The RNA Exosome Component hRrp6 Is a Target for 5-Fluorouracil in Human Cells

Susanne Kammler, Søren Lykke-Andersen, and Torben Heick Jensen

Centre for mRNP Biogenesis and Metabolism, Department of Molecular Biology, University of Aarhus, Aarhus C., Denmark

Abstract
The drug 5-fluorouracil (5-FU) is a widely used chemotherapeutic in the treatment of solid tumors. Recently, the essential 3′-5′ exonucleolytic multisubunit RNA exosome was implicated as a target for 5-FU in yeast. Here, we show that this is also the case in human cells. HeLa cells depleted of the inessential exosome component hRrp6, also called PM/Sc100, are significantly growth impaired relative to control cells after 5-FU administration. The selective stabilization of bona fide hRrp6 RNA substrates on 5-FU treatment suggests that this exosome component is specifically targeted. Consistently, levels of hRrp6 substrates are increased in two 5-FU–sensitive cell lines. Interestingly, whereas down-regulation of all tested core exosome components results in decreased hRrp6 levels, depletion of hRrp6 leaves levels of other exosome components unchanged. Taken together, our data position hRrp6 as a promising target for antiproliferative intervention. (Mol Cancer Res 2008;6(6):990–5)

Introduction
The highly conserved RNA exosome is a multisubunit 3′-5′ exonucleolytic complex that participates in the processing and degradation of a variety of nuclear and cytoplasmic RNAs. Both yeast and human exosome complexes are composed of a core of nine subunits as well as several associated factors. Current data suggest that the exosome core serves as a binding platform for the two active exonucleases hRrp6 (also called PM/Sc100) and hRrp44 (Dis3; refs. 1-3). Whereas Rrp44 is a ubiquitous exosome constituent in both yeast and human cells, yeast Rrp6 (yRrp6) is believed to be exclusively nuclear (4). However, hRrp6 has also been detected in the cytoplasm (5, 6).

The nucleoside analogue 5-fluorouracil (5-FU) is one of the most widely used chemotherapeutics in the treatment of solid colorectal, breast, aerodigestive tract, and head/neck cancers (7). Recent research suggests that the 5-FU effect might be exerted through its incorporation into cellular RNA (7, 8). Consistent with these findings, genome-wide yeast screens for gene products exhibiting 5-FU sensitivity have revealed factors involved in RNA metabolism, including components of the RNA exosome (9, 10). In support, 5-FU disrupts the metabolism of some yeast rRNA precursors (8). Interestingly, altered rRNA metabolism on 5-FU administration has also been reported in human cells, suggesting that the human exosome might also be a drug target (11-13).

Here, we report evidence for this idea. We show a synergistic down-regulatory effect on growth when cells are treated with 5-FU in combination with small interfering RNAs (siRNA), which decrease endogenous hRrp6 levels. Interestingly, human endogenous retroviral (HERV) transcripts that are specifically stabilized on hRrp6 depletion are also stabilized in the presence of 5-FU and in cell lines that are sensitive to low concentrations of the drug. This suggests that 5-FU is specifically targeting hRrp6 of the exosome. Our results highlight the possibility of using 5-FU/hRrp6 depletion combination therapies in cancer treatment.

Results
Specific Depletion of hRrp6p Renders Cells Hypersensitive to 5-FU
Due to the strong evolutionary conservation between yeast and human RNA exosomes, functional similarities are anticipated. To investigate whether this extends to 5-FU sensitivity, we established an efficient siRNA-mediated depletion protocol for various exosomal components in HEK293 and in HeLa cells using double administration of siRNAs. This protocol was found to yield a higher degree of factor depletion than a conventional single-step transfection method (data not shown). In general, we found for both of the cell lines that depletion of one core component was paralleled by the destabilization of other components of the exosome core; for example, cells treated with siRNAs targeting hRrp41 also harbor decreased levels of hRrp40 and hRrp46 (Fig. 1, lane 6; data not shown). Depletion of all core components also resulted in lower levels of hRrp6, whereas, importantly, hRrp6 knockdown in both cell lines specifically depleted only this factor, leaving levels of other exosome factors unchanged [Fig. 1A (lane 3) and B (lane 2)].

We next evaluated the 5-FU sensitivity of cells containing low levels of hRrp6. HeLa cells were either mock transfected or transfected with siRNAs targeting hRrp6, hRrp40, or control siRNAs against the nuclear 5′-3′ exonuclease hXrn2.
 Twenty-four hours after the second siRNA administration, cultures were treated with 0.05 mmol/L 5-FU and cells were counted after 0, 1, and 2 days. Interestingly, cells lacking either hRrp6 or hRrp40 were growth impaired on 5-FU addition compared with the hXrn2-depleted and the mock-transfected control (Fig. 2). Given the specificity of the hRrp6 knockdown, we conclude that low levels of hRrp6 render cells hypersensitive to 5-FU even in the presence of an intact exosome core.

To test whether 5-FU directly affects the stability of the hRrp6 protein, we assayed hRrp6 levels in cells treated with increasing amounts of 5-FU. At 0.1 mmol/L of 5-FU, a slight decrease in hRrp6 levels could be measured (Fig. 1C). This phenotype might explain, at least part of, the hRrp6-specific activity of 5-FU.

**Bona Fide hRrp6 Substrates Are Stabilized in the Presence of 5-FU**

Although hRrp6 functions in several RNA surveillance and maturation processes in conjunction with the exosome core, evidence suggests that hRrp6 might also perform independent activities (14). To address this idea, and to further characterize the link between hRrp6 and 5-FU, we used ENCODE tiling microarrays to identify cellular hRrp6 targets. Interestingly, these analyses identified a HERV cluster 15I on chromosome 16 (ID 41559 in the retrosearch database) among certain HERV RNA levels after 5-FU treatment of HeLa cells. Remarkably, only levels of the HERV15I, envW, envR, envK, and envFRD HERVs, which all contain open reading frames that are expressed in normal tissues to be transcriptionally silent. However, some HERV families still putative hRrp6 substrates were increased on 5-FU addition, whereas the two hRrp40-specific control substrates, Rrp40^#2 (identified on an ENCODE tiling array; data not shown) and envK, were not (Fig. 3C). This observation strongly suggests that 5-FU can target hRrp6 function directly without inhibiting core exosome activity.

Finally, to address the biological relevance of our findings, we monitored relative levels of Rrp40^#2, HERV15I, envW, and envR RNAs in HeLa cells depleted for other enzymes involved in various RNA degradation processes, including cytoplasmic (hXrn1) and nuclear (hXrn2) 5' 3'- exonuclease, decapping (hDcp2), and nonsense-mediated decay (hUpf1). These analyses revealed a marked redundancy in the turnover of most HERV transcripts; for example, envW and envR RNAs are stabilized not only by the depletion of hRrp6 and hRrp40 but also by depletion of the nuclear 5'-3' exonuclease hXrn2 (Fig. 3B, top). Notably, as a special case, envFRD RNAs were only stabilized on hRrp6 and hRrp40 depletion and thus specifically undergo exosomal degradation.

Importantly, for results presented in this study, the HERV expression analyses were also able to detect a difference in the hRrp6 versus hRrp40 degradation profiles; that is, whereas the sensitivities of envW, envR, and envFRD HERVs to both hRrp6 and hRrp40 depletion suggest that these transcripts might be either bona fide hRrp6 substrates or hRrp6-dependent exosome substrates, the envK profile shows that this RNA can be degraded in an exosome-dependent, yet hRrp6-independent, manner (Fig. 3B, bottom left).

The above relationships prompted us to assay the effect of HERV RNA levels after 5-FU treatment of HeLa cells. Remarkably, only levels of the HERV15I, envW, and envR putative hRrp6 substrates were increased on 5-FU addition, whereas the two hRrp40-specific control substrates, Rrp40^#2 (identified on an ENCODE tiling array; data not shown) and envK, were not (Fig. 3C). This observation strongly suggests that 5-FU can target hRrp6 function directly without inhibiting core exosome activity.

Finally, to address the biological relevance of our findings, we monitored relative levels of Rrp40^#2, HERV15I, envW,
envR, and envK RNAs in human cell lines exhibiting differential sensitivities to 5-FU (Fig. 4A). For reasons that are presently unclear, levels of envR were generally high in all three cell lines. However, in the HEK293 and RKO cell lines, which are sensitive to low concentrations of 5-FU, the ratios between hRrp6 target (HERV15I and envW) and hRrp40 target (Rrp40#2 and envK) levels were higher than in the more 5-FU-resistant T47D cell line (Fig. 4B, see ratios below the graph.). Thus, there is the tendency that 5-FU sensitivity correlates with lowered hRrp6 function.

**Discussion**

5-FU was developed to inhibit DNA synthesis by disrupting the activity of thymidylate synthetase (7). Yet, data from the yeast system show a strong effect of the drug on RNA metabolism, and the data reported here suggest that hRrp6 function is directly targeted. This suggestion is consistent with reports showing that cotreatment of cells with uridine, but not thymidine, relieves the cytotoxic effect of 5-FU (17, 18). Thus, it seems likely that 5-FU exerts its effect via incorporation into RNA. It is possible that this in turn leads to hRrp6 sequestration and its increased turnover.

The notion that 5-FU targets cellular RNA metabolism and potentially disturbs the relative stoichiometric balance between components of RNA degradation complexes helps to rationalize previous observations linking this process to cancer. First, the exosome core component hRrp43 (PM/Sc175) as well as hRrp6 are known autoantigens in scleroderma diseases [polymyositis/scleroderma (PM/ScI) overlap syndrome] linked to increased cancer incidence (ref. 19 and references therein). Second, hRrp46, which was identified as an autoantigen in chronic myelogenous leukemia (CML28), is highly overexpressed in a variety of hematopoietic and epithelial tumor cell lines (20, 21). Third, hRrp44, which is linked to mitotic control in yeast, is also overexpressed in human progressive colorectal cancer cells and in murine colon adenocarcinoma where its expression levels correlate with the metastatic potential of the tumor (22).

Although 5-FU is one of the most effective chemotherapeutics in the treatment of solid tumors, the response rates for first-line treatment solely based on 5-FU are limited (23). This is due to unspecific cell toxicity and the development of tumor resistance to the drug. Our data show a significant (>95%) and specific hRrp6 depletion without significant cytotoxic side effects even after exposure times of up to 9 days (data not shown). Therefore, it seems a viable strategy to explore the potential of combining conventional 5-FU therapy with additional hRrp6 targeting agents such as specific siRNAs.

**Materials and Methods**

**Cell Culture, 5-FU Treatment, and RNA Interference**

HeLa cells were cultured in DMEM (Life Technologies) supplemented with 10% fetal bovine serum. HEK293, RKO, and T47D cells were cultivated in RPMI 1640 containing 10% fetal bovine serum, MEM Earle’s salts containing 10% fetal bovine serum, and DMEM containing 2 mmol/L glutamine and 10% fetal bovine serum (Life Technologies), respectively.

To obtain efficient siRNA-mediated knockdown, cells were transfected twice (on days 1 and 4) with 20 nmol/L chemically synthesized duplex siRNAs (Dharmacon or DNA Technology) complementary to hRrp6 (5**G**-GCAAAAUCU-GAAACUUUCCCT/5**G**-GGAAAGUUUCAGAUUUUGCTT or 5**G**-CCAGUUAUACAGACCUAUATT/5**G**-UAUAGGUCU-GUAUACUGGT), hRrp40 (5**G**-CAGCCACAGUACUGATT/5**G**-UGACCUAGUACUGUGCGUGTT), hRrp41 (5**G**-CUAGUAAUCACCCUAATT/5**G**-UAUAGGUCU-GUAUACUGGT), hRrp46 (5**G**-CAACAAGGCCACACUC-GAATT/5**G**-UUCGAGUGUGGCCUUUGUGTT), hDcp2

**FIGURE 2.** Effect of 5-FU treatment on cell viability after exosomal knockdown. HeLa cells were transfected with siRNAs targeting the exosomal components hRrp6 (pink and red circles) or hRrp40 (purple or brown rectangles) or the 5’-3’ exonuclease hXrn2 (light and dark green triangles). Mock-transfected cells (mock, gray and black diamonds) were included as controls. At day 1 after the last transfection, cultures were treated either with 0.05 mmol/L 5-FU (black, red, brown, and dark green symbols) or with H2O (gray, pink, purple, and light green symbols). At days 2 and 3 after 5-FU/H2O addition, cells were counted and plotted relative to the cell count at day 1. Points, mean (n = 3); bars, SD. By applying a paired two-tailed Student’s t test, we found that on day 3 the relative cell counts were significantly (P < 0.1) lower in the 5-FU–treated exosomal knockdown samples (brown rectangle, hRrp40/5-FU; red circle, hRrp6/5-FU) compared with all other samples.
FIGURE 3. Endogenous hRrp6 substrates are up-regulated in the presence of 5-FU. A. Left, top track, screenshot from SignalMap v1.8, showing HERV15I RNA levels in hRrp6-depleted cells as a ratio to the levels in eGFP siRNA-treated control cells. Levels of HERV15I RNA from the individual experiments are depicted in the middle (eGFP) and bottom (hRrp6) tracks, respectively. Red bar, entire length of the HERV15I cluster (HERVID4159; http://www.retrosearch.dk), the major part of which is not present on the ENCODE array due to exclusion of repetitive sequences. Right, relative levels of the up-regulated RNA from the HERV15I cluster after depletion of hRrp6 or hRrp40. HeLa cells were transfected with siRNAs and RNA levels were analyzed by quantitative RT-PCR and plotted as described in A. Columns, mean (n = 3); bars, SD. The difference between a given value and its corresponding control value (here eGFP siRNA) is denoted by asterisks according to its significance.*, P < 0.15; **, P < 0.10; ***, P < 0.05, paired two-tailed Student’s t test. B. Relative levels of HERV envW, envR, envK, and envFRD RNAs after depletion of various mRNA degradation factors. HeLa cells were transfected with siRNAs targeting the exosomal components hRrp6 or hRrp40, the decapping enzyme hDcp2, the 5’-3’ exoribonucleases hXrn1 or hXrn2, or the nonsense-mediated decay factor hUpf1. RNA levels were analyzed by quantitative RT-PCR and plotted as described in A. C. Treatment with 5-FU induces stabilization of hRrp6 substrates. Relative levels of HERV15I, envW, envR, and envK RNAs in 5-FU–treated HeLa cells (black columns) compared with their nontreated counterparts (gray columns). Day 3: 0.1 mM 5FU. Columns, mean (n = 3); bars, SD. Significance levels are plotted as described in A.
(5'-GGACUGGCUUUCUGACAGATT/5'-UCUUCGA-GAAAGCCAGUCCCTT), hXrn1 (5'-AGAUGAACUUACAGUAGAATT/5'-UUCUACGGUAAGUUCAUCUTT), hXrn2 (5'-GAGUACAGAUGAUCAUGUUTT/5'-AACAUGAU-CAUCUGUACUCTT), hUpf1 (5'-GAUGCAGUUCCGCUC-CAUUTT/5'-AAUGGAGCGGAACUGCAUCTT), TEL/AML (5'-GGAGAAUAGCAGAAUGCAUTT/5'-AUGCAUUCUCAUUCUCCCT), and enhanced green fluorescent protein (eGFP; 5'-GACGUAAACGGCCACAAGUTT/5'-ACUU-GUGGCCGUUUACGUCTT). Transfections were Lipofectamine 2000 (Invitrogen) assisted according to the protocol of the manufacturer. Cells were not passaged between the different manipulations.

For 5-FU experiments with siRNA-transfected cells, the drug was added to a concentration of 0.05 mmol/L to cells 1 d after the second siRNA transfection (day 5). The experiment was carried out in multiple replicates for each transfection and trypsinized cells were counted in duplicates in a Bühler-Türck counting chamber on days 5, 6, and 7. In the case of untransfected cells, 5-FU was added to a concentration ranging from 0.001 to 0.1 mmol/L 1 d after passaging of cells.

Real-time RT-PCR

Total RNA for RT-PCR and tiling array experiments was purified from cells on day 6 using Trizol (Invitrogen) according to the manufacturer’s protocol. Total RNA (2.5 μg) was reverse transcribed using random hexamer primers and SuperScript II (Invitrogen) according to the guidelines of the manufacturer. Negative controls without SuperScript II and without templates were included for each experiment. PCRs were carried out in an MX3000P Real-time PCR Cycler (Stratagene) using Platinum SYBR Green qPCR SuperMix (Invitrogen) in a total volume of 25 μL per sample. Primer pairs detecting envW, envK, and envFRD RNAs have previously been published (16): envW, 5'-CCCCATCGTATAGGAGTCTT/5'-CCCCATCAGA-CATCCAGTT; envK, 5'-CCAACAATAAGAAGCTGACG/5'-CATAGGCCCAGTTGGTATAG; envR, 5'-CCATGG-GAAGCAAGGGAAC/5'-CTTCCCCAGCGAGCAATAC; and envFRD, 5'-GCTGCAATAATGCCTCTTT/5'-ATAAGGGCTATCCCATAG. Primer pairs used to detect GAPDH, Rrp40#2, and HERV15I RNAs were as follows: GAPDH, 5'-GTCAGCGCATCCTTTT/5'-GCGCCCAA-TACGACCAATC; Rrp40#2 [sequence on chromosome 2:
220.287.068-220.287.419 (human genome build 35)], 5’-GCGCTTCTATGAAAGAAACT/5’-TCTGAAACCAG-GAAATTCGTT, and HERV15I [sequence on chromosome 16: 1-500000 (human genome build 34)], 5’-TCAGGGACAGGACAGGCGGTC.

ENCODEx Tiling Array
Double-stranded cDNA was synthesized from 50 μg total RNA purified from HeLa cells transfected with either control eGFP siRNAs or hRrp6 siRNAs. Four microarrays of double-stranded cDNA were Cy3/Cy5 labeled and hybridized to Nimblegen ENCODE arrays by the Nimblegen array service. Data were normalized and visualized by the computer program SignalMap v1.8. Repetitive as well as low-complexity DNA sequences are in general masked on the ENCODE arrays in accordance with RepeatMasker.2 Thus, only small sequence fragments of some annotated HERV clusters (positions from the retrosearch database2; ref. 24) were accessible for analysis.

Western Blotting Analysis
Preparation of cell extracts and Western blotting were carried out using standard procedures. The following antibodies were used for immunoblotting analysis: rabbit anti-hRrp6, anti-hRrp40, anti-hRrp41, and anti-hRrp46 (25) and mouse anti-hnRNP C1/C2 (4F4; ref. 26).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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