The Colon Anion Transporter, Down-Regulated in Adenoma, Induces Growth Suppression That Is Abrogated by E1A

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ABSTRACT

The down-regulated in adenoma (DRA) gene is significantly downregulated in adenomas and adenocarcinomas of the colon as well as in colon cancer cell lines. It is also mutated in the disease congenital chloride diarrhea, which is characterized by loss of chloride transport and diarrhea. We now show a second function for DRA relevant to colon tumorigenesis, i.e., growth suppression. Transfection of full-length DRA into various cell lines (DLD-1, HT-29, HCT-15, SW837, SW480, MCF-7, NIH3T3, CaSki, and HeLa) that lack endogenous DRA expression results in a reduced number of drug-resistant colonies compared with vector control, suggesting growth suppression by DRA. In addition, expression of DRA under the control of an inducible promoter reduced the growth rate of DLD-1 cells compared with cells not expressing DRA. The COOH-terminal cytoplasmic domain of DRA is required for growth suppression, but an in-frame deletion (ΔVal317) that causes congenital chloride diarrhea and results in a loss of anion transport had no effect on growth suppression, indicating that anion transport and growth suppression are independent functions of DRA. One cell line, adenovirus-transformed HEK293, exhibited significant resistance to DRA-induced growth suppression, whereas the human papillomavirus-transformed cell lines, CaSki and HeLa, did not. E1A is an adenosival protein required to transform HEK293 cells. DLD-1 cells that stably express 12S E1A are resistant to growth suppression by DRA, similar to HEK293 cells.

INTRODUCTION

Cancers of the colon and rectum will account for ~148,000 new cases in 2002 (1). Colorectal cancer is the third leading cause of mortality from cancer for both men and women, following only lung and breast or prostate cancers. The average person has a 1 in 18 lifetime chance of contracting colon cancer (2).

Vogelstein and colleagues (3–5) have proposed a model outlining the genetic events responsible for colon cancer. Mutations in these genes can be inherited or arise spontaneously through somatic mutations. Among the genes playing a prominent role in colorectal cancer are the tumor suppressor genes APC, p53, and DCC and oncopgenes such as K-ras and c-myc. Other types of genes known to play a role in colon cancer are those responsible for DNA repair, such as hMSH2 and hMLH1 (3, 6). Although mutations of these genes are often the cause for the development of neoplasms, epigenetic changes such as hypermethylation of genes also occur frequently in colon cancers (2, 7, 8).

We have previously described a gene, DRA, that is transcriptionally down-regulated early in the neoplastic process. DRA was found by subtractive hybridization to be underexpressed or absent in colon cancer samples compared with their normal counterparts (9–11). Expression of DRA is primarily on the apical surface of the columnar epithelial cells of the normal colon. Other areas of expression include small intestine, intraprostatic seminal vesicle (12), proximal tubule of the kidney, and eccrine sweat gland (13).

DRA shares significant homology with a family of sulfate transporters. Functional assays in both Xenopus laevis oocytes and SF9 insect cells confirmed that DRA is capable of facilitating transport of sulfate, chloride, and oxalate in vitro (14–18). Mutations in DRA were found to be responsible for a rare disease known as CLD (19). This disease presents as early as in utero as voluminous, watery diarrhea with an extremely high chloride content. This pathology is attributable to lack of chloride uptake in the colon, a major site of chloride uptake in the body (20).

Although the role of DRA in CLD is readily interpreted, its role in colon cancer is not yet clear. The observed underexpression or loss of DRA in colon cancer could be explained by one of two rationales. Down-regulation of DRA could simply be a result of the dedifferentiation process that occurs during cancer progression. Alternatively, down-regulation of DRA might play a role in the stepwise process by which colon cancer progresses. We present data in this report suggesting that loss of DRA does play an active role in colon cancer progression.

MATERIALS AND METHODS

Cell Culture. DLD-1 and HT-15 cells were grown in RPMI 1640 supplemented with 10 and 20% fetal bovine serum, respectively. MCF-7, NIH3T3, HT29, SW837, SW480, CaSki, HeLa, and HEK293 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Incubation was carried out at 37°C in a 5% CO2 atmosphere.

Colony Suppression Assay. Transfections were by the Lipofectin, Lipofectamine (Life Technologies, Inc.), or FuGene 6 (Roche, Indianapolis, IN) manufacturers’ protocols. Typically, 2 μg of DNA were used to transfect one 35-mm well when cells were approximately 50–80% confluent. DLD-1, HT29, HCT-15, and SW837; 600 μg/ml for DLD-1, HT29, HCT-15, and SW837; 600 μg/ml for MCF-7; 800 μg/ml for SW480, HeLa, and HEK293; 80 μg/ml for CaSki) continued for 2 weeks. Colony counts were carried out by first washing the plates once with PBS. They were fixed and stained with 0.2% crystal violet/20% methanol and then counted. DLD-1 cells were selected in 200 μg/ml zeocin for stable E1A expression.

Expression Vectors. The pSGneo vector was constructed by adding the bacterial neomycin resistance gene cassette to the mammalian expression vector, pSG5 (Stratagene, La Jolla, CA). An AelII-DraIII fragment from pSG5 was removed and replaced with an AelII-DraIII fragment from the pOP13CAT vector that contained the neomycin resistance gene. The following deletion mutants of DRA cDNA in the pSGneo vector were constructed: (a) DraΔ606–764 was made by removing an Earl-BglII fragment from the pSGneoDRA construct, filling in the 3′ recessed DNA ends with Klenow fragment and blunt religation of the plasmid. This removes the codons for amino acids 606–764 (end) and adds 16 amino acids (Ile-Leu-Leu-Leu-Asn-Leu-Leu-Asp-Asn-Asp-Gly-Asp-Thr-Lys-STOP) encoded by the vector; (b) DraΔV317 was made by mutagenic PCR (21) using the mutagenic primer pTAAAAACAGGTTTAAAGTGGCTGTGGGGGACATG (5′) phosphorylated with T4 polynucleotide kinase; New England BioLabs, Beverly
MA), along with the exterior primer pair 5'-CATTTGTCTGTGCTGTCGA-CATT (forward) and 5'-CAGTCCTAACGCTATTACTCTCTG (reverse). PCR was performed with 10 ng of template DNA using a two-step protocol (94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 65°C for 5 min, and then 65°C for 15 min) using Deep Vent DNA polymerase (New England BioLabs, Beverly, MA). The PCR product was digested with PflM, purified by agarose gel electrophoresis, and cloned into the two PflM sites within DRA cDNA in the pSGneo vector. The mutation was verified by direct sequence analysis.

For tetracycline-repressible expression (22) in DLD-1 cells, DRA cDNA was cloned into the BglII site of the vector pUHD10-3hygro (pUHD10-3 containing a hygromycin resistance gene) to generate the plasmid pUHD10-3hygroDRA. This construct was transfected into a DLD-1 clone that had previously been stably transfected with the vector pUHD15-neo (pUHD15-1 containing a neomycin/G418 resistance gene), which constitutively expresses the tetracycline repressor. Stable clones were selected in 200 μg/ml G418 and 200 μg/ml hygromycin and maintained in the presence of 1 μg/ml tetracycline to keep the DRA cDNA from being expressed. Individual clones were tested for DRA mRNA expression upon removal of tetracycline. Most clones showed a dramatic increase in DRA mRNA levels.

E1A in pSgizo was constructed by first removing the neomycin resistance gene from pSGneo and a BsrI-HindIII digest. The zeocin resistance cassette was removed from p3DNA3.1/Zeo (Invitrogen, Carlsbad, CA) with a BsrI-HindIII digest and isolation of the 556-bp fragment. DNA ends were filled in with Klenow enzyme and blunt-end ligation was carried out. 12S E1A (a gift from David Kurtz, Medical University of South Carolina) was removed from pBluescriptKs with a HindIII/ApaI digest and inserted into the HindIII/ApaI site of pSGtizo.

Sulfate Transport Assay. Sulfate transport assays were conducted as described previously with some modifications (23). At confluency, cells were rinsed twice with low-ionic strength buffer (1 mM MgCl₂, 300 mM sucrose, 10 mM Tris-HEPES, pH 7.5) containing various concentrations of Na₂SO₄ (0, 50, 100, and 400 mM). They were then incubated for 10 min at 37°C in 1 ml of buffer with various levels of unlabeled Na₂SO₄ as above plus 0.13 μM of carrier-free [³⁵S]Sulfate (Amersham Pharmacia, Piscataway, NJ). Cells were then washed twice with 3.5 ml of ice-cold wash solution (100 mM sucrose, 100 mM NaNO₃, 1 mM MgCl₂, and 10 mM Tris-HEPES, pH 7.5) and lysed in 1 ml of NETN (0.5% NP40, 20 mM Tris (pH 8.0), 100 mM NaCl, and 1 mM EDTA). Lysates were subjected to scintillation counting, and protein concentration was assessed by the BCA method (Pierce, Rockford, IL). DIDS sensitivity was measured using 1 mM DIDS.

Immunofluorescence. Fluorescence immunohistochemical staining was performed with the polyclonal antibody I20F to the COOH terminus of DRA protein upon removal of tetracycline. DRA protein expression caused a significant decrease in the growth rate of the clone when compared with the same clone grown in tetracycline (i.e., no DRA expression). These data provided further evidence that DRA may act as a growth suppressor.

Expression of DRA Mutants in Mammalian Cell Lines. Several deletion mutants were constructed in an attempt to map the functional region(s) of DRA responsible for growth suppression. The mutant DRAΔ606–764 removes the last 158 amino acids from the protein. The mutant DRAΔV317 is a disease-causing mutation in CLD that has been shown to prevent anion transport (16, 19). The growth-suppression capabilities of these mutants were assessed in a colony-suppression assay using DLD-1 cells as described for Fig. 1.

RESULTS

Expression of Full-Length DRA in Mammalian Cell Lines. To delineate the function of DRA, we transfected both colon and non-colon cell lines (DLD-1, HT-29, HCT-15, SW837, SW480, MCF-7, and NIH3T3) with a selectable mammalian expression vector containing full-length DRA. Before transfection, each cell line was confirmed by Northern blot to lack endogenous DRA expression (data not shown). After a 2-week drug selection, G418-resistant colonies were counted. Fig. 1 shows the percentage of colonies obtained from the DRA transfection relative to the number obtained with vector control in the colony suppression assay. In each case, the DRA transfection produced significantly fewer stable transfectants than the vector control. Nonetheless, some G418-resistant colonies from the DRA transfection would usually appear. Some of these colonies were expanded and screened for DRA expression, but none were found to express DRA protein, suggesting that the colonies were simply G418 resistant.

Inducible Expression of DRA in DLD-1 Cells. To further investigate the possibility that DRA played a role in growth control and because we could not obtain stable expression of DRA, we made an inducible construct, pUHD10-3hygroDRA, to be used in the tetracycline-repressible system. This construct was transfected into a DLD-1 cell line that stably expressed the tetracycline repressor plasmid. Independent clones were picked and expanded. The effect of DRA expression on the growth rate of this clone is shown in Fig. 2. The inset shows immunofluorescence of a clone that expresses DRA protein upon removal of tetracycline. DRA protein expression caused a significant decrease in the growth rate of the clone when compared with the same clone grown in tetracycline (i.e., no DRA expression.) These data provided further evidence that DRA may act as a growth suppressor. The growth rate remained fairly constant between days 2 and 6 when the DLD-1 cells approached confluence. Expression of DRA caused growth suppression without causing cell death, and restoration of tetracycline resulted in resumed growth (data not shown).

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Fig. 1. Transfection of full-length DRA into various cell lines. The indicated cell lines were transfected with 2 μg of DRA DNA and subsequently cultured in the presence of G418 for 2 weeks. G418-resistant colonies were fixed, stained, and counted. Columns, means; bars, SD.
shows that the DRAΔ606–764 mutant gave colony counts similar to those for vector control, as did the mutant containing only the COOH-terminal amino acids 606–764. This suggests that the COOH-terminal tail of DRA is required but not sufficient for growth suppression. In contrast, the DRAΔV317 mutant gave colony counts similar to those obtained for full-length DRA, suggesting that the growth control and anion transport functions of DRA are independent. Similar results were obtained when the transfections were performed in NIH3T3 cells (data not shown).

**Expression of DRA in HEK293 Cells.** In a paper reporting that DRA was responsible for Cl⁻/HCO₃⁻ exchange, Melvin et al. (17) also showed stable expression of mouse Dra in HEK293 cells. Therefore, we determined whether human DRA could also be stably expressed in HEK293 cells. Fig. 4 shows stable expression of DRA protein in HEK293 cells by Western blot (A) and immunofluorescence (B). To demonstrate that the DRA protein expressed in the HEK293 cells was also functional, we performed a sulfate transport assay on a stable clone (DRA #11). Fig. 4D shows that the clone expressing DRA exhibited DIDS-sensitive sulfate transport, whereas HEK293 cells do not show sulfate transport.

**Colonies Suppression by DRA in CaSki and HeLa Cells.** Constitutive, stable expression of DRA had previously been unattainable in our laboratory using various cancer-derived cell lines. HEK293 cells are of human embryonic kidney origin and have been transformed by introduction of sheared genomic DNA from adenovirus type 5. This was the first virally transformed cell line into which we had attempted to express DRA. Therefore, we wanted to assess whether we could express DRA in other virally transformed cell lines. For this, we chose two cell lines transformed by HPV, CaSki (HPV-16) and HeLa (HPV-18). Fig. 5 shows colony-suppression experiments using CaSki, HeLa, and HEK293 cells. CaSki and HeLa cells exhibited levels of DRA-induced colony suppression consistent with the previous cell lines tested. HEK293 cells, however, exhibited a decreased level of DRA-induced growth suppression (~60% in HEK293 cells versus 10–30% in other cell lines). Therefore, partial abrogation of growth suppression by DRA was observed in HEK293 cells and suggested that one or both of the adenovirus-transforming proteins (E1A and E1B) may be involved.
Adenovirus Type 2 12S E1A Abrogates DRA-induced Growth Suppression in DLD-1 Cells. HEK293 cells express two transforming proteins, E1A and E1B. To assess whether expression of the E1A protein contributed to the observed abrogation of DRA-induced growth suppression, we constructed stable cell lines expressing the 243-amino acid 12S E1A protein in DLD-1 cells. Expression of E1A was confirmed by immunofluorescence (data not shown). We then performed a DRA colony-suppression transfection assay. Fig. 6A shows colony counts from two independent DLD-1/E1A clones. The number of G418-resistant colonies in DLD-1 cells expressing E1A is significantly higher than in parental DLD-1 cells (compare 10% in Fig. 1 versus ~50% in Fig. 6B). Furthermore, these stable transfecants express the DRA protein in a Western blot assay (Fig. 6B, inset). The addition of E1B expression to DLD-1 cells did not alter the effect attributable to E1A alone (data not shown). This suggests that the pathway by which DRA suppresses cellular growth may be interrupted by E1A expression.

DISCUSSION

The function of DRA was initially investigated by attempting stable transfections of full-length protein into cell lines that lacked endogenous expression. However, these experiments consistently resulted in fewer drug-resistant colonies from the DRA transfection than that of a vector control. Among the few colonies that survived G418 selection, Northern and/or Western blot analyses were performed. Some of these colonies did express DRA mRNA, but none were found to express protein (data not shown). Therefore, DRA protein expression apparently caused growth arrest (or death), preventing colony formation. This pattern of DRA-induced growth suppression was observed in cell lines of various origins and cell types. Several colon cancer cell lines (DLD-1, SW480, SW837, HT-29, and HCT-15), the breast cancer cell line MCF-7, as well as the nontransformed mouse fibroblast cell line NIH3T3 were all apparently growth suppressed upon transfection with full-length human DRA cDNA. These results suggest that growth suppression by DRA is a general phenomenon, rather than a characteristic of a specific cell line.

Inducible expression of DRA provided further evidence that it is a growth suppressor. Upon DRA induction (by tetracycline removal) in a stable transfectant, its growth rate was dramatically decreased as seen by cell counts over a 7-day period (see Fig. 2). This could conceivably be attributed to cell death rather than growth arrest. However, there were no obvious signs of cell toxicity or apoptosis. No increased amount of cell debris or floating cells indicative of cell death was observed. Also, no obvious signs of apoptosis, such as membrane blebbing, were observed. Furthermore, the cells survived several rounds of growth in tetracycline, followed by growth cessation in the absence of the tetracycline (data not shown.) This indicates that cell death was not a major factor contributing to the flat growth curve during DRA protein expression. Importantly, the observed growth suppression by DRA is precisely consistent with our previous report showing that the only cells in the colonic mucosa to express DRA are those that have already withdrawn from the cell cycle (10).

We designed several deletion mutants to map the functional regions of DRA. ΔV317 is a disease-causing mutation in CLD (19) confirmed to be defective in both chloride and sulfate transport (16). Results from the colony-suppression assay show that ΔV317 is still capable of inhibiting colony formation. This suggests that the anion transport and growth-suppression functions of DRA are independent. However, it does not rule out indirect effects on neoplasia attributable to loss of anion transport function. Indeed, patients with CLD attributable to the ΔV317 mutation appear to be more likely to develop colorectal cancer than the general population (24). One possible mechanism for this may be attributable to lower levels of sulfation of heparan sulfate proteoglycans. Heparan sulfate proteoglycans modulate a number of
receptor tyrosine kinase signaling pathways (including those pathways involving the ligands FGF, transforming growth factor β, Wnt/Wingless, and Hedgehog), the aberrant regulation of which contributes to neoplasia (reviewed in Ref. 25). A Drosophila mutant, sulfateless (sfl), which completely lacks sulfated disaccharides from hepanan sulfate (26), is defective in its FGF signaling but can be rescued by a constitutively active form of an FGF receptor (27). This suggests that proper sulfation is obligatory for FGF signaling at the ligand/receptor level. In human colon cancer cell lines, altered patterns of sulfation and decreased overall sulfation levels of hepanan sulfate occur in carcinoma versus adenoma (28). These changes result in a 10-fold decrease in the affinity of carcinoma-derived hepanan sulfate for basic FGF and correlate with a reduced biological response to basic FGF in the carcinoma cells (29).

The mutant that removes the COOH terminus of DRA (Δ606–764) failed to suppress growth in the colony-suppression assay. The current structural model of DRA indicates that this mutation removes at least one transmembrane domain from DRA as well as the entire cytoplasmic tail. This may indicate that the COOH terminus of DRA contains one or more functional region(s) required for growth suppression. A construct containing only the amino acids removed in this mutant (DRA606–764) was tested in a colony-suppression assay. The COOH terminus alone did not suppress growth (see Fig. 3). This indicates that, although the COOH terminus is necessary to facilitate growth control, it is not sufficient to do so. It suggests that interaction with other parts of the DRA protein may also be required for growth suppression, perhaps involving some of the transmembrane architecture. Indeed, the transmembrane architecture of pendrin, a protein most closely related to DRA, cannot substitute for DRA in a colony-suppression assay.6

While some of the experiments described here were in progress, an intriguing report appeared describing the stable expression of mouse Dra in HEK293 cells (17). We were able to repeat this result using our human DRA cDNA. Both DRA protein expression and DRA protein function could be demonstrated in stably transfected HEK293 cells (see Fig. 4). Immunofluorescence of one of these clones (see Fig. 4) revealed strong staining in the Golgi in addition to the membrane where DRA is normally expressed. The Golgi localization is consistent with earlier transient transfections performed in COS-1 cells (10) and most likely represents the nascent polypeptide undergoing glycosylation. However, the demonstration of sulfate transport indicates that the DRA localized to the membrane was functionally active. We conclude that the observed reduction in DRA-induced growth suppression is not likely attributable to misexpression or a nonfunctional protein.

The demonstration of stable DRA expression in HEK293 cells causes us to consider possible explanations for the partial abrogation of growth suppression. HEK293 cells are human embryonic kidney cells that were transformed by introduction of adenovirus type 5 DNA. Specifically, this cell line expresses two transforming proteins, E1A and E1B. To determine whether the reduction of DRA-induced growth suppression was attributable to viral transformation, we transfected DRA into two other virally transformed cell lines, CaSkii and HeLa. CaSkii and HeLa cells are transformed by the HPV types 16 and 18, respectively. Both cell lines express two HPV-transforming proteins, E7 and E6. Although these proteins transform cells by a mechanism similar to E1A and E1B, respectively, we did not observe a reduction in DRA-induced growth suppression in CaSkii and HeLa cells. It is unclear whether the failure to partially abrogate DRA-induced growth suppression in CaSkii and HeLa cells is attributable to subtle differences in the mechanisms of the transforming proteins involved or may be attributable to cell line differences independent of the transforming proteins. However, we could demonstrate that the partial abrogation of DRA-induced colony suppression by E1A could be duplicated in DLD-1 cells, a cell line susceptible to growth control by DRA. This indicates that the 243-amino acid 12S E1A product is capable of mediating the abrogation.

There are numerous E1A-mediated effects resulting in cellular transformation (reviewed in Ref. 30). Because E1A is an early viral gene product, it is responsible for trans-activation of later viral genes, as well as controlling host cell growth upon infection. The latter is accomplished by targeted disruption of the host cell’s growth control machinery. This involves activation of positive growth regulators and inactivation of negative regulators. The most notable effect of E1A expression is the binding and effective inactivation of the retinoblastoma tumor suppressor protein, pRb. pRb negatively regulates cell growth by binding to the transcription factor E2F, which is responsible for transcription of genes involved in cell cycle progression (31). E1A disrupts this interaction by direct binding to pRb, thus freeing E2F and leading to unchecked cell cycle progression. Although one might speculate that DRA controls growth through an Rb pathway, this begs the question of why abrogation was not observed in CaSkii and HeLa cells, where E7 ostensibly blocks pRb function in a manner similar to E1A. Furthermore, if DRA failed to suppress growth in HEK293 cells because of E1A’s disruption of pRb function, it would follow that cell lines that do undergo DRA-induced growth suppression might maintain functional (hypophosphorylated) pRb. However, at least for some cell lines, this is not the case. For example, DLD-1 and SW480 cells have been reported to lack the hypophosphorylated form of pRb (32). Presumably, with no hypophosphorylated pRb, E2F is constitutively unbound and free to transactivate positive cell cycle regulators. In addition, DLD-1, SW480, and HT-29 cell lines lack p16 expression and exhibit high levels of type D cyclins (32). These conditions result in cyclin-dependent kinase 4-mediated phosphorylation of Rb, thus inactivating it. Therefore, a role for pRb is unclear at this time.

It has been reported that the other Rb family members, p130/Rb2 and p107, have an aberrant phosphorylation pattern in HEK293 cells. This is a direct result of E1A expression (33). E1A disrupts the interaction between p130 or p107 and their respective E2F family members while preventing their hyperphosphorylation. The phosphorylation of pRb, however, is not blocked by E1A expression. For this reason, it is believed that p130/Rb2 and p107 may have roles in addition to those defined for pRb. There is some evidence that p130/Rb2 can act as a cyclin-dependent kinase inhibitor (34). One could envision a scenario wherein DRA depends on p130/Rb2 function as a cyclin-dependent kinase inhibitor to effectively inhibit growth. If E1A expression in DLD-1 cells interferes with this function of p130/Rb2, then a reduction in DRA-induced growth suppression might be expected. However, this explanation for the observed reduction is confounded by an important observation; E1A blockade of p130/Rb2 hyperphosphorylation occurs in HeLa cells (33), a cell line that we confirmed to be susceptible to DRA-induced growth suppression. Therefore, an E1A abrogation mechanism mediated through a pathway involving Rb family members is either a much more complicated matter or does not apply to DRA-induced growth suppression.

Other E1A-mediated events with widespread effects on the cell cycle exist. This leaves more avenues to be investigated to uncover the explanation for the observed reduction in DRA-induced growth suppression observed in HEK293 cells and in DLD-1 cells expressing E1A. Direct interaction between DRA and E1A is unlikely because DRA is a membrane protein and E1A functions in the nucleus. This would rule out E1A possibly binding to and masking functional

regions of the COOH terminus of DRA. Alternatively, E1A might inhibit expression of a downstream effector of DRA through its trans-activation region. This could be assessed by mutation or deletion of this region in an E1A construct, followed by expression in DLD-1 cells and “challenge” by DRA transfection. If DRA-induced growth suppression was restored in the absence of E1A trans-activation, then proteins induced or inhibited by E1A expression would be candidates for members of the pathway by which DRA controls cell growth. Although the E1A-mediated abrogation of DRA-induced growth suppression cannot yet be definitively explained, it will hopefully provide clues to the mechanism of this protein with such diverse roles as anion transporter and growth suppressor.

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