Cytosolic RNase Inhibitor Only Affects RNases with Intrinsic Cytotoxicity

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Daria Maria Monti and Giuseppe D’Alessio
From the Department of Biological Chemistry,
University of Naples Federico II, Via Mezzocannone, 16, 80134 Napoli, Italy

Cytosolic RNase inhibitor binds to and neutralizes most members of the pancreatic type RNase superfamily. However, there are a few exceptions, e.g. amphibian onconase and bovine seminal RNase, and these are endowed with cytotoxic activity. Also, RNase variants created by mutagenesis to partially evade the RNase inhibitor acquire cytotoxic activity. These findings have led to the proposal that the cytosolic inhibitor acts as a sentry to protect mammalian cells from foreign RNases. We silenced the expression of the gene encoding the cytosolic inhibitor in HeLa cells and found that the cells become more sensitive to foreign cytotoxic RNases. However, foreign, non-cytotoxic RNases remain non-cytotoxic. These results indicate that the cytosolic inhibitor neutralizes those foreign RNases that are intrinsically cytotoxic and have access to the cytosol. However, its normal physiological role may not be to guard against foreign RNases in general.

The cytosolic ribonuclease inhibitor (here termed cRI) is a 50-kDa protein present in all mammals investigated thus far but absent in other classes, such as amphibia (1, 2). It is a leucine-rich repeat protein, with a high content of cysteine residues and a three-dimensional structure that resembles a horseshoe (3). It avidly binds (with few exceptions, see below) the extracellular RNases of the pancreatic type superfamily (4) and inhibits their enzymatic activity.

The physiological role of cRI has not been ascertained conclusively. It has been proposed that cRI could regulate cellular RNA levels or modulate RNases with a specific biological action, e.g. angiogenins, or protect cellular RNAs from extracellular RNases (2).

This last hypothesis, that cRI protects against foreign RNases, has recently received increasing consensus based on the following observations. Cytotoxic RNases of the pancreatic type superfamily, namely onconase from amphibia and bovine seminal RNase (BS-RNase), are resistant to the inhibitory action of cRI (5). Furthermore, non-cytotoxic RNases with high affinity for cRI, such as bovine (RNase A) or human (HP-RNase) pancreatic RNases, or monomerized BS-RNase, become cytotoxic when engineered through directed mutagenesis into RNases with a decreased affinity for cRI (6–8). Cytotoxic, dimeric BS-RNase, which is completely resistant to cRI inhibition, loses its cytotoxicity when it is dissociated into stable, active monomers, which are cRI-sensitive (9, 10). Recently, it has been reported that cells manipulated to increase their cRI levels become more resistant to RNase cytotoxicity (11).

However, the results of other investigations cast doubt on the conclusion that the cytotoxic action of an RNase depends, mainly or exclusively, on its ability to evade the neutralizing action of cRI and that the physiological role of cRI is to protect cells from extra-cellular RNases. Cases have been reported of RNases of the pancreatic type superfamily that have been engineered into cytotoxic RNase variants, yet are inhibited by cRI (12, 13). It has been reported that when RNase A is dimerized through phosphorylation from acetic acid solutions, it acquires cytotoxic properties (14), but it maintains its high affinity for cRI (15).

Thus, it seemed of interest to investigate the role of cRI in RNase cytotoxicity by suppressing cellular cRI through the silencing of the mRNA transcribed from the cRI gene. This technology, based on the phenomenon of RNA interference (16), involves the use, or the intracellular production by DNA engineering (17), of a double-stranded RNA complementary to the mRNA encoding the protein programmed to be suppressed. The results of this investigation indicate that the cytosolic inhibitor may not be a guardian against foreign RNases in general, as it only affects those foreign RNases that are intrinsically cytotoxic but not those that are non-cytotoxic.

EXPERIMENTAL PROCEDURES

Materials—BS-RNase (18), HP-RNase (19), and onconase (20) were prepared and characterized as described. RNase A (type XII) was purchased from Sigma. MCA-BS-RNase is an active and stable monomer of BS-RNase prepared by selective reduction of the protein intersubunit disulfides followed by alkylation of the exposed sulfhydryls with iodoacetamide (21). RE-HP-RNase (N88R/G89E-HP-RNase) was prepared in our laboratory. AR-RNase A (K7A/G88R-RNase A) was prepared and characterized as described (22).

Salmon alkaline phosphatase was obtained from Promega (Madison, WI). Restriction enzymes, T4 DNA ligase, and the Klenow fragment DNA polymerase were purchased from New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by Primm (Milano, Italy). DNA sequences were obtained from MWG Biotec (Ebersberg, Germany). Plasmid pBSU6 was from Ambion (Austin, TX). Plasmid pEGFP was from Invitrogen.

The Plasmid Encoding cRI-silencing RNA—The sequence of nucleotides 430—449 of the cRI gene (GenBank accession number M22414) was used to produce a siRNA directed to cRI mRNA. The chosen sequence was analyzed by a BLAST search and found to be unique to the cRI gene. The sequence was selected for its high GC content (68%) and its position at more than 100 bases downstream to the AUG (16, 17). Upon cloning, the G doublet at the 5’ end of the sequence contributed to the GGG triplet required for transcription initiation. The pBSU6 plasmid, bearing an Apal site downstream to the polymerase III U6 promoter, was digested with Apal and treated with the

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Klenow fragment of DNA polymerase to remove the protruding nucleotides at the 3' end and generate blunt ends. The plasmid was then digested with HindIII. Oligonucleotide 1a (5'-GGTCTGTCGAGCACACTACA-3') was synthesized with the sequence selected from the cRI gene described above and an extra A nucleotide at its 3' end for construction of a HindIII site. Oligonucleotide 1a was annealed to the complementary oligonucleotide 1b (5'-AGCTGTAGTGTGCTGGACGACCCACC-3') carrying an HindIII sequence at its 5'-end and ligated with T4 ligase to the HindIII-digested pBSU6 plasmid.

Through the screening by sequence analyses of transformed Escherichia coli JM101 cells (New England Biolabs), a positive clone was selected, digested with HindIII and EcoRI, and ligated to a double-stranded DNA fragment made up of complementary oligonucleotides 2a (5'-AGCTGTAGTGTGCTGGACGACCCACC-3') and 2b (5'-AAATCAAAGGGTCTGTCGAGCACACTACA-3'), containing the target sequence from the cRI gene flanked by HindIII and EcoRI sites. The resulting recombinant plasmid, termed pBSU6-cRI, was used for transforming E. coli JM101 cells. Restriction analyses of the plasmid confirmed the correctness of the final construct, in which complementary sequences 1a and 2a generate upon transcription the double-stranded siRNA for cRI silencing.

HeLa cells were transfected with plasmid pBSU6-cRI to obtain transient transformants. To obtain stable transformants the neomycin resistance gene was inserted into the BamHI site of pBSU6-cRI using the pGFP plasmid as a source. An anti-cRI rabbit antisera (Bio-Tech, Perdizn, Salerno, Italy) was used at a 1/3,000 dilution. The anti-glycerol-dehyde dehydrogenase (GAPDH) antibody (Ambion) was used at a 1/1,000 dilution. Secondary antibodies were from Pierce and used at 1/20,000 dilution. Detection was performed with the peroxidase-based Super Signal West-Pico procedure (Pierce) according to the manufacturer's instructions.

Cell Culture and Transfections—HeLa cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). Cells (1 x 10^6/well), grown in 6-well plates, were transfected with 1 μg of plasmid DNA in 10 μl of LipofectAMINE (Invitrogen) as described by the manufacturer's instructions. To select stably transfected clones, cells were treated 48 h after transfection with 0.6 mg/ml G418 (Invitrogen).

Cytotoxicity Assays—HeLa cells were seeded in 24-well plates (2.5 x 10^5/well), grown for 24 h, and transfected with pBSU6-cRI. After additional 24 h, RNases were added to the cell cultures. After 24 h, cells were trypsinized, resuspended in growth medium, and counted. Cell survival was determined as the percentage of surviving cells with respect to non-treated cells. Stably transfected HeLa cells were seeded in 96-well plates (5 x 10^4/well) in the presence or absence of RNases under test. After 48 h, cell counts were determined as described above.

RT-PCR—Transiently transfected HeLa cells (see above) were washed with phosphate-buffered saline and lysed with TRIReagent (Sigma) to isolate total RNA as described by the manufacturer. The Access RT-PCR System (Promega) was used with 200 ng of total RNA. Primers are listed in the legend to Fig. 1. The PCR reaction was performed in a PerkinElmer Life Sciences DNA thermal cycler as follows: 1 cycle at 48 °C for 45 min for the reverse transcription followed by 2 min at 94 °C, 40 cycles, each including 20 s at 94 °C, 1 min at 55 °C, and 2 min at 68 °C. After the last cycle, samples were kept for 7 min at 68 °C and then stored at 4 °C. Amplified samples were analyzed on agarose gels.

RESULTS

HeLa cells were transfected with plasmid pBSU6-cRI, containing the DNA template encoding siRNA designed to silence cRI mRNA. The effects of transfection were analyzed at the RNA and protein levels (Fig. 1). Total RNA was used for RT-PCR, with actin mRNA to standardize the procedure; primers are listed in the legend to Fig. 1. Complete inhibition of the expression of the cRI gene was observed 48 h after transfection (Fig. 1A). The cRI protein level was measured with an anti-cRI antibody; GAPDH, measured with an anti-GAPDH antibody, was used as an internal standard. Complete silencing of the cRI protein was obtained at 48 h after transfection (see Fig. 1B).

These results indicate that transfection of HeLa cells with plasmid pBSU6-cRI, which express a dsRNA complementary to a segment of cRI mRNA, is so effective in silencing cRI gene expression that its transcription and translation products are undetectable.

We then tested the effects of cytotoxic RNases on the growth of HeLa cells in which the cRI gene was silenced. The RNases to be tested were: BS-RNase and onconase, natural cytotoxins fully resistant to cRI; RNase A and HP-RNase, inactive as cytotoxins and with high affinity for cRI; RE-HP-RNase, an HP-RNase variant engineered to be cytotoxic and partially evasive to cRI inhibition.

These results indicate that cytotoxic RNases, such as BS-RNase and RE-HP-RNase, display a more powerful cytotoxic effect on cells deprived of cRI. Notwithstanding, the absence of cRI in HeLa cells does not allow a non-cytotoxic RNase, such as RNase A or HP-RNase, to become cytotoxic. Onconase, strongly cytotoxic under the assay conditions, has the same IC_{50} value on wild-type HeLa as on HeLa-cRI cells.

Additional analyses were carried out on stably transfected HeLa cells with a longer RNAse treatment (48 h), which allowed a closer evaluation of the cytotoxicity of less cytotoxic RNases. Isolated, stably transfected clones were selected on the basis of the absence in lysates of cRI mRNA and cRI protein and tested for sensitivity to RNAse cytotoxicity. In these experiments additional RNases were used: MCA-BS-RNase, a monomeric variant of BS-RNase with no cytotoxic activity and very sensitive to cRI inhibition; KSRE-BS-RNase, a monomeric variant of BS-RNase; and AR-RNase A, a variant of
RNase A, both moderately cytotoxic and moderately evasive of cRI inhibition.

The cytotoxicity tests, summarized in Table I, confirm and expand the results obtained with transiently transfected HeLa cells. They reveal that non-cytotoxic RNases, namely RNase A, HP-RNase, and MCA-BS-RNase, are equally ineffective on wild-type and cRI-deprived HeLa cells. Cytotoxic RNases, on the other hand, have more pronounced cytotoxic effects on cRI-deprived HeLa cells.

**DISCUSSION**

We report here the silencing with dsRNA of the gene encoding the cytosolic RNase inhibitor, obtained when the cells were transiently or stably transfected with a plasmid encoding a dsRNA complementary to cRI mRNA. Transfected HeLa cells were found to have no detectable cRI mRNA nor cRI protein. No significant changes were observed in morphology or growth rate of transfected cells.

When HeLa cells lacking detectable cRI were treated with natural or recombinant cytotoxic RNases, strongly or weakly cytotoxic, the cytotoxic activity of these RNases was enhanced. In contrast, non-cytotoxic RNases remained non-cytotoxic in cells deprived of cRI.

These results indicate that the cytosolic RNase inhibitor may not have the proposed protective role against foreign RNases as it only affects the activity of RNases that are intrinsically cytotoxic. On the other hand, the absence of cRI did not render HeLa cells susceptible to any other foreign RNases. As non-cytotoxic RNases are strongly inhibited by cRI, one would have expected that cells deprived of cRI, hence lacking cRI protection, would become susceptible to these RNases.

The results on the effects of onconase and BS-RNase can clarify the relationships between cRI and cytotoxic RNases. Onconase does not bind and is not inhibited by cRI. This may not be surprising, as onconase is an amphibian RNase, and amphibia do not have cRI, a mammalian protein. Thus, its cytotoxic activity is not affected by the presence or absence of cRI in target cells.

BS-RNase is not inhibited by cRI because it is a dimeric protein, a structural feature that renders this RNase unable to fit in the cRI horseshoe cavity. Its dimeric structure is maintained by non-covalent forces but also by two intersubunit disulfides. Furthermore, the protein exists in two quaternary isoforms: an MXM form characterized by the exchange of the cytosolic or "swap" between monomers of their N-terminal arms and an M=M form with no exchange (23). Under conditions of mild reduction, the intersubunit disulfides are cleaved, which generates either a non-covalent dimer from the MXM form, with monomers still held together by the exchange of terminal arms, or free monomers from the M=M form (23). The reduction of intersubunit disulfides of BS-RNase very likely occurs also in the reducing environment of the cytosol, where internalized BS-RNase is directed and degrades rRNA (24). Under these circumstances, M monomers are inhibited by cRI, whereas the non-covalent dimers are not inhibited (9). Thus, the cytotoxic activity of BS-RNase can be attributed to its resistance to cRI as a dimeric non-covalent dimer (9, 10), while the free monomers liberated in the cytosol are neutralized by cRI. In cells deprived of cRI, free monomers would not be neutralized by cRI, so their cytotoxicity would add to that of the non-covalent dimer, thereby enhancing BS-RNase cytotoxicity.

Clearly, an RNase can interact with the cytosolic inhibitor only if it reaches the cytosol. Naturally cytotoxic RNases, i.e. BS-RNase and onconase, do reach the cytosol and degrade rRNA (24) or tRNA (25), respectively. Non-cytotoxic RNase A, once introduced into cells through membrane entrapment, is directed to lysosomes and is not cytotoxic (26). However, if it is directly microinjected into the cell cytosol, it degrades rRNA and becomes cytotoxic (25).

Although the cytosol has proved to be the final intracellular destination only for intrinsically cytotoxic RNases, we can assume that RNases engineered to be cytotoxic by increasing their resistance to cRI must also have access to the cytosol.
their cytotoxicity (5). We can speculate that the structural alterations generated by the engineering of RNases, besides rendering them (partially) resistant to the cytosolic inhibitor, also enable them to reach the cytosol. Obviously, once an RNase reaches the cytosol, it would not escape the action of cRI. Hence, non-cytotoxic RNases are non-cytotoxic, not because they are susceptible to cRI neutralization but because they most likely do not have access to the cytosol. We believe that it is worth testing this hypothesis, especially because if cRI is not, or it is not primarily, an anti-RNase guardian, we should still search for its yet unknown physiological role.

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