The Rev/Rex homolog HERV-K cORF multimerizes via a C-terminal domain

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Received 8 January 2001; revised 26 February 2001; accepted 28 February 2001

First published online 13 March 2001

Edited by Hans-Dieter Klenk

Abstract Expression of human endogenous retrovirus K (HERV-K) is associated with germ-cell neoplasia. HERV-K encodes a protein of the Rev/Rex family, cORF, that supports cellular transformation and binds the promyelocytic leukemia zinc finger (PLZF) protein implicated in spermatogenesis. Rev/Rex function invariably depends on multimerization. Here we show that cORF likewise self-associates to form higher-order oligomers. Amino acids (aa) 47–87 in cORF are sufficient, aa 75–87 essential for self-association. Consistently, this domain is predicted to form a hydrophobic α-helix that may represent an oligomerization interface. The existence of a dimerization-competent cORF mutant lacking PLZF-binding activity (cORF47–87) suggests a way of dominant negative inhibition of the proposed tumor susceptibility factor cORF.

Key words: Human endogenous retrovirus K cORF; Rev/Rex; Oligomerization

1. Introduction

Human endogenous retroviruses (HERVs) are the results of germ-line infections of our simian and human ancestors by exogenous retroviruses. Retroviral elements account for ~0.6–1% of the human genome. The only HERV family known to still express all essential retroviral proteins leading to release of the most probably uninfectious human teratocarcinoma-derived virus-like particles from defined tissues is the family HERV-K, represented by the most intact member HERV-K (HML-2.HOM) located on chromosome 7 [1,2]. Besides the basic proteins required for formation of retroviral virions (e.g. a functional protease [3,4]) HERV-K expresses an accessory 14.7 kDa gene product termed cORF. This protein is of particular interest as it is – without showing any apparent sequence homology at the amino acid level – a functional homolog of the Rev protein encoded by the human immunodeficiency virus (HIV) and the Rex protein of the human T-cell leukemia virus (HTLV). Like Rev and Rex, cORF is a mainly nucleolar protein at steady state and performs nucleocytoplasmic shuttling via the Crm1 pathway to mediate nuclear export of unspliced viral RNA species required for the synthesis of structural proteins and enzymes [5,8].

HIV and HTLV are pathogens that can still not be controlled. HIV causes a severe and incurable immunodeficiency [9]. HTLV-1 is the causative agent of the aggressive malignancy adult T-cell leukemia and is associated with a chronic neurodegenerative disorder termed tropical spastic paraparesis [10]. Rev/Rex activity is essential for HIV/HTLV replication and thus for viral pathogenicity. In the absence of Rev/Rex, viral transcripts are retained in the nucleus and are subjected to the cellular splicing machinery. Because of the lack of full-length RNAs in the cytoplasm no infectious virus can be produced [11].

HERVs have also been discussed to bear a pathogenic potential, e.g. they have been linked to human autoimmune diseases [12]. Furthermore, HERV-K expression is associated with the occurrence of germ-cell tumors (GCTs) in that a significant percentage of patients suffering from GCTs generate HERV-K Gag- and Env-reactive antibodies. Abundant Gag expression is observed exclusively in the GCT cells but not in the surrounding tissue [13]. The presence of HERV-K cORF in GCTs is suggested by the observations that (i) cORF activity seems to be a prerequisite for the efficient expression of HERV-K structural proteins and (ii) cORF protein is expressed in GCT-derived cell lines [5,6]. Hence, the active implication of HERV-K in tumorigenesis cannot be ruled out. In fact, we could recently demonstrate that the cORF protein encoded by HERV-K induces tumors in nude mice. Furthermore, cORF interacts with the promyelocytic leukemia zinc finger (PLZF) protein implicated in limb morphogenesis, cellular differentiation and spermatogenesis [14–17]. To further analyze the effect of cORF on cellular metabolism, a biochemical characterization of the protein and its functions is indispensable.

Rev and Rex have different functional domains. A nuclear localization signal (NLS) mediates nuclear import by binding to importin β [18,19]. The RNA-binding domain (RNAbd) interacts with a highly conserved RNA secondary structure within all incompletely spliced HIV or HTLV RNA species (termed Rev or Rex response element, RRE or RxRE, respectively). Furthermore, Rev and Rex use leucine-rich nuclear export signals (NES) for direct binding to the Crm1 export receptor and subsequent nuclear export of Rev/Rex and their associated substrates. A further aspect of Rev/Rex function is the potential to form oligomers. Multimer formation seems to be necessary at the import step as well as in the process of interaction with the RRE and the export machinery [20–22].

Like Rev and Rex, cORF possesses a NLS and a NES, and

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0014-5793/01/$20.00
PII: S0014-5793(01)02280-3

binds directly to Crm-1 [7,8,23]. Its function as a RNA export protein requires the presence of a RNAbd, which has, however, not been mapped yet [6]. Hints towards a possible dimerization came from mammalian two-hybrid experiments [7]. As multimerization seems to be fundamental for the biological activity of Rev-like proteins, we further investigated this feature of cORF.

2. Materials and methods

2.1. Plasmids

Plasmids pEG-cORF, pJG-cORF, pCep4-cORF and pGEX-cORF contain the full-length cORF gene in a pEG202, pJG4-5 (kind gifts of R. Brent, Boston, MA, USA, distributed by Clontech), pCep4 (Invitrogen) or pGEX-4T-1 (Pharmacia Biotech) backbone, respectively. pJG-cORF deletion mutants comprise the amino acids in cORF given with the name. All plasmids have been previously described [14]. The pCep4-cORFre vector contains the cORF gene in reverse orientation and was used as a negative control.

2.2. Cell culture, transfection and antibodies

Raji cells were cultured in RPMI 1640 plus 10% fetal calf serum and were transfected with pCEP4-cORF or pCep4-cORFre vectors by electroporation [14]. Transfected cells were selected by 0.3 mg hygromycin B/ml to generate stable cell lines (Raji-cORF and Raji-cORFre). Polyclonal rabbit antisera and monoclonal rat antibodies were as previously described [14].

2.3. Yeast two-hybrid system

To test for protein interactions in yeast two-hybrid assays, yeast strain EGY48 (carrying the lacZ reporter plasmid pSH18-34) was co-transfected with pEG-202 and pJG4-5 derivatives using the lithium acetate/polyethylene glycol method (according to the manufacturer's protocol, Matchmaker LexA, Clontech). Yeast clones were classified positive if reporter genes (lacZ, LEU2) were activated only in the presence of galactose but not in the presence of glucose, which inhibits expression from the plasmid pJG4-5 (internal negative control). The yeast strain EGY48 as well as reporter and control plasmids were generous gifts of Roger Brent.

2.4. GST pulldown assays and Western blot analysis

Glutathione *S*-transferase (GST) or GST–cORF fusion protein was generated by transforming *Escherichia coli* with plasmid pGEX-4T-1 or pGEX-4T-1-cORF, respectively. Exponentially growing cultures were induced with isopropyl-1-thio-β-D-galactopyranoside for 4 h at 37°C and cell pellets of 50 ml bacterial culture were resuspended in 5 ml GST lysis buffer (20 mM Tris–HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) and stored at −20°C. Thawed suspension was sonicated for 1 min, cleared supernatant was added to glutathione-Sepharose beads (1/20 volume) and incubated for 1 h at 4°C with gentle shaking. Beads were collected by centrifugation and washed three times in GST lysis buffer. 35 μl of protein-coated beads (GST or GST–cORF) were incubated with 220 μl of native lysate (cells washed in phosphate-buffered saline (PBS) were lysed for 30 min on ice in lysis buffer (100 mM Tris–HCl pH 8.0, 100 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% Nonidet P40, 1 mM dithiothreitol, 10 μg/ml aprotinin) and cellular debris was removed by centrifugation) from 2.2×10^{-6} Raji or Raji-cORF cells for 1 h on ice. Pellets were washed six times with GST lysis buffer and boiled for 5 min in SDS gel loading buffer (125 mM Tris–HCl pH 6.8, 6.3% (w/v) SDS, 10% (v/v) 2-mercapto-1,2-propanediol, 10% (v/v) glycerol) for release of bound protein. The supernatant was loaded onto a 15% SDS–polyacrylamide gel and blotted onto nitrocellulose membrane to test for cORF protein co-precipitation (following standard protocols). Blots were blocked in 5% non-fat dried milk in PBS pH 7.4 for 30 min and incubated overnight at 4°C with primary antibodies (polyclonal α-cORF antiserum, diluted 1:100 in 5% milk–PBS or monoclonal antibody in a dilution of 1:15). After three washes for 20 min each in PBS, blots were incubated with the secondary antibody (peroxidase-conjugated goat anti-rabbit or goat anti-rat antibody, Sigma) in a dilution of 1:500 in 5% milk–PBS for 1 h at 4°C. Blots were subjected to enhanced chemiluminescence immunodetection (Amersham) after further washing according to the manufacturer's protocol.

2.5. Glutaraldehyde crosslinking assay

Native extracts from 6×10^{-5} Raji-cORF or Raji-cORFre cells (25 μl) were mixed and incubated with 5 μl of glutaraldehyde (GA) solutions (diluted in water to give final concentrations as indicated) or water as a control (−). After 15 min incubation at room temperature the samples were tested for crosslinking of cORF oligomers in a Western blot analysis using a cORF-specific monoclonal antibody.

3. Results and discussion

3.1. HERV-K cORF dimerizes in vivo and in vitro

In a first approach to test for the self-association of cORF we used the yeast two-hybrid system. Co-transfection of the pEG-cORF vector encoding a fusion protein of cORF and the

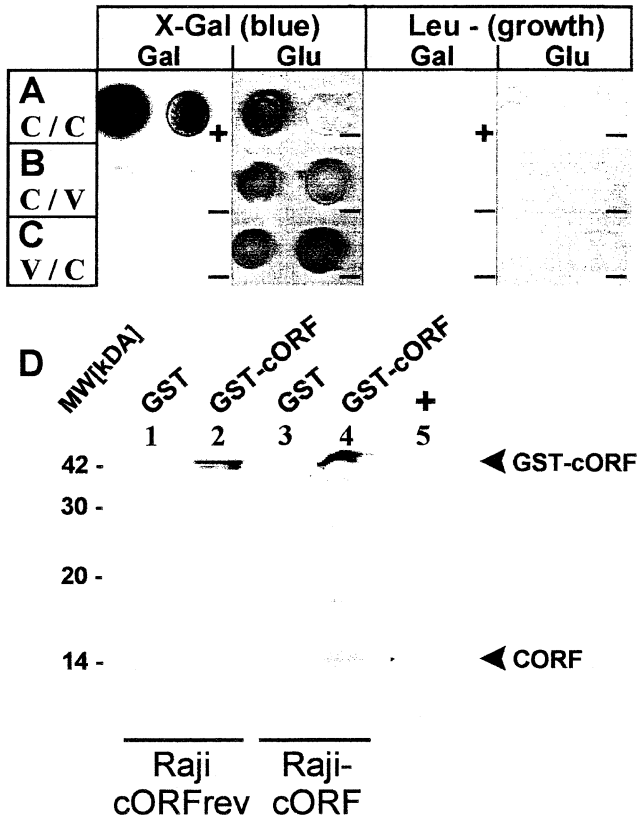

Fig. 1. In vivo and in vitro self-association of cORF. A–C: β-Galactosidase reporter activity (blue) on medium containing X-Gal (X-Gal, left side) and leucine-independent (Leu−, right side) growth of colonies co-transfected to express interacting proteins in the yeast two-hybrid system. Specific interaction is only expected on galactose medium (Gal) as glucose (Glu) represses expression from the vector pJG4-5. Glucose-containing medium was therefore used as an internal negative control. The pictures show drop spots of two independent, representative yeast clones, grown on the indicated medium composition; activation (+) or failing activation (−) of the respective reporter gene is shown in each panel. Yeast cells were co-transfected with pEG-cORF plus pJG-cORF (A), pEG-cORF plus pJG4-5 (B) or pEG202 plus pJG-cORF (C). D: Immobilized GST (lanes 1+3) or GST–cORF fusion protein (lanes 2+4) was incubated with native extracts from Raji cells expressing (lanes 3+4) or not expressing (lanes 1+2) cORF. cORF binding was investigated in a Western blot of Sepharose-bound protein using polyclonal cORF-specific rabbit antiserum. Extract from Raji-cORF cells was used as a positive control in lane 5, positions of the cORF protein and the GST–cORF fusion protein as well as of marker proteins are indicated; cORF-reactive bands in lanes 2 and 4 are degradation products of GST–cORF.

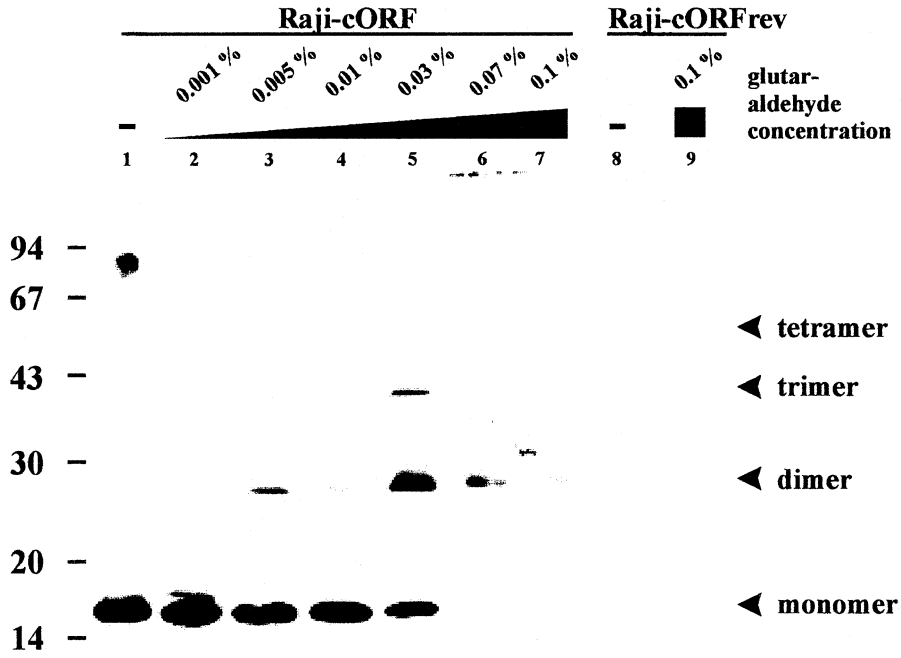

Fig. 2. cORF multimers are stabilized after GA crosslinking. Before (lanes 1+8) and after (lanes 2–7 and 9) incubation of native extracts of cORF expressing (lanes 1–7) or not expressing (lanes 8+9) Raji cells in increasing concentrations of GA (as indicated), cORF-specific signals were detected in a Western blot analysis with a cORF-specific monoclonal antibody. The positions and molecular masses of marker proteins and the cORF-specific protein species are indicated.

LexA DNA-binding domain and of pJG4-5-cORF, encoding a fusion of cORF and the transactivation domain of the Gal4 transcription factor, led to specific activation of a LexA-dependent reporter system in yeast. Activation of the reporter genes is due to interaction of the two cORF hybrid proteins reconstituting a functional LexA transactivator and is visualized by blue staining of the colonies on medium containing X-Gal and growth of the colonies on medium lacking leucine. In contrast, no activation was observed in control transfections (i.e. in combination with the respective parental vectors, Fig. 1B,C).

To confirm the homo-multimerization of cORF we performed GST pulldown assays. GST or GST–cORF fusion protein was coupled to glutathione-Sepharose beads and incubated with native lysate of Raji cells stably transfected to produce cORF (Raji-cORF) or of control cells (Raji-cORFre). Matrix-associated protein was subjected to Western blot analysis using a cORF-specific polyclonal rabbit antiserum. As can be seen in Fig. 1D, only Sepharose beads loaded with GST–cORF fusion protein precipitated cORF protein from Raji-cORF cell extract while Sepharose beads loaded with the GST moiety alone did not (compare lanes 3, 4 and positive control in lane 5), demonstrating that cORF specifically self-associates in this assay. No significant amount of cORF protein was precipitated by either Sepharose beads loaded with GST–cORF or GST from Raji cORFre cell extract lacking cORF protein (lanes 1 and 2). Since we never detected the expression of cORF in Raji cells that were not stably transfected with a cORF expression vector beforehand, the very faint cORF signal in lane 2 most likely results from cleavage of an – obviously very small – part of the GST–cORF protein (thrombin cleavage site between GST and the fusion moiety resulting from usage of vector pGEX-4T-1) resulting in the release of cORF protein that can subsequently bind to GST–cORF. The higher-molecular cORF-reactive species in lanes 2 and 4 represent GST–cORF and degradation products of the latter. In conclusion, these experiments clearly show that cORF self-associates in vivo and in vitro.

3.2. cORF forms di-, tri-, and tetramers

To analyze the oligomerization order, i.e. the number of

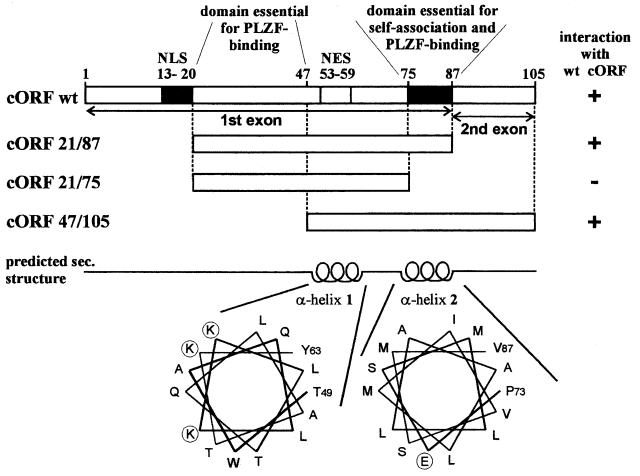

Fig. 3. Mapping of interaction sites using the yeast two-hybrid system. Shortened fragments of cORF covering the indicated amino acids (given with the names) were tested for interaction with the full-length cORF protein. Results of specific activation of the yeast two-hybrid reporter system (+) are shown on the right. Functional domains are indicated above: the NLS as defined by Magin et al. [23] and the NES defined by Boese et al. [8]. The black bar represents the domain identified to be essential for dimerization. Additionally, α-helices (α1 and α2) in the predicted secondary structure are indicated with hydrophobic amino acids as plain letters, hydrophilic amino acids as letters in open circles.

cORF monomers that associate, we performed GA crosslinking experiments. GA is a reagent that connects proteins situated in close proximity, which is only given if proteins are interacting. We therefore incubated native extracts from cORF expressing cells with GA solution appropriately diluted to give final concentrations of 0.001, 0.005, 0.01, 0.03, 0.07 and 0.1% GA. Samples were then tested for crosslinking of cORF oligomers in a Western blot analysis using a cORF-specific monoclonal antibody. As can be seen in Fig. 2, at GA concentrations higher than 0.001% additional bands appear besides the cORF monomer band at 14.7 kDa with molecular weights of about 28, 41, and 53 kDa. At a GA concentration of 0.07% the cORF monomer band is diminished, at a GA concentration higher than 0.2% no cORF-specific bands are observed any more (not shown). This is most likely because of massive crosslinking leading to the stabilization of large protein complexes that are excluded from the gel matrix, indicated by cORF staining at the upper gel border in these samples. As, in contrast, no bands are observed in extracts from Raji cells lacking cORF (in the presence and the absence of GA), all bands observed in Raji-cORF extracts are cORF-specific. It cannot be ruled out that the high-molecular cORF species arise due to interactions with heterologous proteins. However, as their laddering matches very well the predicted molecular weights of cORF mono-, di-, tri- and tetrameric forms, they most likely represent cORF oligomers.

3.3. Identification of the self-association domain

To define the dimerization domain we used the yeast two-hybrid system. Deletion mutants of cORF were expressed as fusion proteins from the vector pJG4-5 and tested against the full-length wild-type protein (pEG-cORF). While the first 46 amino acids (aa) and the most C-terminal 18 aa are dispensable for self-association, a mutant lacking the C-terminal 30 aa no longer binds to full-length cORF (Fig. 3). These results confine the dimerization domain to the C-terminal part of the protein comprising aa 47–87 with the presence of aa 75–87 being absolutely essential for dimer formation. Ongoing studies using a larger number of partially overlapping deletions and point mutations will help to acquire an even more defined picture of the amino acids mediating self-association.

Remarkably, we could identify two domains in cORF to be substantive for binding to PLZF, one encompassing aa 21–47 and a second one at aa 75–87 [14]. The whole central part of cORF is thus necessary for PLZF interaction. This stretch is too large to display a simple linear association epitope. However, the necessity for this relatively large domain could be explained if cORF only binds to PLZF as a dimer. In this suggestive model, the N-terminal part of the interaction domain would then mediate direct interaction with PLZF while the C-terminal part (aa 75–87) confers dimerization. Self-association would then be necessary but not sufficient for PLZF interaction, as a mutant unable to bind PLZF can still dimerize.

3.4. Secondary structure prediction of the cORF dimerization domain

The finding that aa 75–87 in cORF may be part of a dimerization domain is further substantiated by the fact that aa 73–87 are predicted to form a hydrophobic helical structure (Fig. 3). Two independent secondary structure prediction methods were applied (PHD, Columbia University, and Predator, EMBL, Heidelberg) to the cORF sequence which both indicate two α-helical regions within the protein. The first predicted helical segment encompasses Thr49 to Tyr63 (Trp50–Asn66, Predator) which would result in an amphipathic α-helix. Assuming a helical conformation the side chains of residues Trp50, Leu53, Leu56, and Leu59 point in one direction thereby forming a hydrophobic surface, while the positively charged lysine residues Lys54, Lys55, and Lys62 form the opposing hydrophilic and preferentially solvent-exposed area. This structural model is in agreement with our previous observation that the leucine-rich region in cORF forms a NES [3]. The second α-helical secondary structure is predicted to reach from Pro73 to Val84 (Ser75–Val87, Predator). This secondary structure element would form a homogeneously hydrophobic core fragment with isoleucine, leucine, valine and methionine residues exposed throughout the entire helix (Fig. 3). An accumulation of that many hydrophobic residues is very likely to form the central scaffold of the folded protein or – since cORF is small-sized – to form a seed for oligomerization. Future studies will reveal whether the proposed correlation of structure and function of the dimerization domain can be confirmed.

The detection of further characteristics and functions of cORF, besides HERV-K RNA export, is of particular interest because of recent evidence that (i) HERV-K expression is associated with GCTs [13], (ii) cORF has the capacity to transform cells, and (iii) cORF interacts with PLZF [14]. PLZF is involved in functions such as cell differentiation and testis development [16,17], suggesting that deregulation of PLZF, possibly by cORF, might contribute to germ-cell transformation. We show here the multimerization potential of cORF. Multimerization is crucial for the activity of Rev and Rex and thus for the pathogenicity of the respective virus. Comparably, cORF dimerization may be a prerequisite for PLZF binding by cORF. With a cORF mutant at hand that can still dimerize but does not bind to PLZF one could test for the possibility of a dominant-negative effect of this mutant on wild-type cORF. If PLZF deregulation by cORF is indeed implicated in germ-cell tumorigenesis, this could be a first approach to inhibit cORF function.

Acknowledgements: This work was supported by a grant from the German Research Foundation (DFG) to N.M.L. (SFB399-B2) and an EMBO long-term fellowship to M.G. We thank Peter Sommer for stimulating discussions and critical reading of the manuscript.

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