorylated on S591 than the Y208-595 SHP-1 mutant. This suggests that the difference in electrophoretic mobility between the Y208-595 SHP-1 mutant and the 45-kDa SHP-1 peptide generated from endogenous proteasome activity could be attributable to phosphorylation. Interestingly, the Y208-595 SHP-1 mutant demonstrated similar phosphatase activity as that observed for wtSHP-1 (Fig. 7C). Moreover, In-gel phosphatase assays confirmed that the endogenously produced phosphorylated 45-kDa fragment is active in 293 cells (Fig. 7D).

Because S591 in SHP-1 protein is a target for Cdk2-associated complexes (Fig. 7E), this serine was therefore mutated to alanine and western blot analyses with anti-SHP-1, anti-FLAG and anti-
phosphoS591SHP-1 were performed to compare expression of full-length SHP-1 and SHP-1 (S591A) proteins. As shown in Fig. 7E, the 45-kDa peptide was not detected in cells expressing SHP-1(S591A) mutant (lanes 3 of each western blot), in contrast to cells expressing full-length SHP-1 (lanes 2). Thus, these data confirm that SHP-1 degradation is triggered by direct phosphorylation of SHP-1 on S591.

**DISCUSSION**

The importance of SHP-1 expression for the maturation and function of hematopoietic cells has been addressed by studies of motheaten (me/me) and motheaten viable (mev/mev) mice carrying mutations of the SHP-1 gene. Homozygous me and mev mice exhibit multiple abnormalities including neutrophilia, lymphopenia, splenomegaly and/or elevated serum immunoglobulin, severe combined immunodeficiency and systemic autoimmunity, due to the dysregulation of leukocyte development (54, 55). Whereas multiple targets for SHP-1 have been identified in hematopoietic cells (56), very little is known regarding the function, partners and regulation of SHP-1 in epithelial cells. In this report, we demonstrate for the first time a functional interaction of SHP-1 and the key cell cycle regulatory protein, Cdk2, in intestinal epithelial cells.

In a previous report, we suggested for the first time the involvement of SHP-1 in the negative control of intestinal epithelial cell proliferation. Indeed, increased SHP-1 expression and phosphatase activity coincided with cell cycle arrest and induction of differentiation in intestinal epithelial cells. In addition, ectopic expression of SHP-1 in human intestinal crypt cells inhibited E2F-dependent transcriptional activity and decreased the expression of *c-myc* and *cyclin D1* genes, the activation of which represents one of the earliest cell cycle-regulated events occurring during the transition from G0/G1 to S phase (27). In the present study, we identified Cdk2 as a SHP-1 binding protein. Indeed, the specificity of SHP-1 and Cdk2 interaction was confirmed through co-immunoprecipitation assays from intestinal epithelial cells. Of particular interest, and similarly to Cdk2 (41), SHP-1 was primarily localized in the nucleus of growing intestinal epithelial cells. Our *in vitro* experiments suggested that SHP-1 does not directly regulate tyrosine phosphorylation of Cdk2 (data not shown) and its kinase activity. Conversely, the fact that Cdk2-associated complexes may play a role in the regulation of SHP-1 function is suggested by the following: 1) cyclin E/Cdk2 as well as cyclin A/Cdk2 complexes efficiently phosphorylated and modestly activated SHP-1, *in vitro*; 2) inhibition of Cdk2 activity with the roscovitine, a small molecule that specifically targets and inhibits the ATP binding site of Cdk's (57), increased SHP-1 protein expression. Hence, Cdk2-associated complexes may be targeted to SHP-1 by a docking domain which is distinct from the phosphoacceptor motifs. In fact, we observed three docking domains for Cdk2 homologous to the general consensus sequence R-X-L (58) and localized between amino acids 7-9, 223-225 and 400-402 of the SHP-1 protein. Ongoing experiments are currently in progress in order to determine whether site-directed mutagenesis of one of these sequences impairs the association of Cdk2 to SHP-1.

Our results also indicate that S591 in SHP-1 protein is a substrate for Cdk2. It has previously been reported that S591, situated close to the very end of the SHP-1 C-terminal tail, is potentially a very good phosphorylation site for basophilic kinases. In addition, increased S591 phosphorylation of SHP-1 was observed following TCR stimulation of Jurkat cells (43) and following thrombin stimulation of platelets (34). Phosphorylation of S591 was also reported to inhibit nuclear translocation of SHP-1 (43). Interestingly, S591 localizes directly in the middle of the identified NLS, which contains a series of positively charged residues. Hence, phosphorylation of S591 may change the positive charge required in a NLS, impairing NLS-based nuclear localization of SHP-1 (59).

Our data strongly suggest that SHP-1 degradation may be triggered by direct Cdk2-dependent phosphorylation of SHP-1. Indeed, the steady-state level of SHP-1 protein was increased in the presence of MG132, a proteasome inhibitor,
or with roscovitine, indicating that both inhibition of proteasome function or Cdk2 activity enhances SHP-1 protein. Cdk2-mediated phosphorylation is often involved in proteolytic degradation of other cell cycle and differentiation regulators such as p27Kip1 (45), MyoD (46), Ctd1 (60), β-catenin (61), Cdx2 (37) and cyclin E (62). Hence, our data support the model that SHP-1 undergoes proteasome-dependent degradation in cells in which Cdk2 is highly activated, such as proliferative intestinal epithelial undifferentiated crypt cells and colon cancer cells. This concept is also supported by the observation herein that SHP-1 expression levels significantly decreased when HIEC entered the cell cycle e.g when Cdk2-associated complexes became activated. Accordingly, our previous observations indicated that SHP-1 expression and activity levels were much more elevated in confluent growth-arrested intestinal epithelial cells (HIEC and Caco-2/15 cells) as well as in differentiated enterocytes (Caco-2/15 and primary cultures of differentiated enterocytes) (27). This was further corroborated by a greater SHP-1 expression in the lower third of the villi in the small intestine, which are in essence a reflection of the distribution of cells that have ceased proliferation. Taken together, these data demonstrate that high levels of SHP-1 protein expression and phosphatase activity are observed in conditions of diminished cell proliferation and reduced Cdk2-associated complex activity such as quiescence (serum deprivation, confluence) and differentiation. The mechanism of SHP-1 regulation appears primarily related to protein stability, since inhibition of proteasome activity increased SHP-1 protein levels, whereas no change in SHP-1 mRNA levels was found during HIEC cell cycle progression and Caco-2/15 cell differentiation.

In our view, an interesting and novel finding of the present study is that SHP-1 protein can accumulate into splicing speckles of the nucleus. Speckles are subnuclear structures that are enriched in pre-messenger RNA splicing factors such as SC35 and are located in the interchromatin regions of the nucleoplasm of mammalian cells (63). Of note, speckles are often observed close to highly active transcription sites, suggesting that they may have a functional relationship with gene expression, and some genes have been reported to preferentially localize in close proximity to speckles (64-66). Concomitantly, several kinases such as CLK/STY, PRP4 and PSKH1 (68-71) and phosphatases such as PP1 (72) that can phosphorylate and dephosphorylate components of the splicing machinery have also been localized to nuclear speckles. More importantly, it has been previously reported that the β-catenin/TCF4 complex contains several classes of RNA-binding proteins including Fus and heterogeneous nuclear ribonucleoproteins (hnRNPs), while also regulating the pre-messenger (mRNA) splicing reaction (73). For example, it has been demonstrated that β-catenin transfection changes the splicing pattern of the E1A minigene and ER-β gene (73). However, the precise molecular mechanisms causing alternative splicing by accumulation of β-catenin remains to be elucidated. We previously reported that β-catenin is both a binding partner and a substrate for SHP-1 in human epithelial cells (27). Furthermore, SHP-1 expression significantly decreases β-catenin/TCF4-dependent transcription in intestinal epithelial crypt cells (27). Hence, one could speculate that SHP-1 also controls the pre-mRNA splicing activity of β-catenin. However, this remains the subject of future study.

Taken together, our findings suggest that SHP-1 undergoes a limited proteolysis by the nuclear proteasome in a Cdk2-dependent manner, through phosphorylation of S591. Then, SHP-1 protein seems targeted for specific endoproteolytic cleavage by the proteasome that removes its first N-terminal 208 amino acid-containing SH2 domains. The resulting 45kDa peptide which conserves the phosphatase catalytic activity appears to be accumulated in the cytoplasm where it could have an altered biological function. It has already been reported that phosphorylation of S591 changes the positive charge required in a NLS, impairing NLS-based nuclear localization of SHP-1 (59). Hence, Cdk2 by targeting SHP-1 for proteolysis, may counteract the ability of SHP-1 to block nuclear β-catenin/TCF4 complex activity and cell cycle progression of intestinal epithelial cells (27). Such limited proteolysis catalyzed by the proteasome has also been reported for other proteins (74) such as YB-1 transcription factor (75) and the eIF4G and eIF3a translation initiation factors (76). Thus, proteasome-mediated
endoproteolytic cleavage of SHP-1 may therefore be considered as a highly specific mechanism regulating SHP-1 localization and function in the cell.

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**FOOTNOTES**

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**FIGURE LEGENDS**

Figure 1: SHP-1 associates with Cdk2 in human epithelial cells. A. 293 cells (left panel). Cotransfection of wtSHP-1 or SHP-1 C453S with HA-Cdk2 expression vectors was performed in 293 cells. Thirty-six hours after transfection, cell extracts (500 µg) were immunoprecipitated with anti-HA followed by Western blotting with specific antibodies against SHP-1 and HA tag. Proteins were analyzed by Western blotting in order to confirm the amount of SHP-1 and Cdk2 in the immunoprecipitates. Caco-2/15 cells (middle panel). Cdk2 was immunoprecipitated from 800 µg of lysates of subconfluent (sc) and confluent (c) Caco-2/15 cells. Irrelevant control antibody (α-Na+K+ATPase) was used in the last line (-). Proteins from the immunoprecipitates were solubilized in Laemmli's buffer, separated by SDS-PAGE and subsequently analyzed by Western blotting in order to determine SHP-1 and Cdk2 content. HIEC cells (right panel). Subconfluent HIEC were serum-starved for 36 h and then stimulated with 5% FBS for 8, 16 and 24 h. SHP-1 was immunoprecipitated from 800 µg of lysates and proteins from immunoprecipitates were solubilized in Laemmli's buffer and separated by SDS-PAGE. Proteins were analyzed by Western blotting in order to determine SHP-1 and Cdk2 content. B. Subconfluent and 3 days post-confluent Caco-2/15 cells were fixed for immunofluorescence and stained for SHP-1 or Cdk2 proteins. Arrows indicate nuclear localisation; asterisk indicates cytoplasmic localisation. Scale bars, 100 µm.
Figure 2: Cdk2 phosphorylates SHP-1. **A.** Co-transfection of SHP-1 or SHP-1 C453S (both in pcDNA3 vector) with Cdk2-HA or Cdk2DN-HA expression vectors was performed in 293 cells. Thirty-six hours after transfection, cell extracts (500 µg) were immunoprecipitated with anti-SHP-1. The expression of Cdk2 was analyzed by Western blotting by using an anti-HA antibody. The Cdk2 activity was measured by the phosphorylation of histone H1. **B.** Transfections of Cdk2-HA or empty expression vector were performed in 293 cells. Thirty-six hours after transfection, cells were labeled with 32P-phosphate during 2 h. Cells were subsequently lysed and endogenous 32P-phosphate-labeled SHP-1 isolated by immunoprecipitation with SHP-1 antibody and analyzed by Western blot and autoradiography. **C.** Kinase assays were performed by incubating active recombinant cyclin A/cdk2 complex (0.25 and 0.5 µg) or cyclin E/cdk2 complex (25 and 50 ng) for 30 min with 5 µg of GST-SHP-1 fusion protein or 3 µg of Histone H1 (as positive control) as described in Experimental Procedures. **D.** Kinase assays were performed by incubating active recombinant cyclin E/cdk2 complex (50 ng) for 30 min with 100 ng of GST-SHP-1. The reaction was stopped by addition of Laemmli’s buffer and the expression of phosphorylated SHP-1 on S591 was analyzed by Western blotting by using an antibody recognizing SHP-1 phosphorylated on S591. Western blotting using an antibody against GST was also performed as a control. **E.** Kinase assays were performed by incubating active recombinant cyclin A/cdk2 complex (0 or 62.5 ng) for 5 min with 4 µg of human GST-SHPI fusion proteins as described in Experimental Procedures. Radiolabeled proteins were separated on SDS-PAGE and autoradiographed. Thereafter, the gel was transferred onto nitrocellulose membrane and Ponceau staining was shown as control.

Figure 3: SHP-1 protein expression inversely correlates with Cdk2 activity. **A.** Subconfluent HIEC were serum-starved for 36 h and then stimulated with 5% FBS for 8, 16 and 24 h. **Upper panel:** Equal amounts of whole cell lysates were separated by 10% SDS-PAGE, and proteins analyzed by Western blotting with specific antibodies against pRb, Cdk2 and SHP-1 (Rb-P: hyperphosphorylated Rb; the appearance of a faster migrating species of Cdk2 is known to be indicative of phosphorylation of the enzyme on threonine 160 by Cdk-activating kinase (42)). **Lower panel:** RT-PCR was performed for the analysis of SHP-1 expression with total RNA from HIEC as templates. hTBP was used as gene of reference. **B.** Caco-2/15 cells were harvested at subconfluence (sc), 100% confluence (day 1), 5 and 12 days post-confluence. **Upper panel:** Cell extracts were separated by 10% SDS-PAGE, and proteins analyzed by Western blotting with specific antibodies against pRb, Cdk2 and SHP-1. **Lower panel:** Total RNA was extracted from Caco-2/15 cells at different stages of confluence (sc, 1, 5 and 12 days post-confluence). RT-PCR was performed for analysis of SHP-1 expression with total RNA from Caco-2/15 as templates. hTBP was used as gene of reference.

Figure 4: Cdk2 activity promotes SHP-1 degradation through the proteasome. **A.** Subconfluent proliferating Caco-2/15 cells were treated with DMSO (control), 50 µM roscovitine or 50 µM MG132 for 1, 2, 4 and 8 h. Cell extracts were prepared and subjected to Western blot analysis with specific antibodies against SHP-1 and actin. Densitometric analyses were performed and fold-induction was calculated relative to actin and to DMSO-treated cells at the same time points. **B.** Subconfluent proliferating Caco-2/15 cells were transfected with expression vectors encoding HA-SHP-1 and Cdk2-HA or Cdk2DN-HA as indicated, followed by treatment with vehicle or 10 µg/ml cycloheximide for 2, 4, 6 and 8 h. Cell extracts were prepared and analyzed by immunoblotting with HA-specific antibodies. **C.** Subconfluent HIEC were serum-starved for 36 h and then stimulated with 5% FBS for 16 h in the presence or absence of 50 µM roscovitine or 50 µM MG132. Thereafter, cells were fixed and permeabilized for immunofluorescence and staining of SHP-1. Lower panels are inset magnifications of boxed areas in upper panels. Arrows indicate nuclear punctuated localisation. Scale bars, 100 µm.

Figure 5: SHP-1 protein accumulates in splicing speckles when Cdk2 is inhibited. Subconfluent HIEC were serum-starved for 36 h and then stimulated with 5% FBS for 16 h in the presence of 50 µM roscovitine or of 50 µM MG132 (not shown). Thereafter, cells were fixed and permeabilized for
immunofluorescence and co-staining of SHP-1, B23 (a nucleolar protein), PML or SC35 (a signature protein of splicing speckles). Scale bars, 100 µm. Only pictures of cells that were treated with roscovitine+FBS were shown.

**Figure 6: An amino-truncated 45-kDa SHP-1 protein is detected in cells expressing SHP-1.**

A. First panel: 293 cells were transiently transfected with pcDNAneoI expressing SHP-1. Forty-eight hours after transfection, proteins were solubilized in Laemmli's buffer and separated by SDS-PAGE. Second panel: Proteins from HIEC cells, stably transfected with empty vector, SHP-1 or SHP-1 C453S, were solubilized in Laemmli's buffer and separated by SDS-PAGE. Third panel: Proteins from HT29, LoVo, DLD-1 and HCT116 colon cancer cell lines were also solubilized in Laemmli's buffer and separated by SDS-PAGE. All total cell extracts were prepared and analyzed by immunoblotting with anti-SHP-1 antibody. Fourth panel: Nuclear (N) and cytoplasmic (C) extracts from Caco-2/15 cells were prepared as described in Experimental procedures. Protein expression levels of SHP-1, lamin B (nuclear marker) and calpain (cytoplasmic marker) were analyzed by Western blotting. B. 293 cells were transfected with the empty vector pcDNAneoI (EV), SHP-1 or SHP-1 C453S. After 48h, SHP-1 was immunoprecipitated from 800 µg lysates. Proteins from immunoprecipitates were solubilized in Laemmli’s buffer, separated by SDS-PAGE and analyzed by immunoblotting using anti-SHP-1 antibody. C. 293 cells were transfected with pcDNAneoI expressing SHP-1 vector. Forty-eight hours after transfection, proteins were treated with 50 µM MG132 for 0, 4, 6 or 8h. Proteins were then solubilized in Laemmli’s buffer, separated by SDS-PAGE and analyzed by immunoblotting with anti-SHP-1 antibody. D. 293 cells were transfected with SHP-1-FLAG construct. Forty-eight hours after transfection, proteins were solubilized in Laemmli’s buffer, separated by SDS-PAGE and analyzed by immunoblotting with anti-SHP-1 antibodies recognizing the C-terminus portion of SHP-1 (Santa Cruz, C. Nahmias) or the N-terminus portion of SHP-1 (GeneTex).

**Figure 7: Y208 and S591 residues are important for SHP-1 proteolysis.** A. 293 cells were transfected with SHP-1-FLAG or SHP-1(208-595)-FLAG or SHP-1(Y208F)-FLAG mutants. Forty-eight hours after transfection, proteins were solubilized in Laemmli’s buffer, separated by SDS-PAGE and analyzed by immunoblotting with anti-FLAG. B. 293 cells were transfected with SHP-1-FLAG or SHP-1(208-595)-FLAG mutant. Forty-eight hours after transfection, proteins were solubilized in Laemmli’s buffer, separated by SDS-PAGE and analyzed by immunoblotting with anti-SHP-1 or anti-SHP-1 phosphorylated on S591. C. 293 cells were transfected with HA-SHP-1 or SHP-1(208-595)-FLAG mutants. After 48 h, cells were harvested and cell extracts (800 µg) were immunoprecipitated with an antibody against SHP-1. Representative Western blot was shown. Phosphatase activity of SHP-1 was assayed by using pNpp as substrate. The increase in phosphatase activity (absorbance) was calculated relative to the level observed in empty vector-transfected cells which was set at 1. Data shown are representative of that obtained in two independent experiments. D. 293 cells were transfected with SHP-1-FLAG or SHP-1(208-595)-FLAG mutant. After 48 h, cells were harvested and cell extracts (800 µg) were immunoprecipitated with an antibody against SHP-1. Representative Western blot was shown. Phosphatase activity of SHP-1 was assayed by performing an In-gel phosphatase assay as described under Experimental procedures. E. 293 cells were transfected with SHP-1-FLAG or SHP-1(S591A)-FLAG constructs. Forty-eight hours after transfection, proteins were solubilized in Laemmli’s buffer, separated by SDS-PAGE and analyzed by immunoblotting with anti-FLAG antibodies or anti-SHP-1 antibodies recognizing the C-terminus portion of SHP-1 (Santa Cruz) or recognizing SHP-1 phosphorylated on S591.
Figure 1
Figure 2
**Figure 3**

A. **HIEC**

| FCS 5% | 8h | 16h | 24h |
|--------|-----|-----|-----|
| Western blots | ![Western Blots](image1) | ![Western Blots](image2) | ![Western Blots](image3) |

B. **Caco-2/15**

| Days of post-confluency | se | 1 | 5 | 12 |
|-------------------------|----|---|---|----|
| ![Western Blots](image4) | ![Western Blots](image5) | ![Western Blots](image6) | ![Western Blots](image7) |

- Rb<sup>p</sup> → Rb
- Cdk2 → SHP-1
- TBP →
Figure 4
Figure 5
Figure 6
Figure 7
Activation of CDK2 stimulates proteasome-dependent truncation of tyrosine phosphatase SHP-1 in human proliferating intestinal epithelial cells
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On page 25551, a Western blot for another protein (not SHP-1) was inadvertently printed in Fig. 4B. The correct figure is shown below. The results were not affected by this error.