In Vitro Evaluation of Cyanovirin-N Antiviral Activity, by Use of Lentiviral Vectors Pseudotyped with Filovirus Envelope Glycoproteins

Laura G. Barrientos,1 Fatima Lasala,2 Joaquin R. Otero,7 Anthony Sanchez,1 and Rafael Delgado2

1Special Pathogens Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; 2Laboratory of Molecular Microbiology, Hospital 12 de Octubre, Madrid, Spain

Cyanovirin-N (CV-N) has been shown to inhibit Ebola Zaire virus (EboZV) infection, both in vitro and in vivo, through its ability to bind to oligomannoses-8/9 on the EboZV surface glycoprotein (GP). Here, we report the in vitro potency of CV-N to inhibit EboZV GP– and Marburg virus GP–pseudotyped viruses (EC50 –40–60 nmol/L and –6–25 nmol/L, respectively) from mediating gene transduction into HeLa cells. In addition, we provide evidence that CV-N can effectively inhibit DC-SIGN–mediated EboZV infection. Our data emphasize both the utility of GP-pseudotyped vectors in the assessment of compounds that affect cell entry by filovirus and the use of CV-N as a reagent for the probing of carbohydrates-dependent interactions at viral entry.

Both Ebola virus (EboV) and Marburg virus (MbgV) are enveloped, single negative–stranded RNA viruses belonging to the family Filoviridae. These viruses are biosafety level–4 pathogens (the most deadly human-disease–causing agents), and infection in humans is often associated with severe hemorrhagic fever and high mortality. No effective prophylactic or therapeutic agents are currently available. EboV and MbgV each contain a unique but related transmembrane glycoprotein (GP); the GPs share 31% amino acid sequence identity and are heavily glycosylated with both N-linked carbohydrates (including high-mannose oligosaccharides) and O-linked carbohydrates [1]. Because GP forms the trimeric spikes that occupy the entire surface of the virion, it is likely to play a central role in cellular binding and fusion [1]. It is interesting to note that in vitro studies have demonstrated that GP can bind to DC-SIGN, a calcium-dependent C-type lectin highly expressed in dendritic cells, which can function, both in cis and in trans, as an efficient attachment factor for EboV-promoting infection ([2], [3] and references therein). GP is, therefore, a key target for pharmacologic intervention, and substances that directly interact with GP might interfere with filovirus infection by preventing viral entry into and/or fusion with the target cells [4].

The cyanobacterial lectin known as “cyanovirin-N” (CV-N) [5] is perhaps the first molecule (other than antibodies) known to bind to Ebola virions and block cell entry, demonstrating proof of concept for the idea that glycoprotein interactions on the surface of virions provide a new avenue for EboV therapeutic research [4]. Both in vitro and in vivo CV-N antiviral activity against the Zaire strain of EboV (EboZV) have been reported [4]: the addition of CV-N to cell-culture medium at the time of EboZV infection has been shown to inhibit the development of viral cytopathic effect; and in vivo studies have shown that CV-N is able (1) to attain systemic circulation and to exhibit measurable activity, although the distribution patterns are still unknown, and (2) to delay the death of EboZV-infected mice, either when administered in a series of daily subcutaneous injections or when inoculations are performed after virus and protein are mixed ex vivo. The mode of action of CV-N has been found to be similar to that of a broad range of HIV strains [5], influenza virus [6], and other envelope viruses [5–7]. In all cases, antiviral activity implicates carbohydrate moieties on viral surface proteins as common viral molecular targets for this novel protein; in particular, CV-N has high affinity for the epitope Manα1→2Man, in the form of a dimannoside (Manα1→2Man) and a linear trimannoside (Manα1→2Manα1→2Man) located at the terminal arms of N-linked high-mannose oligosaccharides (Man-8 and Man-9) on viral surface glycoproteins ([8], [9] and references therein).

The precise inhibitory mechanism of CV-N is still largely unknown, but further research should shed light on the steps involved at the early stages of EboV infection and facilitate the development of therapies. A major obstacle to the advancement of this research is the requirement of biosafety level–4 containment for work with infectious EboV particles. The use of recombinant systems to generate GP-pseudotyped virus particles has provided a means for the study of the biology of the filovirus.
peplomer under less restrictive conditions; these studies are related to tropism, receptor utilization, and viral entry ([2], [10] and references therein). In the present study, we examined the potential use of (1) pseudotyped vectors bearing native EboZV GP, to further investigate the interesting nature of inhibition by CV-N, and (2) CV-N as a tool to investigate carbohydrate-mediated events at the level of viral entry. In addition, we extended these studies to Marburg virus GP (MbG V GP).

Methods. CV-N was produced and purified as described elsewhere [11]. The protein sample used in the present study was monomeric; the integrity and purity of the sample were assessed as described elsewhere [11].

EboZV GP– and MbG V GP–pseudotyped lentiviruses were produced according to a transient-transfection protocol using 293T cells, as described elsewhere [12]. The lentiviral vector pNL4-3.Luc.R.E+ (obtained from N. Landau [13] through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, National Institute of Health, Rockville, Maryland) was used for the production of EboZV GP– and MbG V GP–pseudotyped lentiviruses. Supernatant fluids were obtained 48 h after transfection, were filtered (by use of a 0.45-μm-pore screen), and were stored at −80°C. Infectious titers were estimated by serial dilution of HeLa cells.

Transduction experiments were performed as follows: Supernatant fluids containing either EboZV GP– or MbG V GP–pseudotyped lentiviruses were treated with different concentrations of CV-N for 20 min at room temperature and were added onto HeLa-cell monolayers in 24-well plates at a MOI of 0.1, by use of Dulbecco’s MEM with 10% fetal bovine serum. Jurkat cells stably expressing DC-SIGN [2] were infected with pseudotypes in 24-well plates, as described above, by use of RPMI 1640 with 10% fetal bovine serum. Infectivity was measured 48 h after infection, by luciferase assay using reagents from Promega, in a Berthold Sirius luminometer with a dynamic range of 10^2–10^7 relative luminescence units.

Results and discussion. Virus vectors pseudotyped with native filovirus GP molecules have become valuable tools in the investigation of viral-entry pathways. These viruses allow one to study, independently of other steps in the viral life cycle, mechanisms of entry, and studies can be performed in a safe, fast, and quantitative manner ([10] and references therein). The pseudotyped vectors described here consist of lentivirus pseudotypes bearing native EboZV GP or MbG V GP. This system has previously been used to demonstrate that DC-SIGN mediates, both in cis and in trans, cell entry by EboV [2]. To assess the application and reliability of pseudotyped vectors in the study of inhibitors to viral entry, we first tested CV-N for antiviral activity and compared our results with those of studies using the highly lethal EboZV, for which both in vitro and in vivo data have been reported [4].

Figure 1A shows that the transduction of HeLa cells by EboZV GP–pseudotyped virus was inhibited by CV-N at an EC<sub>50</sub> similar to that determined for the live agent on Vero E6 cells. The EC<sub>50</sub> for CV-N was in the range of ∼40–60 nmol/L in the pseudotype assay (figure 1A), whereas that due to EboZV
infection was ≈80–100 nmol/L [4]. Because the pseudotype assay used here monitors viral entry that is GP dependent, these results provide further evidence that the previously reported CV-N inhibitory effect on EboZV infection [4] indeed involves interaction with GP. Therefore, differences in CV-N potency are likely influenced by the type of cellular host used in the assays, given that the activity is carbohydrate dependent and the composition of N-glycans on GP is host dependent. It is worth mentioning that similar variations in EC50 values (range, 1–54 nmol/L) have been observed, in anti-HIV assays, when a wide spectrum of viruses and cells have been compared [5, 11]. We have also assayed a CV-N variant that had the carbohydrate binding site depleted but that maintained the same global fold and stability as did wild-type CV-N (L.G.B., unpublished data). In the pseudotype assay, no inhibitory effect was detected for this mutant, which further supports the theory that the antiviral property of CV-N is linked to its interaction with carbohydrate moieties on GP.

CV-N antiviral activity was also tested, with the same cellular target, against MbgV GP–pseudotyped virus particles and was found to be slightly more potent (EC50 ≈6–25 nmol/L, compared with ≈40–60 nmol/L for EboZV GP–pseudotyped virus). This difference, if significant, might be related to the greater amounts of Man-8 and Man-9 on MbgV GP than on EboZV GP, given that these glycoproteins have a predicted 24 and 17 N-glycosylation sites, respectively.

We also examined the effect that CV-N has on DC-SIGN-mediated transduction, using pseudotyped viruses in a system in which viral entry is presumably dependent on contact between DC-SIGN and GP [2]. Jurkat cells expressing DC-SIGN were inoculated with EboZV GP–pseudotyped virus preincubated with CV-N, and, as shown in figure 1B, it is clear that CV-N interferes with the interaction between DC-SIGN and EboZV GP (EC50 ≈40–110 nmol/L). The observed inhibitory effect is not due to a true competition between CV-N and DC-SIGN; both lectins recognize the high-mannose oligosaccharides that are N-linked to GP but that do not bind to the same epitope within the glycan: CV-N is unique in its high specificity for the Manα1–2Man moiety at the termini of the D1 and D3 arms of N-linked glycans ([8], [9] and references therein), whereas DC-SIGN binds an internal core of the sugar, the trimannose branch point (Manα1–3Manα1–6Man) [14]. Our results suggest that CV-N and DC-SIGN can compete for the binding of GP, presumably via steric interference. A similar phenomenon has been described in a competition experiment between CV-N and 2G12 (a neutralizing anti–human HIV antibody that recognizes a cluster of the α1–2 mannose residues on the outer face of HIV viral surface gp120 [15]).

The efficient binding of GP with either of the 2 lectins is highly dependent on the content of the N-linked high-mannose oligosaccharide in GP, and this variable needs to be taken into account in the assessment of in vitro and in vivo studies. In this context, it is worth noting that MbgV GP does not seem to interact with DC-SIGN under the conditions used for experiments with EboZV GP and DC-SIGN Jurkat cells (R.D., unpublished data); this phenomenon might be related to the greater content of complex carbohydrate structures in the MbgV GP used in this particular experiment [3]. Therefore, investigating other lectins for the targeting of alternative carbohydrate structures present on viral glycoproteins may represent yet another avenue in the search for novel antiviral agents.

The present study’s findings regarding CV-N antiviral activity against EboZV GP–pseudotype infectivity are in agreement both with the results of those studies performed in biosafety level-4 facilities on the highly virulent EboZV (from the 1976 Ebola outbreak) [4] and with the current knowledge of CV-N binding properties [9]. The ability of CV-N to inhibit DC-SIGN from binding to EboZV GP highlights the importance of GP-mediated virus attachment to cells, as being the first step of Ebola infection, and reinforces the notion of the potential utility of CV-N in blocking important steps in cell entry/infection by filoviruses. To further characterize the individual steps involved in Ebola infections, the mapping of those N-linked glycosylation sites in EboZV GP that are involved in the binding of DC-SIGN and CV-N to GP will be required. Comparison with parallel studies on MbgV GP can be used to identify processes that are peculiar to entry that is mediated by native proteins. By using the biological assays described in the present study, one can safely and rapidly perform these and other experiments to assess compounds that affect cell entry by filovirus.

Acknowledgments

We thank A. M. Gronenborn for critical reading of the manuscript; MbgV GP was provided by V. Volchkov (University of Lyon, France).

References


