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Biogenesis of Y RNA-derived small RNAs is independent of the microRNA pathway

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ABSTRACT

Y RNAs are approximately 100 nucleotide long conserved cytoplasmic non-coding RNAs, which produce smaller RNA fragments during apoptosis. Here we show that these smaller RNA molecules are also produced in non-stressed cells and in a range of human cancerous and non-cancerous cell types. Recent reports have speculated that the cleavage products of Y RNAs enter the microRNA pathway. We tested this hypothesis and found that Y5 and Y3 RNA fragments are Dicer independent, they are in different complexes than microRNAs and that they are not co-immunoprecipitated with Ago2. Therefore we conclude that Y RNA fragments do not enter the microRNA pathway.

1. Introduction

Y RNAs are a family of non-coding RNAs discovered following two investigations into the autoimmune disorder systemic lupus erythematosus (SLE) [1,2], which is characterised by the inflammation of connective tissue. There are four principal Y RNA genes denoted Y1, Y3, Y4 and Y5 (Y2 is now considered to be a truncated form of Y1) [3]. Y RNAs show high evolutionary conservation within the animal kingdom, and there is evidence that orthologs also exist in prokaryotes [4,5], suggesting that these genes may have an important function.

All Y RNAs are expressed in human cells [6], although many other animal species do not contain the full Y RNA complement. For example, many rodents only possess Y1 and Y3, but both the mouse and rat genomes have a redundant 'fossil' Y5 RNA gene that is no longer expressed [7]. Y RNA genes are transcribed by RNA polymerase III into short, 84–113 nt RNAs which fold back on themselves to form distinct hairpin-containing structures [3]. Although the biological function of Y RNAs is not well understood, a defining trait of Y RNAs is their ability to bind the 60 kDa protein Ro60 [2,8,9] and the 47 kDa La protein [8] to form the Ro-RNP (Ro60 containing ribonucleoprotein complex). These interactions were shown to influence the sub-cellular localisation of Ro [10] and its association with other proteins [11,12]. Y RNAs also bind to Ro in bacteria, which inhibits other proteins to access Ro [4]. It was found by several groups that the majority of Y RNAs in both mouse and human cells are bound by Ro [3,13], but a small proportion of Y RNAs is not associated to Ro and this fraction is involved in the initiation of chromosomal DNA replication [14,15].

The RNA component of Ro-RNPs appears to be altered during apoptosis. Human Y RNAs are rapidly and specifically cleaved during apoptosis in a caspase-dependant manner and this occurs under the duress of a range of apoptotic stimuli [16]. The degradation products of the Y RNAs were found to remain bound to Ro60, and to a lesser extent, the La protein, during the degradation process [16]. The Y RNA degradation products were 22–36 nts in size and were grouped into two classes: a smaller fragment with about 24 nt (22–25 nt) and a larger fragment around 31 nt (27–36 nt) [16]. The smaller fragments were immunoprecipitated with Ro60 but not with La, while the larger fragments were detected in both anti-Ro60 and anti-La immunoprecipitation [16].

Another class of small non-coding RNAs is the microRNAs (miRNAs) [17]. They are generated by Dicer from precursor-miRNA molecules that are folded into a hairpin structure, similar to Y RNAs. MiRNAs are around 22 nt and a recent study has shown that 25 nt small RNAs derived from hY3 and hY5 RNAs are present in solid tumour samples at levels comparable with the cancer-associated miRNA, miRNA-21 [18]. These 25 nt hY3 and hY5 derived RNAs had been annotated in miRBase as hsa-miR-1979 and hsa-miR-1975, respectively, but have since been removed as they originate from Y RNAs. However, it raised the possibility that Y RNAs are processed by Dicer and that the smaller Y RNA fragments are actually

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miRNAs. Meiri et al. attempted to find a miRNA-like function for the smaller Y RNA products via luciferase assay, but no silencing activity was observed [18], although miRNA function could not be ruled out based on that, as discussed in a recent review [19].

In this work, we show that the generation of smaller fragments from Y5 RNA are not dependant on Dicer, and that these Y5-derived small RNAs (Y5 sRNAs) are not found in the Ago2 RISC complex. In addition, RNP complexes containing miRNAs and Y5 sRNAs were in separate fractions following biochemical purification, we therefore conclude that the Y RNA fragments are not miRNAs.

2. Materials and methods

2.1. Cell lines and cell culture

MCF-10A cells were purchased from the American Type Culture Collection (ATCC) and were cultured in MEGM Bulletkit medium (Lonza, cc-3150) supplemented with 100 ng/ml cholera toxin (Sigma, C8052). DLD-1 wild type and DLD-1 dicer−/− were purchased from Horizon Discovery. Both the DLD-1 cell lines, and HCT 116 were cultured in DMEM/F-12 + Glutamax (Gibco, 31331), supplemented with 10% FBS and 1% penicillin–streptomycin.

2.2. Poly(I:C) treatment

Polyinosinic:polycytidylic acid potassium salt (PolyI:C) (Sigma, P9582) was used by transfecting into cells at 10 μg/ml in purified water. Poly(I:C) transfection media was removed from cells after 5 h of incubation and replaced with fresh media. Cells were harvested for RNA/protein extraction 3 h after this media change.

2.3. Northern blotting

Total cellular RNA was extracted from cells using Trizol reagent (Invitrogen, 15596) according to the manufacturer's instructions. For Northern analysis was carried out as previously described [20]. Antisense DNA oligonucleotide probe sequences used were: Y5 3′ end (5′-AGCTAGTCAAGCGCGGTTGTGGGGG-3′), Y3 (5′-TAGTCAAGTGAAGCAGTGGGAG-3′), miR-21 (5′-TCAACATCAGTCTGATAAGCTA-3′), U6 snRNA (5′-GCTAATCTTCTCTGTATCGTTCC-3′).

2.4. Anion chromatography

Native extracts of MCF7 cells were prepared at 24 h after poly(I:C) induction in extraction buffer (20 mM Tris/HCl pH 7.5, 300 mM NaCl, 5 mM MgCl2, 5 mM DTT freshly added). Insoluble material was removed by centrifugation for 10 min at 16000 g at 4 °C, and the supernatant was filtered through a 0.22 lm syringe filter. The extracts were separated using a DEAE-anion exchange column (Pharmacia, http://www4.gelifesciences.com). Fractions from the anion exchange column were eluted in different salt concentration, which was increased as depicted in Fig. 4. RNA was extracted from every second fraction and analysed on a gel blot.

2.5. Ago2 pull-down assay

Cells were lysed using Lysis Buffer (Promega, E3971) plus 2.5 mg/ml of heparin (Sigma), spun down and the supernatant was incubated with 10 μl of Ago2 primary antibody (Abcam, ab57113) for 4 h at 4 °C. Protein A beads were blocked with heparin and then incubated with the lysate 1 h at 4 °C. After four washes the beads were eluted three times with 100 μl of glycin/HCl pH 2.3 100 mM. These three elutions were pooled and neutralized with 75 μl of Tris/HCl pH 9.0 (1 M).

3. Results

3.1. Y5 RNA cleavage occurs in a range of cancerous and non-cancerous cell types under stress induced and non-induced conditions

In order to investigate whether Y5 RNA cleavage is unique to specific cell types, we selected a range of cell lines and treated them with the double-stranded RNA mimic immunostimulant chemical, poly(I:C). Fig. 1A shows that the longer and shorter Y5 sRNAs are produced upon poly(I:C) treatment in both cancerous cell lines MCF 7 (mammary adenocarcinoma cells), HeLa (cervical cancer cells), DLD-1 (epithelial adenocarcinoma cells) and HCT 116 (colorectal carcinoma cells) and the non-cancerous HEK 293 (human embryonic kidney cells) and MCF 10A (mammary epithelial cells) cell lines, although the smaller fragments were produced at a much higher level in MCF7 than in any other cells. The levels of the longer Y5 sRNA is significantly greater in all cells types than the shorter product as found previously [16].

Owing to the short exposure time that is sufficient to detect Y5 RNA and its cleavage products on a phosphorimager screen after poly(I:C) treatment, we asked whether Y5 cleavage products could be detectable at significant levels in non-stressed cells. Using a synthetic RNA oligonucleotide corresponding to the longer Y5 sRNA, we made a dilution series and quantified the level of Y5 sRNA in MCF 7 cells by Northern analysis in the absence of poly(I:C) (Fig. 1B). We calculated that the level of the longer Y5 sRNA in the absence of poly(I:C) was approximately 0.27 femtomoles per μg of total RNA. To determine whether this level is similar to other short non-coding RNAs, we carried out similar experiments for miRNAs and found that the level of miRNA-25 (0.34 femtomoles per μg of total RNA) and miRNA-93 (1.4 femtomoles per μg of total RNA) was similar to the longer Y5 sRNA.

3.2. Processing of Y5 sRNAs is Dicer independent

Having demonstrated that Y5 sRNAs are present in human cells under normal conditions, we wanted to investigate the biogenesis of these molecules. As recent work on Y RNAs has hypothesised that these small RNAs may be microRNAs [18,19], we analysed the accumulation of Y5 sRNAs in the presence and absence of Dicer. Both wild-type DLD-1 and DLD-1 Dicer−/− cells were treated with poly(I:C) and analysed by Northern blot. Fig. 2 shows that the Y5 sRNAs were present in Dicer−/− cells following poly(I:C) treatment, and in fact were produced at a higher level to that found in DLD-1 parental cells, which was reproducible in several experiments. The full length Y5 RNA is at a lower level in the Dicer−/− cells and there were a higher rate of cell death (data not shown), which is also suggested by the lower level of U6 RNA. One possible explanation for these observations is that Dicer may process some of the transfected poly(I:C), since it is a dsRNA mimic and Dicer processes dsRNA. Therefore in the absence of Dicer the concentration of intact poly(I:C) could be higher leading to a stronger effect.

3.3. Y5 sRNAs are not in complex with Argonaute 2

After establishing that Y5 sRNAs were not Dicer dependent and were therefore not involved in the canonical miRNA pathway, we considered whether they might be loaded directly into the RNA-induced silencing complex (RISC) in a Dicer-independent fashion. Therefore, we immunoprecipitated Argonaute 2 (Ago2), a key component of the miRISC complex (Fig. 3). The complete lysate from MCF 7 cells shows that the longer and shorter Y5 sRNAs were present following poly(I:C) treatment, but not in untreated cells and that a miRNA, miR-21, was present under both conditions. Ago2