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AP1G1 is involved in cetuximab-mediated downregulation of ASCT2-EGFR complex and sensitization of human head and neck squamous cell carcinoma cells to ROS-induced apoptosis

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Abstract

In this study, we expanded our recent work showing that ASCT2, a Na⁺-dependent neutral amino acid transporter that plays a major role in glutamine uptake in cancer cells, is physically associated with EGFR in human head and neck squamous cell carcinoma cells and in several other types of cancer cells. We found in our current study that ASCT2 can be downregulated by cetuximab, an approved anti-EGFR therapeutic antibody, via cetuximab-induced EGFR endocytosis independently of cetuximab-mediated inhibition of EGFR tyrosine kinase. We further found that ASCT2-EGFR association involves the adaptor-related protein complex 1 gamma 1 subunit (AP1G1), a subunit of clathrin-associated adaptor protein complex 1, which plays a role in membrane protein sorting in endosomes after receptor-mediated endocytosis. We found that AP1G1 is physically associated with both ASCT2 and EGFR and, together with those molecules, forms a heterotrimeric molecular complex. Knockdown of AP1G1 lowered the level of ASCT2-EGFR association, inhibited cetuximab-mediated internalization of ASCT2-EGFR complex, and decreased intracellular glutamine uptake and glutathione biosynthesis. These findings suggest a new therapeutic strategy to overcome cetuximab resistance in cancer cells through combination of cetuximab, which co-targets ASCT2 along with EGFR, with an ROS-inducing agent.

Keywords

AP1G1; ASCT2; EGFR; endocytosis; cetuximab

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CONFLICTS OF INTEREST

None.

1. Introduction

Both epidermal growth factor receptor (EGFR) and the alanine-serine-cysteine transporter 2 (ASCT2, encoded by *SLC1A5*) are membrane-associated proteins, and both EGFR and ASCT2 play important roles in regulating and maintaining growth and proliferation of cancer cells [1–4]. EGFR is the prototype receptor for class I transmembrane receptor with intrinsic tyrosine kinase activity that is activated via homodimerization of the receptor upon ligand binding [5]. EGFR regulates cell growth and proliferation through triggering a cascade of well-characterized signal transduction pathways after receptor activation [6–8]. Overexpression of EGFR is common in certain types of human cancer, including head and neck squamous cell carcinoma (HNSCC) [9,10]. Targeting EGFR is a well-established approach for cancer treatment. EGFR-targeted therapies include blocking ligand binding to the extracellular domain of EGFR with monoclonal antibodies, such as cetuximab, and competitively inhibiting natural ATP docking to the ATP-binding pocket in the intracellular kinase domain of EGFR with small molecule inhibitors, such as gefitinib [11–14]. By contrast, ASCT2 is a Na⁺-dependent plasma membrane transporter that maintains amino acid homeostasis via importing neutral amino acids into cells in exchange for intracellular amino acids [15]. ASCT2 engages in bidirectional transport of glutamine, serine, asparagine, and threonine but only inward transport of alanine, valine, and methionine [15]. ASCT2 is a major transporter of glutamine uptake in cancer cells [16–18], and glutamine uptake and metabolism (e.g., through glutathione biosynthesis and glutaminolysis) are critical for survival and proliferation of cancer cells that are metabolically addicted to glutamine [19–23]. Directly targeting ASCT2, however, is technically challenging because ASCT2 also has important physiological functions in normal tissues and the crystallographic structure of human ASCT2 remains to be fully elucidated [24,25].

We recently reported that ASCT2 is physically associated in close proximity to EGFR in HNSCC cells and in several other types of cancer cells [26]. We found that the level of ASCT2 can be downregulated upon treatment with cetuximab, which is approved for treatment of metastatic HNSCC and metastatic colorectal adenocarcinoma [27–32]. Downregulation of ASCT2 by cetuximab lowered glutamine uptake and decreased the intracellular glutathione level in HNSCC cancer cells and thereby sensitized HNSCC cells to induction of apoptosis by reactive oxygen species (ROS)-inducing drugs [26]. These novel findings indicate that ASCT2 in cancer cells could be co-targeted by cetuximab; however, further molecular insights into the association between ASCT2 and EGFR are needed before the new findings can be translated into an innovative, effective cancer therapy.

In our recent work [26], we demonstrated that the cetuximab-mediated downregulation of ASCT2 depends on expression of Rab5, a regulatory protein required for biogenesis of early endosomes [33], indicating that cetuximab downregulates ASCT2 via cetuximab-mediated EGFR endocytosis. However, further and more direct evidence is needed to support this proposed mechanism. This line of research is important because new knowledge regarding the mechanisms regulating ASCT2-EGFR association in cancer cells could facilitate development and optimization of the therapeutic strategy of co-targeting ASCT2 by cetuximab and facilitate identification of biomarkers predictive of response to this therapeutic strategy.

product was subcloned into pLex lentiviral vector (Open Biosystem). Lentivirus containing ASCT2-GFP fusion cDNA was packaged in HEK 293T cells after transfection of the ASCT2-GFP fusion plasmid vector, along with psPAX2 and pMD.2D packaging plasmids, using Lipofectamine-2000 transfection. Conditioned medium from the transfected HEK 293T cells was used for infecting the cells of interest.

2.5. Western blotting and immunoprecipitation

Cell lysates were prepared as we previously reported [26]. Western blot analysis was performed using the enhanced chemiluminescence detection kit (Amersham Biosciences) after incubation of the nitrocellulose membrane with various primary antibodies and horseradish peroxidase-labeled secondary antibodies [39–41]. Immunoprecipitation was performed by subjecting cell lysates to reaction with respective primary antibodies and protein A sepharose beads (Amersham Biosciences) at 4°C overnight. The resulting immunoprecipitates were subjected to Western blotting with various primary antibodies. The primary antibodies and sources were as follows: ASCT2 (D7C12) and PARP, Cell Signaling Technology; ASCT2 (H-52) and AP1G1 (F10), Santa Cruz Biotechnology; EGFR (12020), BD Transduction Co.; and EGFR (F4) and β -actin, Sigma-Aldrich Co.

2.6. Intracellular glutathione assay

Intracellular glutathione was measured using a glutathione assay kit (Cayman Chemical) according to the manufacturer's protocol as we previously described [26].

2.7. ROS detection

Intracellular ROS were detected by using a total ROS detection kit (Enzo Life Sciences) according to the manufacturer's protocol as we previously described [26]. At the end of treatment, cells were stained with ROS detection solution at 37°C for 1 h and then observed under a fluorescence microscope.

2.8. Immunocytochemistry

Immunocytochemical staining was performed using the DAKO IHC staining kit. Cells were first fixed with 95% acetone and 5% ethanol at 4°C for 10 min. This step was followed by 1-h incubation with 1% rabbit serum to block nonspecific binding, overnight incubation with ASCT2 antibody (D7C12, 1:200) or a control antibody at 4°C, and 1-h incubation with HRP-labeled goat anti-rabbit IgG at room temperature. The cells were rinsed with cold PBS between steps. Diaminobenzidine was used as a substrate of HRP for color development. Hematoxylin was used for cell nuclear counterstaining. ImageJ image processing tool was used to quantify relative ASCT2 expression in cells after various treatments [41–46].

2.9. Cross-linking

Cells were washed with PBS three times and then incubated with 2 mM disuccinimidyl suberate (DSS, Pierce Chemical Co.) in PBS for 30 min at 4°C. The reaction was quenched by washing the cells three times with 20 mM Tris-buffered saline, pH 7.5.

2.10. Protein identification by mass spectrometry

HN5 cell lysates were subjected to immunoprecipitation with cetuximab and ASCT2 antibody (D7C12), respectively. The immunoprecipitates were separated by SDS-PAGE. Coomassie blue-stained protein bands of interest were cut for protein identification by the Mass Spectrometry Proteomics Core of Baylor College of Medicine.

2.11. Endosome labeling and image processing

CellLight Early Endosomes-RFP, BacMam 2.0 (Thermo Fisher Scientific), a fusion construct of Rab5a and TagRFP packaged in a recombinant insect baculovirus, was used for endosome labeling in cells of interest. Briefly, when a desired culture confluence was reached, the cells were incubated overnight in fresh medium containing the CellLight reagent, the volume of which was based on the number of cells to be treated according to the manufacturer's protocol. The cells were then counterstained with Hoechst 33258 before observation under a fluorescence microscope.

2.12. MTT cell proliferation assay

For measurement of cell proliferation, cells were incubated in a 24-well plate with 1 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2 h at the end of the desired treatments in cell culture [47–49]. Cells were then lysed with a lysis buffer (500 μ l/well) containing 20% SDS in dimethyl formamide/H₂O (1:1, v/v; pH 4.7) at 37°C for at least 6 h. Optical density of the cell lysates was measured at an absorbance wavelength of 570 nm.

3. Results

3.1. ASCT2 can be downregulated by cetuximab independently of EGFR kinase inhibition

To expand our recent work showing that the level of ASCT2 can be downregulated upon cetuximab treatment [26], we performed new experiments to examine the dose- and time-dependent effects of cetuximab and gefitinib, a small molecule inhibitor specific for EGFR tyrosine kinase, on the level of ASCT2 in three HNSCC cell lines (HN5, UMSCC1, and FaDu). Cetuximab downregulated ASCT2 level in both a dose-dependent and a time-dependent manner (Fig. 1A and B). The extent to which cetuximab downregulated ASCT2 level in both dose- and time-dependent manners was much less than the extent to which cetuximab inhibited cell signaling downstream of EGFR, as demonstrated by the dose- and time-dependent reductions in the level of phosphorylated ERK (Fig. 1A and B). At the shortest cetuximab treatment time examined (2 h) and at the lowest cetuximab dose examined (5 nM), the level of phosphorylated ERK was strongly inhibited but the level of ASCT2 was only moderately downregulated; only at longer treatment times and higher doses did cetuximab produce substantial downregulation of ASCT2 level. The dose- and time-dependent effects of gefitinib on the level of phosphorylated ERK were similar to the effects of cetuximab; however, whereas cetuximab produced a dose-dependent decrease in the level of ASCT2, gefitinib had no effect on downregulating ASCT2 level but actually upregulated ASCT2 level in a dose-dependent manner (Fig. 1C and D). We also examined the level of c-Myc, which was reported to be a key upstream regulator of ASCT2 expression

[50]. We found that both cetuximab and gefitinib produced dose- and time-dependent decreases in the level of c-Myc in all three HNSCC cell lines and that the effects of gefitinib were similar to or stronger than the effects of cetuximab (Fig. 1A – D). Together, these data indicate that effective inhibition of EGFR kinase activation is not required for cetuximab-induced downregulation of ASCT2 and that downregulation of ASCT2 is independent of the level of c-Myc after cetuximab treatment.

3.2. ASCT2-EGFR association involves AP1G1 in a heterotrimeric molecular complex

We recently reported detection of physical association between ASCT2 and EGFR using the Duolink-based proximity ligation assay [26], which can detect molecular interactions in close proximity (< 40 nm) [51]. To gain further molecular insight into the association between ASCT2 and EGFR, we attempted to determine whether EGFR and ASCT2 are directly associated or not. As shown in Fig. 2A, whereas EGFR dimerization was readily detected in the region around 340 kDa by Western blotting using an EGFR antibody in cultured HN5 cells upon treatment with DSS, a membrane-permeable covalent cross-linker, no signal was detected by either an EGFR antibody or an ASCT2 antibody in the region around 250 kDa in the blotting, where an EGFR-ASCT2 heterodimer would be expected if it existed. This negative finding suggested that ASCT2 and EGFR might not be directly associated. Interestingly, however, Western blotting using ASCT2 antibody H52 detected a protein with an apparent molecular weight of 85 - 95 kDa that was co-immunoprecipitated both with EGFR by the EGFR antibody cetuximab and with ASCT2 by the ASCT2 antibody H52 (Fig. 2B). The fact that this protein was detected by Western blotting by H52, an ASCT2 antibody that recognizes a peptide sequence near the C-terminus of ASCT2, indicated that this unidentified protein had some sequence homology with ASCT2. This protein was not ASCT2 because it was larger than ASCT2 and because it was not detected by D7C12, an ASCT2 antibody that recognizes a peptide sequence near the N-terminus of ASCT2.

Next, we performed mass spectrometry analysis to identify this protein that contained an amino acid sequence analogous with the amino acid sequence of the C-terminus of ASCT2. Among several candidates with molecular weight between 85 kDa and 95 kDa that were co-immunoprecipitable by both cetuximab and ASCT2 antibody H52, AP1G1 has the closest molecular weight (92 kDa) and has an amino acid sequence homologous to the C-terminus of ASCT2. AP1G1 is a component of clathrin-coated vesicles that transport ligand-receptor complexes from the plasma membrane to endosomes [36]. We next performed co-immunoprecipitation experiments followed by Western blotting using an AP1G1 antibody, which confirmed that AP1G1 was physically associated with both EGFR and ASCT2 (Fig. 2C).

Next, to determine whether AP1G1 existed in a heterotrimeric complex with ASCT2 and EGFR in addition to existing in separate EGFR-AP1G1 and ASCT2-AP1G1 dimeric complexes, we examined the level of ASCT2-EGFR association with and without expression knockdown of AP1G1. As shown in Fig. 3A, each of two different AP1G1 siRNAs successfully knocked down the level of AP1G1 without affecting the total cellular levels of EGFR and ASCT2. We found that the level of ASCT2 co-immunoprecipitated with EGFR

by cetuximab was markedly decreased in cells with AP1G1 expression knockdown compared to cells with control siRNA treatment (Fig. 3B). In a reciprocal experiment, the level of EGFR co-immunoprecipitated with ASCT2 by ASCT2 antibody D7C12 was much lower in the cells with AP1G1 knockdown than in the cells with control siRNA treatment (Fig. 3C). Moreover, cross-linking experiments in which we treated HN5 cells with DSS revealed a molecular complex with apparent molecular weight of 330 kDa, which is the expected molecular weight of ASCT2-AP1G1-EGFR heterotrimeric complex, by Western blotting using antibodies against EGFR, AP1G1, and ASCT2, respectively (Fig. 3D).

Together, these data indicate that the protein association between EGFR and ASCT2 involves AP1G1 in a heterotrimeric complex.

3.3. Cetuximab induces internalization of ASCT2 into early endosomes in an AP1G1-dependent manner

We showed recently that the downregulation of ASCT2 by cetuximab was EGFR level-dependent and was mediated via cetuximab-mediated EGFR endocytosis, as indicated by the finding that knockdown of Rab5, a key regulator of motility of early endosomes, resulted in marked decrease in the level of ASCT2-EGFR association [26]. To further elucidate the mechanism by which cetuximab downregulates ASCT2, we looked for direct evidence of cetuximab-induced ASCT2 internalization via EGFR endocytosis and direct evidence of the role of AP1G1 in cetuximab-mediated ASCT2 internalization. We generated an ASCT2-GFP cDNA fusion construct, which allowed us to track ASCT2 routing into early endosomes after cetuximab treatment. As shown in Fig. 4A, HN5 cells were stably transduced to express ASCT2-GFP fusion protein and then were treated with CellLight Early Endosomes-RFP, BacMam 2.0, a virus-based molecular marker (Rab5a and red fluorescent protein [RFP] fusion protein) that labels early endosomes with RFP in live cells. Treatment of control siRNA-treated cells with cetuximab for 2 h led to translocation of ASCT2-GFP fusion protein into the early endosomes, shown by the characteristic appearance of an orange color in the endosomes after merging of the ASCT2-GFP image and the CellLight Early Endosomes-RFP image (Fig. 4A). In contrast, in cells with AP1G1 knockdown, the same cetuximab treatment resulted in remarkably less translocation of ASCT2-GFP fusion protein into early endosomes (Fig. 4A). Figure 4B shows that compared to the levels of ASCT2-GFP fusion protein and endogenous ASCT2 in untreated cells (left lanes), levels of these proteins were unchanged after cetuximab treatment in AP1G1 siRNA-treated cells (middle lanes) but markedly decreased in control siRNA-treated cells (right lanes).

To further investigate the biological role of AP1G1 in cetuximab-mediated downregulation of ASCT2, we stained ASCT2 protein immunocytochemically using an ASCT2 antibody (D7C12), which allowed us to observe changes in ASCT2 level after various treatments in intact cells rather than just measure changes in protein level by Western blotting. An isotype control antibody was used as background and negative control. Of note, only a fraction of HN5 and UMSCC1 cells had high expression of ASCT2 detected by immunocytochemical staining (Fig. 5). Compared to the results in untreated control HN5 and UMSCC1 cells, cells with ASCT2 knockdown by siRNA exhibited a marked decrease in the level of ASCT2

expression, which was expected and served as a technical control of detection using the immunocytochemical staining protocol. Compared to the percentage of ASCT2 high expression in HN5 and UMSCC1 cells, the percentage was lower in the cells treated with cetuximab but not noticeably altered in the cells treated with gefitinib. Importantly, AP1G1 knockdown again diminished cetuximab-induced decrease in the percentage of ASCT2-high expressing cells (Fig. 5, see quantitative graphs on the right).

Together, these data strongly support a role of AP1G1 in cetuximab-mediated EGFR endocytosis, which co-internalizes EGFR-associated ASCT2 into early endosomes, leading to a lower level of ASCT2 in cells after cetuximab treatment.

3.4. Knockdown of AP1G1 decreases the therapeutic effects of cetuximab-mediated co-targeting of EGFR-associated ASCT2 in HNSCC cells

We recently reported that cetuximab downregulates ASCT2 and thereby inhibits intracellular uptake of glutamine and subsequent biosynthesis of glutathione [26]. Thus, we next examined the impact of AP1G1 knockdown on cetuximab-mediated decrease in intracellular glutathione level and cetuximab-mediated sensitization of HNSCC cells to ROS-induced apoptosis. As shown in Fig. 6A, cetuximab decreased intracellular glutathione level in HN5 and MDA1986 cells but not in the cells with AP1G1 knockdown. Cetuximab sensitized HN5 and MDA1986 cells to dichloroacetate (DCA)-induced overproduction of ROS, and the effect was substantially diminished in the cells with AP1G1 knockdown (Fig. 6B). Cetuximab also sensitized cells to DCA-induced induction of apoptosis and inhibition of cell survival (Fig. 6C and D). Neither DCA nor cetuximab alone for 24 h was able to induce PARP cleavage, a marker of apoptosis, in HN5 or MDA1986 cells, but DCA and cetuximab in combination induced PARP cleavage, which was not detected in the cells with AP1G1 knockdown (Fig. 6C). Cell proliferation assay showed enhanced cell survival inhibition with the combination of DCA and cetuximab, and the effect was diminished in the cells with AP1G1 knockdown (Fig. 6D).

Together, these data provide strong support for the conclusion that AP1G1 is required to mediate the therapeutic effects of co-targeting of ASCT2 by cetuximab in EGFR-overexpressing HNSCC cells.

4. Discussion

The findings reported here are consistent with our recent work showing that ASCT2 and EGFR are physically associated in a molecular complex and that the molecular complex can be targeted by cetuximab, leading to downregulation of ASCT2 [26]. In the current work, we found that ASCT2 and EGFR are physically associated with AP1G1 in a heterotrimeric molecular complex, which provides further evidence supporting our earlier conclusion that cetuximab induces ASCT2 downregulation via cetuximab-induced EGFR endocytosis [26].

During receptor-mediated endocytosis, clathrin-coated pits and vesicles are assembled; after receptor-mediated endocytosis, adaptor protein complexes function in the intracellular sorting and vesicular transport of membrane proteins [34–36]. AP1G1 plays a role in protein sorting to endosomes [37,52]. AP1G1 expression has been found to be high in some types of

human cancers [38]. Although the biological significance of increased AP1G1 expression in cancer is not clear yet, AP1G1 is known to play a critical role in early development because a null mutation of *AP1G1* was embryonic lethal when the mutation was homozygous [53]. Hundreds of proteins have been predicted to interact with AP1G1 [54], so it is not surprising that EGFR and ASCT2 are on the list. Interestingly, however, we found that AP1G1 is associated not only with ASCT2 and EGFR individually but also with ASCT2 and EGFR together in a heterotrimer. This finding suggests that AP1G1 plays a role in ASCT2-EGFR association. At present, the biological significance of ASCT2-EGFR association is unclear. Whereas the mode of activation and mechanisms of regulation of EGFR are well known, little is known about how the transporter function of ASCT2 is regulated. Our data suggest that ASCT2 may be regulated in part through its physical association with EGFR and therefore be subject to EGFR-mediated regulatory mechanisms. Our current findings justify further in-depth work to address these unknowns.

With respect to the mechanism by which cetuximab downregulates ASCT2, we provide here new, direct experimental evidence that an ASCT2-GFP fusion protein can be routed into early endosomes after cetuximab treatment. Given the role of AP1G1 in transporting ligand-receptor complexes from the plasma membrane to early endosomes, our finding of involvement of AP1G1 in ASCT2-EGFR association is consistent with our earlier conclusion that cetuximab downregulates ASCT2 via cetuximab-mediated EGFR endocytosis [26]. Moreover, although we cannot completely rule out the role of cetuximab-mediated inhibition of EGFR signaling in cetuximab-mediated downregulation of ASCT2, our data show that inhibition of EGFR tyrosine kinase by gefitinib, which, unlike cetuximab, does not induce EGFR endocytosis, did not downregulate ASCT2. In our earlier work [26], we showed that cetuximab downregulated ASCT2 in HN5 cells transduced to overexpress an oncogenic mutant of Ras (Ras-G12V), which conferred resistance on the cells to cetuximab-induced inhibition of cell signaling as a result of Ras-G12V-induced constitutive activation of cell signaling downstream of EGFR. Together, these data indicate that cetuximab downregulates ASCT2 at least mainly via inducing EGFR endocytosis.

Another interesting question is whether and how AP1G1 expression level regulates the level of association between ASCT2 and EGFR. Our experimental data showed that AP1G1 knockdown decreased the levels of ASCT2 and EGFR. In our preliminary studies (data not shown), we found that only a fraction of ASCT2 protein was associated with EGFR and vice versa; i.e., significant percentages of ASCT2 and EGFR were not associated with each other at basal level. It will be interesting to learn whether an increase in AP1G1 expression leads to an increase in ASCT2-EGFR association. If so, it could have translational significance for enhancing the extent to which ASCT2 can be downregulated by cetuximab. This could be a future direction in which to expand this project.

Cetuximab is currently approved for use in combination with chemotherapy or radiation therapy for treatment of metastatic HNSCC or metastatic colorectal cancer. However, the response to such combination treatment varies by patient. Ionizing radiation and some chemotherapeutic agents, including cisplatin, induce ROS in cancer cells. It will be interesting to determine whether the response to combination treatment depends on the level

of ASCT2-AP1G1-EGFR heterotrimeric complex and whether a high level of AP1G1 predicts favorable response.

In summary, we found that ASCT2 is associated with EGFR in a molecular complex involving AP1G1 and that the expression level of AP1G1 impacts cetuximab-mediated downregulation of ASCT2 and the associated therapeutic effects. Our data justify additional preclinical studies to validate that downregulation of ASCT2 by cetuximab offers a unique opportunity to sensitize cancer cells to combination treatment with cetuximab and ROS-inducing agents. Such studies could facilitate development of a new therapeutic strategy to overcome cetuximab resistance through co-targeting of ASCT2, a process that does not require effective inhibition of the cell signaling pathways downstream of EGFR by cetuximab, the failure of which is the major reason for the cetuximab resistance currently seen in patients.

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Abbreviation

AP1G1	adaptor-related protein complex 1 gamma 1 subunit
ASCT2	alanine-serine-cysteine transporter 2
ATP	adenosine triphosphate
DCA	dichloroacetate
EGFR	epidermal growth factor receptor
GFP	Green fluorescent protein
HNSCC	head and neck squamous cell carcinoma
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
RFP	Red fluorescent protein
ROS	reactive oxygen species

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Highlights

- AP1G1 is physically associated with ASCT2 and EGFR in a heterotrimeric complex
- Knockdown of AP1G1 decreases the level of ASCT2-EGFR association
- Cetuximab-induced downregulation of ASCT2-EGFR complex is AP1G1-dependent
- Cetuximab-induced inhibition of GSH and sensitization to ROS are AP1G1-dependent

Fig. 1. Cetuximab, but not gefitinib, downregulates ASCT2 level in dose- and time-dependent manners in HNSCC cells

A and B, HNSCC cells were treated with or without cetuximab at the indicated concentrations for 2 h (A) or treated with or without 20 nM cetuximab for the indicated time periods (B). C and D, HNSCC cells were treated with or without gefitinib at the indicated concentrations for 2 h (C) or treated with or without 0.125 μM gefitinib for the indicated time periods (D). Cell lysates were subjected to Western blotting with the indicated antibodies. ASCT2 antibody D7C12 was used in all ASCT2 blots.

Fig. 2. Both EGFR and ASCT2 are physically associated with AP1G1

A, HN5 cells were treated with or without 10 nM EGF for 30 min in the presence and absence of 2 mM DSS cross-linker. Cell lysates were prepared and subjected to Western blotting with EGFR antibody (upper) and ASCT2 antibody (bottom). The arrows indicate absence of protein bands in the expected region (~250 kDa) of an ASCT2-EGFR heterodimer by Western blotting with the EGFR antibody (upper) or the ASCT2 antibody (bottom). B, HN5 cell lysates were subjected to immunoprecipitation (IP) with cetuximab or ASCT2 antibody H52, followed by Western blotting of the immunoprecipitates with ASCT2 antibody H52 or D7C12. Upper, the arrows indicate a protein detected by ASCT2 antibody H52 that was co-immunoprecipitated both by cetuximab and by H52. Bottom, the arrows indicate that the same co-immunoprecipitated protein could not be detected by ASCT2 antibody D7C12. C, HN5 cell lysates were subjected to IP with cetuximab and ASCT2 antibody D7C12, followed by Western blotting of the immunoprecipitates with antibodies directed against AP1G1, EGFR, and ASCT2, respectively, as shown. The inset summarizes information of the antibodies used in this figure and others figures.

Fig. 3. ASCT2, AP1G1, and EGFR are associated in a molecular complex and knockdown of AP1G1 decreases ASCT2-EGFR association in HN5 cells

A, HN5 cells were subjected to knockdown of AP1G1 with siRNA for 72 h, followed by Western blotting with antibodies against AP1G1 (F10), EGFR (E12020) and ASCT2 (D7C12). B and C, The cell lysates in (A) were subjected to immunoprecipitation (IP) with cetuximab (B) and with ASCT2 antibody D7C12 (C), respectively. The immunoprecipitates were subjected to Western blotting with the indicated antibodies as in A. D, HN5 cells were incubated with or without 2 mM DSS for 30 min. Cell lysates were then subjected to EGFR IP with cetuximab, followed by Western blotting with indicated antibodies directed against EGFR, AP1G1, and ASCT2, respectively. The arrows indicate a protein of approximately 330 kDa that was detected by all three antibodies.

Fig. 4. Cetuximab induces internalization of ASCT2 into early endosomes in an AP1G1-dependent manner

A, HN5 cells were transduced with a lentiviral ASCT2-GFP fusion construct as described in Materials and Methods. The HN5 cells with stable expression of ASCT2-GFP fusion protein were either untreated or treated with control siRNA or AP1G1 siRNA for 72 h. The cells were then incubated in culture medium overnight with CellLight Early Endosomes-RFP, BacMam 2.0. The same areas of cells were photographed under a fluorescence microscope before and after treatment with 80 nM cetuximab for 2 h. B, Lysates of cells prepared and treated as described in (A) were subjected to Western blotting with antibodies against ASCT2 (D7C12), AP1G1 (F10) and β -actin as indicated.

Fig. 5. Knockdown of AP1G1 decreases cetuximab-induced downregulation of ASCT2 in HNSCC cells

HN5 cells and UMSCC1 cells were subjected to knockdown of ASCT2 or AP1G1 expression and treatment with gefitinib (0.125 μ M for 24 h) or cetuximab (20 nM for 24 h) as indicated in the figure. After various treatments, the cells were stained immunocytochemically with ASCT2 antibody D7C12. Representative areas (with high and low magnifications) of ASCT2 staining of each treatment group are shown. ASCT2 expression was analyzed quantitatively by ImageJ image processing tool as described in Materials and Methods.

Fig. 6. Knockdown of AP1G1 decreases the therapeutic effects of cetuximab-mediated co-targeting of ASCT2 in HNSCC cells

A, HN5 and MDA1986 cells with or without AP1G1 knockdown for 72 h were treated with cetuximab (10 nM for 24 h) or not. Intracellular glutathione level was measured using the Cayman glutathione assay kit as described in Materials and Methods. All error bars, SD. B–D, HN5 cells and MDA1986 cells with and without AP1G1 knockdown as in A were treated with 10 mM DCA, 20 nM cetuximab, or both for 24 h. In B, the cells were stained with Enzo's ROS detection kit and observed under a fluorescence microscope. Representative areas are shown. In C, cell lysates were subjected to Western blotting with the indicated antibodies. In D, cell cultures were subjected to MTT assays.