Glyceraldehyde-3-Phosphate Dehydrogenase Binds to the AU-Rich 3′ Untranslated Region of Colony-Stimulating Factor–1 (CSF-1) Messenger RNA in Human Ovarian Cancer Cells: Possible Role in CSF-1 Posttranscriptional Regulation and Tumor Phenotype

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Abstract

The overexpression of the colony-stimulating factor–1(CSF-1) by epithelial ovarian cancer cells enhances invasiveness and metastatic properties, contributing to the poor prognosis of the patients. It has been suggested that CSF-1 3′ untranslated region containing AU-rich elements (ARE) could regulate CSF-1 posttranscriptional expression and be responsible for its aberrant abundance in such cancer cells. In this study, normal (NOSE.1) and malignant (Hey) ovarian epithelial cells were used to examine CSF-1 expression and regulation. CSF-1 overexpression in Hey cells was found to associate with increased invasiveness, motility, urokinase activity, and virulence of tumorigenicity, compared with NOSE.1 cells, which expressed little CSF-1. CSF-1 ARE was further found to serve as an mRNA decay element that correlates with down-regulation of protein translation. Moreover, such down-regulation was found more prominent in NOSE.1 than in Hey cells, suggesting differences in posttranscriptional regulation. As a variety of *trans*-acting factors [AU-binding protein (AUBP)] are known to modulate messenger stability through binding to such elements, we examined the protein content of both cell lines for their ability to bind the CSF-1 ARE. Our results strongly suggested the abundance of such AUBP activity in Hey cells. We isolated a 37-kDa AUBP, which was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To summarize, our study identified GAPDH as an AUBP abundant in Hey cells, where it binds to CSF-1 ARE that imparts mRNA decay. These data suggest that GAPDH binding to CSF-1 ARE sequence prevents CSF-1 mRNA decay and subsequent down-regulation of CSF-1 protein translation, leading to CSF-1 overexpression and increased metastatic properties seen in ovarian cancer. (Cancer Res 2005; 65(9): 3762-71)

Introduction

Epithelial ovarian cancers are usually characterized by an advanced stage of tumor invasion into the lymphatic channels and in widespread peritoneal metastases (1–3). Although progress has been made in the diagnosis of this disease, there has been no significant improvement in the 10-year survival of the patients and the molecular basis for epithelial ovarian cancer (EOC) remains largely unknown.

Studies of invasive cancers showed high expression of many proteins involved in the regulation of cell cycle and cell differentiation such as growth factors, onco-proteins, lymphokines, and cytokines (3). One common feature of epithelial cancers of breast, lung, pancreatic, endometrial, and ovarian origin is the overexpression of the secreted form of macrophage colony-stimulating factor, CSF-1 (4-kb mRNA isoform) and its receptor, c-fms (3–6). The coexpression of CSF-1 and c-fms is usually a sign of poor prognosis and can be used as a diagnostic marker of the stage of tumor growth (7–9). The elevated levels in the serum of CSF-1 indicate the progression or the recurrence of the disease (10). Moreover, the increase of CSF-1 concentration in both the serum and the ascites is usually correlated with a poor outcome (11).

We have previously established that the ability of EOC cells to invade extracellular matrix correlates with CSF-1 expression, and that CSF-1–stimulated invasiveness of EOC is mediated through urokinase-type plasminogen activator (uPA; ref. 12). Like CSF-1, uPA has been found present in elevated levels in many cancers, including those of breast and ovary where it is associated with poor prognosis (13, 14). Because uPA gene expression is also modulated by CSF-1 (15, 16), it was not surprising to observe that both factors are overexpressed in ovarian cancer. The up-regulation of uPA and uPA receptor (uPAR) is due in part to the stabilization of their mRNAs through AU-rich elements (ARE) in their 3′ untranslated region (3′UTR; ref. 17). Therefore, it would be likely to observe the same type of regulation in ovarian cancer for the CSF-1 messenger. The human CSF-1 gene is differentially spliced (18, 19). It has been proposed that the most abundant 4-kb CSF-1 transcript, which encodes for the secreted CSF-1 form, contains instability determinants within the AREs of its 3′UTR exon 10, believed to be involved in the posttranscriptional regulation of CSF-1 expression.

In the last decade, many studies have implicated AU-binding proteins (AUBP) in either activating or blocking the rapid degradation of ARE-containing messages of growth factors (20, 21). One of the well-characterized AUBP is the RNA-binding protein HuR (22). HuR is known to interact with the AREs of many mRNA leading to their stabilization and to their rapid export from the nucleus to the cytoplasm. Recently, it was observed that HuR associates with both uPA and uPAR mRNA through their AREs leading to their stabilization (17). HuR is ubiquitously expressed and has been shown to play a key role in many processes such as cell growth (23–27) and cell differentiation (28). These observations suggest that a generic protein such as HuR could have functions

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that are normally attributed to cell-specific proteins. One explanation for these results is that such protein-RNA interaction could be differentially regulated in different cell types and under different growth conditions. Therefore, it would not be surprising that a protein that is ubiquitously expressed in tissues could play a fundamental role in processes like cell cycle, differentiation, and metastasis.

To delineate the molecular mechanisms linked to the metastatic phenotype of EOC cells, we first established a cellular model in which to study CSF-1 mRNA regulation. We used Hey epithelial ovarian carcinoma cells that are highly invasive and overexpress both uPA and CSF-1 and their normal counterparts, NOSE.1 cells, which do not. We then studied the AU-rich sequences contained in the 3′UTR exon 10 of CSF-1, which we showed as being mRNA decay elements and potential targets for specific regulatory RNA-binding proteins. A 37-kDa protein was identified that bound to that sequence in both cell lines with a much higher activity observed in Hey cells, supporting our hypothesis that such a factor could be responsible for increased CSF-1 protein translation with subsequent increased virulence of tumorigenicity. This factor turned out to be overexpressed in Hey cells and other ovarian carcinoma cells such as Bixler, DK2NMA, and Bix3, and was identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry.

Materials and Methods

Cell culture. Both primary (Bixler, DK2NMA, and Bix3; ref. 29) and established (Hey; ref. 30) human epithelial ovarian carcinoma cells were maintained in DMEM/F12 Ham's medium (Sigma, St. Louis, MO) containing 10% FCS (Life Technologies, Gaithersburg, MD), 100 units/mL penicillin/100 μg/mL streptomycin (P/S; Life Technologies), 10 ng/mL insulin (Sigma), and transferrin (Sigma). Normal human ovarian surface epithelial cell line (NOSE.1), spontaneously immortalized cells from the ovary of a postmenopausal woman (gift of Dr. Andrew Berchuck, Duke University) were cultured in M199 and MCDB1051 medium (v/v; Sigma) supplemented with 15% FCS and P/S. BT20 human breast carcinoma cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 (Sigma) in the presence of 10% FCS and P/S.

Immunohistochemical staining with anti-CSF-1 antibody. Hey and NOSE.1 cells were serum starved for 48 hours (12, 31) before cytospin preparation and the slides were processed as previously described (12).

Measurements for CSF-1 and urokinase activity. The amount of CSF-1 secreted in serum-starved medium was measured by sandwich ELISA as previously described (31) and reported as pg CSF-1/mL. Urokinase activity was measured as described (32) and reported as mPU ± SE. Three independent experiments were done.

Invasion and motility assays. Quantitative studies of degree of invasion of NOSE.1 and Hey cells were carried out as previously described (31, 33). Before the invasion assay, the cells were grown in 1% NuSerum (BD Biosciences, San Diego, CA) for 24 hours to limit the presence of protease inhibitors. The invasion assay was carried out in 1% NuSerum for the same reason. The results were reported as mean percent invasion ± SE. Nonrandom motility studies towards the chemoattractant, fibronectin (Sigma), were carried out as described (33). The results were reported as mean percent motility ± SE. At least three independent invasion and motility experiments were done for calculation of SE.

Virulence of tumorigenicity studies. Tumor burden was measured *in vivo* after inoculation of NOSE.1 or Hey cells (1×10^6 cells in 100 μL serum-free medium) into the peritoneal cavity (which represents the orthotopic location for ovarian cancer) of 6- to 8-week-old female NIH athymic NCr-nu mice (six per condition). The mice were observed for evidence of clinically apparent disease, at which time they were sacrificed. The extent of macroscopic metastasis was assessed. In the absence of such clinical evidence, microscopic examination was done to search for subclinical evidence of cells. The mice were sacrificed at 7 months after cell inoculation. The studies were done in accordance with Yale University IACUC protocol 07744.

Chloramphenicol acetyl transferase assays. For preparation of the constructs, we used the p2518 chloramphenicol acetyl transferase (CAT) reporter vector (Dr. Carl Baker, NIH, Bethesda, MD; ref. 34), which does not contain AREs. We directionally cloned into the *Eco*RV and *Bgl*II sites (within the poly-linker situated 3′ to the CAT coding sequences) the terminal 144 bp of CSF-1 exon 10 (nucleotides [nt] 3,829-3,972; obtained by PCR from p3aCSF69, Genetics Institute, Cambridge MA; ref. 18), or the large majority of exon 9 sequences (568 bp, nt 1,013-1,580; obtained by PCR from pcCSF-17; Dr. Martha Ladner, Chiron Corp., Emeryville CA; ref. 35). Stable transfections of NOSE.1 and Hey cells were carried out according to standard procedures, using LipofectAMINE (Life Technologies) with the p2518 CAT reporter vector or the p2518 CSF-1 constructs described above, and pWLneo (Stratagene, La Jolla, CA). Several colonies expressing neomycin resistance were isolated and grown. CAT protein expression in the cell extracts was quantified using a CAT ELISA protocol (Roche, Indianapolis, IN). Results were reported as pg CAT/25 μg total protein.

RNA analyses. Total cellular RNA was extracted from NOSE.1 or Hey cells using standard methods. The RNAs (20 μg per well) were electrophoresed in a 1% agarose-formaldehyde gel, and transferred onto Gene Screen Plus (New England Nuclear, Boston, MA). The Northern blots were then hybridized with a ^{32}P-labeled 1.8-kb fragment of the human CSF-1 coding region, purified from p3aCSFR1 (Genetics Institute; ref. 18) or a ^{32}P-labeled 780-bp fragment of the CAT coding region purified from pMSG (Pharmacia, Piscataway, NJ). In actinomycin D chase experiments, 5 μg/mL actinomycin D (Sigma) was added to inhibit new transcription at time 0. CAT mRNA was followed for 2 hours; cells were harvested at various intervals after drug treatment, total RNA extracted, and CAT mRNA level analyzed by Northern blot as described above. Graphs of relative CAT mRNA were derived by densitometry and half-lives determined, with the intensity normalized for each half-life to 100% at time 0.

Protein extraction. Cell lysates were prepared from adherent cells collected with a cell scraper, harvested by gentle centrifugation, and washed with cold PBS. Total extracts were prepared by incubating the cell pellets directly in gentle lysis buffer [25 mmol/L HEPES (pH 7.9), 150 mmol/L KCL, 1 mmol/L EDTA, 10 mmol/L NaF, 0.1% NP40, 1 mmol/L DTT, and 1× protease inhibitor cocktail; Calbiochem, La Jolla, CA], for 1 to 2 hours on ice followed by centrifugation for 5 minutes at 14,000 rpm. In the case of S_{100} extract preparation, the cell pellets were washed in ice-cold buffer A [10 mmol/L triethanolamine (pH 7.9), 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L DTT, and 1× protease inhibitor cocktail], centrifuged at 4°C for 5 minutes at 1,200 rpm, resuspended in one cell volume of buffer A, incubated on ice for 5 minutes, and centrifuged again. The cells were lysed in two initial cell volumes of buffer A with 20 strokes of a type B pestle in a Dounce homogenizer and the lysate was centrifuged at 4°C for 10 minutes at 3,000 rpm. The supernatant was mixed with 0.11 volume of buffer B [300 mmol/L HEPES (pH 7.9), 1.4 mol/L KCl, and 30 mmol/L $MgCl_2$] and centrifuged in a TL100 centrifuge (Beckman, Fullerton, CA) for 15 minutes at 50,000 rpm. The supernatant (soluble fraction, S_{100}) and the pellet (membranes) were separately stored. Protein concentrations were determined by the Bio-Rad protein assay with bovine serum albumin as a standard. Because different protein preparations were used (S_{100} for Hey cells and total protein extracts for NOSE.1 cells), no quantitative comparison in protein activity could be determined in Results.

Gel shift assays. The terminal 144-nt sequence of CSF-1 exon 10 was PCR amplified using two overlapping oligonucleotides (5′-CCCGGGGTACCCCATTGGCTCACGCACTGTGAGATTTTGTTTTTATACTTGCAACTGGTGAATTATTTTTTATAAAGTCATTTAAATATCTATTTA-3′ and 5′-CCTGCTCTAGAGCGTCAACGGCAGCTTGTGCACTTCTTTTATTATTAAATATATAAGCAGCTTCCTATCTTTTAAATAGATATTTAAATGACT-3′) and subcloned into the *Kpn*I and *Xba*I restriction sites of the PGEM-3Z (Promega, Madison, WI) transcription vector (PGEM-3Z-WT). The mutated

sequence was inserted into PGEM-3Z (PGEM-3Z-MT) according to the same protocol using oligonucleotides that differed from the wild-type sequence in most AU-rich stretches (5′-CCCGGGGTACCCCATTGGCTCACGCACTGTGAGCGGGGGGGGGGCTACTTGCAACTGGTGAATTCGGGGGGCTAAAGTCATTTAAATATCTCGGGC-3′ and 5′-CCTGCTCTAGAGCGTCAACGGCAGCTTGTGCACTTCTTTTATGCCCGATATAAGCAGCTTCCTATCTTTGCCCGTGATATTTAAATGACT-3′). PGEM-3Z-WT and PGEM-3Z-MT constructs were linearized with *Hind*III endonuclease and processed according to standard procedures. Synthesis and purification of high specific activity ^{32}P-radiolabeled RNA probes were done according to the manufacturer's protocol (Promega), using [α-^{32}P] CTP (50 μCi at 10 μCi/μL per reaction) and T7 RNA polymerase; 10^4 to 10^5 cpm (1 μL) were used per gel shift sample (10 μL total volume) along with 8 μg of protein sample from Hey, NOSE.1, or BT20 cells, and cold riboprobe as a competitor (1,500×) in 5 mmol/L HEPES, 40 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 3.8% glycerol, 0.5 μg/μL yeast tRNA, 0.1 mmol/L DTT, 0.5 μg/μL heparin, and 1.5 mmol/L ATP. Protein samples were first premixed with cold riboprobe (when applied) for 10 minutes at room temperature followed by addition of radiolabeled riboprobe. The reaction was run for 15 minutes at 30°C, before addition of RNA loading buffer (2 μL/10 μL, 50% glycerol, 1 mmol/L EDTA, and 0.4% bromophenol blue). Free probes and protein-RNA shifts were separated on a 6% acrylamide gel and autoradiographed.

In the depletion experiment, protein extracts were premixed with different antibodies at 1:250 ratio: anti-GAPDH, (Santa Cruz Biotechnology, Santa Cruz, CA), anti–poly(A)-binding protein (Dr. Gideon Dreyfuss, University of Pennsylvania School of Medicine; ref. 36), anti-HUR or anti-hnRNPD (Dr. Joan Steitz, Yale University School of Medicine, New Haven, CT; refs. 37, 38), nutated for 1 hour at room temperature, placed in presence of 10 μL of protein A-Sepharose beads for another hour, and spun down before the 5.5 μL of the supernatant were added to the radiolabeled riboprobe for a gel shift assay. Human GAPDH was purchased from Sigma-Aldrich (St. Louis, MO).

Northwestern analysis. One hundred micrograms of protein were loaded per lane on a 12% SDS-PAGE gel, electrophoresed, and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were either stained with Ponceau red (Sigma), or blocked for 2 hours at room temperature in PBS 1×, 0.1% Tween 20, 5 mg/mL BSA, washed twice for 5 minutes each in binding buffer (5 mmol/L HEPES, 40 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 3.8% glycerol, 0.1 mmol/L DTT, and 0.5 μg/mL heparin), before incubation for 1 hour at 30°C in binding buffer containing 1 μg/mL of tRNA (Sigma) and 1.5×10^6 cpm/mL of ^{32}P-labeled riboprobe described in the above section. The membranes were finally washed twice for 5 minutes each in binding buffer and autoradiographed.

Immunoblot. Proteins were loaded on a 10 or a 12% SDS-PAGE gel, electrophoresed, and transferred to Immobilon P membranes (Millipore, Bedford, MA). Anti-HuR monoclonal antibody (Dr. Joan Steitz; ref. 37) was used at dilution 1:30,000. As anti-GAPDH antibodies, we used either a polyclonal antibody from Santa Cruz Biotechnology or a monoclonal antibody from Abcam, Inc. (Cambridge, MA) at 1:10,000. Actin monoclonal antibody (Lab Vision, Fremont, CA) was used at 1:400. Immunoblot processing and chemiluminescence protein detection were done according to the manufacturer's instructions (ECL detection system, Amersham Biosciences, Piscataway, NJ) using horseradish peroxidase–conjugated secondary antibodies (Roche).

Chromatography analysis. A S_{100} fraction was prepared from 0.7×10^9 Hey cells, dialyzed against a binding buffer [10 mmol/L sodium phosphate (pH 7.1), 40 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 3.8% glycerol, 0.5 mmol/L DTT, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail; Calbiochem] containing 0.2 mmol/L EDTA and loaded on a 5-mL HiTrap Heparin HP column (Amersham Biosciences). The column was washed with 3 × 10 mL of binding buffer and proteins were gradually eluted with a series of 5-mL solutions made of binding buffer with increased concentrations of KCl (0.2, 0.4, 0.6, 0.8, 1, and 2 mol/L). A total of 30 × 1 mL fractions were collected and subsequently dialyzed overnight at 4°C against the binding buffer containing 40 mmol/L KCl. The dialyzed samples were then all tested in a gel shift experiment as described above (5.5 μL per assay).

Results

CSF-1 overexpression in malignant ovarian cancer cells correlates with *in vitro* and *in vivo* phenotypes. We previously showed that overexpression of CSF-1 confers invasive, metastatic properties and thereby a poor prognosis in ovarian cancer patients (9, 12). We investigated CSF-1 expression and regulation in normal (NOSE.1) and malignant ovarian epithelial cells (Hey). Expression of CSF-1 was first studied by immunohistochemical staining of cytospins prepared from NOSE.1 and Hey cells (Fig. 1*A*). Results confirmed a significant overexpression of CSF-1

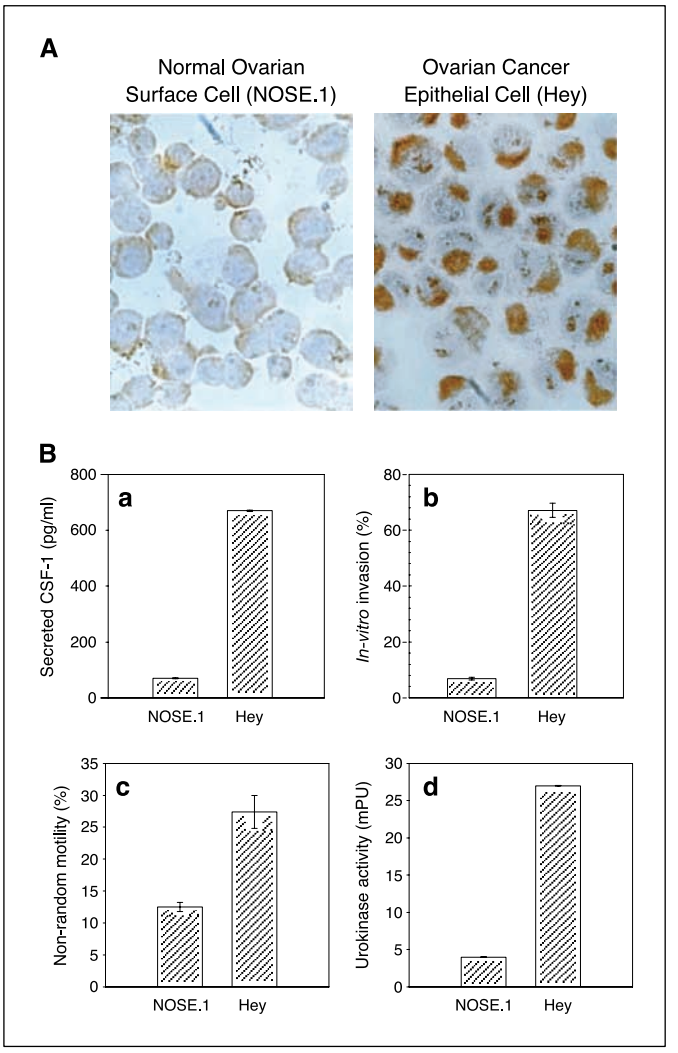

Figure 1. CSF-1 expression and tumor phenotype correlation studies in normal (NOSE.1) and malignant (Hey) ovarian epithelial cells. *A,* CSF-1 immunohistochemical staining of cytospins prepared from NOSE.1 or Hey cells. Antibody specificity for CSF-1 was previously assessed as its signal is competed by molar excess of a (4 kb encoded) CSF-1 antigen (12). Minimal cytoplasmic staining for CSF-1 in NOSE.1 cells was observed, which contrasts with intense cytoplasmic staining (*brown*) in Hey cells. *B,* comparative analyses for CSF-1 secretion, invasion, cellular motility, and urokinase activity. *a,* levels of CSF-1 secreted in the conditioned media of NOSE.1 or Hey cells (pg CSF-1/mL). Hey cells secrete 9.5 times more CSF-1 than NOSE.1 cells. *b,* degree of invasion of NOSE.1 versus Hey ovarian cancer cells, through a human extracellular matrix barrier (% invasion ± SE). Hey cells proved 10-fold more invasive than NOSE.1 cells. *c,* results of nonrandom motility experiments performed towards fibronectin (% ± SE). Hey cells appeared twice more motile than NOSE.1 cells. *d,* urokinase (*uPA*) activity was measured from both cell lysates (mPU ± SE). Results showed a 7-fold uPA overexpression in Hey cells.

in the metastatic Hey cell line. Secreted CSF-1 was subsequently assayed by sandwich ELISA in the conditioned media of those cells. Hey cells showed a 9.5-fold overexpression of secreted CSF-1 compared with NOSE.1 cells (Fig. 1*B*, *a*). Because CSF-1 expression and activity are usually correlated with the ability for cells like macrophages to invade and for malignant cells to metastasize, we studied the invasion and motility abilities of these cells. The difference in CSF-1 expression closely paralleled that obtained in an *in vitro* invasion assay comparing the two cell lines (Fig. 1*B*, *b*). Furthermore, the degree of nonrandom motility of Hey cells proved >2-fold greater than that of NOSE.1 cells (Fig. 1*B*, *c*). Because CSF-1 is also known to promote uPA activity, we measured uPA activity in the cell lysates and observed that it was 7-fold higher in Hey than in NOSE.1 cells (Fig. 1*B*, *d*). Lastly, whereas the genesis of tumorigenicity is complex and unlikely due to a single genetic event, we showed that Hey cells exhibited virulent tumorigenicity with massive i.p. spread of tumor and clinical deterioration of the majority of nude mice in <30 days (data not shown), confirming previous reports (29). In contrast, 100% of the mice implanted with NOSE.1 cells exhibited complete absence of macroscopic or microscopic tumorigenicity after 7 months of observation. All together, these results suggest that NOSE.1 and Hey cells represent a valid model with which to study CSF-1 regulation, as these studies correlate with the findings of the clinical role of CSF-1 overexpression in human ovarian cancers (9, 13, 31).

The terminal 144-nt of CSF-1 3′ untranslated region serves as an mRNA decay element. Regulation of the most abundant 4-kb CSF-1 transcript has previously been investigated in human ovarian carcinomas and evidence was obtained that AU-rich stretches in its 3′ UTR exon 10 may be responsible for mRNA decay (18, 19). Treatment with several different protein synthesis inhibitors resulted in marked overexpression of CSF-1 transcript levels, suggesting a potential role for labile proteins in the regulation of CSF-1 expression (19). Due to alternative splicing, the exon 10 sequences comprise the 3′ UTR of the most abundant transcript (4-kB CSF-1); exon 9 sequences are not included. However, the untranslated exon 9 sequences do comprise the 3′ UTR of the 1.6-, 1.9-, and 2.3-kb minor CSF-1 transcripts. In contrast to exon 10, exon 9 CSF-1 (35) does not contain any recognizable AREs (39, 40).

To study the possible role of the AU-rich exon 10 sequences in mRNA stability, we proceeded to a series of CAT assays, focusing on the terminal 144 nucleotides, as this region contains an abundance of ARE (Fig. 2*A*; ref. 18). We first compared the effects of the exon 10 ARE and/or that of the entire exon 9 (non-AU–rich containing control), cloned 3′ to the *CAT* gene to that of the vector control, on CAT reporter expression, after stable transfection of NOSE.1 and Hey cells. In Table 1, we described the results of CAT protein expression in the cell lysates from pooled stably transfected clones for each condition. The results were confirmed on individual clones and also with transient transfections (data not shown). Interestingly, we found that in neither cell type does exon 9 down-regulate CAT protein expression. In contrast, we observed a significant down-regulation of the CAT protein expression by the exon 10 ARE sequences, which suggested the presence of a strong negative regulatory element in the sequence. The effect was particularly prominent in NOSE.1 cells where CAT is down-regulated 8-fold (104 ± 3 to 13 ± 0.5 pg CAT/25 μg protein), against 2.1 fold in Hey cells (42 ± 9 to 19 ± 5 pg CAT/25 μg protein).

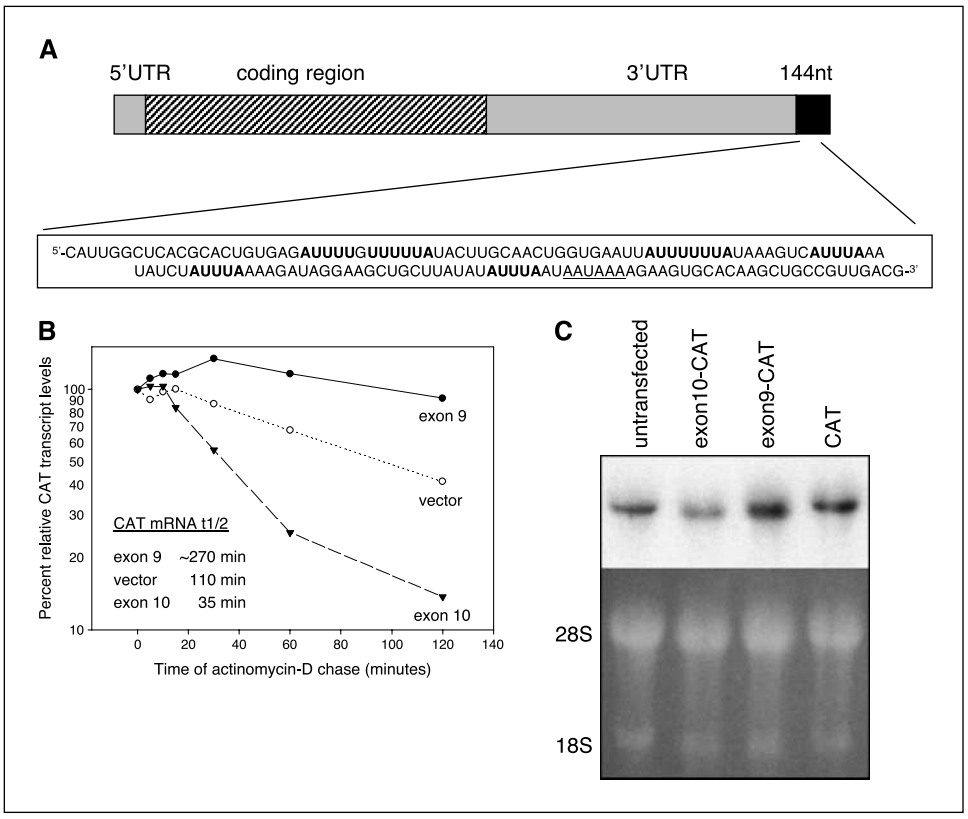

Figure 2. CSF-1 AU-rich 3′UTR in mRNA stability. *A,* schematic representation of the 4-kb CSF-1 mRNA, which consists of a 145-nt long 5′UTR (*gray*) followed by a coding region of 1,662 nt (*black stripes*), and a 3′UTR of 2,200 nt (*gray*) containing AU-rich motifs (*black*). Terminal 144-nt sequence (*text box*); AU stretches (*bold*) and the consensus polyadenylation signal (*underlined*). *B,* effect of exon 10 AU-rich sequences on CAT mRNA stability. Actinomycin D chase experiments done to measure CAT mRNA stability in Hey ovarian cancer cells stably transfected with the CAT vector, or the CAT constructs containing the non–AU-rich containing exon 9 or the CSF-1 exon 10 sequences cloned 3′ to CAT. The relative differences in CAT mRNA half-lives (minutes) are of 2.1-fold between exon 10 CAT and CAT vector and 5.8-fold between exon 10 CAT and exon 9 CAT. *C,* Northern blot analysis of total RNA from NOSE.1 cells, or those stably transfected with the CAT vector, or either of the CAT constructs, for endogenous CSF-1 expression. The probe used contains CSF-1coding region sequences only and none of the 3′ UTR. The ethidium bromide stained gel reflects the load of the RNA.

Table 1. CAT assays in NOSE.1 or Hey stable transfectants

CAT construct	CAT protein expression*	
	NOSE.1	Hey
Vector alone	104 ± 3	42 ± 9
Exon 9	333 ± 11	113 ± 2.0
Exon 10	13 ± 0.5	19 ± 5

*Results reported in pg CAT/25 μg total protein ± SE.

To investigate further the mechanism for CAT protein down-regulation by the exon 10 ARE, we did actinomycin D chase experiments and determined CAT mRNA half-lives in the stably transfected Hey cells described above (Fig. 2*B*). CAT mRNA half-life was estimated at ~270 minutes in the presence of exon 9 sequences, 110 minutes with the vector sequences, and 35 minutes in the presence of the exon 10 ARE sequences, which clearly showed their importance in mRNA decay. Importantly, the relative differences in CAT mRNA half-lives seem to entirely account for the differences observed in CAT protein expression in Hey cells (Table 1).

Taken together, our data clearly showed that the 144-nt ARE is responsible for CAT mRNA decay, leading to CAT protein down-regulation in both Hey and NOSE.1 cells. Because the biological effect of such 3′ UTR sequences is thought to be regulated by RNA protein binding, we postulated that there could be *trans*-acting factors expressed in Hey cells, which mitigate the negative regulatory effect of the AU-rich exon 10 sequence, and may be present at lower levels or absent from NOSE.1 cells, explaining a greater RNA instability in those cells.

To verify this hypothesis and to understand the possible implications of the exon 10 ARE on CSF-1 mRNA posttranscriptional regulation, we studied the *trans*-effect of exon 10 ARE sequences on endogenous CSF-1 mRNA level, in both NOSE.1 and Hey stable transfectants. In NOSE.1 cells (Fig. 2*C*), we observed an 8-fold decrease in the level of CSF-1 mRNA when the 144-nt ARE was expressed in *trans*, compared with the vector alone control. Similar findings were observed in the Hey ovarian cancer stable transfectants, but to a lesser degree, with only a 2-fold decrease (data not shown). Because there is no discernable effect of control CAT sequences on CSF-1 compared with untransfected cells, we thought to attribute the reduction in CSF-1 mRNA observed to excess 144-nt ARE sequences. Interestingly, this *trans*-effect on CSF-1 mRNA level is very similar to that observed on CAT expression in cells expressing ARE sequences in *cis*, with greater degree of RNA instability/down-regulation in NOSE.1 cells. Our data are consistent with our postulate that RNA-binding proteins specific for the 144-nt ARE could exist, in larger amount in Hey ovarian cancer cells than in NOSE.1 cells, and that possible excess exon 10 ARE could compete for CSF-1 mRNA protein binding, leading to a more unstable CSF-1 mRNA, especially in cells of normal ovarian epithelium.

CSF-1 ARE/RNA–binding proteins are more abundant in Hey carcinoma cells than in normal ovarian epithelial cells. To assess the presence of potential CSF-1 mRNA-binding proteins in ovarian epithelial carcinoma cells, gel shift RNA-protein binding experiments were done using a riboprobe corresponding to the terminal 144-nt AU-rich region described in Fig. 2*A* that we tested in presence of protein extracts from Hey or NOSE.1 cells (Fig. 3*A*). The results showed the existence of RNA-binding proteins in the S_{100} fraction of Hey cells (*left*, *lanes 2* and *3*). Their binding to the ^{32}P-labeled riboprobe was significantly reduced in the presence of excess cold riboprobe (*lane 4*). A significantly less important shift occurred when we used NOSE.1 cell total extracts (*right*, *lanes 2* and *3*), suggesting the presence of RNA-binding factors in NOSE.1 cells but either at a low concentration or at a low activity. Furthermore, excess cold riboprobe completely prevented protein binding in the NOSE.1 extract (*right*, *lane 4*). These data suggest the relative abundance of protein factor(s) interacting specifically with the AU-rich 3′UTR of CSF-1 mRNA in ovarian carcinoma Hey cells.

A 37-kDa protein binds to the CSF-1 AU-rich exon 10 with high affinity in Hey cells. To further characterize the RNA-binding factors, we did a Northwestern analysis with the same protein extracts from Hey and NOSE.1 cells and the same exon 10 riboprobes used in the RNA gel shift studies. In Fig. 3*B*, 100 μg of protein extracts isolated from Hey cells (S_{100}, lane 1), NOSE.1 cells (total protein, lane 2), and BT-20 cells, a breast carcinoma cell line which does not express CSF-1 (ref. 41; total protein, *lane 3*) were size fractionated on two identical 12% SDS-PAGE gels, and transferred to nitrocellulose membranes. Protein loading was assessed by Ponceau red staining (*top*). The membranes were hybridized with the ^{32}P-labeled wild-type riboprobe (*bottom left*) or its mutant counterpart (*bottom right*). Interestingly, hybridization to the wild-type RNA revealed two major proteins in Hey cells: a 70-kDa protein common to all three cell lines and a 37-kDa protein specific to Hey cells (*bottom left*). The specificity of the interaction was confirmed upon hybridization with the mutant riboprobe, because under identical binding conditions, there was total absence of protein interaction (*bottom right*).

One of the most common AU-rich RNA-binding proteins is HuR, which has a molecular weight of 37 kDa. We therefore checked our extracts for the presence of HuR by immunoblot analysis with an anti-HuR monoclonal antibody (Fig. 3*C*). Interestingly, HuR was equally detected in all three extracts, suggesting that most likely our 37-kDa RNA-binding candidate was not HuR. It then became critical to determine the identity of this ovarian epithelial carcinoma factor.

Purification of a 37-kDa CSF-1 mRNA binding protein from Hey cells. To identify our candidate protein, we adopted a biochemical strategy to enrich the protein extract in RNA-binding proteins. Because Hey cells could be grown in abundance, we were able to prepare S_{100} extract from 0.7×10^9 cells. The extract was dialyzed against the appropriate binding buffer and loaded on a HiTrap Heparin HP column. After washes in the binding buffer, a salt gradient elution (0.2-2 mol/L KCl) was applied. The various fractions were individually dialyzed against the binding buffer and examined in gel shift assays for binding to the CSF-1 ARE (Fig. 4*A*) and in SDS-PAGE followed by silver staining (data not shown). Figure 4*A* displays the binding activity throughout the salt gradient (fractions 1-30), including samples of S_{100}, flow through (F), and washes (W).

The most active fractions (4–10) were eluted with 0.2 and 0.4 mol/L KCl. When samples from the S_{100} extract, and fractions representative of high activity (fraction 4), medium activity (fraction 9), and no activity (fraction 23) were separated in 12%

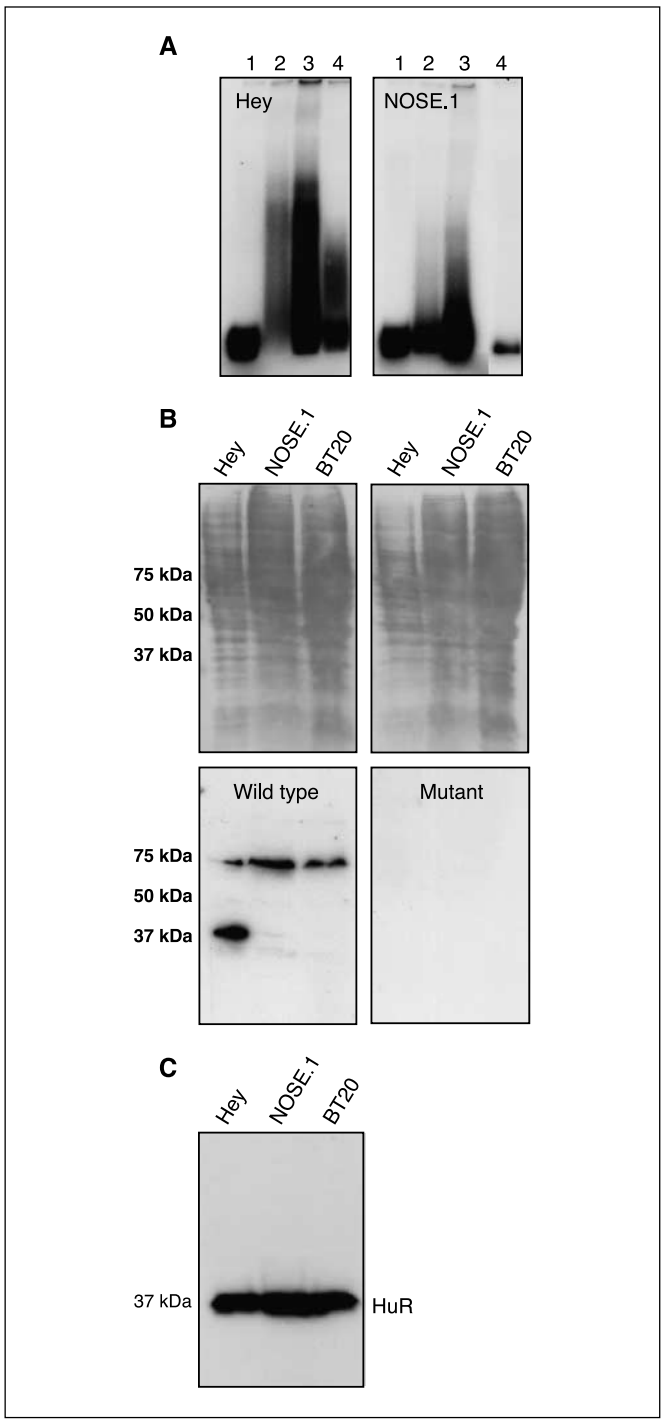

Figure 3. Evidence for relative abundance of CSF-1 3′UTR RNA-binding proteins in epithelial ovarian cancer cells. *A,* gel shift RNA binding assays on a cytoplasmic (S_{100}) fraction prepared from Hey cells and a total extract from NOSE.1 cells, using a CSF-1 3′UTR riboprobe (144-nt sequence). *Lane 1,* free probe; *lane 2,* 8 μg of protein extract plus probe; *lane 3,* 8 μg of protein extract plus 3× probe; *lane 4,* 8 μg of protein extract plus 1× probe in presence of 1,500× cold probe. *B,* Northwestern RNA protein-binding assay on S_{100} prepared from Hey cells and total extract from NOSE.1 and BT20 cells, using a CSF-1 3′UTR riboprobe or its mutant counterpart. Membranes were either Ponceau red stained (*top*) or hybridized with the wild type or the mutant riboprobe (*bottom*). Relative migration of 37-, 50-, and 75-kDa molecular weights (*left*). *C,* Western blot analysis of 37-kDa HuR in Hey (S_{100}), NOSE.1 (total extract), and BT20 (total extract) protein extracts, using a monoclonal antihuman HuR antibody.

SDS-PAGE gel that was stained with a solution of colloidal blue (Sigma; Fig. 4*B*), we noticed in fractions 4 and 9 a very distinct 37-kDa band that was not visible in fraction 23 where the activity was reduced to minimal. Finally, a Northwestern analysis was done on active fractions 4 and 5 using the radiolabeled CSF-1 ARE riboprobe (Fig. 4*C*). The results confirmed in both fractions very strong binding to a 37-kDa protein and to another protein of 70 kDa, already identified in Fig. 3 but not specific to ovarian cancer cells. At that point, a Western blot analysis of the same samples was done using anti-HuR and anti-hnRNPD antibodies and confirmed that the 37-kDa band was neither HuR nor hnRNPD (data not shown). Although both proteins were detected in large quantities in S_{100} and fraction 9, they were considerably reduced in fraction 4.

We therefore decided to take advantage of the apparent purity of the protein shown in Fig. 4*B*, to cut the 37-kDa band from the gel and to determine its identity by MALDI-MS (Keck Laboratory, Yale University). To our surprise, the analysis resulted in the positive identification of the protein GAPDH.

GAPDH binds specifically to CSF-1 exon 10 ARE and is overexpressed in EOC cells but not in normal ovarian or breast cancer epithelial cells. To characterize the specificity of this binding activity, human GAPDH purified from erythrocytes was examined for its binding ability to the exon 10 ARE sequences in a gel shift experiment (Fig. 5*A*). Addition of GAPDH to the radiolabeled riboprobe resulted in a gel shift (*lane 3*) comparable with that observed upon addition of Hey S_{100} extract (*lane 2*). To confirm that result, we proceeded with a series of gel shift experiments involving Hey S_{100} extract previously treated with antibodies specific to GAPDH and other known RNA-binding proteins (Fig. 5*B*). Interestingly, we were able to show that depleting GAPDH from the S_{100} extract prevented the usual gel shift to occur (*lane 2*), whereas depleting poly(A)-binding protein, HuR or hnRNPD did not; the extracts retained their full ability to shift (*lanes 3*, *4*, and *5*, respectively). Finally, by Western blot analysis, we looked at the relative abundance of GAPDH in a few epithelial cell lines (100 μg of proteins were loaded on a 12% SDS-PAGE; Fig. 5*C*) and noted that the protein seemed more abundant in ovarian malignant cells, Hey (S_{100}, *lane 1*) than in a breast malignant line, BT20 (total extract, *lane 3*) or in normal epithelial ovarian cells, NOSE.1 (total extract, *lane 2*), despite the differences between S_{100} and total extract discussed in Materials and Methods. We then verified this finding by analyzing three other epithelial ovarian carcinoma cell lines (16 μg of proteins were loaded on a 10% SDS-PAGE; Fig. 5*D*) and found that GAPDH was overexpressed in Bixler, DK2NMA, and Bix3 cells (total extracts, *lanes 2-4*) compared with control NOSE.1 cells (total extract, *lane 1*). These last results suggested a potential correlation between the up-regulation of GAPDH and the increased binding activity to CSF-1 mRNA in ovarian cancer cells. Ultimately, this could explain the increased stability of CSF-1 mRNA leading to increased CSF-1 translation and metastasis in ovarian epithelial cancers.

Discussion

The goal of this study was to investigate CSF-1 mRNA regulation in relation with CSF-1 overexpression in highly invasive human EOC cells. In this paper, we report the identification of a 37-kDa protein purified from ovarian carcinoma Hey cells that binds to the AU-rich 3′UTR of CSF-1 mRNA as GAPDH. We show that this ARE

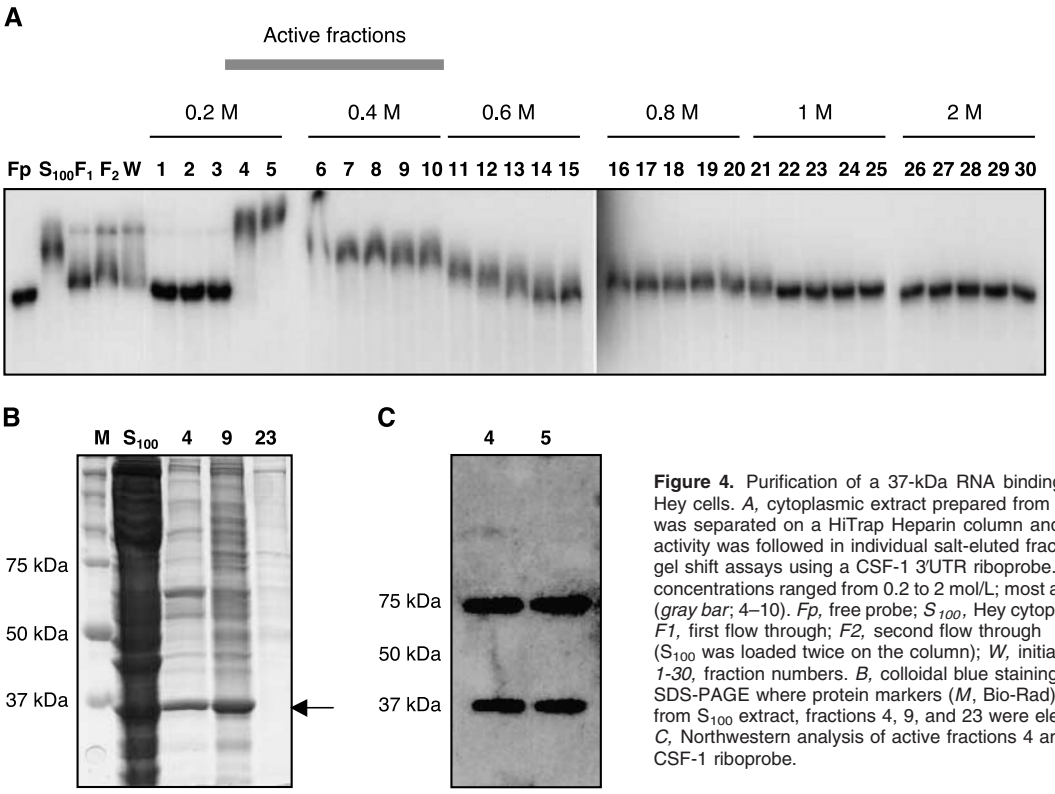

Figure 4. Purification of a 37-kDa RNA binding protein from Hey cells. *A,* cytoplasmic extract prepared from Hey cells was separated on a HiTrap Heparin column and RNA-binding activity was followed in individual salt-eluted fractions by gel shift assays using a CSF-1 3′UTR riboprobe. Salt concentrations ranged from 0.2 to 2 mol/L; most active fractions (*gray bar*; 4–10). *Fp,* free probe; *S 100,* Hey cytoplasmic extract; *F1,* first flow through; *F2,* second flow through (S_{100} was loaded twice on the column); *W,* initial wash; *1-30,* fraction numbers. *B,* colloidal blue staining of a 12% SDS-PAGE where protein markers (*M*, Bio-Rad), and samples from S_{100} extract, fractions 4, 9, and 23 were electrophoresed. *C,* Northwestern analysis of active fractions 4 and 5 using CSF-1 riboprobe.

region is a natural target for mRNA decay. In addition, we show a differential mRNA regulation of CSF-1 in Hey cells where the ARE-binding activity and the relative amount of GAPDH are more abundant than in normal ovarian epithelial cells (NOSE.1), which could explain the increased CSF1 protein found in Hey cells and other ovarian carcinoma cells.

CSF-1 is best known as a hematopoetic cytokine controlling the proliferation and invasive differentiation of the macrophage and the monocytic progenitors (42). The effects of CSF-1 are mediated by a high-affinity binding to a tyrosine kinase receptor that is encoded by the *c-fms* proto-oncogene product. In our laboratory, we focused on the role of the secreted 4-kb form of CSF-1 and its receptor in human epithelial malignancies where their abnormal expression and importance in tumor progression have been documented (4). In benign ovarian neoplasms, CSF-1 is occasionally expressed, whereas its expression increases considerably in invasive cancers. Here we developed a model in which to study this difference of expression and its regulatory mechanism. We found that as in tumors, CSF-1 is expressed at a low level by normal ovarian surface epithelial cells (NOSE.1) and at a higher level by Hey cells, derived from a xenograft of a metastasis from papillary cystadenocarcinoma of the ovary (Fig.1*A* and *B*, *a*).

We previously showed that CSF-1 expression strongly correlates with invasion of ovarian cancer cells (12) and our results confirmed that finding when comparing the two cell lines in a series of assays for *in vitro* invasion and nonrandom motility (Fig. 1*B*, *b-c*). Such augmented invasion also correlated with increased uPA activity in Hey cells (Fig. 1*B*, *d*), supporting the established evidence for invasion mediation by uPA in tumor progression (43). On binding of its homodimeric ligand, the CSF-1 receptor (c-fms) dimerizes and becomes phosphorylated on a number of tyrosine residues. After the initiation of a signaling cascade that induces *uPA* gene transcription, among others, the receptor is covalently cross-linked and both ligand and receptor become internalized and degraded. Interestingly, the maintenance of high level of uPA mRNA is also dependent upon the continuous presence of CSF-1 in primary macrophages (16, 44), which are also highly invasive cells. Thus, CSF-1, c-fms and uPA collaborate very intimately in this tumor function.

It is well established that the expression of many genes involved in growth regulation, including proto-oncogenes (such as *c-fos*, *c-myc*, and *c-jun*), growth factors, and their receptors (*GM-CSF* and *VEGF*), cytokines (*TNF*), and cell cycle regulatory genes (*cyclin A*, *B1*, *D1*, and *p21*), is mainly controlled by modulation of their mRNA stability (40). This regulation is largely exerted through the interaction of RNA-binding proteins with the ARE contained in their 3' UTR. The ARE in the 3′ untranslated region of unstable mRNAs mediate their rapid degradation; AUBPs have been described that either stabilize or otherwise degrade ARE mRNAs. In our case, we showed that CSF-1 mRNA contains instability-determining AREs (Fig. 2*A*) in its 3′UTR, using CAT assay experiments measuring CAT protein expression (Table 1) and CAT mRNA half-lives (Fig. 2*B*). Moreover, we clearly observed a differential down-regulation of CAT protein in ovarian carcinoma Hey versus normal NOSE.1 cells (Table 1), suggesting that CAT messenger is less susceptible to decay in Hey cells than in NOSE.1 cells, leading to increased protein translation. Similar conclusions of differential regulation were drawn after endogenous CSF-1 mRNA was measured in Hey versus NOSE.1 CAT

transfectants, with also clear evidence that endogenous CSF-1 mRNA level was down regulated in presence of excess exon 10 (Fig. 2*C*). These results strongly suggested the presence of AUBP(s) which stabilized the CSF-1 mRNA in Hey cells. Interestingly, we were able to prove without ambiguity the existence of such factors binding to CSF-1 ARE in gel shift assays (Fig. 3*A*). However, at this point no cellular proteins known to bind to these CSF-1 ARE sequences had been described in the literature. We showed that a 37-kDa protein not only was generally more efficient in ARE binding in extracts from Hey cells than in those from other cells used (Fig. 3*B*) but also presented a lower affinity for a mutated form of ARE where most A and U were replaced by C and G, respectively (Fig. 3*B*). We proceeded to isolate (Fig. 4) and identify the CSF-1 ARE binding protein, which to our surprise was GAPDH. Binding specificity was successfully assessed in Fig. 5 showing clear evidence for gel-shift CSF-1 ARE interaction with GAPDH (human purified; Fig. 5*A*) and lack of such interaction when an extract in which GAPDH was depleted was used (Fig. 5*B*). Moreover, by Western blot analysis (Fig. 5*C-D*) we showed for the first time that GAPDH is overexpressed in several carcinoma cell lines compared with their NOSE.1 "normal" cell counterpart. Finally, we formulated a model to explain the GAPDH RNA-binding effect on CSF-1 expression in ovarian epithelial cells (Fig. 6).

The finding of GAPDH overexpression in cancer cells or GAPDH as an AUBP would not have been surprising but showing GAPDH as an AUBP for CSF-1 mRNA whose protein overexpression has been linked to uPA-based metastasis in epithelial ovarian malignant cells is novel. Furthermore, GAPDH has not previously been suggested to impart mRNA stability. GAPDH had been known primarily for its function as a glycolytic enzyme, a key component in energy production. It has also served as a model for enzyme structure analysis. However, GAPDH has been recently shown to display a number of diverse activities unrelated to its glycolytic function. These activities include roles in membrane fusion, microtubule bundling, phosphotransferase activity, nRNA export, DNA replication, and DNA repair. Moreover, these functions depend on the subcellular localization and oligomeric structure of GAPDH (45). Early investigations reported the interaction of GAPDH with tRNA and rRNA but not in mRNA until Nagy and Rigby (46) identified GAPDH in human spleen as a cytoplasmic protein capable of binding ARE stretches of lymphokine mRNA 3′UTR, such as that of *GM-CSF*, *c-myc*, or *IFN-γ*. This leads to the hypothesis that GAPDH could very well be regulating multiple mRNAs in the cells, aside from that of CSF-1, contributing to an emphasis on the malignant phenotype in ovarian cancer.

Rapidly growing malignant cells have been known for a long time to have a high rate of aerobic glycolysis. Likewise, elevated GAPDH mRNA levels in malignant tissues (lung, pancreatic, prostate, and breast) have previously been reported (47), and have somehow been attributed to GAPDH protein function in glycolysis. Our results suggest that GAPDH might be playing multiple roles in neoplasms.

Recently, other AUBPs have been implicated in cancer. For instance, Blaxall et al. (48), referring to earlier work, have observed an increased cytoplasmic expression of both hnRNPD (AUF1) isoform and HuR in mouse lung neoplasia in correlation with

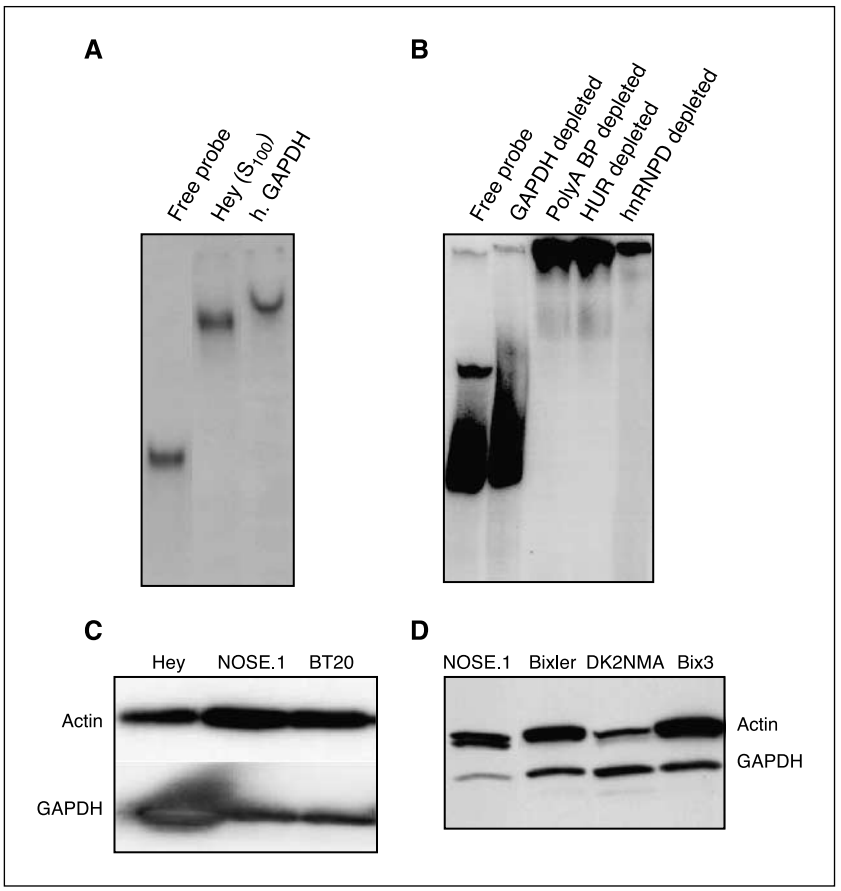

Figure 5. GAPDH associates with CSF-1 3′UTR and is overexpressed in ovarian carcinoma cells. *A,* gel shift CSF-1 RNA binding assay with a Hey S_{100} fraction, or purified human GAPDH. *B,* gel shift CSF-1 RNA-binding assay with a Hey S_{100} fraction, respectively, antibody depleted for GAPDH, poly(A)-binding protein, HuR, or hnRNPD. *C,* Western blot analyses of Hey (S_{100}), NOSE.1 (total extract), and BT20 (total extract) protein extracts for GAPDH and actin as a loading control. *D,* Western blot analyses of NOSE.1, Bixler, DK2NMA, and Bix3 total protein extracts for GAPDH and actin as a loading control.

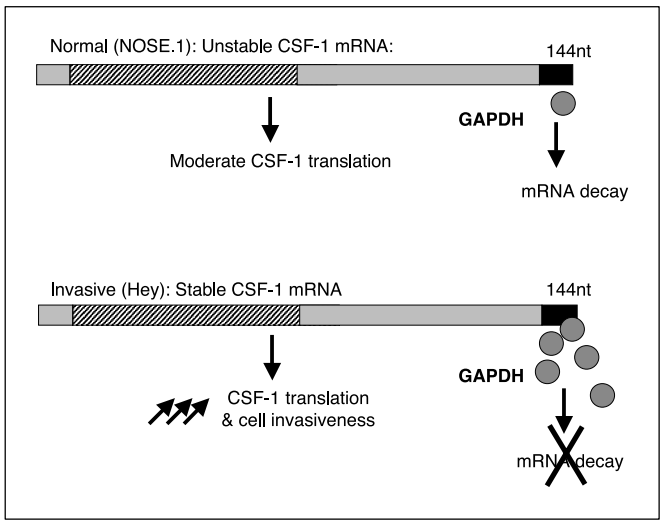

Figure 6. Model for the involvement of CSF-1 mRNA-binding protein, GAPDH, in malignant and in normal epithelial ovarian cells. The terminal 144 nt of CSF-1 3′UTR is target for mRNA decay (Fig. 2*B*), which correlates with protein down-regulation (Table 1). A differential effect is observed in NOSE.1 versus Hey cells (Table 1), where CSF-1 is particularly overexpressed in invasive carcinoma ovarian cells (Fig. 1*A*), suggesting the implication of trans-acting factors, regulating mRNA stability in malignant cells. GAPDH was identified as an RNA-binding protein, which specifically bound to these 3′UTR sequences and appeared more active and more abundant in malignant cells (Figs. 3 and 5), suggesting its role in mRNA stabilization leading to high CSF-1 protein translation and invasiveness (Fig. 1*B*).

growth rate. The authors suggest that the AUBP relative abundance may impact the maintenance/progression of the neoplastic phenotype by causing pleiotropic effects on the expression of regulated messengers. This was experimentally confirmed by Gouble et al. (49) in transgenic mice overexpressing one isoform of hnRNPD/AUF1 (p37). The authors showed that such AUBP overexpression modifies the accumulation level of several ARE-containing mRNAs *in vivo*, such as *c-myc*, *c-jun*, *c-fos*, *GM-CSF*, and *tumor necrosis factor*.

We identified GAPDH as AUBP being overexpressed in ovarian cancer cells in correlation with CSF-1 mRNA stabilization, CSF-1 protein overexpression and increased virulence of tumorigenicity. Whereas it is known that the glycolytic enzyme is a tetramer of identical subunits of M_r 36,000, little is known about the structure of the protein in the various activities recently uncovered (45). Likewise, whereas the *GAPDH* gene has been extensively studied in the 1980s (50) and shown extraordinarily complex, there has been no direct correlation between the various genes, pseudogenes, their transcripts, and the cellular functions of the resulting gene products.

In conclusion, beyond the identification of GAPDH as an AUBP possibly involved in the CSF-1 mRNA regulation in ovarian epithelial cells, this study opens new avenues for better understanding of GAPDH as a multifunctional protein with novel roles in cancer biology and also understanding the role of AUBPs generally in the regulation of genes affecting cell growth, differentiation, and tumor invasiveness.

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