FEBS Letters 585 (2011) 613–617

FEBS Letters

journal homepage: www.FEBSLetters.org

The ribonuclease/angiogenin inhibitor is also present in mitochondria and nuclei

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ARTICLE INFO

Article history:
Received 8 October 2010
Revised 20 December 2010
Accepted 21 January 2011
Available online 26 January 2011

Edited by Vladimir Skulachev

Keywords:
Ribonuclease/angiogenin inhibitor
Reactive oxygen species
Reactive oxygen species scavenger
Mitochondria
Nucleus

ABSTRACT

The data presented here show for the first time that the protein known as "ribonuclease (RNase) inhibitor" (RI or RNH1) is present not only in the cell cytosol, but also in mitochondria, the central organelles in cell redox homeostasis. This finding directly correlates with the reported ability of RI to protect the cell from oxidative stress, with its sensitivity to oxidation and reactivity as a reactive oxygen species scavenger. While this study was carried out we also surprisingly discovered the presence of RI in the cell nucleus. We deem that these data open new views in the investigation on the cellular role(s) of the RI.

Structured summary:
RI physically interacts with **ATP synthase sub Alpha mitochondrial, Stress 70 Protein, Mitochondrial, Calcium-binding mitochondrial carrier protein Aralar 2 ADP/ATP Translocase 2 Elongation factor Tu** and **Cytochrome b-c1 complex Sub 2** by anti bait coimmunoprecipitation (View interaction)
PARP and **RI** colocalize by cosedimentation (View interaction)

1. Introduction

The label of "ribonuclease (RNase) inhibitor" (RI, also termed RNH1) to a ubiquitous protein of about 50 kDa, acidic, very abundant in the cell cytosol, with a high content of Cys residues, consisting of 15 Leu repeats (LRR), has been for more than half a century certainly correct. RI inhibits the great majority of the extracellular RNases from the vertebrate superfamily, bound by RI with very high affinity and catalytically neutralized. The structure of RI, free or complexed to an RNase, has been determined [1,2]. Extensive reviews have been published on RI structure and function [3,4].

However, the physiological role of this protein has not been conclusively defined. It has been proposed that the role of RI in the cell is that of a "sentry", to protect its cytosolic RNA from extracellular RNases that may enter that compartment [5]. This hypothesis is based on the following evidence: RI resistant RNases, such as bovine seminal RNase [6] and onconase [7], are cytotoxic; non-cytotoxic RNases, strongly inhibited by RI, become cytotoxic when they are engineered into relatively RI-resistant RNases [8]; cells manipulated to increase the RI levels become more resistant to RNase cytotoxicity [5,8,9].

In contrast with the "sentry" hypothesis, it has been reported that the removal of RI from cells, obtained through RNA silencing, does not render these cells sensitive to non-cytotoxic RNases [10]. Moreover, cytotoxic RNases have been described [11] that bind RI with high affinity [12]. On the other hand, data have been accumulating on a different research line. Hofsteenge and co-workers have early reported that in cultured cells an oxidative insult with H_2O_2, a general oxidant, or with diamide, a thiol specific oxidant, engenders a severe cellular loss of RI as a protein [13]. More recently, it has been reported that cells over-expressing RI become more resistant to H_2O_2 stress [14], and that RI has a strong scavenging activity on reactive oxygen species (ROS) [15]. These data, and those from a parallel investigation of the effects of oxidants and antioxidants on normal and RI deprived cells, has led to the proposal that RI plays the physiological role of a defensive system against oxidative stress [16]. Furthermore, in cells stressed with arsenite

Abbreviations: RI, RNase inhibitor; RNase, ribonuclease; ROS, reactive oxygen species; PBS, Phosphate Buffered Saline; PBS GS, PBS containing 0.2% Gelatin and 0.075% Saponin; MPTP, mitochondrial permeability transition pore; ANT, ADP/ATP Translocase; SDS–PAGE, polyacrylamide gel electrophoresis in sodium dodecyl-sulfate; TRITC, tetramethylrhodamine isothiocyanate

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doi:10.1016/j.febslet.2011.01.034

as an oxidant RI inhibits the action of angiogenin, leading to inactivation of tRNAs and translational arrest [17,18].

The data presented here, collected through biochemical and mass spectrometric analyses, as well as inspections by immunofluorescence at the subcellular level, support the hypothesis of a key physiological role of RI in redox homeostasis. They show for the first time that RI is present and reactive as a ROS scavenger not only in the cell cytosol, but also in mitochondria, the organelles central in the cell redox homeostasis. Furthermore, they have enabled us to illustrate how RI is surprisingly abundant also in the cell nucleus.

These data and conclusions do not provide a complete, exhaustive picture of the physiological significance of RI in the cell, but certainly open the way to completely novel approaches to the investigation of the cellular role of the RI.

2. Materials and methods

2.1. Cell culture and extracts

HeLa and HCT cells were cultured in DMEM supplemented with 10% bovine fetal serum in standard conditions. Cell viability was assessed with the Trypan Blue exclusion method. Cell extracts were prepared in 1% NP-40 in Phosphate Buffered Saline (PBS) with protease and phosphatase inhibitors (Roche). Surnatants were collected after centrifugation at 18 000×*g* for 30 min 4 °C, then stored at −80 °C. In some experiments, cells were transfected at 80% confluence with Metafectene–Pro (Biointex) coated plasmid DNA, and incubation lasted o/n to allow expression of the recombinant protein. Cytosolic and nuclear extracts were prepared as described [19]. Cytosolic and mitochondrial fractions were prepared with the Qproteome Mitochondria Isolation Kit (Qiagen). Protein concentration was measured by the Bradford method (Bio-Rad).

2.2. Immunoprecipitation and mass spectrometry analyses

The sequence encoding human RI was amplified with GoTaq DNA polymerase (Promega) using the following primers:

5′-GGGGGAATTCAATGAGCCTGGACATCC-3′ (forward);
5′-GGGGGGTCGACTCAGGAGATGACCCTC-3′ (reverse);

bearing EcoRI and SalI restriction sites, respectively, at 5′ ends, and the plasmid pTRP-cRI as a template [20]. The amplified fragment was then directionally inserted into the EcoRI/SalI sites of p3xFlag-CMV-7.1 to facilitate selection of immune complexes with M2 Anti-Flag Affinity Gel (SIGMA). Lysates of HeLa cells transfected with p3xFlag-CMV-7.1 or the recombinant p3xFlag-RI (about 2 mg) were challenged with M2 Anti-Flag Affinity Gel, the immune complexes were separated in 10% polyacrylamide gel electrophoresis in sodium dodecyl-sulfate (SDS–PAGE) and stained with colloidal Coomassie Blue (Invitrogen). Relevant protein bands and the corresponding gel slices from control immunoprecipitation were excised and processed for liquid chromatography and tandem mass spectrometry (LC/MS/MS) analyses, performed on an LC/MSD Trap XCT Ultra equipped with a 1100 nanoHPLC system and a chip cube (Agilent Technologies, Palo Alto, CA). Raw data were used to query a non-redundant protein database using in-house MASCOT software (Matrix Science, Boston, MA).

2.3. Western blotting

Cell lysates were separated in 8% or 10% SDS–PAGE, then blotted on PVDF membranes (Bio-Rad) using HEP-1 electroblot apparatus (OWL) at 2.5 mA/cm^2. After blocking, membranes were incubated o/n with relevant primary antibodies, then washed and incubated with secondary peroxidase-linked antibodies. Light emission in the presence of Immobilon Western Chemiluminescent HRP Substrate (Millipore) was detected with the ChemiDoc system (Bio-Rad).

2.4. Antibodies

Antibodies used in these analyses were: anti-RI rabbit polyclonal antibodies, a kind gift of Dr. Guo-Fu Hu, Department of Pathology, Harvard Medical School, Boston, USA; an alternative anti-RI monoclonal antibody (anti-RNH1 M07, Abnova); anti-PARP-1 C2-10 monoclonal antibody (Enzo) directed to poly-ADP-ribose polymerase as nuclear marker. Antibodies anti-GAPDH, directed to glyceraldehyde-P dehydrogenase as cytosolic marker, 6C5 anti-tubulin, COX4 monoclonal antibody, and goat polyclonal antibody anti-B23 as a nucleolar marker, were from Santa Cruz Biotechnology. Alexa Fluor 488 goat anti-rabbit (Molecular Probes). Peroxidase-linked secondary antibodies were anti-mouse IgG (Fc specific) or anti-rabbit IgG goat polyclonal antibodies (Sigma).

2.5. Immunofluorescence and confocal microscopy

Cells growing on glass cover slips (in medium containing or not 250 nM Mitotracker Red as a mitochondrial dye, according to the manufacturer's protocol) were fixed for 20 min in PBS containing 4% paraformaldehyde (Sigma), washed two times in 50 mM NH_4Cl, then with PBS GS (PBS containing 0.2% Gelatin and 0.075% Saponin, from Sigma) and incubated in humidified atmosphere with the relevant antibodies at room temperature. Cells were then washed with PBS GS and incubated in humidified atmosphere at room temperature with the secondary antibodies and with tetramethylrhodamine isothiocyanate (TRITC)–phalloidin as a dye specific for microfilaments (Sigma), as indicated. As a negative control, cells were also stained with secondary antibodies alone (not shown). Nuclei were stained with DNA intercalants Hoechst 33258 or DRAQ5 (Bio status, Alexis Corporation), as indicated. Cells were finally mounted in 50% glycerol in PBS and analyzed with a Zeiss LSM 510 Meta confocal microscope. The lambda of the argon ion laser was set at 488 nm, those of the two HeNe lasers were set one at 546, the other at 633 nm. Fluorescence emission was revealed by BP 505–530 band pass filter for Alexa Fluor 488, by BP 560–615 band pass filter for TRITC–phalloidin or Mitotracker Red and by 615 long pass filter for DRAQ5. Nuclei stained with Hoechst 33258 were acquired as non-confocal images by mean of a light detector and 352/461 nm filter (UV light). Double- and triple staining immunofluorescence images were acquired separately in the green, red and infra red channels and then saved in TIFF format to prevent the loss of information. They had been acquired with a resolution of 1024 × 1024 pixels with the confocal pinhole set to one Airey unit.

3. Results

3.1. Searching for proteins interacting with RI

We have designed co-immunoprecipitation experiments to identify proteins interacting with RI in the cellular environment, as a new approach to the understanding of its biological role. To this end, lysates from HeLa cells transfected with the recombinant vector p3xFlag-RI or control vectors, were challenged with anti-Flag M2 affinity Gel, then bound proteins were analyzed by SDS–PAGE and stained with Coomassie Blue. The outcome revealed, in addition to Flag-RI, a number of protein bands absent in the control

Table 1
Mitochondrial proteins specifically interacting with RI, the RNase/angiogenin inhibitor in HeLa cell lysates as identified by mass spectrometry. Peptides corresponding to proteins listed in the table were totally absent in control immunoprecipitation.

Swiss-Prot #	Protein	MW	Peptides
P38646	Stress 70 Protein Mitochondrial	73 680	8
Q9UJS0	Calcium-binding mitochondrial carrier protein Aralar 2	74 176	10
P25705	ATP synthase sub Alpha mitochondrial	59 751	12
P49411	Elongation factor Tu	49 542	13
P22695	Cytochrome b-c1 complex Sub 2	48 443	5
P05141	ADP/ATP Translocase 2	32 895	9

sample; the most prominent ones were subjected to LC/MS/MS mass spectrometry analysis. The results (see Fig. S1) show that a relevant group of interacting proteins belongs to the mitochondrial inner membrane or matrix (see Table 1), suggesting that, in addition to the cytosolic compartments, RI appears to reside also inside mitochondria.

3.2. RI is localized in mitochondria

We then tried a direct biochemical approach to detect the presence of RI in mitochondria. The mitochondrial and cytosolic fractions of transfected HeLa and HCT cells were analyzed by Western blotting to identify 3xFlag-RI. The results (Fig. 1A and B) show that a substantial amount of recombinant RI is associated to the mitochondrial fractions. In addition, endogenous RI is revealed by specific antibodies in the mitochondrial fractions of untransfected cells (Fig. 1C), while an unrelated control antiserum was unable to produce the signal (not shown).

To confirm this finding, we performed an immunofluorescence confocal analysis of HeLa cells. The results (see Fig. 2) show that endogenous RI largely colocalizes with mitochondria stained with Mitotracker Red. As expected, RI is also present in the cytosol and, surprisingly, also in the nuclear matrix.

3.3. Evidence of RI localization in the nucleus

We have validated the presence of RI in the nucleus by probing HeLa cells nuclear extracts and cytosolic lysates with polyclonal anti-RI antibodies. The results of Western blottings, shown in Fig. 3, confirm the presence of RI in the cell nucleus. The signal generated by a monoclonal anti-RI antibody in confocal immunofluorescence analyses (see Fig. 4A–C) is consistent with the results shown in Figs. 2, 3 and 4D–G, in which the RI nuclear localization is revealed by specific polyclonal antibodies. In confocal immunofluorescence analyses, both antibodies show the same very intense and diffuse staining pattern of the nucleus, with the exception of regions corresponding to nucleolar areas, identified by an antibody

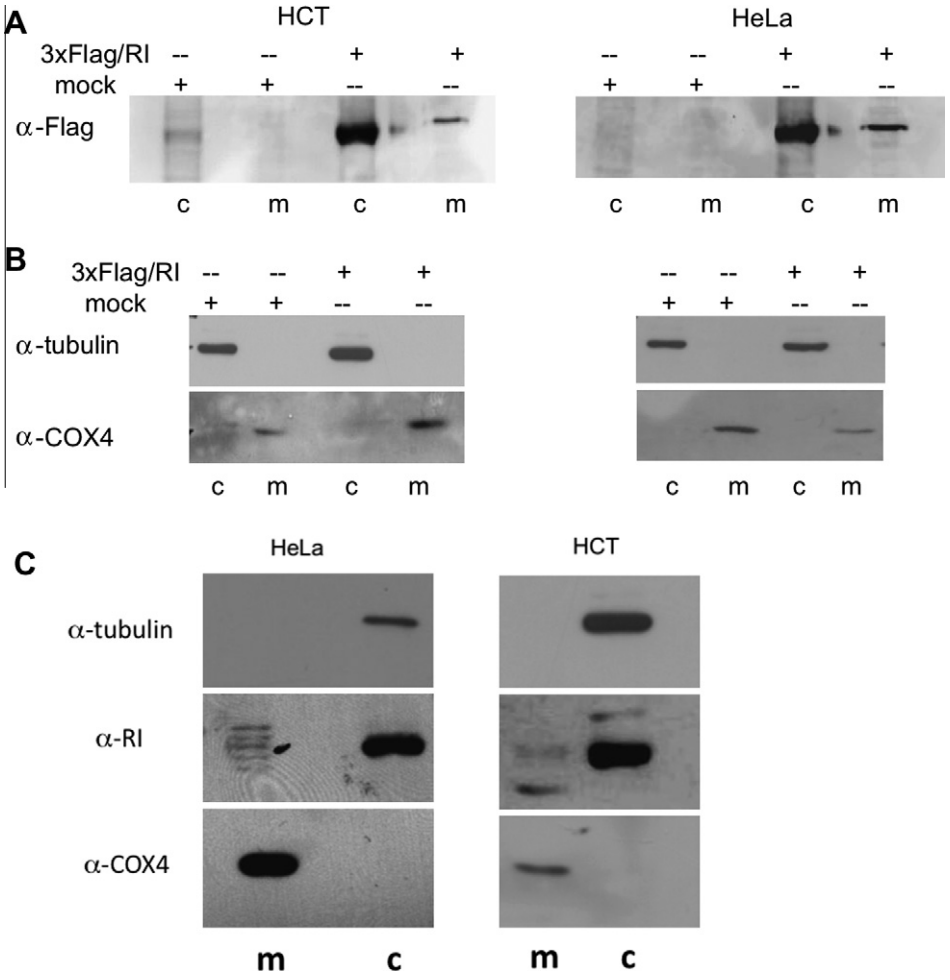

Fig. 1. Subcellular localization of RI in HCT and HeLa cells transfected with p3xFlag or control vector (mock). (A) Recombinant RI, revealed by SDS–PAGE 10% followed by Western blotting with anti-Flag antibody (α-Flag), is detected not only in the cytosolic (c) but also in the mitochondrial (m) fractions. (B) The same lysates were blotted with anti-tubulin (α-tubulin) and anti-COX4 (α-COX4) antibodies as markers for cytosolic and mitochondrial fractions, respectively. (C) The subcellular localization of endogenous RI in HCT and HeLa cells is shown, revealed by Western blotting with anti-RI polyclonal antibodies. Filters were re-blotted with anti-tubulin (α-tubulin) and anti-COX4 (α-COX4) antibodies. 40 μg of each lysate were analyzed.

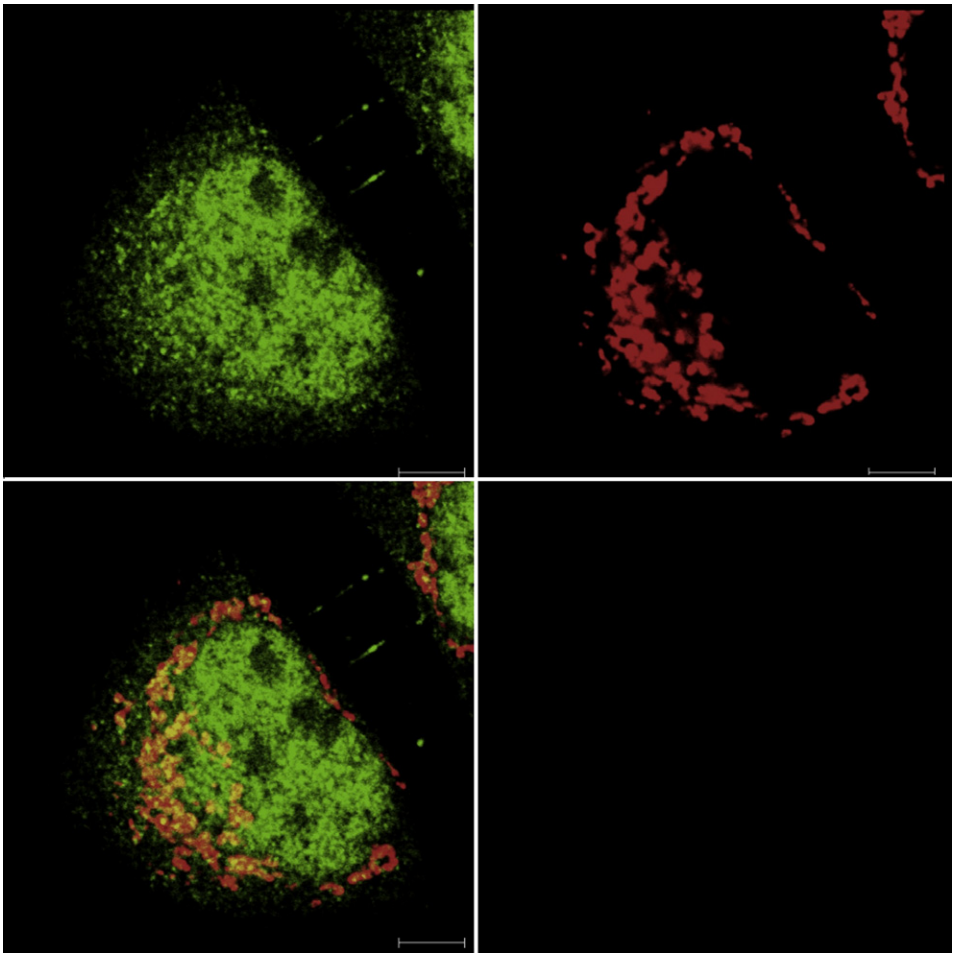

Fig. 2. Confocal microscopy showing significant mitochondrial localization of endogenous RI in HeLa cells. Red: mitochondria stained with Mitotracker Red; green: α-RI followed by Alexa fluor 488-conjugated goat anti-rabbit IgG antibody; yellow: merge. Bars: 5 μm.

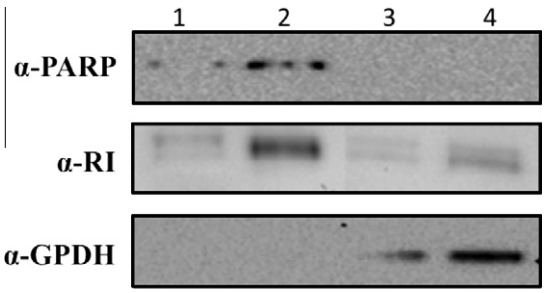

Fig. 3. Evidence of RI localization in the nucleus. Western blot analyses with anti-RI polyclonal antibodies. Lanes 1 and 2: 2.5 or 5 μg of HeLa cells nuclear extracts; in lanes 3 and 4: 2.5 or 5 μg of cytosolic lysates. The blot was re-probed with anti PARP and anti-GAPDH antibodies as markers for nuclear and cytosolic extracts, respectively.

against the nucleolar marker phosphoprotein B23 (data not shown).

4. Discussion

The result of our work uncover novel, previously unnoticed subcellular localizations of RI. We have established that RI, known as a cytosolic protein, is also present in the cell nucleus and mitochondria. Association of RI with proteins of the mitochondrial inner membrane and matrix place this protein in a major environment of ROS production. The ability of RI to protect genome integrity and cell viability from oxidative stress injury is documented in the literature, and correlates well with the sensitivity to oxidation and the in vitro activity as ROS scavenger of the protein [14–16].

Singh et al. have shown that apoptosis induced in HeLa cells by H_2O_2 induced oxidative stress, depends on ROS generated in mitochondria [21]. HeLa cells on the other hand show increased resistance to H_2O_2 induced mortality when RI is overexpressed [14]. The evidence reported here provides a new direct basis, through experimental methodologies distinct from the others so far proposed on the role of RI as a ROS scavenger. Interesting to note protein ADP/ATP Translocase (ANT), co-immunoprecipitated with RI, is a regulative component of the mitochondrial permeability transition pore (MPTP), which plays a pivotal role in mitochondrial induced cell death [21]. Oxidative stress promotes glutathione-mediated oxidation of ANT cysteine residues located in matrix-facing loops, resulting in Ca^{++} binding of the protein and activation of MPTP [22]. Thus, we are tempting to speculate that ANT-associated RI could act as a shield against ROS induced, GSH mediated oxidation of the nucleotide transporter. Oxidation of RI leads to the formation of intramolecular disulfide bridges with an all-or-none pattern, and to its rapid degradation in the cell [13]. We have observed some degradation of endogenous RI in mitochondrial lysates (see Fig. 1C), a finding consistent with the hypothesis that RI is easily oxidized in mitochondria due to the ROS rich environment.

The identification of RI in the nucleus is surprising; its role in this cellular compartment is unknown. In immunoprecipitation

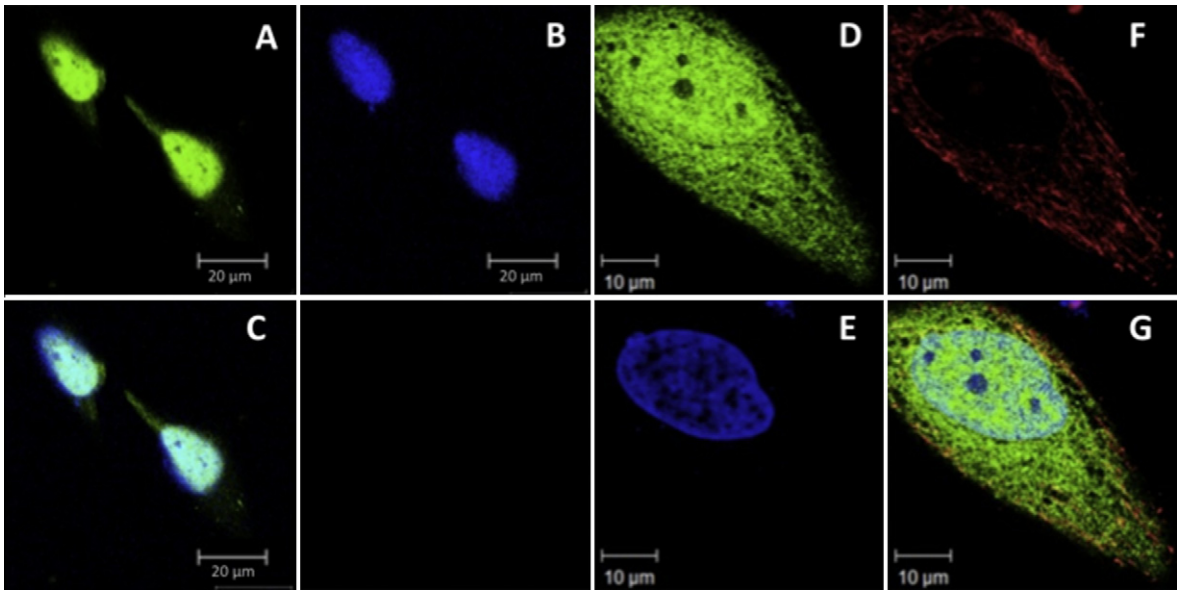

Fig. 4. Confocal microscopic analysis of HeLa cells. (A) Green fluorescence associated with monoclonal anti-RI antibody. (B) Blue fluorescence associated with Hoechst staining, acquired as non-confocal images. (C) Merge between (A) and (B). RI is evident in the nuclear matrix, absent in nucleoli. Bars: 20 μm. (D) Green fluorescence associated with polyclonal anti-RI antibodies. (E) Nuclear blue fluorescence after staining with DRAQ5. (F) Red fluorescence after staining with TRITC-conjugated phalloidin. (G) Merge between (D), (E) and (F). Bars: 10 μm.

assays we failed to identify nuclear proteins associated with RI, except the nuclear pore membrane glycoprotein 210 (accession number Q8TEM1). This finding is in line with the observation that the recombinant 3xFlagRI, perhaps due to the extension of 28 residues at the amino end, does not enter the nucleus (not shown). While the protein appears abundant in the nuclear matrix, it seems definitely excluded from nucleoli. It can be assumed that in the reducing nuclear environment RI is in the thiol form, thus able to bind and inhibit RNases, such as angiogenin. As angiogenin accumulates in HeLa nucleoli, where it plays a key role in rRNA biogenesis [23], the absence of RI, a powerful inhibitor of the enzyme, from the nucleolar areas, would be consistent with this role of angiogenin.

In conclusion, the observations reported in this study offer new evidence supporting the role of RI as a scavenger of ROS, and raise intriguing new questions about its significance and interactions in the nucleus, thus opening new lines of investigation for an advancement in the knowledge of RI functions in cell biology.

Acknowledgements

This work was supported by the Italian Ministry of University and by Associazione Italiana per la Ricerca sul Cancro.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.01.034.

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