A Rev-like NES mediates cytoplasmic localization of HERV-K cORF

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Abstract The human endogenous retrovirus K (HERV-K)-encoded protein cORF has recently been shown to be a functional homolog of the HIV Rev protein. Rev-mediated RNA export requires interaction between a leucine-rich nuclear export signal (NES) in Rev and the nuclear export receptor Crm1/exportin1. Like Rev, cORF binds to Crm1 and cORF-mediated RNA export depends on Crm1 activity. Here we document that mutation of the putative NES in cORF results in trapping of the protein in the nucleus, suggesting that the cORF NES functions in analogy to the Rev NES.

Key words: HERV-K cORF; Nuclear export; Rev-like NES

1. Introduction

The human genome consists to about 1% of retroviral sequences. Most of these human endogenous retroviruses (HERVs) are defective and only the family HERV-K is known to possess intact open reading frames (ORFs) for all essential retroviral proteins [1–3]. Recently, almost complete representatives of this family were identified [4,5]. HERV-K is expressed in a complex splicing pattern in testicular tumors and derived cell lines as well as in normal testis and placenta [1,6]. HERV-K expression is tightly associated with testicular malignancies [7].

The family HERV-K encodes an accessory protein, cORF (central ORF), translated from a doubly spliced RNA [8], in addition to the basic proteins with structural or enzymatic functions encoded by the genes *gag*, *prt*, *pol* and *env*. In complex exogenous retroviruses, accessory proteins often have regulatory functions and are important for efficient virus infectivity. For example, the proteins Tat and Rev of human immunodeficiency virus (HIV) regulate gene expression on the transcriptional or the post-transcriptional level [9]. Rev mediates the nuclear export of incompletely spliced HIV RNA. In the absence of Rev, the intron-containing HIV RNA species that code for the structural proteins are retained to the nucleus unless completely spliced. Rev enters the nucleus via its N-terminal nuclear localization signal (NLS) and uses its NLS-containing protein domain to bind to an RNA secondary structure (Rev-responsive element, RRE) within intron-containing HIV mRNAs. The C-terminal nuclear export signal (NES) of Rev then binds to the cellular export receptor Crm1 (or exportin1, reviewed in [10]), most likely in concert with other Rev- or Crm1-binding co-factors like eukaryotic initiation factor 5A (eIF-5A), the nucleoporin hRIP/Rab or the nucleoporin-like protein 1 (NLP-1) [11–14]. As a consequence, Rev is transported back to the cytoplasm ('nucleocytoplasmic shuttling') with the bound RNA backpacked [15].

The cORF protein resembles the Rev protein with respect to structural features and intracellular localization [8]. cORF possesses putative NLS and NES sequences and has been clearly demonstrated to exert a Rev-homologous function. In analogy to Rev, cORF supports the export of mRNA containing a defined sequence of the HERV-K mRNA. According to the RRE, this sequence was named RcRE. Additionally it was shown that a specific inhibitor of Crm1-mediated export, leptomycin B (LMB), is able to block the cORF-mediated accumulation of RcRE-containing RNA transcripts in the cytoplasm [16]. Furthermore, cORF directly binds to Crm1 [17]. This provides strong evidence that cORF, like Rev, uses the Crm1 export pathway to shuttle between the nucleus and the cytoplasm. However, the involvement of the putative Rev-like NES in this process has not been characterized. We show here that the putative NES in cORF permits a partly cytoplasmic location of the protein while mutation of this sequence or Crm1 inhibition result in nuclear inclusion of cORF.

2. Materials and methods

2.1. Plasmid constructions

The plasmid pEGFP-wtcORF was constructed by inserting full-length cORF in-frame into the *Bam*H1-digested pEGFP-C1 vector (Clontech). pEGFP-cORFMuNES was constructed as follows: An N-terminal and a C-terminal fragment of the cORF gene were amplified by PCR using the primers (1) 5′-AGCAGAGCTCGTTTAGTGAA-3′ (mapping upstream of the multiple cloning site in the pCEP4-cORF vector containing full-length cORF) and (2) 5′-AGCTGCCTGCGTCGCCTTCTTTGCTTGTGC-3′ (mutating the putative NES), and the primers (3) 5′-GCACAAGCAAAGAAGGCGACGCAGGCAGCT-3′ (complementary to (2)) and (4) 5′-CACTGCATTCTAGTTGTGG-3′ (mapping in pCEP4-cORF downstream of the binding site). Purified fragments were mixed and subjected to a PCR generating full-length fragments (by annealing of the overlapping part) that were subsequently amplified using primers (1) and (4), digested and inserted into the *Bam*H1 site of pEGFP-C1.

2.2. Cell culture, transfection and leptomycin B treatment

Tera-1 cells (teratocarcinoma cells expressing HERV-K) were grown in McCoy's 5A medium (GIBCO BRL) supplemented with 10% FCS and were subcultured routinely once per week. Cells were seeded and grown to about 50% confluency on glass cover slips and were then transfected using the Effectene transfection reagent (Qiagen). 48 h after transfection, cells were fixed in paraformaldehyde (4% in PBS), stained with 200 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) in methanol to visualize nuclei, and intracellular localization of EGFP fusion proteins was then studied by fluorescence microscopy. Where required, 50 nM leptomycin B was added to the culture medium 48 h after transfection and 16 h prior to evaluation.

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3. Results and discussion

3.1. HERV-K cORF protein is located in the nucleus and in the cytoplasm

Previous studies have shown that immunohistochemistry detects cORF protein most prominently in the nucleoli of the HERV-K expressing teratocarcinoma cell lines Tera-2 and GH [8]. It was proposed that an arginine-rich stretch of amino acids in the N-terminus of cORF (aa 13–20) may serve as the nuclear (and nucleolar) localization signal, while a central region (aa 50–59) with similarities to the consensus sequence of Rev-type NESs (reviewed in [18]) may act as a nuclear export signal [8]. A comparison of the proposed cORF NES [8] and the consensus Rev-like NES is given in Fig. 1. Hence, the subcellular localization of cORF might reflect the steady-state picture of a protein that shuttles between the cytoplasm and the nucleus, as is the case with Rev. The nucleolar accumulation and the apparent absence of cORF from the cytoplasm in some immunostainings may mirror the difficulties in detecting low protein concentrations with fluorescence labelled antibodies.

Not only teratocarcinoma cell lines, but also Raji cells stably transfected to express cORF display a primarily nucleolar localization of cORF in immunohistochemical stainings using a cORF-specific antiserum. However, Western blot analysis of cellular subfractions revealed that cORF is not only present in the nuclear but also in the cytosolic fraction (data not shown). To investigate intracellular distribution of the cORF protein in a system achieving strong fluorescence we decided for an enhanced green fluorescent protein (EGFP)-based approach. Cells were transfected with the plasmid pEGFP-wtcORF encoding wild-type (wt) cORF protein fused to an EGFP domain (EGFP-wtcORF). Fig. 2A shows the distribution of EGFP-wtcORF protein in the nucleus (made visible with DAPI in Fig. 2B) and in the cytoplasm of transfected Tera-1 cells. This distribution pattern is observed in 92% of the evaluated cells. In all assays presented, only cells were evaluated that showed an intact nuclear morphology and did not show signs of ongoing cell division or apoptosis. For any of the stated percent values at least 1500 cells that meet the evaluation criteria were inspected.

3.2. Efficient nuclear export of cORF protein depends on a leucine-rich domain

To examine whether the cytoplasmic localization of EGFP-wtcORF is a result of nuclear export via the Crm1 pathway, Crm1-dependent nuclear export was blocked by addition of

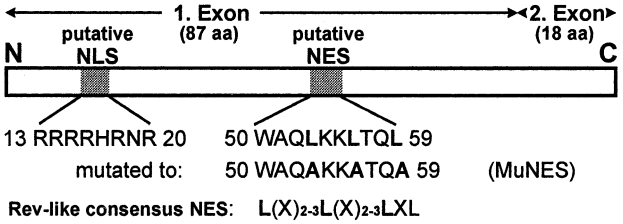

Fig. 1. Domains in cORF representing putative nuclear im- and export signals as proposed in [8]. The putative cORF NES is shown in the wt and in the mutated form with the leucine to alanine exchanges given in bold. The consensus Rev-like NES (leucine residues essential for nuclear export indicated in bold) is shown in comparison.

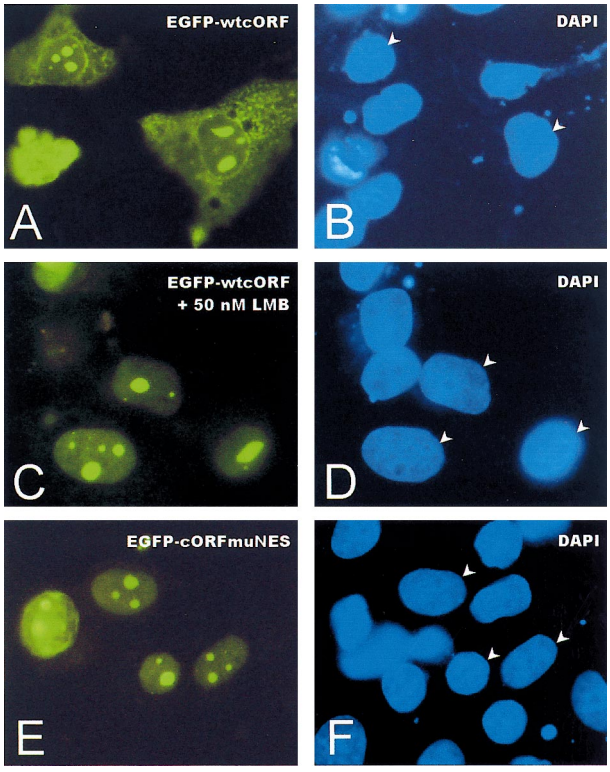

Fig. 2. Intracellular distribution of cORF protein. Tera-1 cells were transfected with EGFP-wtcORF (A, B, C, D) or EGFP-cORF-muNES (E, F) encoding plasmids and were (C, D) or were not (A, B, E, F) treated with LMB. EGFP fluorescence is seen in (A), (C) and (E), DAPI stains of the same cells indicate the nuclei (marked by arrowheads in B, D and F). All observed localization phenotypes are reproducible in various human and murine cell lines.

50 nM LMB. In the presence of LMB, EGFP-wtcORF shows an exclusively nuclear staining in 76% of the evaluated cells (Fig. 2C and D). The absence of wtcORF from the cytoplasm following disruption of the nuclear export machinery supports the notion that the cytoplasmic location of cORF results from active re-export of cORF from the nucleus rather than from cytoplasmic retention of the protein.

Subsequently, the putative NES sequence in cORF was mutated (cORFmuNES) by changing leucine residues that are known to be critical for the export mediated by Rev-like NESs, to alanine residues (Fig. 1). Though the putative cORF NES does not exactly match the proposed consensus sequence, cORFmuNES expressed as an EGFP fusion (EGFP-cORFmuNES) was found to be exclusively localized in the nucleus (Fig. 2E and F) in 83% of the evaluated cells.

In this report we demonstrated that the presence of wtcORF protein in the cytoplasm depends on an active nuclear export mechanism and on a leucine-rich signal in the central part of cORF. Leucine-rich NESs of the Rev-type bind to the nuclear export receptor Crm1 and the NES-containing proteins are subsequently transported to the cytoplasm. cORF, the proposed HERV-K counterpart of Rev, was described to also bind to Crm1. If Crm1 is functionally inhibited, cORF localization is restricted to the nucleus (Fig. 2C, D and [17]) and cORF lacks its RNA export function [16,17]. We show that a NES mutant of cORF (Fig. 2E, F) mimics the intracellular localization seen after blocking of the Crm1 pathway. Therefore, our data provide additional evi-

dence to enforce the model that cORF is not only a functional Rev homolog but shuttles via the same biochemical pathway, involving interaction of a leucine-rich NES and Crm1.

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