Sphingosine kinase 1 overexpression stimulates intestinal epithelial cell proliferation through increased c-Myc translation

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Jiang P, Smith AD, Li R, Rao JN, Liu L, Donahue JM, Wang JY, Turner DJ. Sphingosine kinase 1 overexpression stimulates intestinal epithelial cell proliferation through increased c-Myc translation. Am J Physiol Cell Physiol 304: C1187–C1197, 2013. First published April 10, 2013; doi:10.1152/ajpcell.00271.2012.—Sphingosine kinase 1 (SphK1), the rate-limiting enzyme for S1P synthesis, significantly increased cell proliferation and that this occurred through enhanced expression of c-Myc. Further, we found that the increased pattern of expression of c-Myc occurred predominantly due to its increased translation. The overexpressed SphK1 led to increased checkpoint kinase 2 and enhanced HuR phosphorylation which allowed for increased translation of c-Myc mRNA through HuR binding at the 3′-untranslated regions. Our findings demonstrate that S1P modulates intestinal cell proliferation and provides new insights as to the mechanistic actions of SphK1 and S1P in maintaining intestinal epithelial homeostasis.

 UNDER NORMAL PHYSIOLOGICAL conditions the mammalian intestinal mucosa has the highest cell turnover rate, and maintaining mucosal integrity is vital for many intestinal functions and, ultimately, for survival as well. Cells normally replicate in the intestinal crypts and migrate to the villus tips as they differentiate; replication here is critically balanced with apoptosis; ultimately, for survival as well. Cells normally replicate in the intestinal crypts and migrate to the villus tips as they differentiate; replication here is critically balanced with apoptosis; thus, the exact mechanisms regulating cell proliferation are not completely understood (10).

Sphingosine-1-phosphate (S1P) is a potent bioactive lipid mediator, which can function as both an intracellular signaling moiety and as a ligand of cell surface receptors. S1P is shown to be involved in a variety of physiological and pathophysiological processes, including Ca2⁺ mobilization, cytoskeletal organization, cell growth, differentiation, survival, and motility (28, 36, 45). S1P is produced intracellularly from its precursor sphingosine through the enzymatic activity of sphingosine kinases (SphKs; Ref. 46). Increased intracellular levels of S1P stimulate proliferation of quiescent Swiss 3T3 fibroblasts (45, 39); similarly, overexpression of sphingosine kinase 1 (SphK1) in NIH 3T3 fibroblasts resulted in an increase in cell proliferation (29). Although the precise molecular processes governed by S1P and SphK1 are not fully understood, S1P is shown in several nonintestinal organ systems to regulate epithelial integrity with associated changes in the expression of growth-related genes (36).

The protooncogene c-Myc encodes a nuclear transcription factor involved in the regulation of cell proliferation, differentiation, and apoptosis (38, 6, 34). c-Myc expression is controlled at multiple levels, including transcription (24), stability of both mRNA and protein (33), and translation (15, 20, 41). Although c-Myc upregulation is observed in conditions of increased S1P and SphK (16), a causal relationship is not entirely known nor are any mechanisms whereby S1P regulates c-Myc translation and is central to the current study.

HuR is a 36-kDa RNA binding protein (RBP) possessing two NH2-terminal RNA recognition motifs (RRMs) with a high affinity for AU-rich elements (AREs) and a COOH-terminal RRM that recognizes the poly(A) tail (2). HuR has emerged as a key regulator of genes that are central to cell proliferation, stress response, immune cell activation, carcinogenesis, and replicative senescence (22). HuR is predominantly localized in the nucleus of cells but shows enhanced activity upon translocation to the cytoplasm where it stabilizes specific mRNAs, affects the translation of several target mRNAs, or both (23). Evidence has shown that checkpoint kinase 2 (Chk2) phosphorylates HuR and alters its interaction with several target mRNA transcripts including c-Myc after exposure to oxidative stress (3). In addition, protein kinase C phosphorylates HuR and increases its cytoplasmic abundance (1), whereas the cytoplasmic accumulation of HuR was prevented by cyclin-dependent kinase-1-mediated HuR phosphorylation (14).

In this study we tested the hypothesis that increasing S1P by ectopic SphK1 overexpression stimulates cell proliferation through increased c-Myc expression via HuR activation. In cells stably overexpressing SphK1, cell proliferation was enhanced, as G1 to S phase transition was increased vs. cells transfected with control vector. c-Myc protein was increased in these cells, and this was due to an increase in its translation. Ultimately, the enhanced c-Myc translation was modulated though HuR phosphorylation by Chk2.

MATERIALS AND METHODS

Cell culture and supplies. DMEM and dialyzed fetal bovine serum were from Invitrogen (Carlsbad, CA), and biochemicals were from Sigma (St. Louis, MO). The IEC-6 cell lines are derived from normal rat intestinal crypt cells as described previously (32) and were purchased from the American Type Culture Collection as were HEK cells. IEC-6 cells were maintained in DMEM supplemented with 5%...
heat-inactivated fetal bovine serum and antibiotics. Antibodies recognizing HuR, c-Myc, GAPDH, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and the antibodies against all phosphorylated proteins were obtained from Cell Signaling Technology (Danvers, MA). Chk2 antibody was from BD Biosciences Pharmingen (San Diego, CA).

**Stable cell line production and characterization.** Human full-length SphK1 plasmid (OriGene) was linearized with the restriction enzyme Not I, sequenced, and then subcloned to an expression vector pCMV6-Neo (Fig. 1A). Resulting clones were sequenced for the confirmation of successful subcloning. IEC-6 cells were transfected with the SphK1 expression vectors or control vectors containing no SphK1 cDNA using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. After incubation for 5 h, the transfection medium was replaced by the standard growth medium containing 5% FBS for 2 days before exposure to the selection medium containing 0.6 mg/ml G418. Clones resistant to the selection medium were isolated, cultured, and screened for SphK1 expression by Western blot analysis with specific anti-SphK1 antibody.

**Plasmid construction.** The chimeric firefly luciferase reporter construct containing the c-Myc 3′-untranslated regions (3′-UTR) was generated as described previously (20). The 456-basepair ARE fragment from the c-Myc 3′-UTR was amplified and subcloned into the pGL3-Luc plasmid (Promega, Madison, WI) to generate the chimeric pGL3-Luc-c-Myc-3′-UTR. The sequence and orientation of the fragment in the luciferase reporter were confirmed by DNA sequencing and enzyme digestion. Transient transfections were performed using the Lipofectamine reagent and performed as recommended by the manufacturer (Invitrogen). The luciferase reporter constructs were transfected into cells along with phRL-null, a Renilla luciferase control reporter vector from Promega, to monitor transfection efficiencies as described previously (42). Luciferase activity was measured using the Dual Luciferase Assay System (Promega) following the manufacturer’s instructions. The firefly-to-Renilla luciferase activity ratio was further compared with the levels of each luciferase mRNA.

**Cell cycle analysis.** Cell cycle analysis was performed as described previously (19); after treatment cells were collected by trypsinization and processed using the CycleTHER PLUS DNA Reagent Kit (Beckton Dickinson, San Jose, CA) according to the manufacturer’s instructions. Briefly, after trypsinization the cells were centrifuged, and the cells were washed in a buffer containing sodium citrate, sucrose, and DMSO. Cells were then incubated sequentially for 10 mins each in solution A (containing trypsin in a spermine tetrahydrochloride detergent buffer for the enzymatic digestion of cell membranes and cytoskeletons), solution B (containing trypsin inhibitor and ribonuclease A in citrate-stabilizing buffer with spermine tetrahydrochloride to inhibit the trypsin activity and to digest the RNA), and solution C (containing propidium iodide and spermine tetrahydrochloride in citrate stabilizing buffer for the stoichiometric binding of propidium iodide to the DNA at a final concentration of 125 μg/ml). Flow cytometry analysis was carried out to examine the cell cycle distribution in a Beckton Dickinson FACS Calibur analyzer (Becton Dickinson). Data were further analyzed using the software FLOWJOW Ver. 6.1.1 (Tree Star, San Carlos, CA) with the Watson Pragmatic Model.

**SIP secretion and SphK1 activity assays.** Secretion of SIP and SphK1 activity from stable cell was measured by 3H-incorporation assays as described previously (27, 30). In brief, cells were incubated with 1.5 μM and 0.45 μCi t-erythro-[3-3H]sphingosine for 10 min at 37° to label intracellular sphingosine pools and produce labeled SIP. Alkaline chloroform-methanol extraction was used to extract SIP from the medium and cells as described previously (12). Data are expressed as picomoles SIP released per milligrams of protein, and SphK1 activity was calculated by SIP production in unit time.

**Growth curve assay.** To determine the proliferative ability of the SphK1 stable cell and control vector cell, 1.5 × 10⁴ cells were plated in 12-well plates (4 replicates for each time point), and the number of...
the cells was counted. The growth assay was performed over a period of indicated days, at which time the cells were confluent.

**RNA interference.** The silencing RNA duplexes that were designed to specifically inhibit HuR mRNA were synthesized and transfected into cells as described previously (47). The sequence of small interfering RNA (siRNA) that specifically targets HuR mRNA (siHuR) was AACCACGCTGAAAGCGTTGAG; while the sequence of control siRNA (C-siRNA) was AAGTGTAGTAGACTACACCCG. The siRNA that was designed to specifically inhibit Chk2 mRNA (siChk2) was GAACCUGAGGCCAUACC, to inhibit c-Myc mRNA (sic-Myc) was (CGAGCUAAAACGGAGCUUU), whereas the sequences of its C-siRNA were CAGCGTGTTTGTGACTACACCAACA and GGCUAGCGCCAGGCCA. For each 60-mm cell culture dish, 15 μl of the 20-mM stock duplex siHuR, siChk2, sic-Myc, or C-siRNA was mixed with 300 μl of Opti-MEM medium (Invitrogen). This mixture was gently added to a solution containing 15 μl of Lipofectamine 2000 in 300 μl of Opti-MEM. The solution was incubated for 20 min at room temperature and gently overlaid onto monolayers of cells in 3 ml of medium, and cells were cultured for various assays after 48 h later.

**Western blot analysis.** Whole cell lysates were prepared using 2% SDS, sonicated, and centrifuged at 4°C for 15 min. The supernatants were boiled for 5 min and size-fractionated by SDS-PAGE. After proteins were transferred onto nitrocellulose filters, the blots were incubated with primary antibodies; after incubations with secondary antibodies, immunocomplexes were developed by using chemiluminescence.

**RT followed by PCR and real-time quantitative PCR analysis.** Total RNA was isolated from cells after different treatments by using RNeasy mini kit (Qiagen, Valencia, CA) and used in reverse transcription and PCR amplification reactions as described previously (47). The levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR product were assessed to monitor the evenness in RNA input in RT-PCR samples. Real-time quantitative (q)PCR analysis was performed using 7500-fast real-time PCR systems with specific primers, probes, and software (Applied Biosystems, Foster City, CA).

**Analysis of newly translated protein and polysome analysis.** New synthesis of c-Myc protein was measured by L-[35S]methionine and L-[35S]cysteine incorporation assays as described previously (17). Cells were incubated with 1 μCi (1 Ci = 37 GBq) of l-[35S]methionine and L-[35S]cysteine per 150-mm plate for 20 min, whereupon cells were lysed using radioimmunoprecipitation assay buffer. Immunoprecipitations were carried out for 1 h at 4°C by using either a polyclonal antibody recognizing c-Myc or GAPDH (Santa Cruz Biotechnology). After extensive washes in TNN buffer (50 mM Tris·HCl, pH 7.5, 250 mM NaCl, 15 mM MgCl2, 15 mM Tris·HCl, pH 7.6, 1% Triton X-100, 1 mg/ml heparin, and 0.1 mg/ml cycloheximide) and lysed on ice for 10 min. Nuclei were pelleted (10,000 g; 10 min), and the resulting supernatant was fractionated through a 10%–50% linear sucrose gradient to fractionate cytoplasmic components according to their molecular weights. The eluted fractions were prepared with a fraction collector (Brandel, Gaithersburg, MD), and their quality was monitored at 254 nm by using a UV-6 detector (ISCO, Lincoln, NE). After RNA in each fraction was extracted with 8 M guanidine-HCl, the levels of each individual mRNA were quantified by RT-qPCR in each of the fractions, and their abundance was represented as a percentage of the total mRNA in the gradient.

**RESULTS**

**Stable overexpression of SphK1 increases production of S1P in IEC-6 cells.** To study the potential functions of S1P in IECs, intestinal cell lines stably overexpressing SphK1 or empty vector were established in IEC-6 cells. The structure of the plasmid is shown in Fig. 1A, left. Two clones (SphK1-IEC-C1 and SphK1-IEC-C2) overexpressing SphK1 were selected, whose levels of SphK1 were increased approximately fivefold compared with control vector cells lacking the SphK1 cDNA (Fig. 1A, right). We measured S1P production and SphK1 activity in the stable cell lines, assessing [3H]sphingosine uptake and conversion to [3H]S1P in the cells compared with control vector cells. As shown in Fig. 1B, SphK1 activity was increased significantly in SphK1-IEC-C1 and SphK1-IEC-C2 compared with vector alone. Next, we examined [3H]S1P secretion, as well as total [3H]S1P, which was each increased dramatically in both SphK1 stable cell lines (Fig. 1C).

**SphK1 overexpression increases cell proliferation and induces G1 to S phase cell cycle transition.** Cell cycle analysis was performed in stable SphK1-IEC-C1 and SphK1-IEC-C2 clones and control vector cells. The results demonstrate that overexpression of SphK1 reduced the fraction of cells in G0/G1 phases with an increased proportion of cells in S phase (Fig. 2A). Cell proliferation activity was similarly demonstrated over the long term via a cell growth curve assay. Two clones, SphK1-IEC-C1 and SphK1-IEC-C2, showed a higher proliferation rate, ~1.2- to 1.5-fold higher than control vector cells (Fig. 2B). The growth curve of the SphK1 stable cells were of the typical "S" shape having a detention phase, a logarithmic phase, and a plateau phase. The growth curves in control vector cells and SphK1 stable cells diverged after 4 days and growth of the SphK1 stable cells was markedly enhanced thereafter, demonstrating that the alteration in cell-cycle distribution resulted in increased cell growth rate.

**SphK1 overexpression enhances c-Myc expression.** We examined the effects of overexpression of SphK1 on c-Myc expression. A pronounced increase in c-Myc protein expression (~4-fold higher) was observed in the SphK1 cells (SphK1-IEC-C1 and SphK1-IEC-C2) without appreciable effects on CDK-4 or c-Jun, two other cell cycle regulatory proteins (Fig. 3Aa), showing that the regulation of the expression of c-Myc protein is specific. We next transiently transfected the SphK1 plasmid into HEK cells, showing increased expression of SphK1 and approximately eight times expression of c-Myc protein (Fig. 3Ab). Similarly, we exposed IECs to...
bated in the presence of L-[35S]methionine and L-[35S]cysteine we compared the rate of new c-Myc synthesis between control actions at the translational level. To investigate this directly, comparing SphK1 clones and control vector cells (data not shown), protein stability demonstrated that it was unchanged in comparison of HuR with endogenous c-Myc mRNA in control vector cells and SphK1 stable cells. Polyribosome distribution profiles were examined in control vector cells and SphK1 clone cells as described previously (7). In this study, fractions 1–4 included mRNAs that were not associated with components of the translation machinery or cosedimented with ribosome subunits (monosomes); hence, they were not considered to be translated. Fractions 5–7 included mRNAs that bound to single ribosomes or formed polysomes of low molecular weight, and they were considered to be translated at low-to-moderate levels. Fractions 8–10 included the mRNAs that were associated with polysomes of high molecular weight, and they were thus considered to be actively translated. We found that there were no significant global changes in polysomal profiles between control vector cells and SphK1 stable cells (SphK1-IEC-C1). When the distribution of c-Myc mRNA levels in fractions across the gradient in control vector and SphK1 stable cells (SphK1-IEC-C1) was examined, it was found to be more abundant in high-translating fractions of SphK1 stable cells (Fig. 3D, left). This redistribution of c-Myc mRNA in polyribosomes after overexpression of SphK1 was specific, as the housekeeping GAPDH mRNA distributed similarly in control and stable SphK1 cells (Fig. 3D, right).

**Overexpression of SphK1 increases HuR binding to the c-Myc 3′-UTR.** We also examined the association of endogenous HuR with endogenous c-Myc mRNA in control vector and SphK1 stable cells by RNP IP assays. There was abundant c-Myc mRNA in the RNP complexes immunoprecipitated using anti-HuR antibody, as measured by RT-qPCR analysis (Fig. 4A, left). The association of endogenous c-Myc mRNA with endogenous HuR was increased three- to fourfold in SphK1 stable cells (SphK1-IEC-C1 and SphK1-IEC-C2). Importantly, the c-Myc mRNA was absent in nonspecific IgG IPs (Fig. 4A, right). In this study, GAPDH mRNA was also examined as a negative control (data not shown), because this highly abundant (housekeeping) transcript is present as a low-level contaminant in the IP materials, thus serving to monitor the equal input of lysate as reported previously (1). Together, these findings present that cytoplasmic HuR specifically associates with the 3′-UTR of c-Myc mRNA and that this interaction increased after SphK1 overexpression.

We next studied the interaction of c-Myc mRNA with RNA binding protein HuR. HuR was chosen in this study because c-Myc mRNA contains several computationally predicted hits of the HuR signature motif and has been reported to interact with HuR (18). Our previous studies show that polyamine levels regulate HuR binding to the c-Myc 3′-UTR (47, 21), but normalization of firefly luciferase. To distinguish translational output from changes in mRNA turnover, the luciferase activity assays were normalized to luciferase-reporter mRNA levels to assess the translation efficiency. As shown in Fig. 3C, overexpression of SphK1 enhanced c-Myc translation as indicated by an increase in c-Myc ARE luciferase reporter gene activity. By contrast, no changes in luciferase activity were seen in response to SphK1 overexpression for the control construct without the c-Myc ARE (data not shown). These results indicate that overexpression of SphK1 increases c-Myc mRNA translation through the c-Myc 3′-UTR ARE.

To further elucidate the effect of overexpression of SphK1 on c-Myc mRNA translation, we examined the relative distributions of c-Myc mRNA on individual fractions from polyribosome gradients on control vector cells and SphK1 stable cells. Polyribosome distribution profiles were examined in control vector cells and SphK1 clone cells as described previously (7). In this study, fractions 1–4 included mRNAs that were not associated with components of the translation machinery or cosedimented with ribosome subunits (monosomes); hence, they were not considered to be translated. Fractions 5–7 included mRNAs that bound to single ribosomes or formed polysomes of low molecular weight, and they were considered to be translated at low-to-moderate levels. Fractions 8–10 included the mRNAs that were associated with polysomes of high molecular weight, and they were thus considered to be actively translated. We found that there were no significant global changes in polysomal profiles between control vector cells and SphK1 stable cells (SphK1-IEC-C1). When the distribution of c-Myc mRNA levels in fractions across the gradient in control vector and SphK1 stable cells (SphK1-IEC-C1) was examined, it was found to be more abundant in high-translating fractions of SphK1 stable cells (Fig. 3D, left). This redistribution of c-Myc mRNA in polyribosomes after overexpression of SphK1 was specific, as the housekeeping GAPDH mRNA distributed similarly in control and stable SphK1 cells (Fig. 3D, right).

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whether it was also regulated by SphK1 is unclear, so we used a Biotin pulldown and RNP-IP to detect this binding. First, we detected HuR expression in both the control vector cells and the clone cells and also for both cytoplasmic and nuclear fractions. As shown in Fig. 4B, SphK1 overexpression clones had increased levels of cytoplasmic and whole cell HuR levels with unchanged nuclear fractions.

Next, given the predicted affinity of HuR for the 3′-UTR of the c-Myc mRNA, we hypothesized that HuR bound the c-Myc 3′-UTR and further postulated that this association could be regulated by SphK1. To study the association of HuR with c-Myc mRNA, we used biotinylated transcripts spanning the c-Myc 3′-UTR in RNA pull-down assays using cell lysates prepared from either SphK1 overexpression clones or control
vector cells. The c-Myc 3′-UTR transcript readily associated with cytoplasmic HuR, as detected by Western blot analysis of the pull-down material, binding intensity increasing dramatically when using lysates prepared from SphK1 stable cells (SphK1-IEC-C1 and SphK1-IEC-C2; Fig. 4C). In contrast, transcripts corresponding to the coding region of c-Myc mRNA did not bind to HuR (Fig. 4C). To determine the specificity of binding of the c-Myc 3′-UTR to HuR, competition experiments were carried out. As shown in Fig. 4D, the association of the c-Myc 3′-UTR with HuR was progressively inhibited when increasing concentrations of unlabeled c-Myc 3′-UTR were added to the binding reaction.

HuR phosphorylation by Chk2 is essential for binding to c-Myc mRNA. Recently, Chk2 was shown to influence HuR function by regulating its phosphorylation in response to oxidative stress or polyamine depletion (1, 21). According to our previous results, Chk2 physically interacted with HuR in IEC-6 cells (21). To investigate the possibility that SphK1 overexpression modulated the association of HuR with c-Myc mRNA through Chk2-regulated HuR phosphorylation, we examined changes in Chk2 and HuR levels in control vector and SphK1 stable cells (Fig. 5A, top). As shown, SphK1 overexpression increased the Chk2 and HuR abundance and increased levels phosphorylated HuR (p-HuR; Fig. 5A, bottom), the active kinase. Also seen were increased levels of phosphorylated Chk2 (p-Chk2; data not shown). Increased levels of Chk2 in stable SphK1 cells were associated with a significant increase in the level of p-HuR, whereas silencing of Chk2 resulted in a decrease in the level of p-HuR without an effect on total HuR levels in both SphK1-IEC-C1 and SphK1-IEC-C2 (Fig. 5B).
Silencing c-Myc reverses increases in SphK1-induced cell proliferation. This specificity of c-Myc-induced increases in cell proliferation was investigated through silencing c-Myc expression via transfection with si-c-Myc in the stable SphK1 cells. Silencing c-Myc increased the fraction of cells in G0/G1 phase and reduced the proportion of cells in S phase (Fig. 7, A and B). Similar effects were noted on cell number, which was decreased at day 3 after c-Myc silencing, and maintained at day 5 (Fig. 7C).

DISCUSSION

S1P is a known regulator of cell proliferation, paracellular permeability, and apoptosis, but the exact mechanisms responsible for these events, as well as the exact effects of S1P in intestinal epithelia are not well understood. The present study demonstrates that IECs overexpressing SphK1 possess increased levels of S1P and have higher proliferative activity, accompanied by higher expression of c-Myc. Further experiments showed that the enhanced c-Myc expression occurred at the translational level and that cells with increased SphK1 expression had higher HuR association with c-Myc mRNA. Increased S1P activity enhanced Chk2 activity, leading to abundant HuR with higher c-Myc association, resulting in increased amounts of c-Myc and ultimately increased cell proliferation. This provides new insights into the regulatory actions of S1P, which has known roles in cellular proliferation and apoptosis.

S1P acts as an important mediator of cell survival in several organ systems in physiological and pathological states (36, 45). S1P is a bioactive factor that is ubiquitous, as it is generated in the metabolism of sphingolipids present in eukaryotic cell membranes (1), and also dietary sphingomyelin is converted to sphingosine and ceramide (16). The level of S1P is tightly regulated, and alterations in S1P or of the enzymes regulating its synthesis or destruction often lead to variations in cell proliferation. Intracellular sphingosine phosphatase, sphingosine lyase, and the sphingosine kinases are among the intracellular enzymes that regulate intracellular production and concentration of S1P (35).

S1P is shown to regulate cell proliferation and survival (10). Gene deletion and reverse pharmacology studies have provided evidence that many of the biological effects of S1P are mediated extracellularly via five specific G protein-coupled receptors, formerly termed the endothelial differentiation gene receptors but now designated S1P1–5 (4, 36, 46). Our group has shown that S1P acts via the S1P receptors to confer a protective effect from apoptotic stimuli in IECs (1). Treatment with S1P also stimulates proliferation of quiescent Swiss 3T3 fibroblasts and increases DNA synthesis (45, 39). Platelets are known to be devoid of sphingosine lyase (which catalyzes S1P to ceramide (16, 44), and therefore, platelets are a potential source of extracellular S1P upon platelet destruction.

S1P also mediates other cellular effects via an intracellular signaling pathway that has not yet been fully characterized. Microinjection of S1P mobilizes calcium (40) and enhances proliferation and survival (39). Intestinal adenomas have low sphingosine lyase levels, with the resulting increase in S1P contributing to their phenotypical increased proliferation (16, 37). Alternatively, inhibitors of SphK block formation of S1P and selectively inhibit cellular proliferation induced by plate-

Fig. 5. HuR phosphorylation by Chk2. A: levels of Chk2 and HuR protein were assessed by Western blot analysis after detecting HuR and Chk2. To determine changes in levels of p-HuR, cell lysates were immunoprecipitated with anti-HuR antibody, and precipitates were analyzed by Western blotting with the antibody against all phosphorylated proteins (pProteins). B: cell lysates were IPed by anti-Chk2 antibody, and precipitates were analyzed by Western blotting with the antibody against all phosphorylated proteins (pProteins), whole cell lysate were prepared to measure Chk2 and HuR expression by Western blot. Data are representative from 3 independent experiments showing similar results.
let-derived growth factor nerve growth factor (9), serum (28), activation of protein kinase C (25), and cross-linking of the immunoglobulin receptors FceR1 (8) or FcgR1 (26). Deletion of SphK1 leads to significantly reduced proliferation in intestinal adenomas (16). SphK1 can be stimulated by a variety of growth factors including platelet-derived growth factor, VEGF, epidermal growth factor, TNF-α, cytokines, and even S1P itself (37). In the present study we demonstrate that stable overexpression of SphK1 results in increased cellular proliferative activity in intestinal epithelia in vitro. These clones demonstrated increased S1P, increased intracellular calcium (data not shown), and significantly higher proliferation. The effects of SphK1 overexpression on S1P production were not simply due to clonal variation, because two different clonal populations, SphK1-IEC-C1 and SphK1-IEC-C2, showed similar responses. This is most likely due to actions of the S1P generated intracellularly, which is in keeping with other reports. In one study, it was shown that SphK1 overexpression stimulated growth and survival in wild-type and in S1PR negative cells (31).

Although the precise molecular processes governed by S1P are not fully understood, S1P has been shown in nonintestinal organ systems to regulate epithelial integrity through modulating the expression of growth-related genes (36). The protooncogene c-Myc encodes a nuclear transcription factor involved in the regulation of cell proliferation, differentiation, apoptosis, and cell cycle progression (6, 34, 38). c-Myc expression is controlled at multiple levels, including transcription (24), sta-
bility of both mRNA and protein (33), and translation (15, 20, 41). Although c-Myc is associated with increased S1P signaling (16), the exact mechanisms whereby S1P affects c-Myc remain unknown. The results presented herein clearly show that SphK1-overexpressing cells have increased c-Myc expression, which is clearly associated with cell proliferation, as silencing c-Myc abrogated the stimulation of cell proliferation. This has been seen in tissue specimens, as Kohno et al. (16) have shown lower levels of c-Myc and Cdk4 mRNA and protein in murine intestinal crypts of SphK1/H11002/H11002 mice. Interestingly, the IEC cells in our study showed no difference in CDK4 levels (data not shown) with SphK1 overexpression but did demonstrate increased c-Myc expression.

c-Myc expression is known to be critically regulated via transcription (21), although several recent reports have recognized the importance of translational regulation (20, 41). These studies detail multiple sites of the c-Myc mRNA that are sources of regulational control, some within the 5′-UTR but more recently also within the 3′-UTR (20, 21). The c-Myc 3′-UTR contains AREs with potential interactions with several RBP, including AUFl, TIAR, TIA-1, and HuR. We have reported previously that the 3′-UTR of c-Myc mRNA contains U-rich elements and AREs that interact with RBPs involved in the control of c-Myc translation (18, 21). In the current study we show results indicating that specific interactions with the c-Myc mRNA 3′-UTR confer its translational efficiency, effecting levels of newly synthesized c-Myc protein (Fig. 3), increasing c-Myc luciferase reporter activity (Fig. 3), and shifting the distribution of c-Myc mRNA from low-translating polysome fractions to higher-translating polysome fractions.

Our results further indicate that the increased c-Myc expression results from increased HuR association with the 3′-UTR.
of the c-Myc mRNA (Fig. 4), as the SphK1 clones demonstrated increased cytoplasmic HuR levels. HuR traditionally serves to increase mRNA translation (4, 47), and the results in Fig. 4 demonstrate that HuR specifically binds the c-Myc 3′-UTR. Chk2 is also increased in the SphK1 clones, which has been shown to target HuR among others, with the resulting phosphorylation of HuR modifying its affinity for target mRNAs, in this case the cMyc mRNA. Figure 6 demonstrates the effects of silencing HuR or Chk2, the abolition of the increased c-Myc in the SphK1 clones, and the loss of the enhanced proliferation seen in these cells. As reported previously, there are several potential triggers that can affect HuR binding, including modifications of the transcript itself (43, 47) and the influence of microRNAs that interfere with HuR target binding (5, 15), and current studies in our laboratory are focused on these interactions.

In summary, these results indicate that SphK1 overexpression in intestinal epithelial cells regulates cell proliferation through c-Myc. The current study provides novel evidence that S1P is an important mediator in intestinal mucosa of normal cell growth and proliferation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: P.J., J.-Y.W., and D.J.T. conception and design of research; P.J., A.D.S., and D.J.T. performed experiments; P.J., A.D.S., R.L., J.N.R., J.M.D., J.Y.W., and D.J.T. analyzed data; P.J., A.D.S., R.L., and D.J.T. prepared figures; P.J. and D.J.T. drafted manuscript; P.J., A.D.S., R.L., and D.J.T. revised manuscript; P.J., A.D.S., R.L., and D.J.T. approved final version of manuscript.

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