The Multifunctional Protein Glyceraldehyde-3-Phosphate Dehydrogenase Is Both Regulated and Controls Colony-Stimulating Factor-1 Messenger RNA Stability in Ovarian Cancer

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Abstract

Although glyceraldehyde-3-phosphate dehydrogenase’s (GAPDH) predilection for AU-rich elements has long been known, the expected connection between GAPDH and control of mRNA stability has never been made. Recently, we described GAPDH binding the AU-rich terminal 144 nt of the colony-stimulating factor-1 (CSF-1) 3’ untranslated region (UTR), which we showed to be an mRNA decay element in ovarian cancer cells. CSF-1 is strongly correlated with the poor prognosis of patients with ovarian cancer. We investigated the functional significance of GAPDH’s association with CSF-1 mRNA and found that GAPDH small interfering RNA reduces both CSF-1 mRNA and protein levels by destabilizing CSF-1 mRNA. CSF-1 mRNA half-lives were decreased by 50% in the presence of GAPDH small interfering RNA. RNA footprinting analysis of the 144 nt CSF-1 sequence revealed that GAPDH associates with a large AU-rich-containing region. The effects of binding of GAPDH protein or ovarian extracts to mutations of the AU-rich regions within the footprint were consistent with this finding. In a tissue array containing 256 ovarian and fallopian tube cancer specimens, we found that GAPDH was regulated in these cancers, with almost 50% of specimens having no GAPDH staining. Furthermore, we found that low GAPDH staining was associated with a low CSF-1 score (P = 0.008). In summary, GAPDH, a multifunctional protein, now adds regulation of mRNA stability to its repertoire. We are the first to evaluate the clinical role of GAPDH protein in cancer. In ovarian cancers, we show that GAPDH expression is regulated, and we now recognize that one of the many functions of GAPDH is to promote mRNA stability of CSF-1, an important cytokine in tumor progression.

Introduction

Although glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was originally thought of as an enzyme important only for glycolysis, and as a housekeeping gene appropriate for use as a control in the measurement of equal loading in experiments, over the last decade, every succeeding year has brought to light a new function for this protein. The many and diverse roles for this multifunctional protein (1, 2) include among others: DNA replication and repair, nuclear membrane fusion, apoptosis, microtubule bundling, vesicular secretory transport, and maintenance of telomere structure, with its roles in part related to subcellular localization. GAPDH has been found in nuclear, cytoplasmic, and membrane localizations, and can shuttle between these compartments.

Importantly, GAPDH has also been implicated as having a role in transcriptional control (1), in export of nuclear tRNA (2), and in translational control (3), levels of control which regulate gene expression. GAPDH has not been previously shown to have a role in the regulation of mRNA stability, despite the fact that is has been known to have AU-rich RNA-binding capabilities for over a decade. GAPDH has a predilection for regions of RNA critical for its regulation (2), such as the 5’ sequences involved in hepatitis A viral replication, and the 3’ untranslated region (UTR) sequences including those containing AU-rich elements. Some RNA sequences which are recognized by GAPDH have been described to have a poly-U tract (4), UC-rich region (5), or AU-rich elements (6). RNA footprinting of the GAPDH binding site has shown a UC-rich domain in hepatitis 6 virus RNA (5), and three discontinuous AU-rich regions (one located in the 3’translated and two in the 3’UTR), in hepatitis A virus RNA (7). Computer modeling suggested that these regions were located on stem-loop structures, having the potential for tertiary interactions.

Recently, we described GAPDH to be an RNA-binding protein for the AU-rich terminal 144 nt of the 3’UTR of the macrophage colony-stimulating factor (CSF-1; ref. 8). CSF-1 and/or its receptor (encoded by the c-fms proto-oncogene) are expressed by the large majority of epithelial ovarian cancers (9-11), with 75%
of primary tumors and 69% of the metastases expressing CSF-1,
and 92% of primary tumors and 83% of metastases expressing its
receptor. We further found that strong coexpression of CSF-1 and
receptor by ovarian cancer metastases was an independent
(P = 0.007) poor prognostic factor, with a 2.3-fold increased
relative risk of recurrence (9). The mean time to recurrence for
patients with stage III ovarian cancer was shortened by 11 months,
from 24.1 ± 3.9 months to 13.5 ± 4.0 months. No such coex-
pression of CSF-1 and its receptor was observed in any of the tumors
of low malignant potential, which by definition, are noninvasive.
Moreover, serum CSF-1 levels proved to be a sensitive tumor
marker (12), with elevated levels heralding disease recurrence or
progression. Elevated levels of both serum and ascitic CSF-1
levels at diagnosis correlated with a poor outcome (13, 14). This
correlation with prognosis and aggressive tumor behavior sug-
gests an etiologic role for CSF-1 in ovarian cancer progression.

In our search for novel regulators of CSF-1 expression, we
noted that the terminal 144 nt of the 3'UTR in exon 10,
contained in the most abundant CSF-1 transcript, was
particularly AU-rich. We showed that this 144 nt region served
as an mRNA decay element, and identified GAPDH as an
AU-rich RNA-binding protein which binds with high affinity to
this 144 nt stretch (8). Although we provided evidence by
Northwestern analysis that a mutant riboprobe containing
sequences that differed from the wild-type 144-nt sequence in
most, but not all, AU-rich stretches, did not bind the 37 kDa
protein which we subsequently identified to be GAPDH, the
region or regions within the 144 nt CSF-1 RNA sequence which
were recognized by GAPDH remain unknown, and the
functional consequence of such binding has not been delineated.

Elevated levels of GAPDH RNA have been shown in
several human cancers, including lung, pancreas, cervical,
breast cancer (as summarized in refs. 15, 16), and of GAPDH
protein in prostate cancer (17). Its role in prognosis has only
previously been investigated in breast cancer, where the level of
GAPDH RNA from breast cancer specimens correlated with
decreased survival on univariate analysis. To our knowledge,
the clinical role of GAPDH protein has not been explored in
any cancer. We did so by immunohistochemical analysis of
GAPDH RNA from breast cancer specimens correlated with
its protein expression.

In this report, using RNA interference, we showed that
GAPDH regulates CSF-1 RNA and protein expression, which we
found to be the basis of stabilization of CSF-1 mRNA. Fur-
thermore, CSF-1 RNA footprinting of its 144 nt 3'UTR suggested that
binding by GAPDH involves a large AU-rich region, which
contains several stem-loop structures predicted by computer
modeling. We showed that mutating or deleting targeted areas
within this region mitigates GAPDH binding. Lastly, we found
that GAPDH is regulated in ovarian and fallopian tube
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that GAPDH is regulated in ovarian and fallopian tube
cancers, and that GAPDH is coexpressed with CSF-1 in ovarian cancers.

Results

Down-Regulation of GAPDH by RNA Interference Reduces Steady-State CSF-1 mRNA and Protein Levels

To investigate the functional significance of GAPDH's association with CSF-1 3'UTR, small interfering RNA (siRNA)
targeted to GAPDH mRNA was delivered into normal human
ovarian surface epithelial (NOSE.1) cells and Hey epithelial
ovarian cancer cells. Results showed the ability of GAPDH
siRNA to specifically down-regulate GAPDH protein in both
cell types, when compared with the effect of control siRNA,
which does not alter the expression of either GAPDH or β-actin
(Fig. 1A). We then studied the effect of GAPDH siRNA on
CSF-1 mRNA and protein levels. In both cell types, GAPDH
down-regulation by siRNA resulted in a significant reduction
in both CSF-1 mRNA (Fig. 1B) and secreted CSF-1 levels
(Fig. 1C). CSF-1 mRNA levels decreased to the same degree
when compared with control conditions in both cell types; in
fact, in NOSE.1 cells, CSF-1 RNA levels decreased to almost
an undetectable level. Secreted CSF-1 protein levels decreased
by 40% in both cell types. In NOSE.1 cells, CSF-1 protein

![FIGURE 1. Silencing GAPDH and its effect on CSF-1 mRNA and protein expression. A. Immunoblot analysis of total protein extracts from
cell types, when compared with the effect of control siRNA,
which does not alter the expression of either GAPDH or β-actin.
GAPDH siRNA significantly down-regulated CSF-1
RNA compared with controls. B. Reverse transcription-PCR
analysis of the CSF-1 and β-actin RNA expression in GAPDH or control
siRNA-treated NOSE.1 or Hey cells. The RNAs were harvested 24 h post-
siRNA transfection. GAPDH siRNA significantly down-regulated CSF-1
RNA compared with controls. C. Levels of CSF-1 secreted in the
conditioned medium of GAPDH or control siRNA-treated NOSE.1 or Hey
cells (ng/mL). The medium was harvested 57 h post-siRNA transfection.
There was no significant effect of control siRNA on secreted CSF-1 levels
compared with untreated Hey cells (P = 0.15). GAPDH siRNA was able
to significantly decrease secreted CSF-1 protein levels when compared with
controls.](image-url)
levels decreased from 0.0806 ± 0.0078 to 0.0482 ± 0.0064 ng/mL by GAPDH siRNA (P = 0.005). In Hey cells, CSF-1 protein levels decreased from 0.1568 ± 0.0116 to 0.0972 ± 0.0091 ng/mL by GAPDH siRNA (P = 0.002). Thus, GAPDH positively regulated both CSF-1 mRNA and secreted protein levels. If the regulation of steady-state CSF-1 mRNA by GAPDH was mediated by 3’UTR AU-rich CSF-1 mRNA binding, then this should be at a posttranscriptional level.

**CSF-1 mRNA Stability Is Enhanced by GAPDH Protein**

Because the majority of posttranscriptional events mediated by AU-rich regions are on the basis of altered mRNA stability, we studied the effect of GAPDH on CSF-1 mRNA half-life. Actinomycin D chase experiments were done in Hey ovarian cancer cells treated with either GAPDH or control siRNA. In Fig. 2, we show that in the presence of GAPDH siRNA, the CSF-1 mRNA half-life was only 1.8 hours, whereas in the cells treated with control siRNA, the predicted CSF-1 mRNA half-life was >3 hours. A total of three independent experiments of CSF-1 mRNA half-life were done. Each experiment confirmed the decrease in CSF-1 mRNA stability with GAPDH siRNA treatment. However, the measured mRNA half-life under these complex conditions differed from experiment to experiment. Collectively, the mean (± SE) CSF-1 mRNA half-life with GAPDH siRNA was reduced by 52%, from 3.87 ± 0.72 hours (control siRNA) to 1.87 ± 0.52 hours (GAPDH siRNA). The finding of an approximate CSF-1 mRNA half-life of 3.9 hours in Hey ovarian cancer cells after control siRNA treatment is in line with our previous report of CSF-1 mRNA half-lives of 4.5 hours in two other untreated ovarian cancer cell lines (18). We concluded that the reduction in steady-state CSF-1 RNA expression is on the basis of a decrease in CSF-1 mRNA stability because the magnitude of the difference in CSF-1 mRNA half-lives resulting from treatment with GAPDH or control siRNA (Fig. 2) seemed to correlate with the difference in secreted CSF-1 protein levels (Fig. 1C).

**A Large AU-Rich Region Within the 3’UTR of CSF-1 RNA Is Identified for GAPDH Binding**

To further understand the nature of the interaction between GAPDH and the binding domains of CSF-1 mRNA within the 144 nt terminal 3’UTR, enzymatic RNA footprinting analyses were done. We had previously shown that this 144 nt 3’UTR CSF-1 RNA region served as an mRNA decay element (8). First, we chemically sequenced the 3’-end labeled 144 nt CSF-1 RNA (Fig. 3A) in order to help interpret the footprinting experiments which followed. For the footprinting experiments, 3’-end labeled 144 nt CSF-1 RNA was subjected to a standard binding reaction in the presence or absence of GAPDH, followed by treatment with RNase A or T1. Two regions for GAPDH binding lying in close proximity were identified (Fig. 3B; 3’ region; and Fig. 3C, 5’ region). Because the two regions lie in such close proximity to each other, being separated by 2 nt (C3906/U3907) only, it seems that these regions represent one large footprint with those two positions being susceptible to RNase A in this in vitro reaction.

The 5’ region. The 5’ region of GAPDH binding was described by the following findings. First, in the presence of GAPDH, RNase A (specifically cleaves the 3’ of U and C residues) decreased sensitivity in U/C residues starting from 3’ to G3855 (arrowhead, Fig. 3C). There is a run of 5U starting at U3856, therefore, the exact start site of this protected region could not be discerned. Decreased sensitivity to RNase A is again clearly shown at position C3868 and at all the U and C positions depicted in Fig. 3B, ending at U3905 (arrow, Fig. 3C). The footprint disappears at C3906/U3907. This 5’ region is also captured in Fig. 3B, as the footprint 5’ to U3907. Thus, the 5’ region protected by GAPDH binding seems to span positions from (3856-3868 nt) to 3905 nt.

The 3’ region. The 3’ region of GAPDH binding was described by the following findings. First, in the presence of GAPDH, the CSF-1 RNA was susceptible to RNase A at positions U3907 and C3955 (Fig. 3B), and to RNase T1 at G3949 (data not shown). Starting at 3’ to U3907 (arrowhead, Fig. 3B), the footprint affects all the U and C positions depicted in Fig. 3B, including U3918 and U3936-U3938. RNase T1 (specifically hydrolyses single-stranded RNA after G) showed decreased sensitivity at positions G3916 and G3927, including the G residues in between (data not shown). The footprint ends before G3949. Thus, the 3’ region protected by GAPDH seems to span positions from (3908-3916 nt) to (3939-3948 nt) nucleotides.

The results from treatment with RNase A and T1 indicates a large region with reduced sensitivity to RNases, which is very AU-rich (3856-3948 nt; Fig. 3D). Therefore, footprint analyses showed that the interactions between GAPDH and 3’UTR of CSF-1 lie within this AU-rich region. Our findings may reflect an interaction which results from direct hindrance by GAPDH protein with or without an indirect effect secondary to altered RNA folding by GAPDH binding.

The wild-type 3’UTR CSF-1 riboprobe sequence was subjected to computer-assisted modeling with the MFold program. One of the most thermodynamically stable structures with a calculated free energy of −46.2 kcal/mol is shown in Fig. 4A. As expected, this AU-rich 3’UTR region is predicted to
form several stem-loop structures. Notably, the midportion of the footprint predicts the most complex of the secondary structures, with two stem-loops forming each arm of a “v” structure.

In order to explore whether this large region is important for GAPDH binding, deletions as described in Fig. 4A were introduced to assess their effects on GAPDH binding in vitro. Those AU-rich deletion regions were chosen based on the footprinting data (Fig. 3), combined with information from the predicted secondary structure (Fig. 4A).

Results of RNA gel shift assays (Fig. 4B) showed that the binding between CSF-1 RNA and GAPDH protein is successfully competed with a molar excess (6.7- to 60-fold) of unlabeled wild-type RNA sequences (Fig. 4B, lanes 3-5). The binding was partially competed in reactions with a molar excess (6.7- to 60-fold) of either one of the unlabeled mutant RNA sequences (Fig. 4B, lanes 6-11), indicating that each mutant had weaker binding than the wild-type sequence. This data also suggested that both regions are required for efficient 3'UTR CSF-1 RNA binding to GAPDH protein.

Before we performed footprinting analyses, we empirically created a mutant of the 144 nt 3’UTR CSF-1 RNA (8), in which within four AU-rich regions (3850-3861, 3881-3888, 3908-3912, and 3935-3939 nt), the A was mutated to a C, and T to a G. Northwestern analysis showed that this mutant abolished binding visualized by wild-type CSF-1 RNA to a 37 kDa protein in Hey ovarian cancer cells which we subsequently identified to be GAPDH (8). Comparison of the mutant sequence with that of the region identified by enzymatic footprinting shows that three of the four regions mutated and part of the fourth lies within this region. One (3881-3888 nt) partially overlaps with deletion I, and the other (3935-3939 nt) was completely included in deletion II. We did RNA gel shift assays with this mutant in Hey and NOSE.1 cells (Fig. 5), and show that these mutations largely abrogate 3’UTR CSF-1 RNA binding, when compared with the wild-type 144 nt 3’UTR CSF-1 sequence in NOSE.1 cells, and partly interfere with such binding in Hey cells. Our results in Fig. 4 suggest that a large AU-rich region is important to GAPDH binding. Our results in Fig. 5 along with the previously published Northwestern analysis (8) are in line with this. They also suggest, in Hey ovarian cancer cells, that other proteins such as the thus far unidentified 75 kDa protein visualized on Northwestern analysis (8) may have different binding affinities or sequence requirements within the 144 nt CSF-1 RNA. Alternatively, mutations of most but not all of the AU-rich stretches may not be adequate to abrogate GAPDH binding in Hey ovarian cancer cells.

GAPDH Immunohistochemical Staining of a Tissue Microarray Cohort of Epithelial Ovarian and Fallopian Tube Cancer Specimens

The levels of expression of nuclear and cytoplasmic GAPDH in ovarian cancer cells were examined in an epithelial

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**FIGURE 3.** RNA footprinting analysis of the interaction of GAPDH protein with the 3'UTR of CSF-1 RNA. **A,** 3'-end–labeled 3'UTR CSF-1 RNA (3829-3972 nt) sequenced chemically. The pattern derived was used to help interpret the footprinting experiments. **B,** 3' region of GAPDH binding to the 3'UTR CSF-1 RNA. Lane 1, labeled CSF-1 RNAs (3828-3972 nt) are partially hydrolyzed by RNase T1 (cleavagesite3'of single-stranded G's) to provide a reference ladder. CSF-1 RNA (3828-3972 nt) was subjected to a standard binding reaction in the presence (+; lanes 3, 7, 8, and 9) or absence (−; lanes 2, 4, 5, and 6) of 1.4 μg of human GAPDH, followed by cleavage with RNase A (lanes 4-9). Partial cleavage with the decreasing amounts of RNase A [0.1 ng/mL (lanes 4 and 7), 0.02 ng/mL (lanes 5 and 8), 0.004 ng/mL (lanes 6 and 9)] or control reactions without RNase (lanes 2 and 3) was done. The approximate boundaries of the 3' region of the footprint between 3908/3916 nt (arrowhead) and 5' to 3955 nt (arrow; the 3' end of the footprint is better defined as 3939-3948 nt in the text). **C,** 5' region of GAPDH binding to the 3'UTR CSF-1 RNA. The conditions for each lane are as described in **B.** The approximate boundaries of the 5' region of the footprint between (3856-3868 nt) (arrowhead) and 3905 nt (arrow). The 5' region of the footprint can also be visualized in **B,** but with less detail. **D,** AU-rich footprint for GAPDH binding of 3'UTR CSF-1 RNA.
ovarian and fallopian tube cancer tissue microarray (TMA) consisting of 322 specimens. This array also contained benign epithelial ovarian neoplasms (n = 4), ovarian tumors of low malignant potential (n = 5), and duplicate tissue cores (n = 16). All cases were reviewed for histologic diagnosis by one gynecologic pathologist, as described previously. The primary peritoneal cancers were included as part of the invasive epithelial ovarian cancers.

The analysis in this report was restricted to invasive ovarian (n = 243) and fallopian tube cancer (n = 13) patients who underwent primary surgery, and excluded those who were treated with neoadjuvant chemotherapy or who had concurrent cancers. Thus, 256 invasive cancer specimens were available for analysis of the clinical role of GAPDH. Of these 256 cores, 248 were interpretable for nuclear and cytoplasmic immunoreactivity. Tissue cores which were deemed noninterpretable usually had insufficient tumor cells, or the core was not representative of the cancer. Follow-up information was missing in only one patient. The median follow-up time was 38 months (range, <1-222 months). Of the 256 invasive cancers, the primary adjuvant treatment was known in 82% of cases. Of these cases, 14% received no adjuvant treatment, and 62% were treated with platinum-based adjuvant chemotherapy, of which 46% also received a taxane. The remaining 24% received either non–platinum-based chemotherapy or radiation therapy. Within this array were 16 duplicate cores. There was perfect concordance in the scoring for GAPDH in 14 of 16 of these duplicates; in only one case would the discrepancy have resulted in a change in the grouping of the score.

Analysis of Clinical Prognostic Factors. Analysis of standard prognostic factors in epithelial ovarian and fallopian tube cancer was done to confirm that studies using this tissue array would have clinical validity. As expected, we found that stage (I and II versus III and IV), grade (1 and 2 versus 3), age (<60 versus >60 years), and histologic subtype (serous versus nonserous) were all highly significant factors for overall and progression-free survival (P < 0.009 for all analyses).

GAPDH Staining of Noninvasive Cases. Regarding GAPDH immunostaining, we found that all four benign ovarian tumors had a nuclear and cytoplasmic score of 0. Among the five ovarian tumors of low malignant potential, three had nuclear and cytoplasmic scores of 0, whereas one had a nuclear score of 20 and a cytoplasmic score of 100, and the other had a nuclear score of 180 and a cytoplasmic score of 90.

GAPDH Staining of Invasive Cases. Overall, 52% of specimens expressed GAPDH in cytoplasmic and/or nuclear locations, with 24% of the whole group expressing GAPDH in both compartments. Expression of cytoplasmic GAPDH was highly associated with that of nuclear GAPDH (P < 0.0001), in line with the known ability of GAPDH to shuttle between compartments. Only 23% of specimens had moderate to strong immunoreactivity (score >100) for cytoplasmic GAPDH, and 9% for nuclear GAPDH. Notably, 55% of ovarian cancer cases had no staining (score = 0) for GAPDH in the cytoplasm, and 70% with those findings in the nucleus.

Forty-eight percent had no staining for GAPDH in either compartment.

We studied GAPDH staining in another cohort of 53 primary epithelial ovarian cancer cases using conventional slides. In this cohort, 92% had zero nuclear staining. We found 26 of 53 cases (50%) to have a GAPDH score of 0 to 50 out of a total score of up to 300, i.e., zero to very weak staining. Out of those 26 cases, 15 (29% of the entire cohort) had zero cytoplasmic and nuclear staining for GAPDH.

Thus, the findings in the second cohort validate the findings of the TMA, in that the large majority of cases (70-90%) have no nuclear staining for GAPDH, and that approximately half of the cases have zero to very weak GAPDH staining in the cytoplasm. This implies that if GAPDH was expressed by these tumors, it is at a very low level that is undetectable or barely detectable by immunohistochemistry. Representative examples of GAPDH staining in the TMA are shown in Fig. 6.

Analysis of Association of GAPDH with Clinicopathologic Factors and with Survival. In the TMA, among all stages, the presence of either cytoplasmic or nuclear GAPDH staining (scores >0) was associated with nonserous histologic subtype (P = 0.0007 for cytoplasmic, and P = 0.0073 for nuclear staining), with cytoplasmic GAPDH staining also associated with early stage disease (P = 0.0004). Neither cytoplasmic nor nuclear GAPDH was associated with any other clinicopathologic factor, such as grade or age. Moreover, neither nuclear nor cytoplasmic GAPDH proved to be a prognostic factor for either progression-free or overall survival.

Association of GAPDH with CSF-1. Thirty-eight of the second cohort of 53 ovarian cancer cases had previously been analyzed for CSF-1 and c-fms expression (9). We analyzed this subset for association between GAPDH and CSF-1. We found a highly significant association (P = 0.008) between a low cytoplasmic GAPDH score and a low CSF-1 score (Table 1). In fact, we found that 93% of ovarian cancer cases with low CSF-1 staining had a low GAPDH score. In contrast, and as expected, there was no significant association between GAPDH and c-fms expression.

Summary of Immunohistochemical Findings for GAPDH. Thus, our findings show the significant regulation of GAPDH protein in ovarian and fallopian tube cancers. Furthermore, our observation that GAPDH and CSF-1 were coexpressed in ovarian cancer cases is in line with our finding that GAPDH positively regulates CSF-1 expression.

FIGURE 5. RNA gel shift assays comparing binding of wild-type and mutated forms of CSF-1 144 nt 3′UTR to protein extract from Hey or NOSE.1 cells. Mutations were created in four AU-rich regions within the terminal 144 nt CSF-1 3′UTR as described in the text. A and B, results of binding of labeled wild-type CSF-1 riboprobe to Hey (A) or NOSE.1 (B) protein extracts; C and D, results of binding of labeled mutant CSF-1 riboprobe to Hey (C) or NOSE.1 (D) protein extracts. Lane 1, free riboprobe control; lane 2, 1× probe mixed with 8 μg of protein extract; lane 3, 3× probe mixed with 8 μg of protein extract; lanes 4, 5, and 6, 1× probe mixed with 8 μg of protein extract in the presence of 1,500×, 770×, or 390× excess cold probe, respectively.

FIGURE 6. Representative pictures of GAPDH from an ovarian and fallopian tube cancer TMA. A, C, and E, original magnification, ×40; B, D, and F, magnified from insets. A and B, a case of ovarian serous carcinoma with both cytoplasmic and a nuclear GAPDH score of zero. Hematoxylin was used as a counterstaining agent (blue, negative staining). C and D, an ovarian serous carcinoma case which had cytoplasmic (score 300) but no nuclear GAPDH staining. E and F, an ovarian serous carcinoma case which had nuclear (score 100) but no cytoplasmic GAPDH staining.
Discussion

Although GAPDH’s predilection for AU-rich elements has long been known, the expected connection between GAPDH and control of mRNA stability has never been made. Our results now document a functional role for GAPDH in controlling mRNA decay rates. The key observation supporting this claim is that siRNA-mediated knockdown of GAPDH levels leads to increased degradation of the CSF-1 mRNA. Moreover, GAPDH binds to a region in CSF-1 mRNA 3′UTR that has been shown to promote mRNA degradation. Taken together, this suggests that GAPDH can bind to the CSF-1 3′UTR and enhance the stability of the mRNA. We do not believe that this effect of GAPDH on control of mRNA stability is limited to CSF-1 as a target. Indeed, GAPDH can bind the AU-rich regions of the 3′UTR of c-myc and granulocyte macrophage-CSF RNA (6). It would not be surprising to find that GAPDH also regulates these and other AU-rich-containing messages. A role for GAPDH in regulating mRNA turnover is also important in the context of the increasing recognition of the contribution of mRNA stability to the control of gene expression. It is estimated that at least 50% of steady-state gene expression is controlled by stability regulation (19), and GAPDH now adds this critical level of regulation to its repertoire.

We show by enzymatic footprinting that there is a large AU-rich region within the 144 nt 3′UTR of CSF-1 RNA which is protected by GAPDH protein, and seems to be necessary for GAPDH binding. As previously suggested, it is likely that an RNA consensus binding motif for GAPDH does not exist (20). Instead, localization of binding to secondary structures such as stem-loop regions likely plays a strong contributory role (7, 20, 21). The predicted secondary structure of the AU-rich 3′UTR of CSF-1 RNA contains several stem-loop structures, in line with other reports showing GAPDH predilection for AU-rich regions and those with complex secondary structures (6, 7, 20). We show that deletions or mutations targeting AU-rich regions within these CSF-1 3′UTR structures mitigate binding to GAPDH protein and ovarian cell extracts.

Our results show that GAPDH expression levels vary in ovarian and fallopian tube cancers, with almost 50% of tumors showing no detectable staining. It makes sense that GAPDH is regulated specifically in cancers. It has been proposed that cancers may have a glycolytic phenotype (22), in that hypoxia, a common feature of malignancy, can stimulate anaerobic glucose metabolism. Our finding that GAPDH is not detectable by immunohistochemistry in both nuclear and cytoplasmic compartments in almost 50% of ovarian and fallopian tube cancers deserves some consideration. We are the first to make this observation in a large array of cancers, in part, because there is a surprising paucity of literature on the study of GAPDH protein levels in cancer specimens. In one study of 13 prostate cancer specimens, heterogeneous nuclear and/or cytoplasmic GAPDH staining was observed, with GAPDH not detectable in the normal secretory epithelium (23).

In our TMA, the results of a highly significant association between nuclear and cytoplasmic expression of GAPDH are in line with GAPDH’s known shuttling function between the nuclear and cytoplasmic compartments. GAPDH has numerous functions, some specifically related to its nuclear localization, such as in transcriptional control, DNA repair, and apoptosis (1). In the cytoplasm, GAPDH also has many roles, such as in translational control (2, 3), and we now add regulation of mRNA turnover as one of GAPDH’s cytoplasmic functions.

Our finding that GAPDH staining is largely associated with the early stage of disease may be counterintuitive, in that we show one of its functions is to stabilize CSF-1 mRNA, an important factor in tumor progression. However, among advanced stage disease, GAPDH was still detectable almost 50% of the time, and its functions in the cell are innumerable. Thus, it comes as no surprise that GAPDH protein has little prognostic value. Prior to this report, the only study to examine prognosis related to GAPDH expression measured GAPDH RNA by reverse transcription-PCR in breast cancers, and found it to be associated with reduced survival (15). Importantly, we showed that cytoplasmic GAPDH is significantly coexpressed with CSF-1 in ovarian cancer cases, which is in line with our finding that CSF-1 RNA and protein is regulated by GAPDH.

In conclusion, (a) we are the first to show that GAPDH, a multifunctional protein, has the capacity to regulate gene expression at the level of mRNA stability. We found that GAPDH promotes CSF-1 RNA and protein expression in ovarian cancer cells by stabilizing its message; (b) we show that GAPDH protein interacts with a large region of AU-rich CSF-1 RNA, which is predicted to have a complex secondary structure; (c) we are the first to evaluate the clinical role of nuclear and cytoplasmic expression of GAPDH protein in any cancer. Our finding that GAPDH is not detectable by immunohistochemistry in both nuclear and cytoplasmic compartments in almost 50% of ovarian and fallopian tube cancers is notable, and underscores that fact that GAPDH protein is regulated in cancers. We also found that GAPDH is coexpressed with CSF-1 in ovarian cancers, which makes sense because we now recognize that one of the many functions of GAPDH is to promote the mRNA stability of CSF-1, an important cytokine in tumor progression.

Materials and Methods

Cell Culture and Protein Extraction

Hey (8) human epithelial ovarian carcinoma cells were maintained in DMEM (American Type Culture Collection) supplemented with 1.5 g/L of sodium bicarbonate and 10% FCS (Life Technologies). The NOSE.1 cell line (8) was cultured in M199 and MCDB1051 medium (vol/vol; Sigma-Aldrich) supplemented with 15% FCS and 1.5 g/L of sodium bicarbonate. Total cellular protein extract for immunoblot analysis was prepared from cells using 50 mmol/L of Tris-C1 (pH 7.4), 100 mmol/L of NaCl, 2 mmol/L of EDTA, 1% Igepal (Sigma-Aldrich), and Protease Inhibitor Cocktail Set 1 at 1:100

| Table 1. The Association between Cytoplasmic GAPDH Score and CSF-1 Staining in Ovarian Cancer Cases (n = 36) |
|--------------------------------------------------|-----------|-----------|
| Score    | CSF-1 ≤200 | CSF-1 >200 |
| Cytoplasm|    ≥80     |    >80    |
| GAPDH    |           |           |
| score    |    26     |    5      |
|          |    2       |    5      |

NOTE: The association is highly significant (P = 0.008; Fisher’s exact test).
dilution (Calbiochem). S100 and total extracts for gel shift assays were prepared as described (8). Protein concentrations were determined by BCA assay (Pierce) using bovine serum albumin as the standard.

Preparation of RNA Probes

The terminal 144 nt wild-type sequence of CSF-1 exon 10 (3829-3972 nt) was PCR-amplified and subcloned into the KpnI and XhoI restriction sites of the PGEM-3Z (Promega) transcription vector (PGEM-3Z-WT) as described previously (24). The original mutant pGEM-3Z-MT was created as described previously (8). This mutant sequence, which contained mutations within four AU-rich areas within the 144 nt 3'UTR, was designed empirically prior to the footprinting experiments.

The mutated sequences containing deletion I or deletion II, which differed from the wild-type sequence in AU-rich regions contained within the footprint (as depicted in Fig. 4A), were inserted into PGEM-3Z to create PGEM-3Z-MT.1 and MT.2. For construction of the mutant sequences, the deletions were introduced by PCR using two overlapping oligonucleotides (5'-CCCCGGGTACCCCCATTGCTACGCCTGAGTTGGTTTTTATACTTGCAACTGGTGAAATTTATT-TAAAAGATAAGA-3' and 5'-CCTGCTCTAGACGC-GTCAACGGCGCTTTGTGCACCTTTTTATTAAAAATAAT-TATAAGCAGCTTATCTTAAAAATGAAATAAA-3') for deletion I and (5'-CCCCGGGTACCCCCATTGCTACGCGCTGACTTTGATTTTTTATTTTATTTGAAAT-TAAGTAAAGTCAATTAAAA-3' and 5'-CCTGCTCTAGACGC-GTCAACGGCGCTTTGTGCACCTTTTTATTAAAAATAAT-TATAAGCAGCTTATCTTAAAAATGAAATAAA-3') for deletion II. The wild-type and mutant constructs were linearized with HindIII endonuclease. PCR reactions were done using Taq polymerase (Perkin-Elmer) and were carried out at 95°C for 1 min, 42°C for 1 min, and 72°C for 1 min for 40 cycles (72°C at end).

Unlabeled RNAs, which were used for competition experiments and 3'-end-labeling, were generated with the MEGascript In vitro Transcription Kit (Ambion). For sequencing of 3'UTR CSF-1 RNA and footprinting of GAPDH, 50 pmol of the 3'UTR CSF-1 RNA was labeled with [32P]ppcp (Amersham Biosciences) at the 3'-end using T4 RNA ligase (Ambion) according to the manual.

Direct Chemical Method for Sequencing RNA

G, A, C, and U reactions were carried out as previously described (24). For each reaction, 3 × 10^4 cpm of 3'-end-labeled CSF-1 RNA was used. After specific chemical modification of each RNA base, aniline was used for strand scission. Samples containing those labeled fragments were dissolved in 2 to 3 μL of 8 mol/L of urea/20 mmol/L of Tris-HCl (pH 7.4)/1 mmol/L of EDTA/0.05% xylene cyanol/0.05% bromophenol blue. The samples were heated at 90°C for 2 min, chilled on ice immediately, and then layered on 8% polyacrylamide and 8 mol/L urea gel.

Enzymatic Footprinting of GAPDH

For enzymatic footprinting (7, 25), 1 μL (6 × 10^3 cpm) of 3'-end-labeled CSF-1 RNA was incubated with 1.4 μg of purified human heart GAPDH (Advanced Immunochemical, Inc.) in reaction buffer [5 mmol/L HEPES (pH 7.6), 40 mmol/L KCl, 2.5 mmol/L MgCl2, 3.8% glycerol, 1.5 mmol/L ATP, 0.1 mmol/L DTT, and 5 μg yeast tRNA] at 30°C for 10 min, then placed on ice. Next, RNase A (0.1 or 0.02 or 0.004 ng/mL) or RNase T1 (1.25 × 10^-5 or 1.25 × 10^-6 units/μL; Ambion) was added and incubated at 30°C for 10 min. Reactions were stopped with 300 μL of 0.2 mg/mL of proteinase K, 0.03 mg/mL of yeast tRNA, 50 mmol/L of Tris (pH 7.5), 50 mmol/L of NaCl, 5 mmol/L of EDTA, 0.5% SDS, and incubated 30 min at 55°C. Following phenol extraction and ethanol precipitation, samples were fractionated in 8% polyacrylamide and 8 mol/L urea gel.

RNA Gel Shift and Cold Competition Assays

Reaction mixtures (10 μL) containing 1 μL (6 × 10^3 cpm, 2 ng) of 3'-end-labeled RNA and 0.7 μg of purified human heart GAPDH in reaction buffer [5 mmol/L HEPES (pH 7.6), 40 mmol/L KCl, 2.5 mmol/L MgCl2, 3.8% glycerol, 1.5 mmol/L ATP, 0.1 mmol/L DTT, 5 μg yeast tRNA] were incubated for 10 min at 30°C. The complexes were resolved by electrophoresis through nondenaturing 3.2% polyacrylamide gels and autoradiographed. RNA gel shift assays with Hey S100 or NOSE.1 total cell extracts were done as described (8). For competition analysis, excess unlabeled wild-type RNAs or mutant RNAs were used in addition to the labeled RNAs.

siRNA Treatment

The GAPDH siRNA (5'-UGGUUUACAUGUUCUAAUA-UU-3' 5'-UUACCAAAUGUACCAAGGUAUA P-5') used was obtained from Dharmaco, and serves as their positive control for other siRNA experiments. Transfection of siRNA into Hey or NOSE.1 was done in six-well plates or T75 flasks. Cells were plated at a density of 1.7 × 10^4 cells/cm². Pilot dose-response experiments were first done to determine the lowest effective dose of GAPDH siRNA, in order to decrease off-target effects. Transfection of 20 mmol/L of GAPDH siRNA or negative control RISC-Free siRNA (Dharmacon) was done for 24, 48, or 57 h. For all transfections, DharmaFECT-1 siRNA Transfection Reagent was used.

Immunoblot

Twenty micrograms of proteins were loaded per lane on 10% SDS-PAGE gels, electrophoresed, transferred to Immobilon P membranes (Millipore) and Ponceau S (Pierce)–stained. Membranes were probed with GAPDH monoclonal antibody (Abcam, Inc.), and β-actin monoclonal antibody (ACTN05; Lab Vision). Immunoblot processing and enhanced chemiluminescence protein detection were done according to the manufacturer’s instructions using SuperSignal West Pico (Pierce) and horseradish peroxidase–conjugated 2° antibodies (Amersham GE).

Preparation of Total RNA and Analysis by Reverse Transcription-PCR

Total RNA extracts from cells were prepared with a RNeasy Mini kit (Qiagen). Reverse transcription was done using 0.7 μg of RNA according to the manufacturer’s protocol (Fermentas) in a final volume of 20 μL. Subsequently, 3 μL of cDNA was PCR-amplified using HotMaster DNA Taq polymerase.
Actinomycin D Chase Experiment

Hey cells were transfected with GAPDH siRNA or control RISC-Free siRNA (0.02 μmol/L) for 48 h. Total cellular RNA was isolated using RNeasy Mini Kit (Qiagen). The RNAs (10 μg per well) were dissolved in glyoxal/DMSO buffer (Ambion), electrophoresed in a 1% agarose gel, and transferred onto BrightStar-Plus membranes (Ambion). The Northern blots were then hybridized with a (α-32P) dCTP-labeled 500-bp fragment of the human CSF-1 coding region, a kind gift from Eleanor C. Weir (Yale University) and 18S rRNA (Ambion). For actinomycin D chase experiments (8), 5 μg/mL of actinomycin D (Sigma) was added to inhibit new transcription at time = 0. Cells were harvested at various intervals after actinomycin treatment, total RNA extracted, and CSF-1 RNA and 18S RNA levels analyzed by Northern blot. Graphs of relative CSF-1 mRNA were derived by densitometry (Image Quant; Molecular Dynamics, Inc.) and normalization of CSF-1 RNA to its 18S RNA control. Half-lives were determined with the maximum intensity normalized for each half-life to 100%. A representative experiment of three independent experiments was shown.

Detection of CSF-1 in the Culture Supernatants of Post–siRNA-Transfected Cells by ELISA

Hey or NOE.1 cells were plated at a density of 1.7 × 10⁴ cells/cm². Transfection with GAPDH siRNA or control RISC-Free siRNA was done as above. The concentration of secreted CSF-1 in the culture supernatants was measured by ELISA (R&D Systems, Inc.) 57 h post-siRNA transfection.

Construction of TMA

To construct the ovarian and fallopian tube cancer TMA used in this study, formalin-fixed, paraffin-embedded archival tissue blocks and their matching H&E-stained slides were retrieved, reviewed, and screened for representative tumor regions by a gynecologic pathologist as described previously. The tissue cores were 0.6 mm in diameter and spaced 0.8 mm apart using a precision instrument (Beecher Instruments); the TMA was constructed by the Yale University Cancer Center TMA Shared Resource, with approval from the Yale University Institutional Review Board. Microarrays were stored in a nitrogen chamber at room temperature. Samples used in this study were from patients mainly with primary epithelial ovarian cancers who had undergone initial surgery at the Department of Obstetrics and Gynecology of Yale University between 1980 and 2001. The TMA consisted of 322 specimens including 297 conventional slides of primary epithelial ovarian carcinoma served as positive controls for GAPDH staining. Negative controls were prepared by replacing primary antibodies with class-matched mouse IgG immunoglobulins on parallel sections. To confirm the specificity of the GAPDH staining, 10 additional specimens were re-stained with and without the antigenic peptide for GAPDH for 2 h at room temperature prior to the staining procedure.

To validate our findings with this TMA, we studied another cohort of 53 conventional slides of primary epithelial ovarian cancer for GAPDH staining using the same antibody as used for the TMA. The technique was the same with the exception that the final concentration used for the conventional slides was 3 μg/mL. Negative controls were prepared by replacing primary antibodies with class-matched mouse IgG immunoglobulins. Thirty-eight of the 53 cases had been previously analyzed for CSF-1 and c-fms expression (9).
weak staining; 2, moderate staining; and 3, intense staining. The scores used for analysis were generated by multiplying the intensity by the percentage of positive cells in a defined tissue core, yielding scores ranging from 0 to 300.

Statistical Analysis

All analyses were done using SAS Proprietary Software version 9.0 (SAS Institute, Inc.). The difference between levels of secreted CSF-1 was determined by Student’s t test. Survival curves were calculated using the Kaplan-Meier method, with the significance evaluated using the Mantel-Cox log-rank test. The association between GAPDH staining scores and clinicopathologic variables was calculated using the χ² test, or Fisher’s exact test as appropriate. Spearman’s correlation coefficient method was used for validation of the TMA with conventional slides.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The Multifunctional Protein Glyceraldehyde-3-Phosphate Dehydrogenase Is Both Regulated and Controls Colony-Stimulating Factor-1 Messenger RNA Stability in Ovarian Cancer

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