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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.

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1. Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a tick-born 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 [1–3]. 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, 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 αvβ3 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.

2. Materials and methods

2.1. 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 (Carlsabad, 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).

2.2. 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

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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.

2.3. 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% CO_2 incubator. The expression cell line 293 Freestyle was purchased from Invitrogen and cultured in 293 Freestyle medium (Invitrogen).

2.4. 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).

2.5. 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 1 h. Cell lysates were clarified by centrifugation at 13,000*g* 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 10^7 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 five 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 streptavidinr-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).

3. Results

3.1. 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 (Fig. 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 (Fig. 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.

3.2. Identification of a putative RBD domain

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 (Fig. 2). This is consistent with the indication that Gc is the primary binding moiety from the CCHFV [6].

3.3. 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 (Fig. 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 (Fig. 4A). Since nucleolin is predominantly a nucleolar protein, we further tested