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Identification of a Putative Crimean-Congo Hemorrhagic Fever Virus Entry Factor

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Abstract

Entry of enveloped viruses into cells is initiated by binding of their envelope glycoproteins (Envs) to cell surface-associated receptors. The Crimean-Congo hemorrhagic fever virus (CCHFV) has two Envs, Gn and Gc, with poorly understood role in binding to susceptible cells. We expressed codon optimized Gn and Gc, and identified independently folded soluble Env fragments, one of which (Gc residues 180-300) bound CCHFV susceptible cells supposedly by interacting with a putative receptor. This receptor binding domain (RBD) was used to identify its interacting partner by coimmunoprecipitation and mass spectrometry. Thus we identified the human cell surface nucleolin as a putative CCHFV entry factor. Nucleolin was expressed on all susceptible cells tested but not on the surface of cells resistant to CCHFV infection. Further studies are needed to explore the nucleolin function as a plausible CCHFV receptor and the molecular mechanisms of the Gc-nucleolin interactions. The identification of the CCHFV RBD and its binding partner could provide novel targets for therapy and tools for prevention as well as more complete understanding of the mechanisms of CCHFV entry and pathogenesis.

Keywords

CCHFV; Gn; Gc; nucleolin; receptor; receptor-binding domain

Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a tick-borne disease caused by the Crimean-Congo hemorrhagic fever virus (CCHFV), a member of the genus Nairovirus within the family Bunyaviridae. This disease has wide-ranging symptoms such as high fever and diarrhea and in severe cases hemorrhagic symptoms with a fatality rate as high as 30%. Originally identified during an outbreak in Russia during the 1940s, it continues to cause sporadic outbreaks in Africa, Europe, and Asia [1,2,3]. It has been listed as a category C priority pathogen by CDC/NIAID. Treatment options for CCHF are limited partially due to the limited understanding of the pathogenesis of this virus [4]. In particular, the entry mechanism remains ambiguous because the potential roles played by the only two viral membrane proteins, Gn and Gc, in the entry process have yet to be elucidated. Furthermore,

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the human factor(s) involved in this process remains unknown. Attempts to resolve these issues have been impeded by the inability to express and purify soluble and functional Gn and Gc proteins. The only virus from the Bunyaviridae family with a putative human receptor(s) identified is Hantaan virus. Integrin $\alpha\beta 3$ was found to be one possible receptor through functional screening rather than the traditional biochemical approach often used for this purpose [5]. This same functional screening approach so far has not yielded any promising lead for CCHFV and other viruses in this family.

In this study, we report the soluble expression of the full-length ecto-domain of matured Gn and fragments of the ecto-domain of matured Gc, characterization of their binding to human cells, and identification of a possible human factor (receptor) involved in the entry process by CCHFV.

Materials and Method

Plasmid, primers, codon-optimized genes and antibody

Codon-optimized full-length, matured Gn and Gc genes corresponding to the CCHFV isolate IbAr10200 were purchased from Genescript (Piscataway, NJ). All PCR primers used for cloning of Gn and Gc fragments into expression vectors were purchased from Invitrogen (Carlsbad, CA). The mammalian expression vector pSecTag was also purchased from Invitrogen. The monoclonal antibody against human nucleolin (MS-3) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Protein expression

Codon optimized full-length Gn and Gc as well as fragments were cloned into the pSecTag expression vector. In some cases, Fc from human IgG1 was fused to the C termini of the Gn or Gc fragments. All constructs were sequenced to confirm the accuracy of the cloning procedure. The expression plasmids carrying Gn and Gc fragments with or without Fc fusion were expressed in 293 freestyle cells according to the supplier's suggested protocol in protein free medium (Invitrogen). The expressed proteins were either purified using Nickel column (Qiagen, Hilden, Germany) for Gn and Gc fragments without Fc fusion or Protein A column (GE Healthcare, Piscataway, NJ) for Gn and Gc fragments fused with Fc.

Cell lines

The CCHFV susceptible cell lines including the human adrenal gland carcinoma SW-13, African green monkey kidney cell line Vero E6 (purchased from ATCC), and a subclone of the human embryonic kidney cell line 293T, 293T/17 (kindly provided by Robert Doms, University of Pennsylvania). The cells were cultured in DMEM supplemented with 10% FBS in a 37 °C, 5% CO₂ incubator. The expression cell line 293 Freestyle was purchased from Invitrogen and cultured in 293 Freestyle medium (Invitrogen).

Flow cytometry

Gn and Gc fragments fused with Fc at various concentrations were incubated with SW-13, Vero E6 or 293T/17 cells in DMEM+10% FBS on ice for 30 min. Cells were then washed with the same medium three times. After washing cells were re-suspended in the same medium and mouse anti-human Fc IgG-FITC (Sigma, St Louis, MO) was added to the cells to a final concentration of 4 µg/ml. After another 30 min of incubation on ice, the cells were washed three times in the same medium and re-suspended in ice-cold PBS. Cells were then subjected to analysis on a FACSCalibur (Becton Dickinson, Franklin Lakes, New Jersey).

Immunoprecipitation and protein identification

SW-13, Vero E6 and 293T/17 were grown in the DMEM+10% FBS medium. Cells were collected before they reached confluence using the cell disassociation buffer (Invitrogen). Cells were washed twice in ice-cold PBS buffer, surface-labeled using the membrane impermeable reagent EZ-Link® Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) and quenched with 0.5 M glycine according to the protocol suggested by manufacturer. After labeling, cells were washed twice with ice-cold PBS and the cell pellets were re-suspended in a lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5% (v/v) glycerol, 1% (w/v) Cymal-5 (Anatrace, Maumee, OH) and protease inhibitors (Roche, Mannheim, Germany). Cells were incubated on ice for one hour. Cell lysates were clarified by centrifugation at 13000g at 4 °C for 30 min. The supernatant was collected and pre-incubated with protein A-sepharose beads (GE Healthcare) for one hour at 4 °C. The supernatant was again subjected to the same centrifugation step and the recovered supernatant was mixed with 2 µg of Gn-Fc or Gc-Fc fusion protein and 20 µl of protein A-sepharose beads. Each sample contained the lysate made from approximately 10⁷ cells. The bead/lysate mixture was put on a rotator and kept at 4°C to allow for continuous mixing overnight. The beads were then washed thoroughly with the same lysis buffer for 5 times. Reducing SDS-PAGE sample buffer was then added to the beads to elute bound proteins by heating at 90 °C for 4 min. The eluted samples were divided into two halves to run in two identical gels. One of the gels was detected with simple coomassie blue stain and the other transferred to PVDF membrane for Western blot with streptavidin-HRP. The proteins that were visible from both coomassie blue stain and streptavidin-HRP detection were excised from the coomassie blue-stained gel and sent out for identification by a NanoLC-MS/MS peptide sequencing technology (ProtTech, Inc, Norristown, PA).

Results

Soluble expression of Gc and Gn

The expression and cellular transportation of Gn and Gc have been studied extensively. It was found that they are both targeted to the Golgi body after expression and surface presentation is limited if any [6,7]. Furthermore, soluble expression of their ecto-domains has been difficult. We cloned codon-optimized Gn and Gc of various lengths into the pSecTag expression vector and tested their expressions (Figure 1A). The full-length, matured Gn and Gc [8], expressed efficiently, with Gc running as a smear in a conventional reducing SDS-PAGE gel. The full-length ecto-domains of both Gn and Gc without the transmembrane domains and regions C terminal to them also expressed efficiently. However, only Gn ecto-domain was secreted to the growth medium, whereas Gc ecto-domain was retained inside the cells. Subsequent deletions revealed that residues 1-380 of the matured Gc constitute the largest soluble Gc fragment (Figure 1B). Computer analysis using the different programs including Kyte-Doolittle and Chou-Fasman suggested the existence of unusually high content of beta-strand secondary structure downstream of residue 380 (data not shown). This may be an indication of regions involved in membrane fusion after viral engagement of host cells.

Identification of a putative RBD domains

We hypothesized that the receptor binding domain (RBD) of viral Envs should be solvent accessible and readily soluble, and focused on the Gn ecto-domain and soluble fragments of Gc ecto-domain for identification of potential RBD domains. For this purpose, we constructed various Gn and Gc-Fc fusion proteins for use in the flow cytometry. It was found that compared to Gc fragments tested, Gn ecto-domain only had limited binding to CCHFV susceptible cell lines. Gc fragments, particularly the one encompassing residues

180-300 exhibited significant binding (Figure 2). This is consistent with the indication that Gc is the primary binding moiety from the CCHFV [6].

Identification of a putative entry factor for CCHFV

After confirming the specific binding of Gn-ecto and Gc fragments to the susceptible cell lines, we used the Gn-Fc and Gc-Fc fragments in immunoprecipitation assays to identify the possible human cell surface proteins that interact with Gn and/or Gc. The Gn fragment used was Gn-ecto-Fc and the Gc fragment used was Gc-180-300-Fc. There was only one protein that was dominant in both coomassie blue-stained and streptavidin-HRP detected gels in the Gc-180-300-Fc sample. It had a size of approximately 110 kDa (Figure 3). There was also one specific protein immunoprecipitated by Gn, even though it was visible only by coomassie blue stain. Peptide sequencing of both proteins revealed that the protein immunoprecipitated by Gc-180-300-Fc was nucleolin, with 16 distinct peptides identified, representing more than 20% of the nucleolin sequence (Table 1). The protein immunoprecipitated by Gn was a strictly cytoplasmic protein reflecting its lack of biotin labeling and was not pursued further (data not shown). To confirm the finding with nucleolin, biotin-labeling followed by immunoprecipitation was repeated in two CCHFV susceptible cell lines, SW-13 and 293T/17. The immunoprecipitation samples were analyzed by Western blot using both streptavidin-HRP and an anti-nucleolin antibody. Nucleolin was confirmed to be the only dominant protein species immunoprecipitated by Gc-Fc in both cell lines (Figure 4A). Since nucleolin is predominantly a nucleolar protein, we further tested its cell-surface expression by flow cytometry. Its surface expression was detected in all three cell lines with SW-13 having the highest expression level (Figure 4B). This is consistent with the previous finding that SW-13 cell line shows the most prominent cytopathic effect upon CCHFV infection by forming visible plaques and yielding the highest virus titer [9].

Discussion

The soluble expression of Gn and Gc of CCHFV has long been a bottleneck in studying their functions in CCHFV entry as well as their usages in developing vaccines and therapeutics. In this report, we extensively characterized the expression and solubility profiles of both Gn and Gc, and identified Gc as the primary binding moiety to human cells. Furthermore, we narrowed the potential receptor binding domain (RBD) to a Gc fragment slightly longer than 100 residues (Gc180-300). Using this fragment, we co-immunoprecipitated nucleolin, an extremely versatile protein found primarily in the nucleolus of eukaryotic cells.

The exact roles of Gn and Gc in CCHFV entry has not been clearly defined. Center to the debate is which of the two proteins is responsible for binding to the host cells and mediating the entry. For different genus in the Bunyaviridae family, it appears that either Gn, Gc or both could be involved [10]. There is increasing evidence suggesting that Gc is the primary target-binding protein for CCHFV. Panels of monoclonal antibodies have been generated against both Gn and Gc and only those against Gc inhibited virus infection of susceptible cell lines but not those against Gn [6]. This is by far the clearest indication that Gc not only binds target cells but also mediates virus entry. Our findings that Gc seems to be divided into two halves, the first being the hydrophilic N terminal fragment including residues 1-380 and the second being the hydrophobic C terminal fragment including the rest of the Gc residues support this model. The identification of a potential RBD domain will greatly assist in the study of the cellular entry mechanism and the development of treatment of CCHFV as is seen with other emerging viruses [11]. The participation of Gn if any is yet to be determined. The success in soluble expression of both Gn and Gc provide the possibility to test their usefulness as subunit CCHFV vaccines. Protein therapeutics as well as diagnostics including neutralizing antibodies against these two proteins can now be developed, too.

The identification of a potential human factor for CCHFV entry could add significantly to our understanding of CCHFV entry, pathogenesis, and development of therapeutics. The highly restricted access to the CCHFV and the lack of a reliable surrogate in vitro assay approach such as cell-cell fusion or pseudovirus system prevented us from testing the functional role of nucleolin if any in supporting CCHFV entry. However, there are multiple lines of evidence indicating its involvement.

The prerequisite to be a functional viral entry factor is the cell surface expression. Even though nucleolin is primarily a nucleolar protein, accumulating evidences reveal that it is also present on cell surface, shuttling between cell surface and nuclear and carries out a multitude of functions [12], among which are as a putative receptor for several viruses including Parainfluenza virus type 3 [13], HIV [14], coxsackie B viruses [15] and bacterium such as enterohemorrhagic *E. coli* O157:H7 [16]. More specifically, immunohistochemistry and in situ hybridization have shown that mononuclear phagocytes, endothelial cells, and hepatocytes are the main targets for CCHFV infection [17]. Nucleolin was first identified as a cell surface protein in hepG2 cells serving as a lipoprotein receptor [18]. Its functional cell surface expression has also been found in macrophages [19,20] and endothelial cells [21, 22]. There is an excellent correlation between cell-surface expression of nucleolin and CCHFV distribution in human tissues.

CCHFV is a tick-borne disease which utilizes both insect and human cells as hosts. This suggests that the entry factor(s) would have to be present in both cell types, even though they are evolutionarily far apart. Nucleolin is highly conserved among all vertebrates and can be found in all eukaryotic species from yeast to human [23]. This seems to suffice the needs of CCHFV to infect drastically different hosts.

One hallmark of CCHFV infection is hemorrhage in some of the severe cases [1]. Different hypotheses have been given as for the cause of hemorrhage including both direct and indirect injury to the vascular system. Nucleolin has been shown to be involved in the angiogenesis process and expressed exclusively on the angiogenic endothelial cell surface [21,22]. Anti-cancer therapeutics targeting nucleolin have shown effectiveness in reducing angiogenesis and tumor growth [24,25]. This establishes a direct link between CCHFV infection and hemorrhage. It is noteworthy that pathogenic Hantaviruses-induced vascular permeability seems to be the direct consequences of the inhibition of α V β 3 function by virus infection rather than an indirect mechanism [26].

Actively shuttling between the cell surface and nucleolus, nucleolin has been shown to go through the clathrin-mediated endocytic pathway [27], and actin is important for its movement between cell membrane and nucleolus [28]. It has been reported recently that CCHFV entry also uses the clathrin-mediated endocytic pathway [29] and this process is actin-dependent [30].

Finally, our results show that nucleolin expressed on the surface of all susceptible cells tested, albeit at low level. Among the cell lines tested SW-13 has the highest surface expression (Figure 4), even though the total cellular expression is similar among all three cell lines (data not shown). Interestingly SW-13 was found to be the most able to support high level of replication of CCHFV when compared to 293T/17, Vero E6, and BHK-21 [9].

Further studies are needed to explore the nucleolin function as a plausible CCHFV receptor and the molecular mechanisms of the Gc-nucleolin interactions. The identification of the CCHFV RBD and its binding partner could provide novel targets for therapy and tools for prevention as well as more complete understanding of the mechanisms of CCHFV entry and pathogenesis.

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Highlights

> identified putative receptor for the Crimean-Congo hemorrhagic fever virus which allows new exploration of virus entry and development of inhibitors > identified a putative receptor binding domain which also allows new exploration of virus entry and development of inhibitors > expressed and purified soluble viral envelope glycoproteins which can be used as candidate vaccine immunogens

Figure 1. Soluble expression of Gn and Gc

1A, schematic representation of full-length matured Gn and Gc proteins (depicted as thick lines), as well as Gn and Gc sub-fragments (depicted in thin lines) that were expressed. Fragments starting with the residue number 1 of Gn or Gc are shown with only the ending residue numbers; fragments starting down stream of the first residues are shown with both starting and ending residue numbers. The same rule also applies to the Fc fusion proteins. 1B, Western blot of various recombinant Gn and Gc fragments expressed in 293FS cells with anti-His mAb. Fragments described in 1A were expressed, purified with Ni-chelating columns, and analyzed on reducing, SDS-PAGE gels. RT, samples were incubated in reducing, SDS-PAGE sample buffer at room temperature for 5 min. before loading. Boil, samples were boiled in the same loading buffer for 5 min. before loading. Lysate, crude cell lysate was loaded directly for analysis. Medium, culture medium containing secreted proteins was analyzed. IP, culture medium was subjected to immunoprecipitation with anti-His antibody before being loaded to the gels.

Figure 2. Flow cytometry analysis of Gn-Fc and Gc-Fc bindings to cells
Gn-Fc and Gc-Fc fragments as described were used in flow cytometry assays. For each assay, the amount of Gn-Fc used was at least two folds higher than that of the Gc-Fc fragments. The amount was comparable among the three Gc-Fc fragments with Gc-300-Fc having the lowest concentration due to the difficulties in production. The lower panel summarizes the binding of the three Gc fragments to both CCHFV susceptible cell lines and a monoclonal anti-Gc antibody developed in house (54A) (Zhu, Z. et al., unpublished).

Figure 3. Identification of a human factor for CCHFV entry

Immunoprecipitation was performed and samples were analyzed for potential CCHFV interacting proteins. Left and middle panels, biotin labeled cells were subjected to immunoprecipitation by incubation with Epherin B2-Fc (Con) used as control, Gn-175-Fc (Gn), and Gc-180-300-Fc (Gc) fusion proteins and detected with two approaches, including streptavidin-HRP Western blot (left panel) and coomassie blue staining (middle). Lysate represents the starting sample prior to immunoprecipitation. The right panel represents a repeat of the experiment without biotin labeling and the protein highlighted by a “*” was excised and used for protein identification. The symbol “#” represents the protein immunoprecipitated by Gn. M, molecular weight marker. Symbols a, b, and c indicate the input proteins of Eph, Gn, and Gc.

Figure 4. Surface expression of nucleolin in multiple cell lines

4A, immunoprecipitation was performed for two biotin labeled cell lines indicated and samples obtained were subjected to detection with two methods including Western with streptavidin-HRP and anti-nucleolin monoclonal antibody MS-3. Gn and Gc represent Gn-175-Fc and Gc-180-300-Fc fragments used in immunoprecipitation. 4B, flow cytometry assay of surface expression of nucleolin. Flow cytometry was performed as described in Methods. Mouse anti-caspase antibody was used as an isotype control (filled peak) and MS-3 was used to detect the nucleolin expression, as represented by the red lines in the left two panels and green line in the right panel. The X axis represents the fluorescence intensity and the Y axis represents the cell number.

Table 1

Summary of the peptide sequencing. A total of 16 peptides were recovered from the excised protein described in Figure 3 (marked with *) and they constituted ~23% (167 out of 710 amino acids) of the sequence of the human nucleolin protein.

Peptide mass	Peptide sequence
1396.64	TEADAEKTFEEK
1321.62	GLSEDTTEETLK
999.53	NDLAVVDVR
999.53	NDLAVVDVR
1056.60	VTLDWAKPK
1177.56	EVFEDAAEIR
1159.58	SISLYYTGEK
936.49	TGISDVFAK
939.51	GIAYIEFK
2199.02	GLSEDTTEETLKESFDGSVR
1775.82	KFGYVDFESAEDLEK
1647.73	FGYVDFESAEDLEK
1990.98	VTQDELKEVFEDAAEIR
1990.98	VTQDELKEVFEDAAEIR
1593.73	GYAFIEFASFEDAK
2567.30	QKVEGTTEPTTAFNLFVGNLNFNK
2567.30	QKVEGTTEPTTAFNLFVGNLNFNK
2311.15	VEGTEPTTAFNLFVGNLNFNK
2311.15	VEGTEPTTAFNLFVGNLNFNK
2311.15	VEGTEPTTAFNLFVGNLNFNK
2500.26	TLVLSNLSYSATEETLQEVFEK

Amino acid sequence of human nucleolin. The parts of the sequence identified by NanoLC-MS/MS were underlined

1 MVKLAKAGKN QGDPKMMAPP PKEVEEDED EEMSEDEEDD SSGEEVIPQ KKGKKAATS
61 AKKVVVSPTK KVAATPAKK AAVTPGKAA ATPAKKTVTP AKAVTPGKK GATPGKALVA
121 TPGKKGAAIP AKGAKNGKNA KKEDSDEED DDSEDEEDD EDEDEDEI EPAAMKAAA
181 APASEDEDDE DDEDEDDEDD DEEDDSEEEA METTPAKGKK AAKVVPVKAK NVAEDEDEEE
241 DDEDEDDEDD EDEDEDDEDD DEEEEEEEEE EPVKEAPGKR KKEMAKQKAA PEAKKQKVEG
301 TEPTTAFNLF VGNLNFNSA PELKTGISDV FAKNDLAVVD VRIGMTRKFG YVDFESAEDL
361 EKALELTGLK VFGNEIKLEK PKGKDSKKER DARTLLAKNL PYKVTQDELK EVFEDAAEIR
421 LVSKDGKSKG IAYIEFKTEA DAEKTFEEKQ GTEIDGRSIS LYTGEGKQN QDYRGGKNST
481 WSGESKTLVL SNLSYSATEE TLQEVFEKAT FIKVPQNQNG KSKGYAFIEF ASFEDAKEAL
541 NSCNKREIEG RAIRLELQGP RGSPNARSQP SKTLFVKGLS EDTTEETLKE SFDGSVRARI
601 VTDRETSSK GFGFVDFNSE EDAAAKEAM EDGEIDGNKV TLDWAKPKGE GFGGGRGGGR
661 GFGGGRGGGR GGRGGFGGRG RGGFGGRGGF RGGRGGGGDH KPQKKTKFE