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SHC1 sensitizes cancer cells to the 8-Cl-cAMP treatment

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ABSTRACT

8-Chloro-cyclic AMP (8-Cl-cAMP) is a cyclic AMP analog that induces growth inhibition and apoptosis in a broad spectrum of cancer cells. Previously, we found that 8-Cl-cAMP-induced growth inhibition is mediated by AMP-activated protein kinase (AMPK) as well as p38 mitogen-activated protein kinase (p38 MAPK). To identify downstream mediators of the 8-Cl-cAMP signaling, we performed co-immunoprecipitation combined with mass spectrometry using the anti-AMPK or p38 MAPK antibodies. Through this approach, SHC1 was identified as one of the binding partners of p38 MAPK. SHC1 phosphorylation was suppressed by 8-Cl-cAMP in HeLa and MCF7 cancer cells, which was mediated by its metabolites, 8-Cl-adenosine and 8-Cl-ATP; however, 8-Cl-cAMP showed no effect on SHC1 phosphorylation in normal human fibroblasts. *SHC1* siRNA induced AMPK and p38 MAPK phosphorylation and growth inhibition in cancer cells, and SHC1 overexpression re-sensitized human foreskin fibroblasts to the 8-Cl-cAMP treatment. SHC1 phosphorylation was unaffected by Compound C (an AMPK inhibitor) and SB203580 (a p38 MAPK inhibitor), which suggests that SHC1 is upstream of AMPK and p38 MAPK in the 8-Cl-cAMP-stimulated signaling cascade. On the basis of these findings, we conclude that SHC1 functions as a sensor during the 8-Cl-cAMP-induced growth inhibition in SHC1-overexpressing cancer cells.

1. Introduction

Chemotherapeutic treatment with poor cancer cell specificity could lead to severe side effects for patients. Therefore, targeting cancer-specific cellular signal pathways is fundamental to the development of anti-cancer agents. 8-Chloro-cyclic AMP (8-Cl-cAMP) has been known to induce growth inhibition, apoptosis, reverse-transformation, and differentiation of cancer cells [1–3]; whereas, it has been shown to have no or minimal effects on non-transformed cells [4,5]. Besides cyclic AMP-dependent protein kinase (PKA), 8-Cl-cAMP activates protein kinase C, Rap1 GTPase, Akt, AMP-activated protein kinase (AMPK), and p38 mitogen-activated protein kinase (p38 MAPK) during the induction of growth inhibition and apoptosis in cancer cells [4–9]. The conversion of 8-Cl-cAMP to 8-Cl-adenosine, one of the metabolites of 8-Cl-cAMP, is essential to its anti-cancer activity [6,8]. However, the accurate mechanisms pertinent to the cancer cell specificity of 8-Cl-cAMP have not been fully elucidated yet.

To search for signaling molecules regulating the activation of AMPK or p38 MAPK during the 8-Cl-cAMP-induced cancer cell growth inhibition, we performed mass spectrometric analysis of co-immunoprecipitated proteins by using anti-AMPK or p38 MAPK antibodies. Through this approach, we identified SHC1 (Src homology 2 domain-containing transforming protein 1) as one of the interacting partners of p38 MAPK.

SHC1 is an adapter protein containing Src homology 2 (SH2) domains that are conserved among cytoplasmic signaling proteins [10,11]. SHC1 is involved in various receptor-mediated signaling pathways such as growth factors [12,13], antigens [14], cytokines [15,16], hormones [17,18], and G-protein-coupled receptors [19].

Abbreviations: 8-Cl-cAMP, 8-Chloro-cyclic AMP; PKA, cyclic AMP-dependent protein kinase; AMPK, AMP-activated protein kinase; p38 MAPK, p38 mitogen-activated protein kinase; SHC1, Src homology 2 domain-containing transforming protein 1; SH2, Src homology 2; HDF, human dermal fibroblasts; HFF, human foreskin fibroblasts.

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Furthermore, SHC1 is highly phosphorylated and expressed in many types of transformed cells compared with their non-transformed counterparts [20,21]. The cancer-specific induction and activation of SHC1 might regulate tumor progression, implying that SHC1 can be a good prognostic marker and therapeutic target [22,23].

In this paper, we show that 8-Cl-cAMP and its metabolite, 8-Cl-adenosine, suppress SHC1 phosphorylation only in cancer cells, which is an upstream event of AMPK and p38 MAPK activation. We conclude that SHC1 is a crucial determinant of cancer-specific responsiveness to 8-Cl-cAMP.

2. Materials and methods

2.1. Reagents and antibodies

8-Cl-cAMP was purchased from Biolog (Bremen, Germany). Compound C and ABT702 were from Calbiochem (San Diego, CA), and SB203580 was from A. G. Scientific (San Diego, CA). Phospho-SHC1 (Tyr239/240), phospho-AMPKα (Thr172), phospho-p38 MAPK (Thr180/Tyr182), phospho-MAPKAPK2 (Thr334), and total-MAPKAPK2 antibodies were purchased from Cell Signaling Technology (Danvers, MA). The β-actin antibody was purchased from Bioworld Technology (St. Louis Park, MN). Total-SHC1, total-p38 MAPK, total-AMPKα, and normal rabbit IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). *SHC1* and *GFP* siRNAs were purchased from ST Pharm (Seoul, Korea). p46 and p52 *SHC1* cDNA were cloned by reverse transcription-PCR from the total mRNA of HeLa cells. PCR products were inserted into the *Not*I site of pcDNA3.0 vector (Life Technologies, Carlsbad, CA) and then subcloned into the *Eco*RI site of pEGFP-C3 vector (Clontech, Mountain View, CA). *p38 MAPKα* (*MAPK14*) cDNA was purchased from Korea Human Gene Bank (Medical Genomics Research Center, KRIBB, Korea) and subcloned into the *Bam*HI site of pCMV-3Tag-6 vector (Agilent Technologies, Santa Clara, CA). Primer sequences used for *SHC1* cDNA cloning are as follows (*Not*I sites are underlined);

p46 SHC1-Forward: 5′-ACC GCGGCCGC ACC ATGGGACCCGGGG-TTTCCTACTT-3′.
p52 SHC1-Forward: 5′-ACC GCGGCCGC AAG CCTCCTCCAGGA-CATGAACAAG-3′.
p46/p52 SHC1-Reverse: 5′-ACC GCGGCCGC CAC TCACAGTTT-CCGCTCCACAGGT-3′.

2.2. Cell culture

HeLa (human cervical carcinoma) and MCF7 (human breast carcinoma) cells were maintained in Eagle's minimal essential medium (EMEM; Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin G, and 100 μg/ml streptomycin. HDF (human dermal fibroblasts) and HFF (human foreskin fibroblasts) cells were cultured in DMEM (Hyclone) supplemented with 15% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin. Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. *SHC1* siRNA, p46 and p52 expression vectors, and the pcDNA3.0 vector were transfected into HeLa, MCF7, and HFF cells using Lipofectamine™ 2000 (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. The transfected cells were used for experiments after 48 h. Cell numbers were determined using a Coulter™ counter (Beckman Coulter, Fullerton, CA).

2.3. Western blot analysis

Cells were harvested by centrifugation and washed with PBS. The cell pellet was suspended in extraction buffer (50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.5% NP-40, 100 μM PMSF, 0.1 mM sodium pyrophosphate, 2.5 mM NaF, and 1 μg/ml each of Na_3VO_4, benzamidine, aprotinin, antipain, and leupeptin) and incubated on ice for 30 min. After centrifugation at 14,000 × g for 15 min at 4 °C, the supernatant was taken as cell extract. The extracts were separated on 10% SDS/PAGE and transferred onto a PVDF membrane. The protein-bound membrane was incubated with the appropriate antibodies followed by horseradish peroxidase-conjugated anti-mouse or rabbit IgG antibody (Bio-Rad, Hercules, CA). The relevant protein bands were then visualized using the ECL detection kit (GE Healthcare, Piscataway, NJ) and the Fusion Solo chemiluminescence detection system (Vilber Lourmat, Eberhardzell, Germany).

2.4. Immunoprecipitation assay

Cultured cells were washed with cold PBS, resuspended in an extraction buffer (50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1% NP-40, and 1 mM PMSF), then incubated on ice for 30 min. After lysates were clarified by centrifugation at 14,000× *g* for 10 min, cleared protein extracts (2 mg) were incubated with appropriate antibodies at 4 °C overnight with continuous agitation. To collect immune complexes, 100 μl of protein A Sepharose 4B (50% slurry, GE Healthcare) was added to the mixture and further incubated for 2 h. The precipitated complex was washed three times with extraction buffer and then resuspended in 1x SDS-PAGE sample buffer. Following SDS-PAGE, bound proteins were analyzed by immunoblotting as well as mass spectrometry. Mass spectrometric analysis was carried out by Q-Exactive MS (Thermo Scientific, Waltham, MA) at the Proteomics Core Facility in School of Biological Sciences, Seoul National University.

3. Results

3.1. SHC1 interacts with p38 MAPK

We performed co-immunoprecipitation using anti-AMPK and anti-p38 MAPK antibodies to search for the signaling molecules participating in the AMPK and p38 MAPK pathways during the 8-Cl-cAMP-induced cancer cell growth inhibition. We then identified the proteins interacted with AMPK or p38 MAPK by using mass spectrometric analysis (Supplementary Table). Among them, SHC1 was selected for further study, because it interacts with various proteins as an adapter protein [24] and regulates diverse cellular functions such as MAPK activation [25], cell proliferation [26], apoptosis [27] as well as tumor progression and metastasis [28,29]. We confirmed the interaction between SHC1 and p38 MAPK by co-immunoprecipitation followed by Western blotting (Fig. 1A). p38 MAPK was co-immunoprecipitated with p52 SHC1 that was fused to GFP to avoid masking by immunoglobulin heavy chains (~50 kD), suggesting that SHC1 is an interacting partner of p38 MAPK.

3.2. 8-Cl-cAMP suppresses SHC1 phosphorylation in cancer cells

SHC1 is phosphorylated on two tyrosine residues, Tyr239 and Tyr240, within the SH2 and PTB domains for its full activation in response to extracellular signals [30,31]. To test whether 8-Cl-cAMP influences SHC1 phosphorylation and activation, HeLa and MCF7 cells were incubated with 8-Cl-cAMP (10 μM) for 3 days, and then Western blotting was performed using antibodies against phospho-SHC1 and total SHC1 (Fig. 1B and C). The phosphorylation of SHC1 was significantly decreased from 1 to 2 days after the treatment

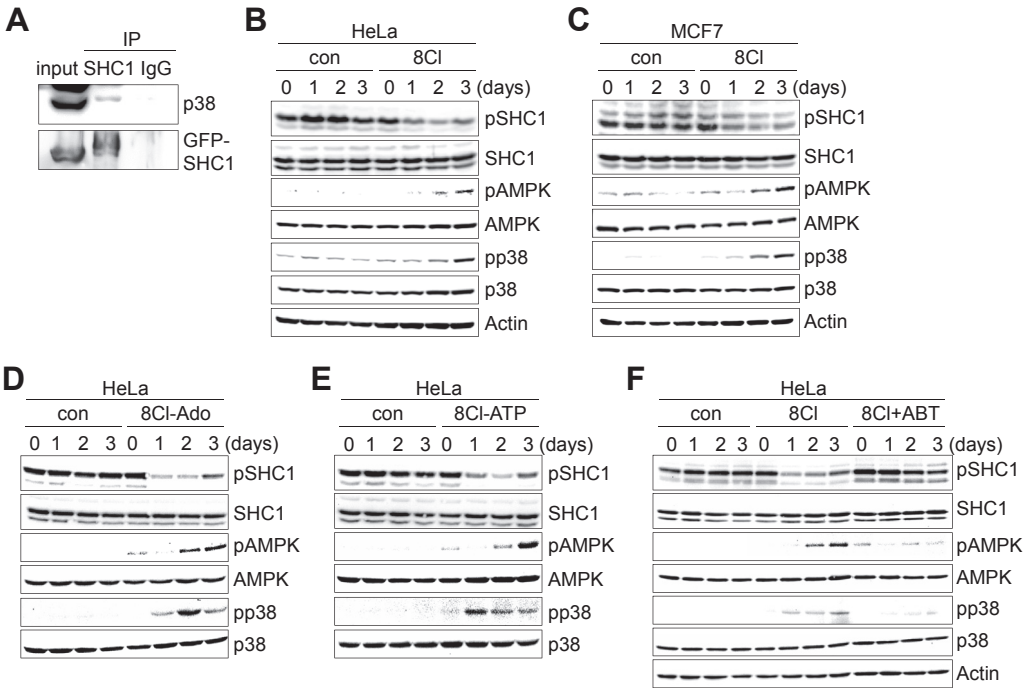

Fig. 1. 8-Cl-cAMP and its metabolites suppress SHC1 phosphorylation. (A) A co-immunoprecipitation assay was performed by using anti-SHC1 antibody in 293T cells transfected with GFP-p52 SHC1 and p38 MAPK. The bound proteins were analyzed by Western blotting using anti-SHC1 and anti-p38 MAPK (p38) antibodies. Normal rabbit IgG was used as a negative control. (B, C) HeLa (B) and MCF7 (C) cells were treated with 8-Cl-cAMP (10 μM) for the indicated times, and Western blotting was performed using anti-phospho-SHC1 (pSHC1, Y239/240), anti-SHC1, anti-phospho-AMPK (pAMPK), anti-AMPK, anti-phospho-p38 MAPK (pp38), anti-p38 MAPK (p38), and anti-actin antibodies. (D, E) HeLa cells were treated with 8-Cl-adenosine (5 μM; D) and 8-Cl-ATP (5 μM; E), and Western blotting was carried out using the indicated antibodies. (F) HeLa cells were treated with 8-Cl-cAMP (10 μM) in the presence or absence of ABT702 (an adenosine kinase inhibitor, 10 μM), and then Western blotting was carried out using the indicated antibodies.

with 8-Cl-cAMP, while the protein expression of SHC1 was unaffected in both HeLa and MCF7 cells (Fig. 1B and C). As published earlier [8,9], 8-Cl-cAMP increased the phosphorylation of AMPK and p38 MAPK in these cells (Fig. 1B and C). Also, 8-Cl-adenosine (5 μM) and 8-Cl-ATP (5 μM), the metabolites of 8-Cl-cAMP, decreased SHC1 phosphorylation and increased AMPK and p38 MAPK phosphorylation (Fig. 1D and E). In addition, pretreatment of ABT702 (an adenosine kinase inhibitor, 10 μM) effectively blocked the effect of 8-Cl-cAMP on SHC1 phosphorylation (Fig. 1F). These data collectively demonstrate that 8-Cl-cAMP inhibits SHC1 phosphorylation (or activation) in cancer cells, which is dependent on the metabolic conversion of 8-Cl-cAMP into its metabolites.

3.3. 8-Cl-cAMP has no effect on SHC1 phosphorylation in normal fibroblasts

8-Cl-cAMP-stimulated growth inhibition and apoptosis is a cancer cell-specific phenomenon [5,7]; therefore, we tested whether 8-Cl-cAMP would inhibit SHC1 phosphorylation only in cancer cells. Both SHC1 protein expression and phosphorylation were very low in two non-transformed cell types, HDF (human dermal fibroblasts) and HFF (human foreskin fibroblasts), when compared with HeLa cells (Fig. 2A and B). SHC1 phosphorylation was also not affected by 8-Cl-cAMP treatment. Inversely, both AMPK and p38 MAPK were already highly phosphorylated in the basal states of HDF and HFF, and 8-Cl-cAMP could not further activate AMPK and p38 MAPK in these low SHC1-expressing cells. These results suggest that the expression level of SHC1 protein is one of the key factors that determine the influence of 8-Cl-cAMP on AMPK and p38 MAPK signaling pathways.

3.4. SHC1 regulates AMPK and p38 MAPK activation and cancer cell growth

On the basis of the results presented above, we hypothesized that SHC1 could mediate the cellular responses to 8-Cl-cAMP treatment; that is, AMPK and p38 MAPK activation and growth inhibition. In order to prove this hypothesis, we first depleted SHC1 in HeLa cells by using *SHC1* siRNA (Fig. 3A). *SHC1* siRNA blocked the phosphorylation and expression of SHC1, and augmented AMPK and p38 MAPK phosphorylation (activation). Moreover, in both HeLa and MCF cells, *SHC1* siRNA suppressed cell growth (Fig. 3B), which mimics the effects of 8-Cl-cAMP treatment.

Next, we overexpressed SHC1 in non-transformed cells to see if it can restore the cells' response to 8-Cl-cAMP. HFF cells were transfected with two SHC1 isoform expression vectors, p46 and p52, which are involved in cellular proliferation and carcinogenesis [20]. With enough SHC1 protein expression, HFF cells started to respond to 8-Cl-cAMP; AMPK and p38 MAPK phosphorylation were enhanced (Fig. 3C) and the growth inhibitory effect became prominent (Fig. 3D). These results support the idea that 8-Cl-cAMP induces growth inhibition only in SHC1-overexpressing cancer cells, and that SHC1 acts as a sensor responding to the 8-Cl-cAMP treatment.

3.5. SHC1 is upstream of AMPK and p38 MAPK during 8-Cl-cAMP-induced growth inhibition

8-Cl-cAMP induces growth inhibition through the phosphorylation/activation of AMPK and p38 MAPK only in cancer cells with high SHC1 expression; however, the signaling cascade between SHC1, AMPK, and p38 MAPK is not clear yet. Given that SHC1

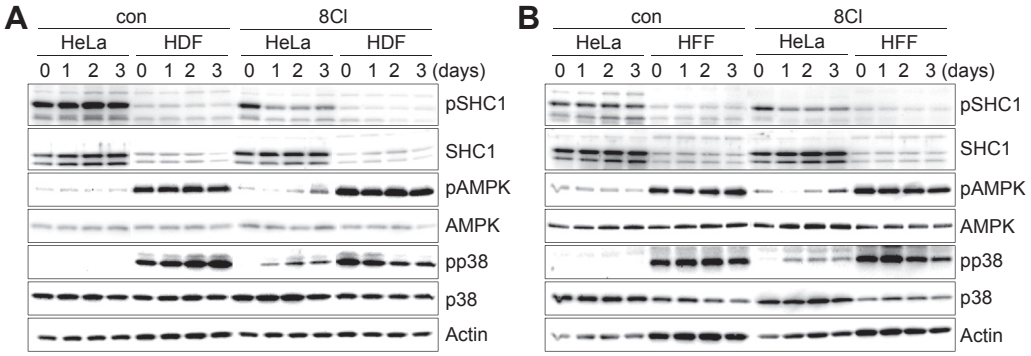

Fig. 2. The decrease of SHC1 phosphorylation by 8-Cl-cAMP is limited to cancer cells. (A, B) HeLa cells, HDF (human dermal fibroblasts; A), and HFF (human foreskin fibroblasts; B) were treated with 8-Cl-cAMP (5 μM) for the indicated times, and Western blotting was carried out using anti-pSHC1 (Y239/240), anti-SHC1, anti-pAMPK, anti-AMPK, anti-pp38, anti-p38, and anti-actin antibodies.

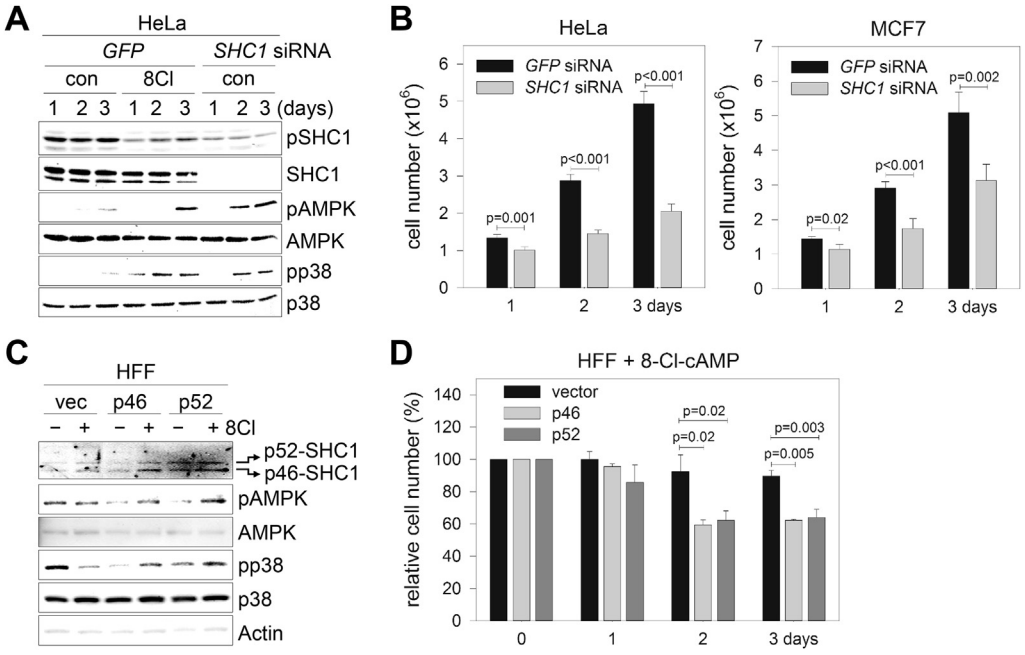

Fig. 3. SHC1 mediates 8-Cl-cAMP-induced AMPK and p38 MAPK activation, and cell growth inhibition. (A) HeLa cells were transfected with *GFP* (negative control) or *SHC1* siRNA, and Western blotting was carried out using anti-pSHC1 (Y239/240), anti-SHC1, anti-pAMPK, anti-AMPK, anti-pp38, and anti-p38 antibodies. (B) HeLa and MCF cells were transfected with *GFP* or *SHC1* siRNA, and 48 h after the transfection, cell numbers were counted daily using a Coulter™ counter (mean ± SD, n = 4). p, two-tailed Student's *t* test. (C, D) HFF cells were transfected with pcDNA3.0 control (vec), p46, or p52 expression vector, and 48 h after the transfection, the transfected cells were treated with 8-Cl-cAMP (5 μM) for 3 days. Then Western blotting was performed with the indicated antibodies (C), or cell numbers were counted daily using a Coulter™ counter (D). Bars denote the relative cell numbers normalized to those of control groups (Day 0), which were set to 100% (mean ± SD, n = 3). p, two-tailed Student's *t* test.

depletion by siRNA increased AMPK and p38 MAPK phosphorylation (Fig. 3A), SHC1 might be upstream of these kinases. To confirm this, we used chemical inhibitors against AMPK (Compound C, 1 μM) and p38 MAPK (SB203580, 1 μM). Compound C attenuated p38 MAPK phosphorylation induced by 8-Cl-cAMP, whereas SHC1 phosphorylation was not affected (Fig. 4A). SB203580 had no effect on either AMPK or SHC1 phosphorylation regulated by 8-Cl-cAMP (Fig. 4B). The phosphorylation of MAPKAPK2, a downstream factor of p38 MAPK, was entirely suppressed by SB203580, showing that this inhibitor functioned effectively. Taken together, these results demonstrate that SHC1 is an upstream signaling molecule of AMPK and p38 MAPK, and AMPK functions between SHC1 and p38 MAPK in the 8-Cl-cAMP-induced signaling cascade.

4. Discussion

8-Cl-cAMP and its metabolite, 8-Cl-adenosine have been investigated as therapeutic agents against several cancers including solid tumors [32], multiple myeloma (ClinicalTrials.gov Identifier: NCT00004902), and leukemia (NCT00714103); however, the precise action mechanism of 8-Cl-cAMP's anti-tumor activity has not been fully identified. Previously, we showed that AMPK and p38 MAPK are essential factors for 8-Cl-cAMP-induced growth inhibition [8,9]. In this study, we revealed that p38 MAPK interacts with SHC1 (Fig. 1A), an adapter protein interacting with various signaling molecules [11,24]. SHC1 phosphorylation was diminished after the treatment with 8-Cl-cAMP in HeLa and MCF7 cells (Fig. 1B and C),

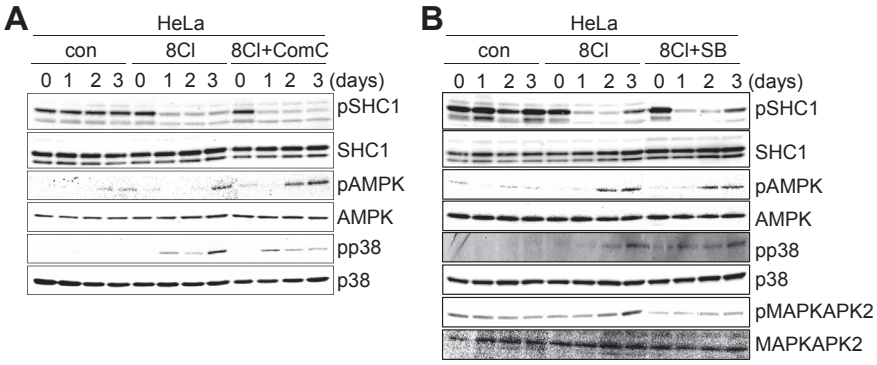

Fig. 4. SHC1 is upstream of AMPK and p38 MAPK during the 8-Cl-cAMP-induced growth inhibition. (A, B) HeLa cells were pre-treated with Compound C (ComC, an AMPK specific inhibitor, 1 μM; A) or SB203580 (SB, a p38 MAPK specific inhibitor, 1 μM; B) for 1 h prior to the treatment with 8-Cl-cAMP (10 μM). Western blotting was performed with anti-pSHC1 (Y239/240), anti-SHC1, anti-pAMPK, anti-AMPK, anti-pp38, anti-p38, anti-phospho-MAPKAPK2 (pMAPKAPK2), anti-MAPKAPK2, and anti-actin antibodies.

which might be necessary for 8-Cl-cAMP to induce growth inhibition and AMPK and p38 MAPK activation in SHC1-overexpressing cells. *SHC1* siRNA induced AMPK and p38 MAPK activation and suppressed cellular growth (Fig. 3A and B), but chemical inhibitors against AMPK and p38 MAPK had no effect on the phosphorylation status of SHC1 (Fig. 4A and B). These results imply that 8-Cl-cAMP first targets SHC1 and its signaling network, and then triggers AMPK and p38 MAPK phosphorylation/activation and the subsequent growth inhibition in cancer cells.

Even though we proved that SHC1 is a key player mediating the cellular responses to 8-Cl-cAMP, we did not elucidate how SHC1 controls the phosphorylation of AMPK and p38 MAPK. We surmise that SHC1 acts like a scaffold protein, holding signaling molecules required for AMPK and p38 MAPK activation [24], and when dephosphorylated by 8-Cl-cAMP, SHC1 releases these molecules to activate AMPK and p38 MAPK. Currently, we are attempting to identify the SHC1-associated signaling molecules regulating the AMPK and p38 MAPK pathway during the 8-Cl-cAMP-induced growth inhibition.

There are three isoforms of SHC1 (46, 52, and 66 kDa), which result from alternative RNA splicing or different translational start sites [10,33]. In HeLa and MCF7 cells that were used in this study, the p46 and p52 isoforms are abundant (Fig. 1B and C), and the p66 isoform is known to be a negative regulator of SHC1 function [33]; hence, we overexpressed the p46 and p52 isoforms in HFF cells to restore their sensitivity to 8-Cl-cAMP. Both p46 and p52 isoforms could enable HFF cells to be sensitive to the 8-Cl-AMP treatment, suggesting that these isoforms function interchangeably during the 8-Cl-cAMP-induced growth inhibition.

It is noteworthy that a slight increase of the SHC1 protein level could sensitize HFF cells to the 8-Cl-cAMP treatment. Although SHC1 protein marginally increased after the transfection with p46 and p52 expression vectors, it was sufficient for 8-Cl-cAMP to increase AMPK and p38 MAPK phosphorylation (Fig. 3C) and to suppress cellular growth in HFF cells (Fig. 3D). Increased SHC1 may recruit more signaling molecules required for the responsiveness to 8-Cl-cAMP, which changes the local signaling network around AMPK and p38 MAPK. In other words, SHC1 can be regarded as a sensor or a balancer that determines cells' sensitivity to 8-Cl-cAMP; thus, only cells with noticeable SHC1 expression can be influenced by 8-Cl-cAMP treatment. This may be the reason why 8-Cl-cAMP exerts its cancer cell-specific effects. Collectively, these findings elucidate the signaling mechanism governing 8-Cl-cAMP-induced growth inhibition (i.e., SHC1-AMPK-p38 MAPK), and provide the experimental basis for the application of 8-Cl-cAMP as a cancer-selective therapeutic agent.

Acknowledgments

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Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.123.

Transparency document

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