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(54) Title: ENGINEERED SPIKE PROTEINS OF HANTAVIRUSES AND USES THEREOF

(57) Abstract: The present invention describes specific engineering of the hantavirus spike proteins with modifications to stabilize (Gn/Gc)_n heterodimer contacts and/or Gc homodimer contacts and/or Gn/Gc oligomer contacts on the spike with the purpose of using them as immunogens in next-generation vaccine design. Said spike proteins have been covalently stabilized by at least one disulphide inter-chain bond between Gn/Gc heterodimers and/or between Gc homodimers and/or between Gn homo-oligomers as they are presented at the surface of infectious virions. It also involves spike stabilization by introduction of cavity-filling amino acids with a bulky side chain at the above-mentioned contacts. Said spike proteins can be soluble Gn/Gc ectodomains in solution and/or incorporated as (Gn/Gc)_n hetero-oligomers onto virus-like particles (VLPs) and/or used for pseudotyping virus vectors and/or form part of a stabilized recombinant virus, wherein said spike proteins can be used to select ligands and/or can be used for preventing or treating infections by one or more hantaviruses.



ENGINEERED SPIKE PROTEINS OF HANTAVIRUSES AND USES THEREOF**FIELD OF THE INVENTION**

[001] The invention relates to the field of preventing and treating hantavirus infections and related antigens and uses.

BACKGROUND OF THE INVENTIONHantavirus disease

[002] Hantaviruses are worldwide spread pathogens that can cause severe disease in humans such as hantavirus pulmonary syndrome (HPS) and hemorrhagic fever with renal syndrome (HFRS). While the former disease occurs principally in America with mortality rates that can reach up to 50%, the latter produces yearly 150-200 thousand cases in Asia and in a milder version in Europe with lethality rates ranging from 1 to 15%. Among the most frequent HFRS-causing viruses, Hantaan virus is the prototype virus and is endemic in Asia, especially in China, Russia, and Korea. Several other HFRS-causing hantaviruses have been identified, among them the most frequent ones are Seoul virus that is endemic worldwide, Dobrava virus primarily in the Balkans and the Puumala virus producing weaker forms of HFRS in Scandinavia, western Europe and western Russia. The higher pathogenic Dobrava virus has been re-emerging during the last years and a permanent risk of expansion in Central Europe has been reported. In America, hantaviruses are the most lethal endemic viruses and have been emerging and re-emerging since 1993, being Sin Nombre virus the most frequent cause of HPS in North America and Andes virus in South America. In this context, the US National Institute of Allergy and Infectious Diseases (NIAID) from the National Institute of Health (NIH), has classified hantaviruses that cause hantavirus pulmonary syndrome as NIAID category A pathogens of highest priority. Such prioritized emerging pathogens represent the highest risk to the national security and public health, since they are easily transmitted from person to person, produce high mortality rates, might cause public panic and social disruption and require special action for public health preparedness in absence of effective therapeutic or prophylactic treatments against hantavirus diseases (NIH, 2016). Given that hantaviruses the hosts of are rodents and insectivores, their eradication is impossible. At the date of patent application, no therapeutic or prophylactic solutions (e.g. vaccines) approved by the NIH are available.

Hantavirus vaccines and antiviral treatments

[003] For hantaviruses that produce HFRS, there exist at least three different vaccine preparations based on formalin-inactivated virus produced suckling-mouse brains or in cell cultures that was only commercialized locally in China and South Korea (Maes et al., 2009). Besides a vaccination scheme consisting of three doses, the neutralizing antibody response has been reported to be short lived and the efficacy of the vaccine must be re-evaluated. These vaccines are not approved in the USA and other countries of America where the highest mortality rates through hantavirus disease exist due to the high risk that is involved in the production of such a vaccine in higher quantities.

[004] Another vaccine approach that has reached a clinical trial study corresponds to a DNA vaccine based on plasmids that encode the hantavirus Gn/Gc spike proteins of the hantaviruses Andes, Puumala, Hantaan and Seoul under the cytomegalovirus promotor. When in a phase I clinical trial these plasmids were administrated into the dermis of humans, neutralizing antibodies were induced in 30 to 56% of the volunteers (Boudreau et al. 2012). However, given that currently only one product has been approved to perform gene therapy in humans, and not a single DNA vaccine has yet been approved, it seems difficult that such an approach will reach acceptance to be used in healthy humans due to the risk of spontaneous integration of DNA into the human genome.

[005] In terms of antiviral treatments, broad spectrum antivirals such as Ribavirin have proven to be inefficient. However, another approach using plasma from surviving patients has been used in an open clinical trial in HPS patients in Chile from 2008 to 2012. In this trial, 29 out of 32 patients were treated and reached a mortality rate of 14%, compared to a 32% rate for non-treated patients during the same period and 28% for non-treated patients in the same geographic zone (Vial et al., 2015). While this treatment seemed safe, it is difficult to standardize given that 1) the titer of neutralizing antibodies in humans varies over time and 2) access to surviving patients' plasmas is generally scarce hence being a variable that is difficult to control. The scarceness of immune sera is the reason why it is currently only used in patients with severe symptoms.

[006] Overall, each here described preventive or therapeutic approach shows that there is an urgent need to develop improved solutions that can be established in the market.

Hantavirus structure

[007] Hantaviruses (*Bunyavirales* order) have a genome composed of three single strand RNA segments of negative polarity that encodes at least four structural proteins; the nucleocapsid (N) protein, the viral RNA-dependent RNA polymerase (RdRp) and the two envelope glycoproteins, Gn and Gc, that project from the virion as surface spikes. The virions are pleomorphic and heterogenic in size ranging from 120-160 nm and also elongated particles up to 350 nm in length have been reported. They contain helical capsids in the interior of the virion composed of N covering the three genomic segments associated to RdRp that are enveloped by a lipid membrane displaying the spikes composed of Gn and Gc. The spike proteins resemble hence the outer most proteins of hantavirus virions, and are therefore crucial to direct hantavirus entry into the cell and are at the same time key for virus recognition by the immune system for viral neutralization. In this context, the Gn/Gc are important antigens for the design of vaccines and effective antiviral treatments.

[008] Previous work by Husikonen et al. 2011 using cryo-electron tomography (Cryo-ET) of hantavirus virions demonstrated that the Gn/Gc spikes form a local four-fold quasi symmetry; however, the orientation of Gn and Gc within these spikes remained to be determined. The later structural characterization of Gn monomers and the improvement of the Cryo-ET map allowed Shi et al., 2016 the fitting of Gn monomers into the most distal volumes of the spike density map; however, the exact orientation and molecular contacts of Gn and Gc still awaited to be solved to obtain a molecular understanding of their assembly.

[0009] An additional difficulty for the development of preventive and therapeutic strategies represents the high metastability and of the hantaviral spikes. Such metastability has been previously reported for Dengue virus and human immunodeficiency virus spike proteins and seems to apply in general to all enveloped viruses since it is crucial for enveloped virus that their spikes can dissociate once the virus enters into a cell. More specifically, the metastability is associated with the pre-fusion conformation of the viral protein on infectious virions, in the case of hantaviruses this protein corresponds to the Gc glycoprotein. Once the virus bound to cellular receptors, it is uptaken into endosomes where under acidic pH the Gc fusion protein is activated, leading to consecutive conformational changes that expose the Gc fusion loops inserting into the endosomal membrane and lead irreversibly to a stable post-fusion conformation (Guardado-Calvo et al., 2016). Such irreversible pre-fusion to post-fusion transitions have been well described for viral fusion proteins with class I or class II folds, whereby the energy that is released to reach the ground state of these proteins is believed to drive the merger of the virus-cell membranes that allows the ingress of the viral nucleocapsids into the cell cytoplasm, resulting ultimately into the infection of the cell (Harrison, SC. 2015). In this line, for hantaviruses it has been well described that mildly low pH decreases their titer over 100 fold (Hepojoki et al., 2010).

[0010] In a wider context, from what is known from other viruses, the viral surfaces have a highly dynamic behavior that leads to the exposure of internal epitopes that are otherwise cryptic. For easy of description the inventors call the conformations opened and closed, in which the closed conformation represents the infectious conformation while the open conformation corresponds to non-infectious conformations (Rey & Lok, 2018). For hantaviruses, there is evidence that the cell culture adapted viruses present mostly open conformations since they are highly labile, losing their infectivity within little hours outside a host cell (Hardestam et al., 2007).

[0011] For the design of therapeutic or prophylactic strategies that block efficiently hantavirus infections, it is therefore of crucial importance to arrest the hantaviral spike arrangement in a conformation that resembles that of infectious virus particles (a closed conformation including Gc in its pre-fusion state). To solve this technical problem, it is imperative to know the molecular contacts that establish the infectious conformation of the hantaviral spikes. These contacts involve those of the Gn/Gc heterodimer, those of the Gc/Gc homodimer and those of the Gn/Gn homotetramer. In this context, the present invention discloses the molecular structure of the hantaviral spike lattices, the key amino acids involved in the molecular contacts, and amino acid residue modifications (e.g. substitutions) that significantly improve the hantaviral spike stability in their infectious conformation. Thus, the present invention provides new solutions for the design of improved therapeutic and preventive strategies against hantavirus infections.

SUMMARY OF THE INVENTION

[0012] The invention relates to an improved and stabilized recombinant hantaviral spike composed of at least one homodimer of mutants Gc, at least one heterodimer of a mutant Gc and a mutant Gn or at least one oligomer of mutants Gn, or a combination thereof.

[0013] The invention relates to the stabilized recombinant hantaviral spike of the present invention comprising at least one homodimer of mutants Gc having each at least one amino acid mutation (substitution) at a position selected from the group consisting of: 676, 677, 678, 679,

680, 681, 682, 683, 832, 833, 834, 835, 836, 837, 838, 839, 840, 841, 842, 843, 844, 845, 846, 847, 951, 952, 953, 954, 955, 956, 957 and 958, the indicated positions being determined by alignment with SEQ ID NO: 1 (using standard alignment online tool BLAST).

[0014] The invention relates to the stabilized recombinant hantaviral spike of the present invention wherein the at least one homodimer of mutants Gc is selected from the group consisting of: a homodimer of mutants Gc having each the substitution G838C, a homodimer of mutants Gc having each the substitution T839C, a homodimer of mutants Gc having each the substitution H953C, and a homodimer of mutants Gc having each the substitution H954C, and wherein the amino acid residues 838C, 839C, 953C and 954C are linked respectively to the amino acid residues 838C, 839C, 953C and 954C through disulphide inter-chain bonds.

[0015] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one homodimer of mutants Gc selected from the group consisting of: a homodimer of mutants Gc having each the double substitution H953C and Q844C, a homodimer of mutants Gc having each the double substitution H954C and Q844C, a homodimer of mutants Gc having each the double substitution S841C and R951C, a homodimer of mutants Gc having each the double substitution E677C and R951C, a homodimer of mutants Gc having each the double substitution D679C and H953C, and a homodimer of mutants Gc having each the double substitution D679C and H954C, and wherein the amino acid residues 677C, 679C, 841C, 844C, 951C, 953C and 954C are linked respectively to the amino acid residues 677C, 679C, 841C, 844C, 951C, 953C and 954C through disulphide inter-chain bonds between the two mutants Gc.

[0016] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one heterodimer of mutants Gn/Gc, wherein the mutant Gn monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 281, 290, 291, 292, 293, 294, 295, 296 and 297, and wherein the mutant Gc monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 729, 730, 731, 732, 733, 734, 735, 736, 737 and 748, the indicated positions being determined by alignment with SEQ ID NO: 1.

[0017] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one heterodimer of a mutant Gn having the substitution H294C and a mutant Gc having the substitution T734C, wherein the amino acid residues 294C and 734C are linked together through a disulphide inter-chain bond.

[0018] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one heterodimer of a mutant Gn having the substitution N290C and a mutant Gc having the substitution T729C, wherein the residues 290C and 729C are linked together through a disulphide inter-chain bond.

[0019] The invention relates to the stabilized recombinant hantaviral spike of the present invention, which comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98 and 99, the indicated positions being determined by alignment with SEQ ID NO: XXX, and wherein the mutant Gc monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 738, 739, 740, 741, 742, 743, 744, 745, 746, 747, 774,

775, 776, 777 and 778, the indicated positions being determined by alignment with SEQ ID NO: XXX.

[0020] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one heterodimer of a mutant Gn having the substitution K85C and a mutant Gc having the substitution P774C, wherein the residues 85C and 774C are linked together through a disulphide inter-chain bond.

[0021] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one heterodimer of a mutant Gn having the substitution N94C and a mutant Gc having the substitution V776C, wherein the residues 94C and 776C are linked together through a disulphide inter-chain bond.

[0022] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one heterodimer of a mutant Gn having the substitution A95C and a mutant Gc having the substitution Y739C, wherein the residues 95C and 739C are linked together through a disulphide inter-chain bond.

[0023] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one heterodimer of a mutant Gn having the substitution T99C and a mutant Gc having the substitution P774C, wherein the residues 99C and 774C are linked together through a disulphide inter-chain bond.

[0024] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one heterodimer of a mutant Gn having the substitution R281C and a mutant Gc having the substitution P748C, wherein the residues 281C and 748C are linked together through a disulphide inter-chain bond.

[0025] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one Gn/Gc heterodimer, wherein mutant Gn comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 203, 204, 205 and 206, and wherein Gc comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 853, 854 and 855, the indicated positions being determined by alignment with SEQ ID NO: 1.

[0026] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one heterodimer of a mutant Gn having the substitution H203C and a mutant Gc having the substitution L855C, wherein the residues 203C and 855C are linked together through a disulphide inter-chain bond.

[0027] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one heterodimer of a mutant Gn having the substitution D206C and a mutant Gc having the substitution P854C, wherein the residues 206C and 854C are linked together through a disulphide inter-chain bond.

[0028] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one homooligomer of a mutant Gn having at least one mutation (substitution) at a position selected from the group consisting of: 332, 333, 334, 335,

336, 337 and 338, and a mutant Gn having at least one mutation (substitution) at a position selected from the group consisting of: 177, 178, 179, 180, 181 and 182, the indicated positions being determined by alignment with SEQ ID NO: 1.

[0029] The invention relates to the stabilized recombinant hantaviral spike of the present invention which comprises at least one homooligomer of a mutant Gn having at least one mutation (substitution) at a position selected from the group consisting of: 332, 333, 334, 335, 336, 337 and 338, and a mutant Gn having at least one mutation (substitution) at a position selected from the group consisting of: 374, 375, 376, 377, 378, 379 and 380, the indicated positions being determined by alignment with SEQ ID NO: 1.

[0030] The invention relates to the stabilized recombinant hantaviral spike of the present invention which is in solution.

[0031] The invention relates to the stabilized recombinant hantaviral spike of the present invention incorporated onto virus-like particles.

[0032] The invention relates to the stabilized recombinant hantaviral spike of the present invention incorporated into the envelope of recombinant viruses, onto pseudotype virus vectors or any non-viral system.

[0033] The invention relates to a pharmacological composition comprising the stabilized hantaviral spike of the present invention.

[0034] The invention relates to a pharmacological composition comprising the stabilized recombinant hantaviral spike of the present invention incorporated onto virus-like particles.

[0035] The invention relates to the stabilized recombinant hantaviral spike or the pharmacological composition of the present invention for use in the preparation of a medicament effective in preventing or treating hantavirus infection.

[0036] The invention relates to a method for preventing and/or treating a hantaviral infection, comprising administering to a subject in need thereof the stabilized recombinant hantaviral spike or a pharmacological composition of the present, in an amount effective to inhibit hantaviral infection of susceptible cells so as to thereby prevent or treat the infection.

[0037] The invention relates to a diagnostic agent comprising or consisting of a stabilized recombinant hantaviral spike of the present invention and an appropriate diagnostic reagent.

[0038] The invention also relates to a kit for diagnosing or monitoring, in a subject, a hantaviral infection, comprising the stabilized recombinant spike of the present invention and an appropriate diagnostic reagent.

[0039] The invention also relates to a kit for treating and/or preventing hantavirus infections, comprising the stabilized spikes or the pharmacological composition that comprise the stabilized hantaviral spikes.

[0040] The invention also relates to the use of the stabilized spikes in the generation or selection of ligands useful to treat and/or prevent hantavirus infections.

[0041] The invention also relates to the use of the stabilized spikes for the identification of epitopes recognized by ligands useful to treat and/or prevent hantavirus infection.

[0042] The invention also relates to the use of the stabilized spikes for prepare monoclonal antibodies (in the case of mice) or immunoglobulin heavy and light chains from animals immunized with the selected Gn/Gc spike mutants. The invention also relates to the use of these antibodies to measure their virus neutralizing efficacy against the infectious virus and to determine their epitopes by X-ray crystallography or by Cryo-ET.

[0043] The invention also relates to the use of the stabilized spikes for assess the survival rate of animals that are vaccinated with the wt or mutant hantaviral spikes and subsequently challenged with Andes virus. Then, by following this methodological strategy, those Gn/Gc mutants can be selected that confer highest survival rates of the animals (Hooper et al., 2001).

[0044] The invention also relates to the use of the stabilized spikes for assess the efficiency of stabilized hantaviral spikes to deplete neutralizing antibodies from patient sera. For this, patient sera with high neutralizing titer are preincubated with wt or stabilized hantaviral spikes and then mixed with infectious hantaviruses on the surface of cells. After the virus-patient sera-spike mixture is removed and virus infectivity titrated as described in Barriga et al., 2016. Alternatively, we perform virus plaque reduction assays. Using this approach, we can select those stabilized spikes that reach highest infection rates.

DETAILED DESCRIPTION OF THE INVENTION

[0045] In the present invention, the inventors were seeking to prepare improved antigens resembling the hantavirus spikes in their infectious conformation, given the high lability of the native hantaviral spikes. Therefore, a novel hantavirus Gn/Gc structure, a Gc prefusion structure and their fitting into a Cryo-EM map was used to identify inter-chain contacts in order to design the improvement of the stability of inter-chain contacts, thereby arresting them into a determined conformation, avoiding conformational changes that dynamically occur on the surface of viruses outside host cells or during the infection of cells and decreasing the lability of the virus particle. Such increased stability is crucial for vaccine design because the antigen must present epitopes that occur on infectious viruses to allow B cell stimulation and as a consequence, the production of virus neutralizing antibodies.

[0046] Therefore, the present invention comprises engineered hantaviral spikes in which the stability of spike proteins has been improved by either introducing at least one disulphide inter-chain bond and/or by at least one cavity-filling amino acids with bulky residues such as for example phenylalanine. The introduction of site-specific residue substitutions into the hantaviral spikes has been designed at inter-chain contacts of the (Gn/Gc)₄ heterodimer and between the (Gn/Gc)₄ heterodimers, consisting specifically of contacts between the Gn/Gc heterodimer, the Gc/Gc homodimer and the Gn/Gn oligomer. The advantage of stabilized spike proteins for the design of vaccines and therapeutics is given by arresting them in a conformation that resembles that of infectious hantavirus virions, thereby avoiding other conformations that are inefficient or ineffective in generating protective immune responses.

[0047] The invention refers to the stabilization of the hantaviral spike through at least one residue substitution or several substitutions at a single contact interphase or different inter-chain contact interphases, which are treated for clarity in separate embodiments, and can be used separately as well as in any combination. Based on the conserved structure of the hantavirus spike proteins said substitution characterized in the Andes virus species, can be easily transferred to analogous positions in any other species of the hantavirus family, including among others, Dobrava virus, Puumala virus, Sin Nombre virus, Hantaan virus and Seoul virus (see Figure 1 for analogous positions).

[0048] For the design of the inter-chain contact-stabilizing residue substitutions, the present invention describes novel structures of the hantaviral spike, and novel contact interphases that allowed for the design of contact-stabilizing modifications. The secondary structure elements of these structures are summarized in Figure 1, while the individual structures and contacts are shown in Figures 2-4.

[0049] The present invention relates to a stabilized improved recombinant hantaviral spike composed of at least one homodimer of mutants Gc, at least one heterodimer of a mutant Gc and a mutant Gn or at least one oligomer of mutants Gn, or a combination thereof.

Gc/Gc homodimer

[0050] In a first aspect of the stabilized recombinant hantaviral spike of the invention, the amino acid modifications suitable to improve the hantaviral spike stability correspond to any residue at the two-fold molecular axis of the Gc/Gc homodimer, that comprise different regions of the Gc protein (see nomenclature Figure 1). Specifically, the invention refers to amino acids comprised of the Gc strands B₀ (amino acids L676-P683), H₀ (amino acids V832-V837), I₀ (amino acids R951-L958) and the linker strand H₀ of Gc domain I and the f strand of Gc domain II comprising amino acids G838-D847 (Figure 1; Figure 2A,B). Preferably, any of the amino acids contained in these regions can be substituted by C (Cys, Cysteine) or any other modification such as A (Ala, Alanine), L (Leu, Leucine), V (Val, Valine), I (Ile, Isoleucine), W (Trp, Tryptophane), Y (Tyr, Tyrosine), F (Phe, Phenylalanine), P (Pro, Proline), M (Met, Methionine), S (Ser, Serine), G (Gly, Glycine), N (Asn, Asparagine), Q (Gln, Glutamine), T (Thr, Threonine), E (Glu, Glutamic acid), D (Asp, Aspartic acid), H (His, Histidine), K (Lys, Lysine) and/or R (Arg, Arginine).

[0051] The present invention relates to a stabilized recombinant hantaviral spike comprising at least one homodimer of mutants Gc having each at least one amino acid mutation (substitution) at a position selected from the group consisting of: 676, 677, 678, 679, 680, 681, 682, 683, 832, 833, 834, 835, 836, 837, 838, 839, 840, 841, 842, 843, 844, 845, 846, 847, 951, 952, 953, 954, 955, 956, 957 and 958, the indicated positions being determined by alignment with SEQ ID NO: 1.

[0052] In some preferred embodiments, at least one amino acid residue at positions 676, 677, 678, 679, 680, 681, 682, 683, 832, 833, 834, 835, 836, 837, 838, 839, 840, 841, 842, 843, 844, 845, 846, 847, 951, 952, 953, 954, 955, 956, 957 and/or 958 is substituted by an amino acid residue selected from the group consisting of: C, A, L, V, I, W, Y, F, P, M, S, G, N, Q, T, E, D, H, K and R.

[0053] According to an additional embodiment, preferred amino acids for introducing site-directed modifications correspond to E677, D679, G838, T839, S841, R951, H953 and H954. The present invention relates to a stabilized hantaviral spike comprising at least one homodimer of mutants Gc having each at least one amino acid mutation (substitution) at a position selected from the group consisting of: E677, D679, G838, T839, S841, R951, H953 and H954, the indicated positions being determined by alignment with SEQ ID NO: 1.

[0054] In a preferred embodiment, the amino acid modifications corresponds to one or more substitutions by C (Cys, Cysteine) and/or any other bulky side chain amino acid residue such as T (Tyr, Tyrosine), W (Trp, Tryptophan), M (Met, Methionine), L (Leu, Leucine) or F (Phe, Phenylalanine) and combinations thereof. In the case of C (Cys, Cysteine) substitutions, one or several disulfide bonds can covalently join the Gc monomers at their two-fold axis into Gc dimers. Said preferred stabilized Gc dimers correspond to the single substitutions G838C and/or T839C and/or H953C, and/or H953F, and/or H954C, and/or H954F, and/or the Gc double substitution Gc H953C/Q844C, H954C/Q844C, S841C/R951C, E677C/R951C, D679C/H953C and/or D679C/H954C.

[0055] In some embodiments, the stabilized recombinant hantaviral spike comprises at least one homodimer of mutants Gc selected from the group consisting of: a homodimer of mutants Gc having each the substitution G838C, a homodimer of mutants Gc having each the substitution T839C, a homodimer of mutants Gc having each the substitution H953C, a homodimer of mutants Gc having each the substitution H954C, and wherein the amino acid residues 838C, 839C, 953C and 954C are linked respectively to the amino acid residues 838C, 839C, 953C and 954C through disulphide inter-chain bonds.

[0056] In a preferred embodiment, the stabilized recombinant hantaviral spike comprises at least one homodimer of mutants Gc having each the substitution G838C, wherein the amino acid residues 838C are linked together through a disulphide inter-chain bond.

[0057] In a preferred embodiment, the stabilized recombinant hantaviral spike comprises at least one homodimer of mutants Gc having each the substitution T839C, wherein the amino acid residues 839C are linked together through a disulphide inter-chain bond.

[0058] In a preferred embodiment, the stabilized recombinant hantaviral spike comprises at least one homodimer of mutants Gc having each the substitution H953C, wherein the amino acid residues 953C are linked together through a disulphide inter-chain bond.

[0059] In a preferred embodiment, the stabilized recombinant hantaviral spike comprises at least one homodimer of mutants Gc having each the substitution H954C, wherein the amino acid residues 954C are linked together through a disulphide inter-chain bond.

[0060] In some embodiments, the stabilized hantaviral spike comprises at least one homodimer of mutants Gc selected from the group consisting of: a homodimer of mutants Gc having each the double substitution Q844C and H953C, a homodimer of mutants Gc having each the double substitution Q844C and H954C, a homodimer of mutants Gc having each the double substitution S841C and R951C, a homodimer of mutants Gc having each the double substitution E677C and R951C, a homodimer of mutants Gc having each the double substitution D679C and H953C, and

a homodimer of mutants Gc having each the double substitution D679C and H954C, and wherein the each double substitution is linked respectively through disulphide inter-chain bonds between the two mutants Gc.

[0061] In some embodiments, the stabilized hantaviral spike is a homodimer of mutants Gc selected from the group consisting of: a homodimer of mutants Gc having each the substitution G838C, a homodimer of mutants Gc having each the substitution T839C, a homodimer of mutants Gc having each the substitution H953C, a homodimer of mutants Gc having each the substitution H954C, a homodimer of mutants Gc having each the double substitution H953C and Q844C, a homodimer of mutants Gc having each the double substitution H954C and Q844C, a homodimer of mutants Gc having each the double substitution S841C and R951C, a homodimer of mutants Gc having each the double substitution E677C and R951C, a homodimer of mutants Gc having each the double substitution D679C and H953C, and a homodimer of mutants Gc having each the double substitution D679C and H954C, and wherein each double substitution is linked respectively together across the dimer interface through disulphide bonds between the double substitutions in Gc.

[0062] In some embodiments, the stabilized hantaviral spike comprises at least one homodimer of mutants Gc having each the substitution H953F.

[0063] In some embodiments, the stabilized hantaviral spike is a homodimer of mutants Gc having each the substitution H953F.

[0064] In some embodiments, the stabilized hantaviral spike comprises at least one homodimer of mutants Gc having each the substitution H954F.

[0065] In some embodiments, the stabilized hantaviral spike is a homodimer of mutants Gc having each the substitution H954F.

Gn/Gc heterodimer

[0066] In a second aspect, the invention refers to the stabilization of hantaviral spike by modifications that comprise the Gn/Gc heterodimer contacts (see nomenclature Figure 1) and can correspond to amino acid modifications through substitution by C (Cys, Cysteine), A (Ala, Alanine), L (Leu, Leucine), V (Val, Valine), I (Ile, Isoleucine), W (Trp, Tryptophane), Y (Tyr, Tyrosine), F (Phe, Phenylalanine), P (Pro, Proline), M (Met, Methionine), S (Ser, Serine), G (Gly, Glycine), N (Asn, Asparagine), Q (Gln, Glutamine), T (Thr, Threonine), E (Glu, Glutamic acid), D (Asp, aspartic acid), H (His, Histidine), K (Lys, Lysine) and/or R (Arg, Arginine). The preferred regions of the Gn/Gc contact stabilization comprise three different contact areas which are all preferred and are here mentioned in separate embodiments.

[0067] One of the preferred embodiments refers to modifications of any amino acid residue of Gn N290-I297 comprised in Gn by: 1) the Gn linker located between helix 2 and the A₈ beta strand, and 2) the region spanned by the Gn A₈ beta strand in combination with any amino acid residue from the Gc a/b strand, comprising T729-H737. In a preferred embodiment, the amino acid modifications correspond to any C (Cys, cysteine) substitutions including Gn H294C/Gc T734C and/or Gn N290C/Gc T729C.

[0068] In some embodiment, the invention relates to a stabilized recombinant hantaviral spike comprising at least one heterodimer of mutants Gn/Gc, wherein the mutant Gn monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 281, 290, 291, 292, 293, 294, 295, 296 and 297, the indicated positions being determined by alignment with SEQ ID NO: 1, and wherein the mutant Gc monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 729, 730, 731, 732, 733, 734, 735, 736, 737 and 748, the indicated positions being determined by alignment with SEQ ID NO: 1.

[0069] In some embodiment, the stabilized recombinant hantaviral spike comprises at least one heterodimer of a mutant Gn having the substitution H294C and a mutant Gc having the substitution T734C, wherein the amino acid residues 294C and 734C are linked together through a disulphide inter-chain bond.

[0070] In some embodiment, the stabilized recombinant hantaviral spike is a heterodimer of a mutant Gn having the substitution H294C and a mutant Gc having the substitution T734C, wherein the residues 294C and 734C are linked together through a disulphide inter-chain bond.

[0071] In some embodiment, the stabilized hantaviral spike comprises at least one heterodimer of a mutant Gn having the substitution N290C and a mutant Gc having the substitution T729C, wherein the residues 290C and 729C are linked together through a disulphide inter-chain bond.

[0072] In some embodiment, the stabilized hantaviral spike is a heterodimer of a mutant Gn having the substitution N290C and a mutant Gc having the substitution T729C, wherein the residues 290C and 729C are linked together through a disulphide inter-chain bond.

[0073] In another embodiment, the modifications include any amino acid residue contained in the Gn loop between strands D_A and E_A that comprises Gn K85-T99 with any Gc amino acid residue either from the Gc cd loop comprising P774-T778, and/or from Gc bc loop comprising C738-Y747. Preferably, the modifications correspond to C (Cys, cysteine) substitutions comprising Gn K85C/Gc P774C and/or Gn N94C/Gc V776C and/or Gn A95C/Gc Y739C and/or and/or T99C/Gc P774C.

[0074] In some embodiment, the invention relates to a stabilized recombinant hantaviral spike comprising at least one Gn/Gc heterodimer, wherein the mutant Gn monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98 and 99, and wherein the mutant Gc monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 738, 739, 740, 741, 742, 743, 744, 745, 746, 747, 774, 775, 776, 777 and 778, the indicated positions being determined by alignment with SEQ ID NO: 1.

[0075] In some embodiment, the stabilized recombinant hantaviral spike comprises at least one heterodimer of a mutant Gn having the substitution K85C and a mutant Gc having the substitution P774C, wherein the residues 85C and 774C are linked together through a disulphide inter-chain bond.

[0076] In some embodiment, the stabilized recombinant hantaviral spike comprises at least one heterodimer of a mutant Gn having the substitution N94C and a mutant Gc having the

substitution V776C, wherein the residues 94C and 776C are linked together through a disulphide inter-chain bond.

[0077] In some embodiment, the stabilized recombinant hantaviral spike comprises at least one heterodimer of a mutant Gn having the substitution A95C and a mutant Gc having the substitution Y739C, wherein the residues 95C and 739C are linked together through a disulphide inter-chain bond.

[0078] In some embodiment, the stabilized hantaviral spike comprises at least one heterodimer of a mutant Gn having the substitution T99C and a mutant Gc having the substitution P774C, wherein the residues 99C and 774C are linked together through a disulphide inter-chain bond.

[0079] In some embodiment, the stabilized hantaviral spike comprises at least one heterodimer of a mutant Gn having the substitution T99C and a mutant Gc having the substitution P744C, wherein the residues 99C and 744C are linked together through a disulphide inter-chain bond.

[0080] In some embodiment, the stabilized hantaviral spike comprises at least one heterodimer of a mutant Gn having the substitution R281C and a mutant Gc having the substitution P748C, wherein the residues 281C and 748C are linked together through a disulphide inter-chain bond.

[0081] In another embodiment, the modifications include any amino acid residue comprised by Gn H203-D206 comprised in the Gn bc loop in combination with any amino acid residue comprised between Gc G853-L855 located in the Gc fg loop. In a preferred embodiment, the modification corresponds to C (Cys, cysteine) substitutions comprising Gn H203C/Gc L855C and/or Gn D206C/Gc P854C.

[0082] In some embodiment, the invention relates to a stabilized recombinant hantaviral spike comprising at least one Gn/Gc heterodimer, wherein the mutant Gn monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 203, 204, 205 and 206, and wherein the Gc monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 853, 854 and 855, the indicated positions being determined by alignment with SEQ ID NO: 1.

[0083] In some embodiment, the stabilized hantaviral spike comprises at least one heterodimer of a mutant Gn having the substitution H203C and a mutant Gc having the substitution L855C, wherein the residues 203C and 855C are linked together through a disulphide inter-chain bond.

[0084] In some embodiment, the stabilized hantaviral spike comprises at least one heterodimer of a mutant Gn having the substitution D206C and a mutant Gc having the substitution P854C, wherein the residues 206C and 854C are linked together through a disulphide inter-chain bond.

Gn/Gn homooligomer

[0085] In a further embodiment, the invention refers also to the hantaviral spike stabilization by improving Gn/Gn homooligomeric contacts (see nomenclature Fig.1) by amino acid modifications that comprise:

[0086] The Gn bc loop P192-D206 comprised between beta strands b and c of one Gn protomer in combination with the region K59-Q75 in the other Gn protomer containing the CA strand and a region comprised between the C_α strand and the D_α strand.

[0087] In some embodiment, the stabilized recombinant hantaviral spike comprises at least one homooligomer of a mutant Gn having at least one mutation (substitution) at a position selected from the group consisting of: 192, 192, 193, 294, 195, 196, 197, 198, 199, 200, 201, 202, 203, 203, 204, 204, 206, and a mutant Gn having at least one mutation (substitution) at a position selected from the group consisting of: 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 79, 70, 71, 72, 73, 74 and 75, the indicated positions being determined by alignment with SEQ ID NO: 1.

[0088] More preferable, the modifications correspond to single or multiple modifications in any combination, comprising residues E61-Q200. In a more preferable embodiment, modifications include residues E61 and Q200 by simple or multiple modifications such as through substitution by C (Cys, Cysteine), A (Ala, Alanine), L (Leu, Leucine), V (Val, Valine), I (Ile, Isoleucine), W (Trp, Tryptophan), Y (Tyr, Tyrosine), F (Phe, Phenylalanine), P (Pro, Proline), M (Met, Methionine), S (Ser, Serine), G (Gly, Glycine), N (Asn, Asparagine), Q (Gln, Glutamine), T (Thr, Threonine), E (Glu, Glutamic acid), D (Asp, aspartic acid), H (His, Histidine), K (Lys, Lysine) and/or R (Arg, Arginine). In a still more preferred embodiment, the modifications correspond to double residues substitutions by C (Cys, cysteine), resulting in Gn E61C/Q200C.

[0089] In certain embodiment, the invention refers to stabilized spike proteins that can correspond to soluble Gn and/or Gc ectodomains in solution. Such an ectodomain of Gn and/or Gc typically does not include the well-defined transmembrane anchors nor the Gn and/or Gc endodomains.

[0090] In another embodiment, the stabilized spike proteins are incorporated onto virus-like particles (VLPs).

[0091] In a further embodiment, the stabilized hantaviral spike proteins are used to pseudotype virus vectors. Said virus vector can correspond to any virus vector, such as those of the family *Retroviridae*, and *Vesiculoviridae* or any other virus family.

[0092] In another embodiment, the stabilized spike proteins are incorporated into the envelope of recombinant viruses. Said recombinant viruses can correspond to viruses from the family *Hantaviridae*, *Vesiculoviridae*, *Togaviridae* or any family of the *Bunyavirales* order, or any other family.

[0093] In another embodiment, the stabilized hantavirus spikes of the invention can include additional amino acids at their N- and C-terminals, such as those used for protein purification and/or protein sorting and/or specific enzymatic digestions and/or as part of a fusion protein. Also, the stabilized hantaviral spike proteins can include amino acid deletions at their extreme N- and C-terminals.

[0094] In another preferred embodiment, the invention refers to stabilized spike proteins codified by nucleotide sequences alone and/or a sequence incorporated into a vector (e.g. viral vectors, plasmids) where said vector is used to be introduced into a cell.

[0095] In another preferred embodiment, the invention refers to stabilized spike proteins that can be used for preventing or treating infections by one or more hantaviruses. Said spike proteins can be used for *in vivo* administration to induce protective immune responses. Such immune responses can include neutralizing antibodies that can be used to prevent or treat hantavirus infections.

[0096] In a further embodiment, the stabilized hantaviral spikes can be used to select ligands that block hantavirus infection.

[0097] In a preferred embodiment, said stabilized hantaviral spikes can be immobilized on any resin and/or any surface and/or any substrate and/or any other known technique for this purpose, in order to identify new ligands from libraries such as those obtained from immunoglobulin heavy and light chain libraries from B cells and/or from aptamer (oligonucleotide) libraries and/or any other libraries and/or other random ligands, including any molecule that can be selected by binding to the stabilized recombinant hantavirus spikes.

[0098] In another embodiment, said stabilized hantaviral spikes can be immobilized on any resin and/or any surface and/or any substrate and/or any other known technique for this purpose, in order to characterize the mode of binding of ligands such as monoclonal antibodies.

[0099] In another embodiment, the invention refers to a pharmacological composition that includes said stabilized recombinant hantaviral spike.

[00100] The terms "pharmacological composition", "vaccine composition", "immunogenic composition" and "pharmaceutical formula" are used interchangeably herein.

[00101] Advantageous said pharmacological composition further comprises a pharmaceutically acceptable excipient, diluent, adjuvant, or carrier.

[00102] As used herein, a "pharmaceutically acceptable excipient, diluent, adjuvant or carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Suitable carriers are described in the most recent edition of Remington's Pharmaceutical Sciences, a standard reference text in the field. Preferred examples of such carriers or diluents include, but are not limited to, water, saline, glycerol, ethanol, Ringer's solutions, dextrose solution, and 5% human serum albumin. Liposomes, cationic lipids and non-aqueous carrier such as fixed oils may also be used. Additionally auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, may be present in such carrier. The use of such media and compounds for pharmaceutically active substances is well known in the art. Except insofar as any such conventional media or compound is incompatible with a therapeutic agent of the present invention, use thereof in a composition of the present invention is contemplated. The pharmaceutically acceptable carrier can be a non-naturally occurring pharmaceutically acceptable carrier.

[00103] Due to the high structural and functional conservation of the hantaviral spike proteins Gn and Gc, it is expected that the pharmaceutical composition is useful in preventing and treating the infection by viruses from the *Hantaviridae* family. These include classified hantaviruses such as the Andes virus, the Araraquara virus, the Bayou virus, the Bermejo virus,

the Black Creek Canal virus, the Caño Delgadito virus, the Choclo virus, the Dobrava-Belgrade virus, the El Moro Canyon virus, the Hantaan virus, the Khabarovsk virus, the Laguna Negra virus, the Lechiguanas virus, the Maciel virus, the Maporal virus, the Muju virus, the New York virus, the Oran virus, the Pergamino virus, the Prospect Hill virus, the Puumala virus, the Rio Mamore virus, the Sangassou virus, the Seoul virus, the Sin Nombre virus, the Topografov virus, and the Tula virus. This genus also includes unclassified hantaviruses, such as the Asama virus, the Catacamas virus, the Cao virus, the Castelo dos Sonhos virus, the Gou virus, the Hokkaido virus, the Fusong-Mf-682 virus, the Limestone Canyon virus, the human/hrp/02-72/bra/2002 hantavirus, hantavirus CGRn8316, hantavirus CGRn9415, hantavirus Jurong, hantavirus AH09, hantavirus Z10, hantavirus Liu, the Montano virus, the Monongahela-2 virus, the Necocli virus, the Oxbow virus, the Rockport virus, the Soochong virus, and the Yuanjiang virus. This classification is in line to the established classification by the International Committee on Taxonomy of Viruses, at the moment of the priority date invoked for this invention.

[00104] In preferred embodiments, the stabilized hantaviral spike proteins and/or a pharmaceutical formula and/or pharmaceutical composition can be used *in vitro* (e.g. cell culture) or *in vivo*, preferably administering them to a living eukaryotic organism. Preferably, the eukaryotic organism is a mammal, and still more preferably, the organism corresponds to a human. The stabilized recombinant hantaviral spike of the present invention and/or the pharmacological composition, which induces neutralizing antibodies against hantaviral infection, is administered to a mammal subject, preferably a human, in an amount sufficient to prevent, treat or attenuate the severity, extent of duration of the infection by hantavirus.

[00105] The therapeutically effective amount varies depending on the subject being treated, the age and general condition of the subject being treated, the capacity of the subject's immune response to synthesize antibodies, the degree of protection desired, the severity of the condition to be treated, the particular stabilized recombinant hantaviral spike selected and its mode of administration, among other factors. An appropriate effective amount can be readily determined by one of skill in the art. A therapeutically effective amount will fall in a relatively broad range that can be determined through routine trials.

[00106] Typically, the pharmacological composition is prepared as an injectable form (either a liquid solution or suspension) or as a solid form suitable for solution or suspension in a liquid carrier prior to injection. The preparation may be emulsified or encapsulated in liposomes for enhanced adjuvant effect.

[00107] Once formulated, the pharmacological composition may be administered parenterally, by injection, such as intravenous, intraperitoneal, intramuscular, intradermal or subcutaneous injection.

[00108] Alternative formulations suitable for other mode of administration include oral and intranasal formulations.

[00109] In a preferred embodiment, the invention uses any of the stabilized recombinant hantaviral spike proteins described above because they enable the preparation of a medicament effective in preventing or treating hantavirus infections.

[00110] The present invention also provides a method for preventing and/or treating a hantavirus infection, comprising administering to a subject in need thereof a stabilized recombinant hantaviral spike or a pharmacological composition as defined above, in an amount effective to inhibit hantavirus infection of susceptible cells so as to thereby prevent or treat the infection.

[00111] The term "treating" includes the administration of a stabilized recombinant hantaviral spike or a pharmacological composition of the present invention to a patient who has a hantavirus infection or a symptom of hantavirus infection, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the hantavirus infection and/or the symptoms of the hantavirus infection.

[00112] The term "preventing" means that the progression of a hantavirus infection is reduced and/or eliminated, or that the onset of a hantavirus infection is delayed or eliminated. The invention also includes an embodiment in which the above described hantaviral spike proteins can be used for diagnostic purposes to detect hantavirus infections.

[00113] The present invention provides a diagnostic agent comprising or consisting of a stabilized recombinant hantaviral spike according to the present invention.

[00114] The present invention also provides a kit for diagnosing or monitoring, in a subject, a hantaviral infection, comprising a stabilized recombinant spike according to the present invention and an appropriate diagnostic reagent.

[00115] The appropriate diagnostic reagent is necessary for performing an assay for diagnosing or monitoring, in a subject, a hantavirus infection. The appropriate diagnostic reagent can be a solvent, a buffer, a dye, an anticoagulant.

DEFINITIONS

[00116] In the description, the residues are designated by the standard one letter amino acid code and the indicated positions are determined by alignment with SEQ ID NO: 1 corresponding to the glycoprotein precursor in which amino acids 1-650 comprise the Gn sequence while amino acids 651-1138 comprise the Gc sequence. . For example, G838C is the G (Gly, Glycine) residue at position 838 of SEQ ID NO: 1. Substitutions are designated herein by the one letter amino acid code followed by the substituting residue in one letter amino acid code; G838C is a substitution of the glycine (G) residue at position 838 of SEQ ID NO: 1 with a Cysteine acid (C) residue.

[00117] By "comprises at least one substitution", it is meant that the hantaviral recombinant spike of the present invention has one or more amino acid substitutions as indicated with respect to the amino acid sequence SEQ ID NO: 1 for mutant Gc and/or mutant Gn, but may have other modifications, including with no limitation substitutions, deletions or additions of amino acid residues. The mutant Gc and/or the mutant Gn can comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or all of the substitutions listed above. All of these possible combinations are specifically contemplated.

[00118] As used herein, the terms "a", "an" and "the" include plural referents, unless the context clearly indicates otherwise. As such, the term "a" (or "an"), "one or more" or "at least one" can be used interchangeably herein.

[00119] As used herein, the term "recombinant" refers to the use of genetic engineering methods (cloning, amplification) to produce the mutant hantaviral Gc and Gn.

[00120] By "stabilized hantaviral spike" or "stabilized hantaviral spike protein" it is meant a that the viral spikes have been improved concerning their thermal resistance and/or acid resistance and/or exposure of otherwise cryptic region, such as the viral Gc fusion loops.

[00121] By "Gc/Gc homodimer", it is meant a dimer of two identical recombinant hantaviral mutants Gc ectodomains proteins.

[00122] By mutant "Gn/Gc heterodimer", it is meant a dimer of a mutant Gn ectodomain having at least one amino acid substitution as defined above and a mutant Gc ectodomain having at least one amino acid substitution as defined above.

[00123] By "Gn/Gn homooligomer", it is meant a dimer of two identical mutants Gn ectodomain having each at least one amino acid substitution as listed above.

[00124] By "inter-chain bonds" it is meant a bond that is formed across an interface formed by two proteins chains, that can be identical or different.

[00125] VLP: The term VLP is an abbreviation for virus-like particle. Hantavirus VLPs are viral particles that resemble those of the native hantaviruses in both, structural and antigenic terms. This type of particle consists of a lipid bilayer membrane in which Gn/Gc glycoproteins are anchored. The VLPs used here lack other viral proteins and viral RNA, and were prepared as described in Chilean patent application CL01085-2011: by expressing viral Gn/Gc glycoproteins in 293FT cells and purifying them from the supernatant of transfected cells by ultracentrifugation.

[00126] Gc fusion loops: The Gc membrane fusion protein contains at the tip of its structure a region that inserts after Gc activation into target lipid membranes. This region is composed of three loop regions of which each exposes at least one aromatic residue (Guardado-Calvo et al., 2016).

[00127] Cryo-ET map: The term cryo-ET map is an abbreviation for cryo-electron tomography map. It refers to a three-dimensional electron density map that has been obtained by imaging technique to produce high-resolution three-dimensional views of a specimen obtained by reconstruction of series of 2D images during tilting of a grid examined by cryo-electron microscopy.

[00128] The present invention comprise among others technical features:

A stabilized hantaviral spike comprising at least one homodimer of mutants Gc, or at least one heterodimer of a mutant Gn and a mutant Gc, or at least one oligomer of mutants Gn, or a combination thereof.

A stabilized hantaviral spike according to Claim 1, comprising at least one homodimer of mutants Gc having each at least one amino acid mutation (substitution) at a position selected from the group consisting of: 676, 677, 678, 679, 680, 681, 682, 683, 832, 833, 834, 835, 836, 837, 838, 839, 840, 841, 842, 843, 844, 845, 846, 847, 951, 952, 953, 954, 955, 956, 957 and 958, the indicated positions being determined by alignment with SEQ ID NO: 1.

The stabilized hantaviral spike according to claim 2, comprising at least one homodimer of mutants Gc is selected from the group consisting of: a homodimer of mutants Gc having each the substitution G838C (SEQ ID NO: 2), a homodimer of mutants Gc having each the substitution T839C (SEQ ID NO: 3), a homodimer of mutants Gc having each the substitution H953C (SEQ ID NO: 4), and a homodimer of mutants Gc having each the substitution H953F (SEQ ID NO: 5), wherein the amino acid residues 838C, 839C and 953C are linked respectively to the amino acid residues 838C, 839C and 953C through disulphide inter-chain bonds.

The stabilized hantaviral spike according to claim 3, wherein each mutant Gc of the at least one homodimer of mutants Gc has the substitution G838C (SEQ ID NO: 2), wherein the amino acid residues 838C are linked together through a disulphide inter-chain bond.

The stabilized hantaviral spike according to claim 3, wherein each mutant Gc of the at least one homodimer of mutants Gc has the substitution T839C (SEQ ID NO: 3), wherein the amino acid residues 839C are linked together through a disulphide inter-chain bond.

The stabilized hantaviral spike according to claim 3, wherein each mutant Gc of the at least one homodimer of mutants Gc has the substitution H953C (SEQ ID NO: 4), wherein the amino acid residues 953C are linked together through a disulphide inter-chain bond.

The stabilized hantaviral spike according to claim 3, wherein each mutant Gc of the at least one homodimer of mutants Gc has the substitution H953F (SEQ ID NO: 5).

The stabilized hantaviral spike according to claim 3, wherein each mutant Gc of the at least one homodimer of mutants Gc has the double substitution Q844C/H953C (SEQ ID NO: 6), wherein the amino acid residues 844C and 953C are linked respectively to the amino acid residues 844C and 953C through a disulphide inter-chain bond.

The stabilized hantaviral spike according to claim 1, comprising at least one heterodimer of mutants Gn/Gc, wherein the mutant Gn monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 281, 290, 291, 292, 293, 294, 295, 296 and 297, the indicated positions being determined by alignment with SEQ ID NO: 1; and wherein the mutant Gc monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 729, 730, 731, 732, 733, 734, 735, 736, 737 and 748, the indicated positions being determined by alignment with SEQ ID NO: 1.

The stabilized hantaviral spike according to claim 9, which comprises at least one heterodimer of a mutant Gn having the substitution H294C and a mutant Gc having the substitution T734C (SEQ ID NO: 7), wherein the amino acid residues 294C and 734C are linked together through a disulphide inter-chain bond.

The stabilized hantaviral spike according to claim 9, which comprises at least one heterodimer of a mutant Gn having the substitution R281C and a mutant Gc having the substitution P748C (SEQ ID NO: 8), wherein the residues 281C and 748C are linked together through a disulphide inter-chain bond.

The stabilized hantaviral spike according to claim 1, comprising at least one heterodimer of mutants Gn/Gc, wherein the mutant Gn monomer which comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 61, 85, 86, 87, 88,

89, 90, 91, 92, 93, 94, 95, 96, 97, 98 and 99, the indicated positions being determined by alignment with SEQ ID NO: 1; and wherein the mutant Gc monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 738, 739, 740, 741, 742, 743, 744, 745, 746, 747, 748, 774, 775, 776, 777 and 778, the indicated positions being determined by alignment with SEQ ID NO: 1.

The stabilized hantaviral spike according to claim 12, which comprises at least one heterodimer of a mutant Gn having the substitution T99C and a mutant Gc having the substitution P774C (SEQ ID NO: 9), wherein the residues 99C and 774C are linked together through a disulphide inter-chain bond.

The stabilized hantaviral spike according to claim 12, which comprises at least one heterodimer of a mutant Gn having the substitution K85C and a mutant Gc having the substitution P774C (SEQ ID NO: 10), wherein the residues 85C and 774C are linked together through a disulphide inter-chain bond.

The stabilized hantaviral spike according to claim 12, which comprises at least one heterodimer of a mutant Gn having the substitution N94C and a mutant Gc having the substitution V776C (SEQ ID NO: 11), wherein the residues 85C and 774C are linked together through a disulphide inter-chain bond.

The stabilized hantaviral spike according to claim 1, which comprising at least one Gn/Gc heterodimer, wherein the mutant Gn monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 203, 204, 205 and 206, the indicated positions being determined by alignment with SEQ ID NO: 1; and wherein the Gc monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 853, 854 and 855, the indicated positions being determined by alignment with SEQ ID NO: 1.

The stabilized hantaviral spike according to claim 1, which comprises at least one homooligomer of a mutant Gn having at least one mutation (substitution) at a position selected from the group consisting of: 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74 and 75; and a mutant Gn having at least one mutation (substitution) at a position selected from the group consisting of: 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205 and 206, the indicated positions being determined by alignment with SEQ ID NO: 1.

The stabilized hantaviral spike according to claim 16, which comprises at least one homooligomer of mutants Gn having each the double substitution E61C/Q200C (SEQ ID NO: 12), wherein the amino acid residues 61C and 200C are linked respectively to the amino acid residues 61C and 200C through disulphide inter-chain bonds between the two mutants Gn.

The stabilized hantaviral spike according to any one of claims 1 to 18, wherein the spike is in solution.

The stabilized hantaviral spike according to any one of claims 1 to 19, wherein the spike is incorporated into the envelope of recombinant viruses, pseudotype virus vectors, virus-like particles or any non-viral system.

The stabilized hantaviral spike according to claims 20, wherein the spike is incorporated onto virus-like particles.

A pharmacological composition comprising the stabilized hantaviral spike according to any one of claims 1 to 21.

The stabilized hantaviral spike according to any one of claims 1 to 21, or the pharmacological composition according to claim 22, for use in the preparation of a medicament effective in preventing and/or treating hantavirus infection.

A method for preventing and/or treating a hantavirus infection, comprising administering to a subject in need thereof the stabilized hantaviral spike according to any one of claims 1 to 21 or a pharmacological composition according to claim 22, in an amount effective to inhibit hantavirus infection of susceptible cells so as to thereby prevent or treat the infection.

A diagnostic agent comprising or consisting of a stabilized hantaviral spike according to any one of claims 1 to 21 and an appropriate diagnostic reagent.

A kit for diagnosing or monitoring, in a subject, a hantaviral infection, comprising the stabilized spike according to any one of claims 1 to 21 and an appropriate diagnostic reagent.

A kit for treating and/or preventing hantavirus infections, comprising the stabilized spikes according to any of claims 1 to 21 or the pharmacological composition according to claim 22.

Use of the stabilized spikes according to any one of claims 1 to 21, in the generation or selection of ligands useful to treat and/or prevent hantavirus infections.

Use of the stabilized spikes according to any one of claims 1 to 21, for the identification of epitopes recognized by ligands useful to treat or prevent hantavirus infections.

BRIEF DESCRIPTION OF THE SEQUENCES

[00129] SEQ ID NO: 1: Sequence corresponding to the wild type glycoprotein precursor of *Orthohantavirus Andes* GenBank accession number AAO86638.1. This precursor sequence comprises glycoprotein Gn within amino acids 1-651 and glycoprotein Gc within amino acids 652-1138.

[00130] SEQ ID NO 3: Sequence corresponding to the single amino acid substitution Gc T839C in SEQ ID NO: 1.

[00131] SEQ ID NO 4: Sequence corresponding to the single amino acid substitution Gc H953C in SEQ ID NO: 1.

[00132] SEQ ID NO 5: Sequence corresponding to the single amino acid substitution Gc H953F in SEQ ID NO: 1.

[00133] SEQ ID NO 6: Sequence corresponding to the double amino acid substitution Gc Q844C/H953C in SEQ ID NO: 1.

[00134] SEQ ID NO 7: Sequence corresponding to the double amino acid substitution Gn/Gc H294C/T734C in SEQ ID NO: 1.

[00135] SEQ ID NO 8: Sequence corresponding to the double amino acid substitution Gn/Gc R281C/P748C in SEQ ID NO: 1.

[00136] SEQ ID NO 9: Sequence corresponding to the double amino acid substitution Gn/Gc T99C/P744C in SEQ ID NO: 1.

[00137] SEQ ID NO 10: Sequence corresponding to the double amino acid substitution Gn/Gc K85C/P774C in SEQ ID NO: 1.

[00138] SEQ ID NO 11: Sequence corresponding to the double amino acid substitution Gn/Gc N94C/V776C in SEQ ID NO: 1.

[00139] SEQ ID NO 12: Sequence corresponding to the double amino acid substitution Gn/Gn E61C/Q200C in SEQ ID NO: 1.

BRIEF DESCRIPTION OF FIGURES

Figure 1. Secondary structure elements of the hantaviral spike structure.

[00140] Multiple sequence alignment of the Gn/Gc proteins of pathogenic hantaviruses compared to hantaviruses harbored in insectivores. The Gn/Gc glycoproteins are synthesized as glycoproteins precursor that is cleaved by a host protease into the N-terminal Gn and the C-terminal Gc glycoproteins, at the signal sequence "WAASA". Strictly conserved and highly similar residues are highlighted in grey. The secondary structure elements obtained from the Gn/Gc crystal structure of Andes virus are displayed above the sequences. For regions missing structural information (to be included). Disulfide bonds are indicated with light grey numbers below the sequence alignment.

Figure 2. The structure of hantavirus Gc and identification of Gc/Gc homodimer contacts.

[00141] A) Top view of the Hantaan virus Gc homodimer structure. To improve visibility, the human single-chain variable domain (scFv) antibody was removed from the Gc/scFv A5 structure complex. One Gc protomer is highlighted in black, while the other is indicated in grey. B) Side view of the Gc homodimer structure. C) Insert showing the Gc/Gc homodimer contacts where the different regions forming the contacts are highlighted. D) Multi-angle light scattering (MALS) of the soluble Gc ectodomain of Hantaan virus used to obtain the Gc crystal structure.

Figure 3. The structure of hantavirus Gn/Gc heterodimer and identification of Gn/Gc heterodimer contacts.

[00142] A) Side view of the Andes virus Gn/Gc heterodimer structure. The Gn protomer is indicated in white, the Gc protomer in black. B-D) Inserts showing the different Gn/Gc contacts in which the different regions forming the contacts are highlighted.

Figure 4. Fitting of the Gn/Gc structure into the Tula hantavirus Cryo-ET map and identification of Gn/Gn contacts.

[00143] A) Top view of four Gn/Gc heterodimer structures fitted into the available Cryo-ET map for the Tula hantavirus spike (Shi et al., 2016). Gn is indicated in white, Gc is indicated in black. B) Side view of two Gn/Gc heterodimers fitted into the spike density of the Tula hantavirus Cryo-ET map. C) Insert showing different Gn/Gn contacts in which the different regions forming the contacts are highlighted.

Figure 5. Characterization of Gn/Gc mutants expression and of their assembly into VLPs bearing the engineered disulfide bonds.

[00144]

(A-B) Characterization of the expression yields and cellular localization of ANDV Gn and Gc proteins that comprise either single mutation at the Gc homodimer interface (A), or double mutations at the Gc homodimer interface, or double mutations at the Gn/Gc interface of the Gn/Gc spikes (B). Western blot analysis using anti-Gc or anti- β -actin MAbs of different cellular fractions obtained from 293FT cells expressing wild type (wt) or mutant Gn/Gc after cell surface biotinylation. The fractions correspond to the non-biotinylated fraction (intracellular proteins) or the biotinylated fraction (surface proteins). C) SDS Page and western blot under reducing and non-reducing conditions of VLPs bearing wt or mutant Gn/Gc spikes comprising single or double

cysteine substitutions at the Gc homodimer interface, using anti-Gc antibody. D) SDS Page and western blot under reducing and non-reducing conditions of VLPs bearing wt or mutant Gn/Gc spikes comprising double cysteine mutations at the Gn/Gc heterodimer interface : H294C/T734C, R281C/P748C, T99C/P744C, K85C/P774C or N94C/V776C using either anti-Gc (left panel) or anti-Gn specific antibodies (right panels). VLPs bearing wt Gn/Gc spikes were used as a negative control for disulfide bond formation, while VLPs bearing Gn/Gc spikes comprising the single mutant G838C at the Gc homodimer interface was used as a positive control for disulfide bond formation of Gc homodimers.

Figure 6. Acid stability of wt and mutant hantaviral spikes.

A) Liposome co-floitation assay to visualize acid-induced activation and membrane insertion of VLPs bearing wt Gn/Gc spikes or Gn/Gc spikes comprising the single mutation H953F at the Gc homodimer interface. VLPs were incubated with liposomes at different pHs at 37°C and the mixture was floated on a step gradient. Fractions taken consecutively from the bottom of the step gradient were examined for the presence of VLPs western blot using anti-Gc MAb. B) Quantification of the presence of wt and mutant VLPs in the fractions of the co-floitation assay. Results from at least n=3 independent experiments were averaged.

Figure 7. Thermal stability of wt and mutant detergent-solubilized hantaviral spikes.

[00145]A-B) The Oligomeric state of the detergent-solubilized hantaviral spikes at different temperatures. Blue native-PAGE and western blot analysis of detergent solubilized VLPs displaying wt or mutant spikes including single Gc mutants at the Gc:Gc interface (A) or double mutations at the Gn/Gc interface (B). The spikes were extracted from VLPs by Triton X-100 and treated at the indicated temperatures of 20-60°C at neutral pH. The presence of Gn or Gc in each lane was detected by western blot analysis by splitting the transferred gel in two parts and revealing with anti-Gn (left panel) and anti-Gc (right panel) antibodies. As internal control for Gc species migration, Gc wt homotrimers were examined in each gel by treatment of VLPs at pH 5.5. To further estimate the oligomerization species of Gn and Gc (indicated on the left side of the blot), the migration of their monomeric and multimeric forms was compared with a native protein standard (indicated on the right side of the blot). (C-E) Graph of the temperature-induced Gn/Gc dissociation of detergent solubilized spikes quantified by densitometry from wt, or (C) mutants at the Gc homodimer interface that do not improve the spike stability, (D) the Gc mutant G838C at the Gc homodimer interface and (E) Gn/Gc mutants at the Gn/Gc interface. Averages $\pm$ s.d. are shown. The curves were fitted using a sigmoidal equation. The melting temperature (T_m) of the detergent-solubilized spikes is indicated for each mutant.

Figure 8. Exposure of the Gc fusion loop of wt and mutant hantaviral spikes.

[00146](A) Liposome co-floitation assay to determine the exposure of the Gc fusion loops and their insertion into membranes. VLPs were incubated with liposomes at different temperatures and pHs and the mixture floated on a step gradient and the presence of VLPs examined in each fraction by western blot using anti-Gc MAb. The double Gc fusion loop mutant W766A/F901A, that does not insert into target membranes was used as negative control. (B) Fusion loop exposure temperature compared to the melting temperature of wt Gn/Gc spikes versus mutant spikes assembled onto VLPs. The quantification of the fraction of Gc interacting with liposomes at different temperatures, superimposed to the fraction of dissociated Gc at the same temperatures. Results from at least n=3 independent experiments were averaged.

Figure 9. Neutralizing antibody responses against VLPs bearing wild type or mutant hantaviral spikes in mice.

[00147] (A-B) Neutralizing activity of sera from Balb/c mice after three immunizations with VLPs bearing wt or mutant hantaviral spikes comprising either the single mutant G838C at the Gc homodimer interface (A) or comprising the double mutation H294C/T734C at the Gn/Gc heterodimer interface. Neutralization of Andes virus was assessed through incubation of ANDV with sera from mice for 1 hr and subsequent adsorption to Vero E6 cells that were immunized with VLPs bearing wt or mutant hantaviral spikes. As a control, sera of mice immunized with VLPs bearing wt Gn/Gc were used as well as sera from mice before immunization. Infection was quantified by flow cytometry 16 h post-infection using anti-ANDV nucleoprotein antibody.

EXAMPLES

Example 1. The selection and design of amino acid modifications for improved stability of the hantaviral spike

[00148] In order to face the technical challenge to improve the spike stability, for the generation of improved immunogens, we used structural information to identify and select key positions and regions in the hantaviral spikes that allow the design of sequence modifications for their stabilization.

[00149] The molecular structures of the ectodomains of the hantavirus Gn and Gc proteins have been described in their monomeric conformations; however information was still missing concerning their orientation in the hantaviral spike and their molecular contacts for a molecular perspective on their assembly and the design of preventive or therapeutic strategies.

[00150] In the present example, the inventors have obtained novel molecular structures that describe the contacts of the Gn/Gc assembly. Among these structures, two particular structures have been obtained from two different expression constructs stably transfected into Drosophila S2 cells:

- a) expression plasmid pMT-rGc-W115H coding the recombinant Gc ectodomain (rGc) (residues 652-1107) including the W115H mutation and two C-terminal strep-tag sequences separated by a (GGGS)₃ linker preceded by an enterokinase cleave site as previously described (Guardado-Calvo et al., 2016).
- b) expression plasmid pMT-rGn-Gc coding for the recombinant Gn (rGn) (residues 21-374) and rGc (residues 652-1107) ectodomains connected by a 42 amino acids flexible linker region and two C-terminal strep-tag sequences separated by a (GGGS)₃ linker preceded by an enterokinase cleave site as previously described (Guardado-Calvo et al., 2016).

[00151] The expression products were purified and crystallized for subsequent X-ray diffraction by standard methods. In the case of rGc, we crystallized this protein by previous incubation with human single-chain variable domain (scFv) antibody fragment A5 as previously described (Guardado-Calvo et al., 2016).

[00152] We determined the Gc crystal structure at 3.0 Å resulting in a Gc homodimer (Figure 2) while we were able to reveal the Gn/Gc crystal structure at 2.7 Å (Figure 3). The structures allowed us to identify the molecular contacts between the Gc/Gc homodimer and the Gn/Gc

heterodimer. When we further fitted the Gn/Gc crystal structure into the available 16 Å resolution electron cryo-tomography reconstruction of Tula hantavirus (Shi et al., 2016), we could also define contacts of the Gn/Gn homooligomers (Figure 4).

[00153] In this context, we selected preferred amino acids for modifications by the following criteria:

- a) forming the contact interphase between the Gc/Gc homodimer, Gn/Gc heterodimer and Gn/Gn homooligomer.
- b) cavity filling mutants.

[00154] Among these amino acids, we selected those for modifications that fulfilled at least one of the following criteria:

- a) The impact of the mutation in the structure to avoid protein missfolding.
- b) The conservation of the residue.
- c) The certainty of the residue position in the model based on the observed electron density.
- d) For the design of inter-chain bonds, the distances between the C_{alpha}-C_{alpha} atoms should be less than 6.5 Å and for C_{beta}-C_{beta} atoms less than 4.5 Å.
- e) For inter-chain disulfide bonds, the quality of the modeled disulphide bond is evaluated using the dihedral angles C_{beta}-S_i-S_j-C_{beta}, C_{alpha}-C_{beta}-S_i-S_j, and C_{alpha}-C_{beta}-S_j-S_i.
- f) For the design of cavity filling mutants, residues were selected with improved affinity score (improved ΔG) by Rosetta.

[00155] To highlight the different selected amino acids, we refer to the Andes virus sequence nomenclature as identified in Figure 1. However, since the structure and sequences of the glycoproteins is highly conserved between hantaviruses (Guardado-Calvo et al., 2016; Wilensky et al., 2016; Shi et al., 2016), the contacts that we observe in the Andes virus and Hantaan virus Gn/Gc structures, can be extended to any Gn/Gc protein of the Hantaviridae family.

[00156] For the ease of understanding, we called each region as we could derive it from the secondary elements in the structure. If hence a region is contained in a beta strand, we termed this region following the strand nomenclature; e.g. "b strand". When a region is contained between two beta strands, for example between beta strand b and c, we termed this region "bc linker" or "bc loop" according to its structural features.

[00157] From these overall criteria, we selected the following possible amino acids for modifications. Among them, the amino acid modification can correspond to any amino acid substitution, including Ala, Leu, Val, Ile, Trp, Tyr, Phe, Pro, Met, Ser, Cys, Sec, Gly, Asn, Gln, Thr, Glu, Asp, His, Lys and/or Arg :

(1) The Gc/Gc homodimer

[00158] The Gc/Gc homodimer contacts spanning amino acids comprised of the Gc strands B₀ (residues L676-P683), H₀ (residues V832-V837), I₀ (residues R951-L958) and the H₀f linker region between strand H₀ of Gc domain I and the f strand of Gc domain II comprising amino acids G838-D847. For clarity, please see Figure 1 and Figure 2.

[00159] This list of amino acid regions resulted in the preferred amino acids: Gc E677, D679, G838, T839, S841, R951 and/or H953 and the contact pairs Gc E677/R951, D679/H953 and/or H953C/Q844C.

[00160] In our preferred realization, the modifications correspond to Cys substitutions to form inter-chain disulfide bonds through the following amino acid substitutions: G838C, T839C, S841C/R951C, E677C/R951C, D679C/H953C and/or Q844C/H953C. A preferred cavity filling mutant includes H953F.

(2) The Gn/Gc heterodimer

[00161] The Gn/Gc heterodimer contacts comprising (Figure 1, Figure 3):

- A) Any Gn amino acid from the helix 2-A₈ linker region and A₈ strand comprising residues N290-I297 in combination with any residue from the Gc a/b strand, comprising residues T729-H737.
- B) Any amino acid from the Gn D_AE_A loop comprising residues K85-T99 with any Gc amino acid either from the Gc cd loop comprising P774-T778, and/or from Gc bc loop comprising C738-Y747.
- C) Any amino acid from the Gn bc loop comprising H203-D206 in combination with any amino acid comprised by the Gc fg loop spanning residues G853-L855.

[00162] This list of amino acid regions resulted in the preferred amino acids:

Gn K85, N94, A95, T99, H203, D206, N290 and/or H294 and/or Gc T734, T729, P774, V776 Y739, P774, Gc L855 and/or P854. From those, we established the following contact pairs: Gn H294/Gc T734, Gn N290/Gc T729, Gn K85/Gc P774, Gn N94/Gc V776C, Gn A95/Gc Y739, T99/Gc P774, Gn H203/Gc L855 and/or Gn D206/Gc P854.

[00163] In our preferred realization, the amino acid modifications correspond to Cys substitutions to form inter-chain disulfide bonds through the following amino acid substitutions: Gn H294C/Gc T734C, Gn N290C/Gc T729C, Gn K85C/Gc P774C, Gn N94C/Gc V776C, Gn A95C/Gc Y739C, T99C/Gc P774C, Gn H203C/Gc L855C and/or Gn D206C/Gc P854C.

(3) The Gn/Gn homooligomer

[00164] The Gn/Gn homooligomer contacts, comprising (Figure 1, Figure 4):

- A) Any amino acid of the Gn bc loop comprising residues P192-D206 of one Gn protomer in combination with any amino acid modification in the Gn region K59-Q75 comprising the CA strand and the region comprised between the CA strand and the DA strand in the other Gn protomer.

[00165] This list of amino acid regions resulted in the preferred amino acids: Gn E61, Q200 From which we established the following contact pair: Gn E61/Q200.

[00166] In our preferred realization, the amino acid modifications correspond to Cys substitutions to form inter-chain disulfide bonds through the following amino acids substitutions: E61C/Q200C.

[00167] Based on the results shown in this example, we identified several key positions and regions that allowed the proposal of specific sequence modifications in such key positions and regions for the design of recombinant hantaviral spikes in order to improve their stability.

Example 2. Expression and folding of hantaviral spike mutants.

[00168] The present example of the invention provides information on how the inventors experimentally assess whether the design of recombinant hantaviral spikes are expressed and properly folded in cells.

[00169] In this example we used the plasmid pl.18/GPC coding for the Gn/Gc glycoproteins of Andes orthohantavirus CHI-7913 isolate GenBank accession number AAO86638.1 (Cifuentes-Muñoz et al., 2010) as a model for all hantaviral Gn/Gc coding plasmids and introduced nucleotide mutations using standard oligonucleotide-based PCR amplification technique.

[00170] The expression and folding of each mutant construct was assessed by transient transfection of 293FT cells (Invitrogen) and subsequent biotinylation of cell surface proteins as previously described (Guardado-Calvo et al., 2016). The presence of the Gn/Gc proteins in the biotinylated (cell surface proteins) and non-biotinylated fractions (intracellular proteins) was tested by western blot using the MAb anti-Gc 2H4/F6. The empty pl.18 plasmid was used as negative control. In Figure 5A the inventors show an example of Gn/Gc single mutants in which the Gc/Gc contacts were modified to stabilize or to weaken the hantaviral spikes. For these mutants the inventors found different expression levels, ranging from high expression levels (H953C, E677Q, R951Q, E677K, D679K, D679N, G838C) to low (D679A, D679S, H953D, R951E, H953E) and non-detectable (E677A, R951A, H953A) (Figure 5A). The Gc mutants that we detected in the intracellular fraction trafficked all to the plasma membrane, confirming that they could exit the ER and enter the secretory pathway of the cell, a measure for proper protein folding (Figure 5A, surface fraction). As example of the expression yields and cellular localization of double mutations in the hantavirus spike at the Gc/Gc or Gn/Gc heterodimer interfaces, the inventors used the same approach. Although expression yields were found to be lower in all cases compared to wt (Figure 5B, intracellular fraction), most mutants were independently detected at the plasma membrane (Q844C/H953C, H294C/T734C, T99C/P774C, N294C/V776C) (Figure 5B, surface fraction), confirming their proper folding and cell trafficking. Hence, these mutants can be used for further analysis of the hantaviral spike characterization and are candidate antigens for vaccine design or for the selection of antiviral compounds.

[00171] With the above techniques, the inventors also introduced other amino acid modifications at additional residue positions. For the following residue substitutions the inventors obtained high expression levels and proper trafficking:

Gc homodimer single mutant GcT839C and Gc double mutant Gc Q844C/Gc H953C.

Gn/Gc heterodimer double mutants: Gn H294C /Gc T734C; Gn R281C/Gc P748C; Gn T99C/Gc P774C and Gn N94C/Gc V776C.

Gn/Gn homodimer double mutants: Gn E61C/Q200C.

To detect Gn mutant proteins, the by us well-established anti-Gn MAb 6B9/F5 was used.

[00172] In this example, the Gn/Gc wt and mutant proteins were expressed in their full length and can be harvested from the cell supernatant in form of virus-like particles. Alternatively, those of skill in the art can also express the Gn/Gc proteins as soluble ectodomains in which the transmembrane anchors and stem regions have been removed as described in Example 1. Also, Gn/Gc proteins can be used to pseudotype virus vectors or to produce recombinant viruses as it has been well described in the field (Ray et al. 2009; Cifuentes-Muñoz et al., 2010; Kleinfelter et al., 2015).

[00173] With the expressed Gn/Gc proteins, the inventors obtained well expressed recombinant hantavirus Gn/Gc proteins, that were incorporated onto virus-like particles.

Example 3. Assessment of inter-chain disulfide bonds in hantaviral spikes

[00174] In this examples, the inventors provide evidence, that the Gn/Gc proteins that contain amino acid substitutions by Cys, are close enough on the viral particles to allow the formation of disulfide bonds across the different Gc/Gc and Gn/Gc interfaces.

[00175] To improve the hantaviral Gc spike stability, we have designed several inter-chain disulfide bonds between Gc/Gc homodimers, Gn/Gc heterodimers and Gn/Gn homooligomers (see Example 1) based on the Gn/Gc and Gc/Gc crystal structures and Gn/Gc structure fitting into the Cryo-EM density map (Shi et al., 2016). Some inter-chain disulfide bonds may involve the substitution of single residues in each monomer (for example the substitutions Gc G838C) since in such case these residues are facing each other at the center of the 2-fold axes of the Gc homodimer. In other cases, we have designed a pair of Cys substitutions of residues that are opposing each other at any of the homodimer and/or heterodimer contact interphases to establish disulfide inter-chain bonds.

[00176] By way of example, the following hantaviral Gc/Gc spike mutants have been used to functionally assay the formation of such inter-chain disulfide bonds; Gc single mutants G838C, T893C, and Gc H953C and Gc double mutant Q844C/H953C (Figure 5C). Therefore, we concentrated the wt and mutant VLPs from 293FT cells expressing these mutants (Figure 5A) and subjected them to SDS PAGE and western blot analysis under reduced and non-reduced conditions. In the case of the addition of β -Mercaptoethanol (reducing condition), we found that all mutants migrated as the wt Gc protein (~50 kDa). However, in absence of a reducing agent, all Gc mutants migrated with a molecular weight that corresponds to the predicted weight of Gc dimers (~100 kDa) (Figure 5C).

[00177] In the same way of example, the same approach was performed with double mutations of the Gn/Gc heterodimer interface; H294C/T734C; R281C/P748C; T99C/P774C; K85C/P774C and N94C/V776C. Under reducing conditions, the Gc mutants migrated at the wt Gc protein (~50 kDa) while the Gn mutants migrated as the wt Gn protein (~70 kDa) (Figure 5D, lower panels). However, under non-reducing conditions, additional migration species were recognized by both, anti-Gn and anti-Gc antibodies, with a molecular weight of (~130 kDa) that corresponds to the Gn/Gc heterodimer (Figure 5D, upper panels).

[00178] Together, in this example the inventors have shown that the cysteine substitution mutants at the Gc/Gc and Gn/Gc interface can be disulfide linked in a biological context, thereby

forming Gc/Gc or Gn/Gc dimer linkage at the surface of viral particles. Hence, the residues forming the Gc dimer contacts in the X-ray structure of a pre-fusion form of Gc and the Gn/Gc contacts in the X-ray structure of the Gn/Gc heterodimer are proximal enough to each other on viral particles to allow for disulfide formation while still forming VLPs. This data also supports the biological relevance of the crystallographic structures proposed in Example 1 of this invention.

Example 4. Improved acid stability of the hantaviral spikes

[00179]In this example, the inventors provide evidence, that bulky residue substitution at the Gc/Gc dimer interface can increase the resistance to different environmental factors, such as mild acidification. This is an important aspect since the administration of antigens to individual involves their suspension into immunologic adjuvants to improve immune responses. Yet, the most frequently used antigens, alum adjuvant in humans and Freund adjuvant in animals, have acidic pH that can perturb the antigen structure. This is of particular importance for the hantaviral spike, since already a mildly acidic pH activates the hantavirus Gc fusion protein inducing its non-infectious post-fusion conformation. Thus, in a preferred embodiment, it is desirable to improve the hantaviral spike stability not only in terms of their inter-chain contacts, but also in terms of their resistance to acidic pH.

[00180]In this invention His residues were substituted since they are molecular sensors of mildly acidic pH, having an acid dissociation constant (pK_a) of ~6.0, coinciding with the pH range of Gc activation. In this context, the inventors have designed and characterized one Gc mutant in which they have substituted His953, located at the Gc homodimer interphase, to Phe (H953F) (Figure 5A). To functionally assess its activation pH compared to wt Gn/Gc, we performed a liposome coflotation assay as a measure for activation by fusion loop exposure and membrane insertion established previously (Guardado-Calvo et al., 2016). Therefore we incubated VLPs bearing the wt or mutant spike H953F with fluorescently labeled liposomes at each pH and loaded the mixture to the bottom of a sucrose step gradient. After centrifugation, we monitored each fraction for the presence of liposomes (by fluorescence) and VLPs (by western blot against Gc). At pH 6.2, the liposomes migrated to the top of the gradient while the wild type VLPs remained in the bottom fractions (Figure 4c and figure supplement 1a), but increasing amounts of the VLPs were observed in the top fractions at more acidic pHs. The inventors found that VLPs bearing the H953F spike mutant for pH-induced liposome coflotation was more resistant to activation, in a way that 50% activation occurred at pH 5.5 while the wt spike activation occurred already at pH 5.9 (Figure 6). With the above techniques, those of skill in the art can routinely design other His substitutions or substitution of other protonable residues such as Asp and Glu at additional positions, and expect hantaviral spike resistance to mild acidification. Combinations of several His substitutions can lead to a still higher resistance to low pH and thus decrease perturbation of the hantaviral spikes when introduced into a pharmaceutical preparation.

[00181]The inventors have shown in this example that it is possible to design residue substitutions that confer the hantaviral spikes a higher resistance to irreversible acid-induced activation that they can face in various environments.

Example 5. Improved thermal stability of the hantaviral spikes

[00182]In order to favor conformations of the hantaviral spikes that correspond to their infectious arrangement, and in order to decrease the exposure of otherwise cryptic regions that may serve as a decoy for the immune system, the inventors subjected the diverse hantaviral spike mutants to temperature gradients in order to assess the melting temperature of each mutant.

[00183]The inventors used blue-native polyacrylamide gel electrophoresis (BN PAGE) combined with its western blotting (native western blot) to compare the stability at increasing temperatures of the detergent-solubilized hantavirus wild type and mutant spike complexes. Previous to the characterization of specific hantaviral spike mutants, the properties of the wt spike had to be established. When we thus incubated VLPs bearing wt Gn/Gc spikes at neutral pH and 20°C, the detergent-solubilized spike was identified as a single band recognized by both, anti-Gn and anti-Gc MAbs (Figure 7A, Gc WT 20°C). This band migrated roughly as expected in BN-PAGE, given the migration of the individual Gn and Gc monomers (see migration at 50°C), of the Gc postfusion homotrimer (see migration at acidic pH), and of the standard reference bands. When the wild type spike complex were treated at temperatures up to 50°C, the dissociation of the Gn/Gc spikes could be visualized on the gel by the gradual disappearance of the corresponding band and the concomitant appearance of faster migrating bands, which corresponded to several oligomeric Gn forms and to a monomeric Gc species (Figure 7A, WT 40-50°C). Quantification of the temperature-induced dissociation of the detergent solubilized wild type ANDV Gn/Gc spike revealed a melting temperature (T_m) of $37,7 \pm 0,4^\circ\text{C}$.

[00184]By using this technique, the inventors characterized different hantaviral spike mutants, particularly those modifying the Gc/Gc homodimer contacts. Among those, we assessed the stability properties of the mutant G838C, in which we engineered a disulfide bond at the Gc dimer 2-fold axes, which thus revealed a strongly increased T_m of 48°C (Figure 7A, D). In this mutant spike, the Gc dissociation resulted in Gc migration species that did not dissociate at any tested condition, and the Gn/Gc complex dissociation resulted into $(\text{Gn/Gc})_2$ heterodimers, Gn homooligomers and Gc homodimers. Thus, the higher T_m that we observed for G838C indicates that the Gc homodimer stabilizes not only the Gc homodimer interphase, instead it stabilizes the entire Gn/Gc spike. Hence, as for the wt spike, the dissociation of the Gc G838C homodimers leads to the disruption of the hantaviral spike. As a whole, our data indicates that the improvement of the Gc homodimer contacts at its 2-fold axis, strengthens the hantaviral spike structure as a whole.

[00185]Another hantaviral spike mutant that the inventors characterized includes an inter-chain disulfide bond at the position H953 located at the interphase of the Gc homodimer by introducing the substitution H953C. Although this mutant forms disulfide linked Gc dimers (Figure 5C), the inventors found that this Gc dimer mutant completely abrogated its simultaneous interaction with Gn since not Gn/Gc migration species of higher weight could be detected. Thus, from this result it can be concluded that not any inter-chain disulfide bond at the Gc homodimer interphase leads to a concomitant improvement of the overall hantaviral spike stability and underlines the importance of this assay for the spike characterization.

[00186]As negative controls, and to further include additional standards into this assay, we have also tested mutants from which we expected to weaken the Gc dimer contacts at its 2-fold axes.

As expected, we could observe an opposite effect on the Gn/Gc spike stability, since the T_m decreased in all cases: Gc E677Q ($T_m=35^\circ\text{C}$), Gc D679S ($T_m=34.5^\circ\text{C}$) and Gc R951Q ($T_m=32.3^\circ\text{C}$). The decreased T_m of the mutants was accompanied by a concomitant decrease in their interactions energies, corroborating the role of the Gc homodimer in the stability of the Gn/Gc heterooligomers and confirming the role of these residues in the homodimeric Gc/Gc interactions.

[00187]The inventors also assessed hantaviral spike complexes bearing the following double residue substitutions at the Gn/Gc interface; H294C/T734C and N94C/N776C. These mutants showed high molecular weight Gn/Gc migration species that did not dissociated up to high temperatures revealing highly increased T_m 's of 79.1°C and 60.4°C , respectively (Fig. 7B and Fig. 7E).

[00188]As a whole, from this example it can be concluded that the introduction of the specific inter-chain disulfide bonds across the Gc/Gc interface located at the Gc homodimer 2-fold axes,(Gc G838) or at the Gn/Gc interface strongly increases the stability of the entire hantaviral spike. Those of skill in the art can perform similar analysis for other mutants and can expect to further improve the hantaviral spike stability by the introduction of residue modification that improve the contacts between Gn/Gc and Gn/Gn as described in Example 1. Combinations of different residue substitutions at different interphases of the hantaviral spike is likely to provide optimal spike stability.

[00189]In a still wider context, hantaviral mutants bearing multiple residue substitutions, including those that increase the dissociation energy, for example by an inter-chain disulfide bond such as G838C, H294C/T734C or N94C/N776C combined with residue substitutions that improve the hantaviral spike resistance to low pH, such as H953F, can confer optimal spike stability.

Example 6. Restriction of molecular fluctuations in hantaviral spikes

[00190]After having established how to assess and select stabilized Gc homodimers through disulfide bonds or by other residue substitutions (Examples 3-5), we also tested whether the hantaviral spike mutants induced a Gn/Gc conformation of lower flexibility concerning its molecular fluctuations. Therefore, we assessed whether the Gn/Gc heterooligomers expose transitorily the Gc fusion loops at physiological temperature (20 to 37°C , inside or outside a host cell, respectively). We further applied higher temperatures to measure whether the Gn/Gc dissociation into Gc monomers (Fig. 7) is related with the exposure of the Gc fusion loops. We measured the fusion loop exposure through their insertion into target membranes by the well-established liposome coflotation assay (Guardado-Calvo et al., 2016).

[00191]When we thus incubated the wt hantaviral spikes assembled onto VLPs at neutral pH at low temperatures (20-30 $^\circ\text{C}$), we observed the VLPs in the bottom fractions of the gradient (Figure 8A), requiring as expected at these temperatures low pH for the conformational change that leads to target membrane insertion as described previously (Acuña et al., 2015; Guardado-Calvo et al., 2016). When we further increased the temperature to 37°C and above, VLPs floated gradually with liposomes to the upper fractions, as temperature increased. To next determine whether membrane insertion at temperatures at 37°C and above are specifically conducted by

the Gc fusion loops or rather by unspecific interactions, we tested liposome coflotation of VLPs bearing the Gc mutant W766A/F900A. This Gc mutant includes two substitutions of the aromatic residues at the tip of the cd and bc fusion loops to alanine, previously proven to be required for insertion into target membranes at low pH (Guardado et al., 2016). Thus, the heating to 50°C at neutral pH of VLPs bearing this double fusion loop mutant, showed absence of liposome insertion (Figure 8A), thereby proofing that exclusively the fusion loops are directly involved in membrane insertion at neutral pH. When we further compared the profiles of the VLP-liposome interaction with that of (Gn/Gc)₄ dissociation, we found that they coincide extremely well (Figure 8B; $T_{50\% \text{ interaction}} = 37,3^\circ\text{C}$; $T_m = 36,3^\circ\text{C}$), confirming that the temperature-induced dissociation of the Gn/Gc heterooligomers into Gc monomers is responsible for the exposure of the Gc fusion loops.

[00192] After establishing the molecular fluctuations of the hantaviral spike in terms of its fusion loop exposure, we analyzed whether stabilized Gn/Gc mutants are more restricted in such fluctuations, providing additional information on the molecular structures that the antigen will adapt upon *in vivo* administration. By way of example, the inventors have characterized two Gc mutants to assess this. In the case of the stabilized hantaviral spike carrying the Gc G838C substitution including a disulfide bond at the Gc homodimer interphase, this mutant showed reduced fusion loop exposure at 50°C compared to wt Gn/Gc (Figure 8A), confirming thus a tighter association of the Gn/Gc complex.

[00193] As a control, we also analyzed the inter-chain Gc disulfide bond mutant H953C that prevents the association of the Gc homodimers into Gn/Gc heterooligomers (Figure 7A). Consistent with the previous results showing that heat-induced liposome insertion is dependent on the dissociation of the Gn/Gc spike complex, this mutant inserts readily into liposomes at any temperature, proofing the specificity of the assay (Figure 8A).

[00194] By using the technical approach of this example, those of skill in the art can perform similar analysis for other spike stabilizing mutants and can observe a decrease in the fluctuation of the spike.

[00195] From these results it can be concluded the hantaviral spike complex exposes a high dynamic behavior at 37°C and above, exposing internal regions that are not functionally involved in entry and act as a decoy to elicit antibodies that are not neutralizing. The design of stabilized Gn/Gc mutants will result in an increase the Gn/Gc dissociation energy in a way that conformational dynamics will be reduced. Thereby, the increase of the spike stability has a direct impact on the antigen presentation to the immune system of a host since it increases structures that are involved in a protective immune response through neutralizing antibodies and represses structures that are involved in the generation of non-neutralizing antibodies.

Example 7. Immune responses the hantaviral spikes with improved stability and antigenicity

[00196] In this examples the inventors provide evidence, that the stabilization of the hantaviral spikes elicits higher neutralizing antibody titers than the wt spikes.

[00197] To determine the efficacy of immune responses to wt or mutant hantaviral spikes, the inventors proceeded to assess the neutralizing antibody titers of animals which was each immunized with a different hantaviral spike mutant.

[00198] In brief, 16 week old Balb/C mice were immunized intraperitoneal with 50 µg of antigen with incomplete Freund adjuvant on day 0 and immunizations repeated on days 7 and 14 with 50 µg of antigen mixed with complete Freund Adjuvant. On day 16 blood was extracted and used to analyze the neutralizing antibody titers. The following antigens were used for immunizations: VLPs bearing wt hantaviral spikes, stabilized VLPs bearing the single mutation G838C at the Gc homodimer interface and stabilized VLPs bearing the double mutation H294C/T734C at the Gn/Gc heterodimer interface.

[00199] Neutralizing antibody titers of sera against Andes virus was assessed by incubation of Andes Orthohantavirus strain CHI-7913 with mice sera for 1 h and subsequent 1 h adsorption of the mixture to Vero E6 cells. As we established previously, using other cell entry inhibitors (Barriga et al., 2016), viral infection was allowed to proceed for 16 h to assess inhibition of the first round of infection. Next, cells were detached and the percentage of infected cells measured by cell cytometry using the anti-nucleoprotein MAb clone 7B3/F7. At a dilution of 1/500, the sera from mice immunized with VLPs bearing stabilized hantaviral spikes G838C showed 90% of viral inhibition while sera from mice immunized with wt VLPs only reduced infection by 45% (Figure 9A). On the other hand, at a dilution of 1/500, the sera obtained from animals immunized with VLPs stabilized by double mutation H294V/T734C blocked viral infection by ~50% while in the same assay, sera from mice immunized with the wt VLPs achieved only 30 % of inhibition (Figure 9B).

[00200] These results provide evidence that stabilized hantaviral spikes induce higher neutralizing antibody responses in animals and hence have a huge potential to be used as improved immunogens or screening for binding of other viral inhibitors.

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KCWFTKSGEWLLGILNGNWIVVVVLVILILSIIMFSVLCPRRGHKKT

SEQ ID NO: 1

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CLAIMS

1. A stabilized hantaviral spike comprising at least one homodimer of mutants Gc, or at least one heterodimer of a mutant Gn and a mutant Gc, or at least one oligomer of mutants Gn, or a combination thereof.
2. A stabilized hantaviral spike according to Claim 1, comprising at least one homodimer of mutants Gc having each at least one amino acid mutation (substitution) at a position selected from the group consisting of: 676, 677, 678, 679, 680, 681, 682, 683, 832, 833, 834, 835, 836, 837, 838, 839, 840, 841, 842, 843, 844, 845, 846, 847, 951, 952, 953, 954, 955, 956, 957 and 958, the indicated positions being determined by alignment with SEQ ID NO: 1.
3. The stabilized hantaviral spike according to claim 2, comprising at least one homodimer of mutants Gc is selected from the group consisting of: a homodimer of mutants Gc having each the substitution G838C (SEQ ID NO: 2), a homodimer of mutants Gc having each the substitution T839C (SEQ ID NO: 3), a homodimer of mutants Gc having each the substitution H953C (SEQ ID NO: 4), and a homodimer of mutants Gc having each the substitution H953F (SEQ ID NO: 5), wherein the amino acid residues 838C, 839C and 953C are linked respectively to the amino acid residues 838C, 839C and 953C through disulphide inter-chain bonds.
4. The stabilized hantaviral spike according to claim 3, wherein each mutant Gc of the at least one homodimer of mutants Gc has the substitution G838C (SEQ ID NO: 2), wherein the amino acid residues 838C are linked together through a disulphide inter-chain bond.
5. The stabilized hantaviral spike according to claim 3, wherein each mutant Gc of the at least one homodimer of mutants Gc has the substitution T839C (SEQ ID NO: 3), wherein the amino acid residues 839C are linked together through a disulphide inter-chain bond.
6. The stabilized hantaviral spike according to claim 3, wherein each mutant Gc of the at least one homodimer of mutants Gc has the substitution H953C (SEQ ID NO: 4), wherein the amino acid residues 953C are linked together through a disulphide inter-chain bond.
7. The stabilized hantaviral spike according to claim 3, wherein each mutant Gc of the at least one homodimer of mutants Gc has the substitution H953F (SEQ ID NO: 5).
8. The stabilized hantaviral spike according to claim 3, wherein each mutant Gc of the at least one homodimer of mutants Gc has the double substitution Q844C/H953C (SEQ ID NO: 6), wherein the amino acid residues 844C and 953C are linked respectively to the amino acid residues 844C and 953C through a disulphide inter-chain bond.
9. The stabilized hantaviral spike according to claim 1, comprising at least one heterodimer of mutants Gn/Gc, wherein the mutant Gn monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 281, 290, 291, 292, 293, 294, 295, 296 and 297, the indicated positions being determined by alignment with SEQ ID NO: 1; and wherein the mutant Gc monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 729, 730, 731, 732, 733, 734, 735, 736, 737 and 748, the indicated positions being determined by alignment with SEQ ID NO: 1.

10. The stabilized hantaviral spike according to claim 9, which comprises at least one heterodimer of a mutant Gn having the substitution H294C and a mutant Gc having the substitution T734C (SEQ ID NO: 7), wherein the amino acid residues 294C and 734C are linked together through a disulphide inter-chain bond.
11. The stabilized hantaviral spike according to claim 9, which comprises at least one heterodimer of a mutant Gn having the substitution R281C and a mutant Gc having the substitution P748C (SEQ ID NO: 8), wherein the residues 281C and 748C are linked together through a disulphide inter-chain bond.
12. The stabilized hantaviral spike according to claim 1, comprising at least one heterodimer of mutants Gn/Gc, wherein the mutant Gn monomer which comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 61, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98 and 99, the indicated positions being determined by alignment with SEQ ID NO: 1; and wherein the mutant Gc monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 738, 739, 740, 741, 742, 743, 744, 745, 746, 747, 748, 774, 775, 776, 777 and 778, the indicated positions being determined by alignment with SEQ ID NO: 1.
13. The stabilized hantaviral spike according to claim 12, which comprises at least one heterodimer of a mutant Gn having the substitution T99C and a mutant Gc having the substitution P774C (SEQ ID NO: 9), wherein the residues 99C and 774C are linked together through a disulphide inter-chain bond.
14. The stabilized hantaviral spike according to claim 12, which comprises at least one heterodimer of a mutant Gn having the substitution K85C and a mutant Gc having the substitution P774C (SEQ ID NO: 10), wherein the residues 85C and 774C are linked together through a disulphide inter-chain bond.
15. The stabilized hantaviral spike according to claim 12, which comprises at least one heterodimer of a mutant Gn having the substitution N94C and a mutant Gc having the substitution V776C (SEQ ID NO: 11), wherein the residues 85C and 774C are linked together through a disulphide inter-chain bond.
16. The stabilized hantaviral spike according to claim 1, which comprising at least one Gn/Gc heterodimer, wherein the mutant Gn monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 203, 204, 205 and 206, the indicated positions being determined by alignment with SEQ ID NO: 1; and wherein the Gc monomer comprises at least one amino acid mutation (substitution) at a position selected from the group consisting of: 853, 854 and 855, the indicated positions being determined by alignment with SEQ ID NO: 1.
17. The stabilized hantaviral spike according to claim 1, which comprises at least one homooligomer of a mutant Gn having at least one mutation (substitution) at a position selected from the group consisting of: 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74 and 75; and a mutant Gn having at least one mutation (substitution) at a position selected from the

group consisting of: 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205 and 206, the indicated positions being determined by alignment with SEQ ID NO: 1.

18. The stabilized hantaviral spike according to claim 16, which comprises at least one homooligomer of mutants Gn having each the double substitution E61C/Q200C (SEQ ID NO: 12), wherein the amino acid residues 61C and 200C are linked respectively to the amino acid residues 61C and 200C through disulphide inter-chain bonds between the two mutants Gn.

19. The stabilized hantaviral spike according to any one of claims 1 to 18, wherein the spike is in solution.

20. The stabilized hantaviral spike according to any one of claims 1 to 19, wherein the spike is incorporated into the envelope of recombinant viruses, pseudotype virus vectors, virus-like particles or any non-viral system.

21. The stabilized hantaviral spike according to claims 20, wherein the spike is incorporated onto virus-like particles.

22. A pharmacological composition comprising the stabilized hantaviral spike according to any one of claims 1 to 21.

23. The stabilized hantaviral spike according to any one of claims 1 to 21, or the pharmacological composition according to claim 22, for use in the preparation of a medicament effective in preventing and/or treating hantavirus infection.

24. A method for preventing and/or treating a hantavirus infection, comprising administering to a subject in need thereof the stabilized hantaviral spike according to any one of claims 1 to 21 or a pharmacological composition according to claim 22, in an amount effective to inhibit hantavirus infection of susceptible cells so as to thereby prevent or treat the infection.

25. A diagnostic agent comprising or consisting of a stabilized hantaviral spike according to any one of claims 1 to 21 and an appropriate diagnostic reagent.

26. A kit for diagnosing or monitoring, in a subject, a hantaviral infection, comprising the stabilized spike according to any one of claims 1 to 21 and an appropriate diagnostic reagent.

27. A kit for treating and/or preventing hantavirus infections, comprising the stabilized spikes according to any of claims 1 to 21 or the pharmacological composition according to claim 22.

28. Use of the stabilized spikes according to any one of claims 1 to 21, in the generation or selection of ligands useful to treat and/or prevent hantavirus infections.

29. Use of the stabilized spikes according to any one of claims 1 to 21, for the identification of epitopes recognized by ligands useful to treat or prevent hantavirus infections.

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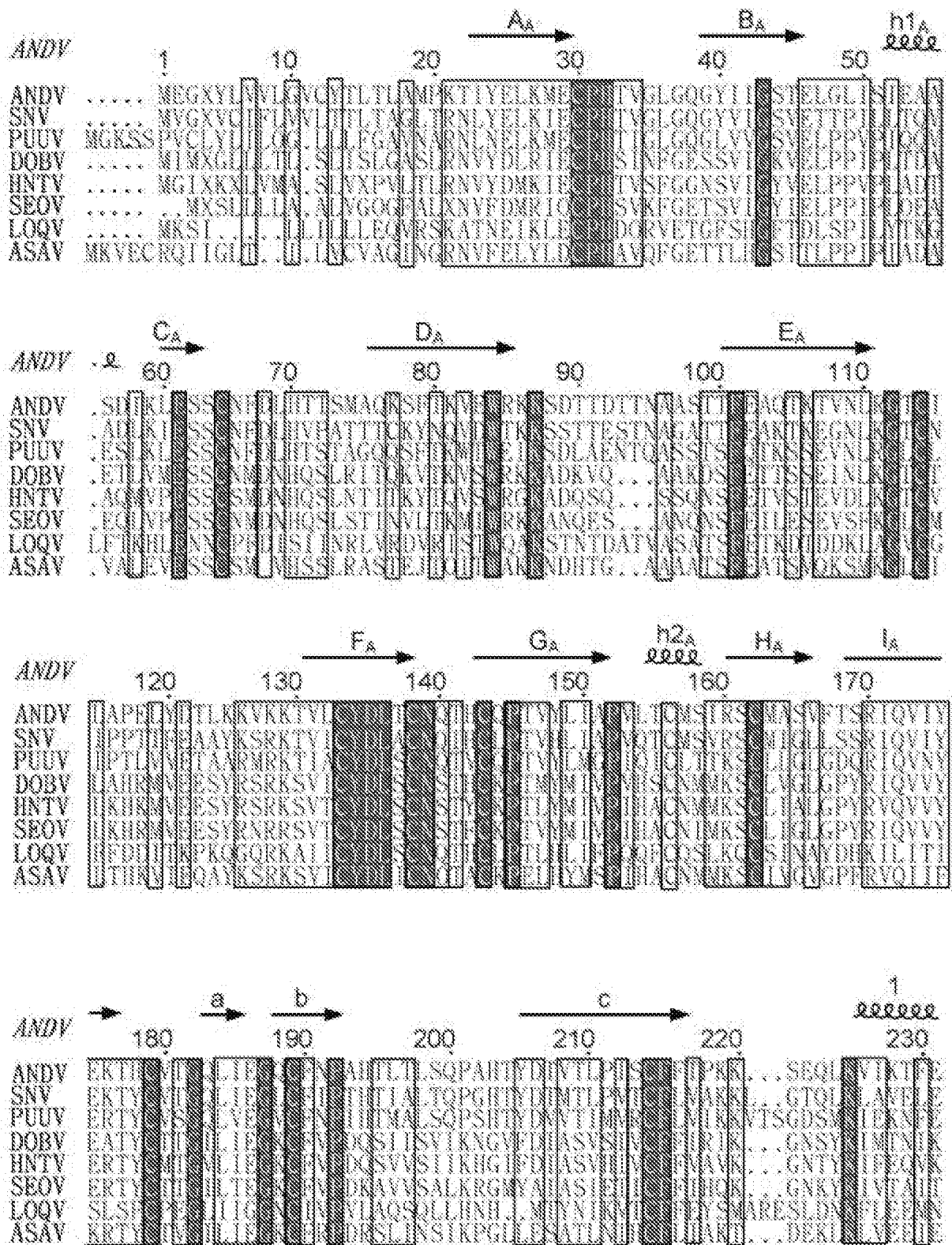


FIG. 1

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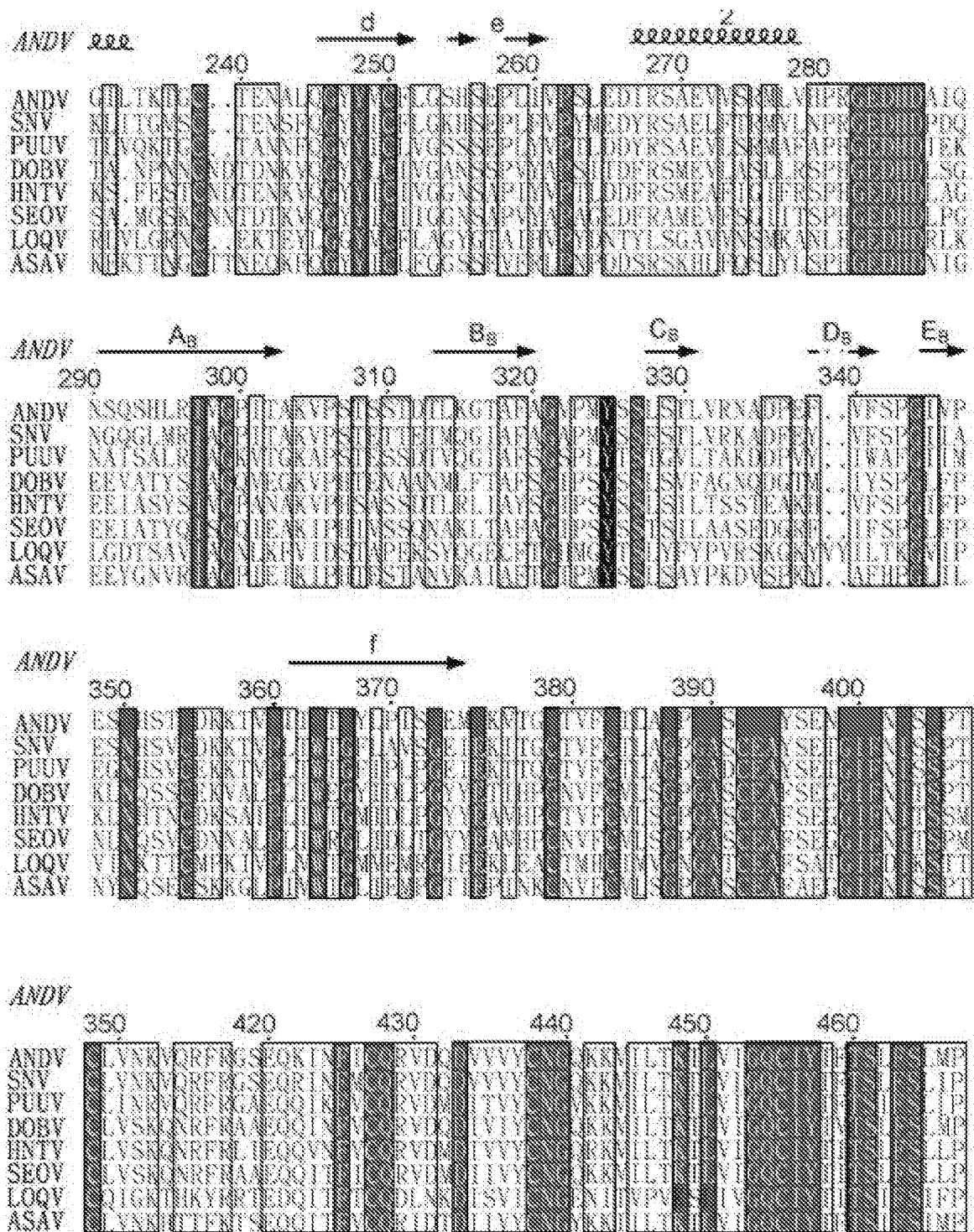


FIG. 1 (cont.)

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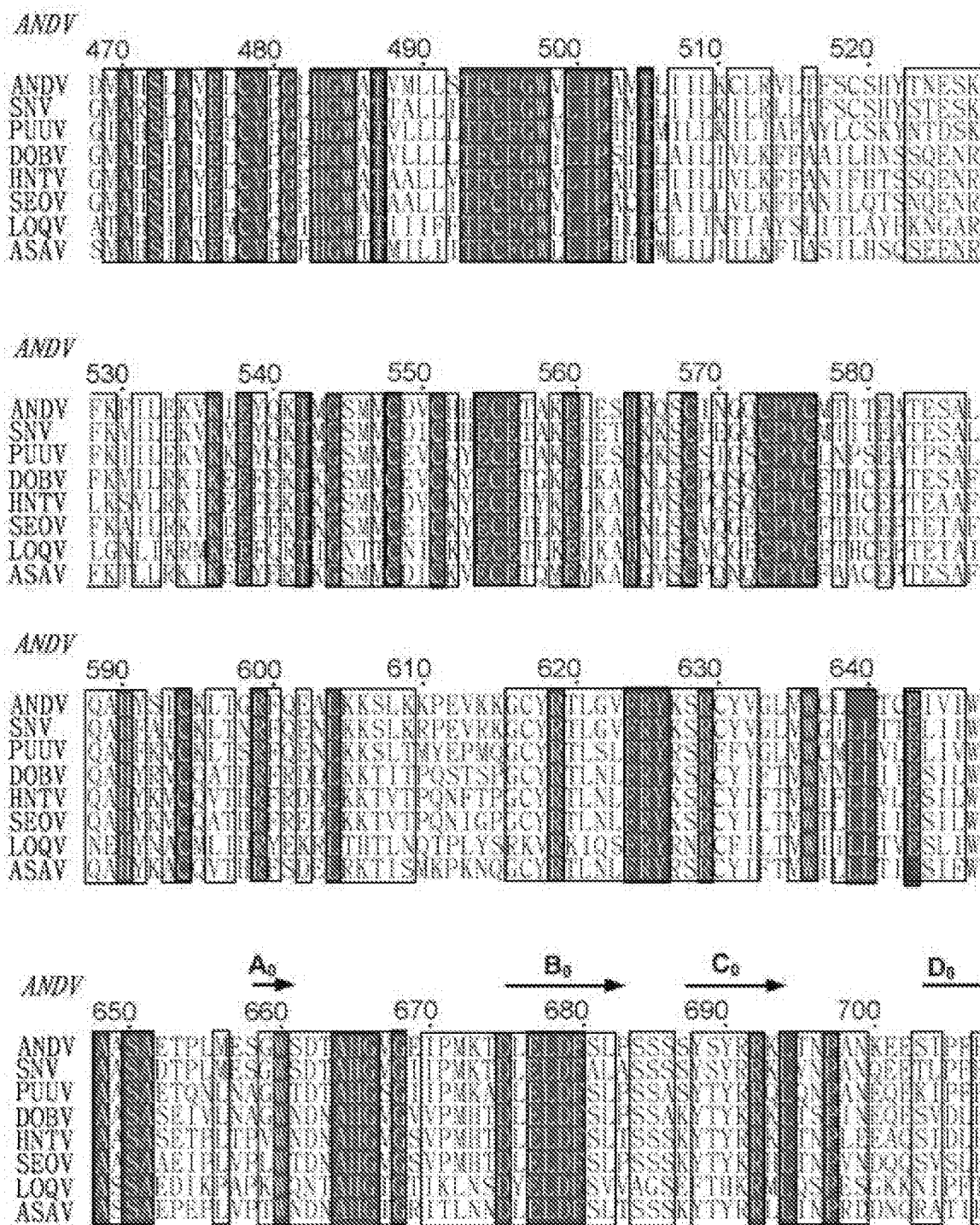


FIG. 1 (cont.)

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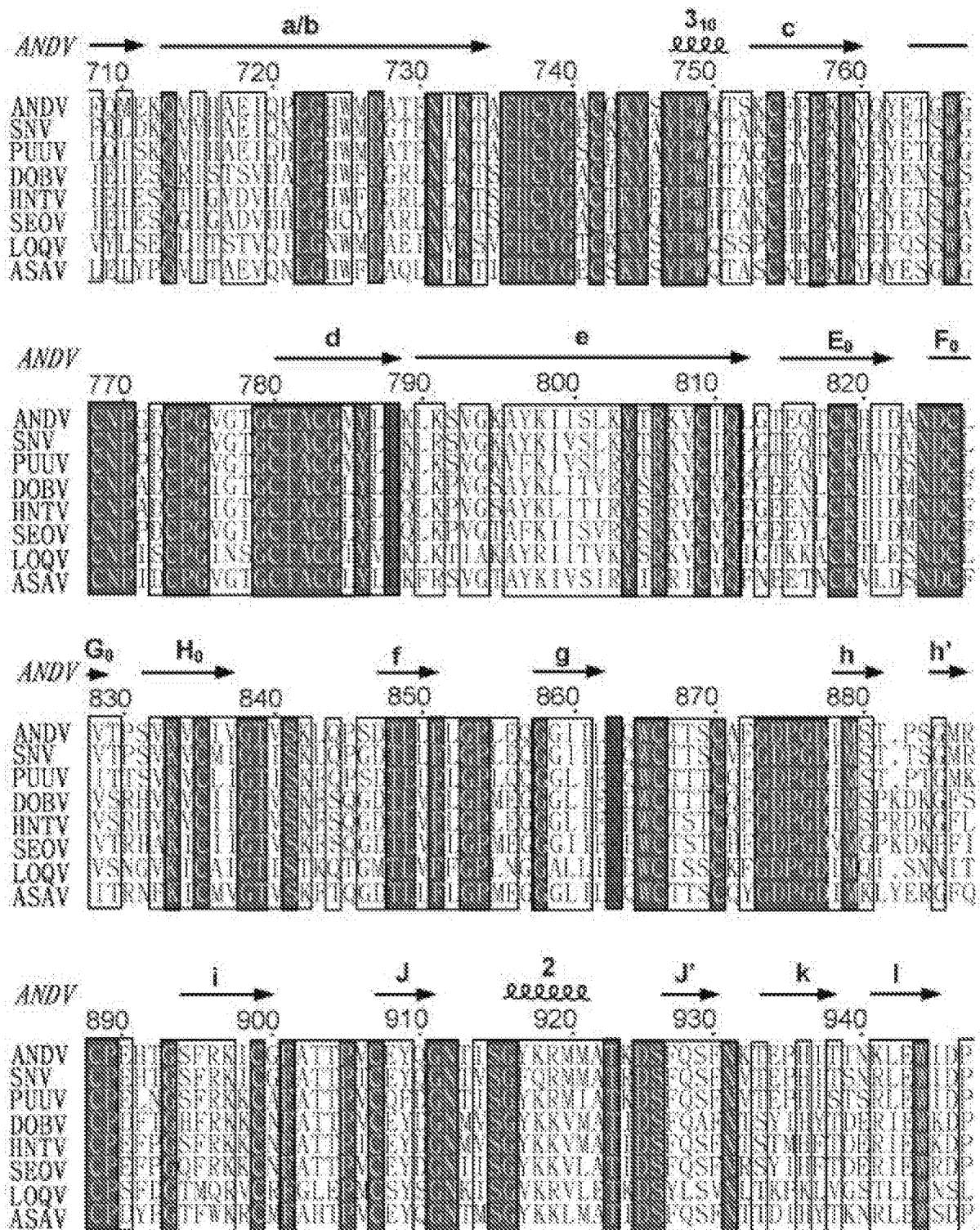


FIG. 1 (cont.)

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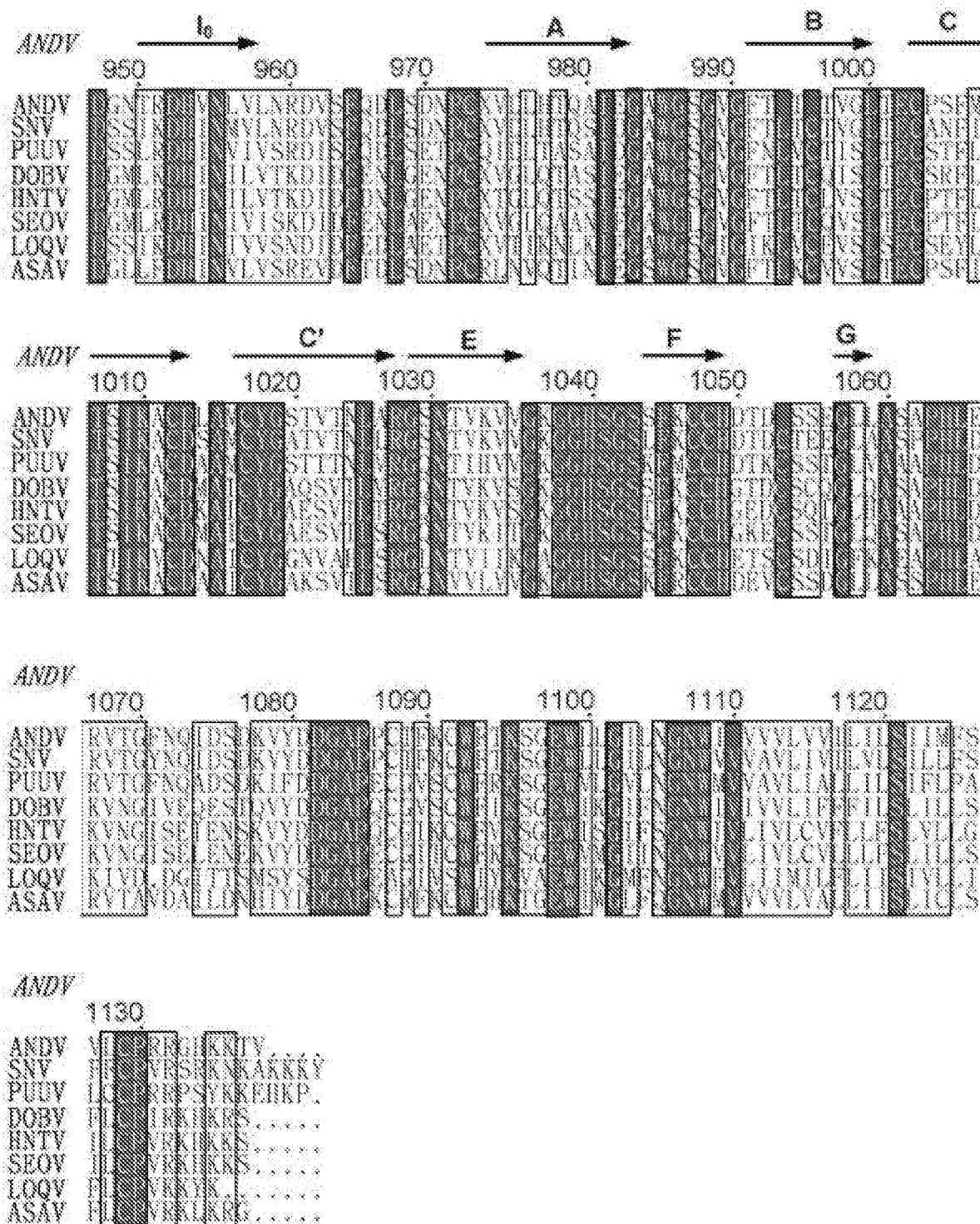


FIG. 1 (cont.)

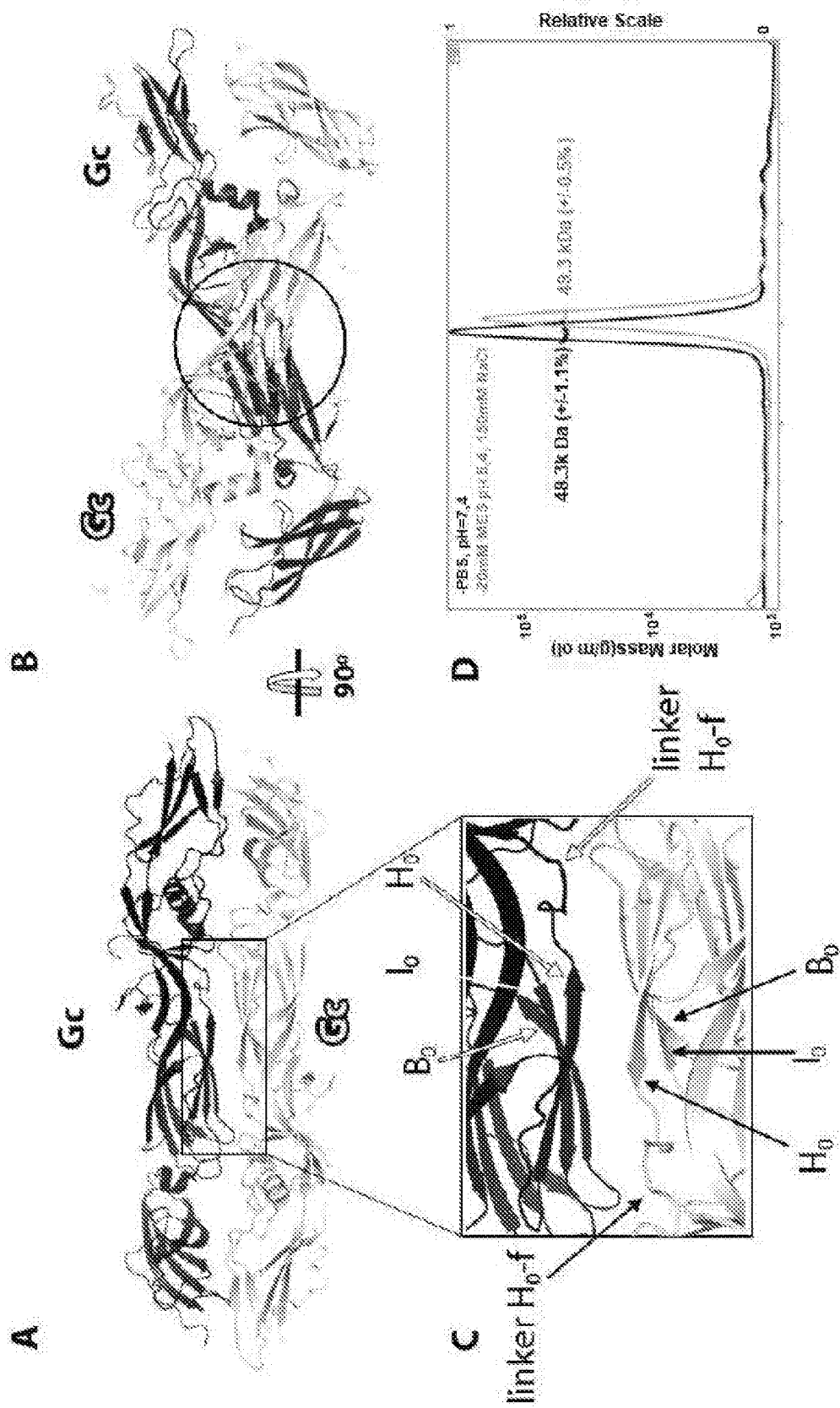


FIG. 2

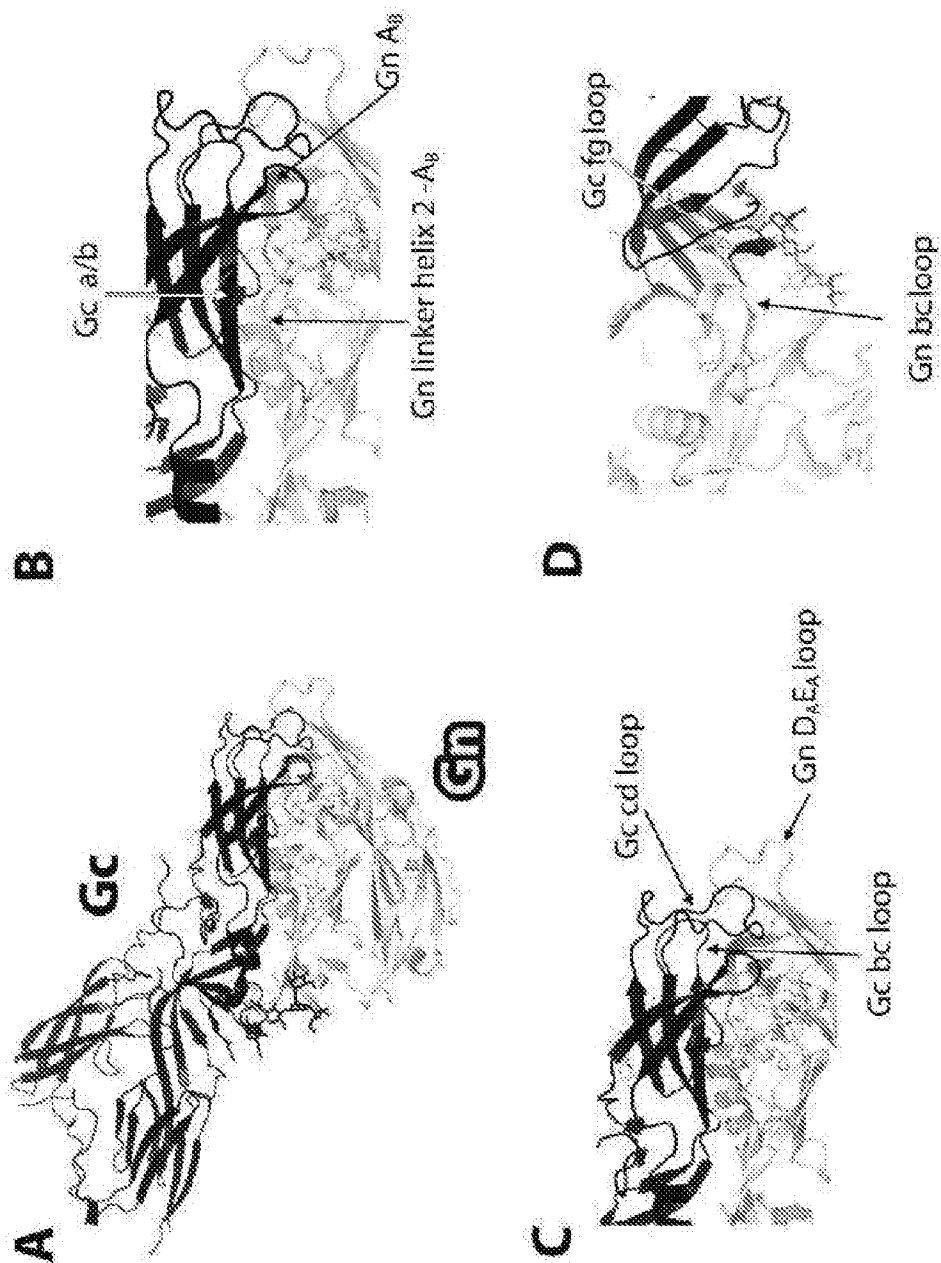


FIG. 3

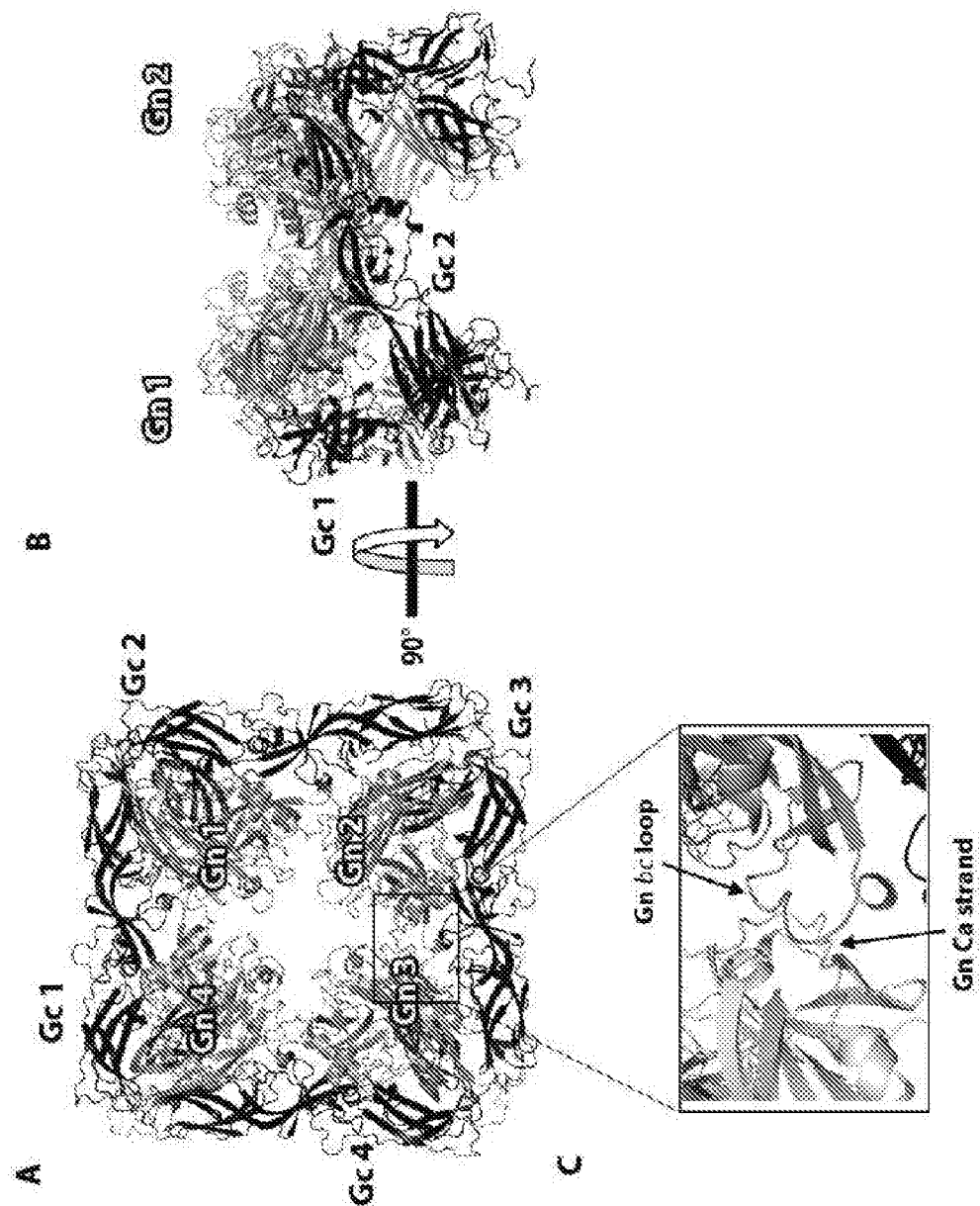


FIG. 4

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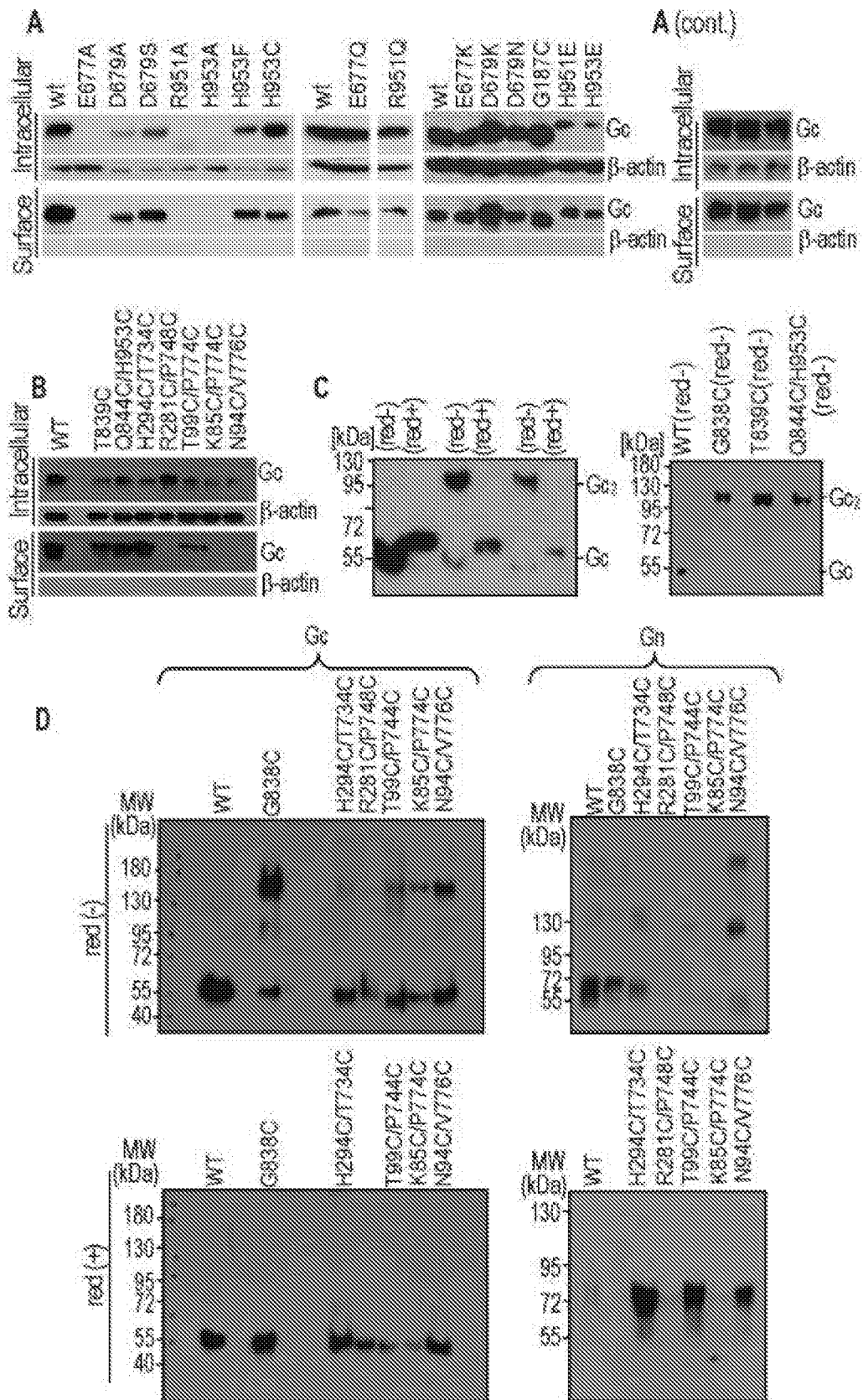


FIG. 5

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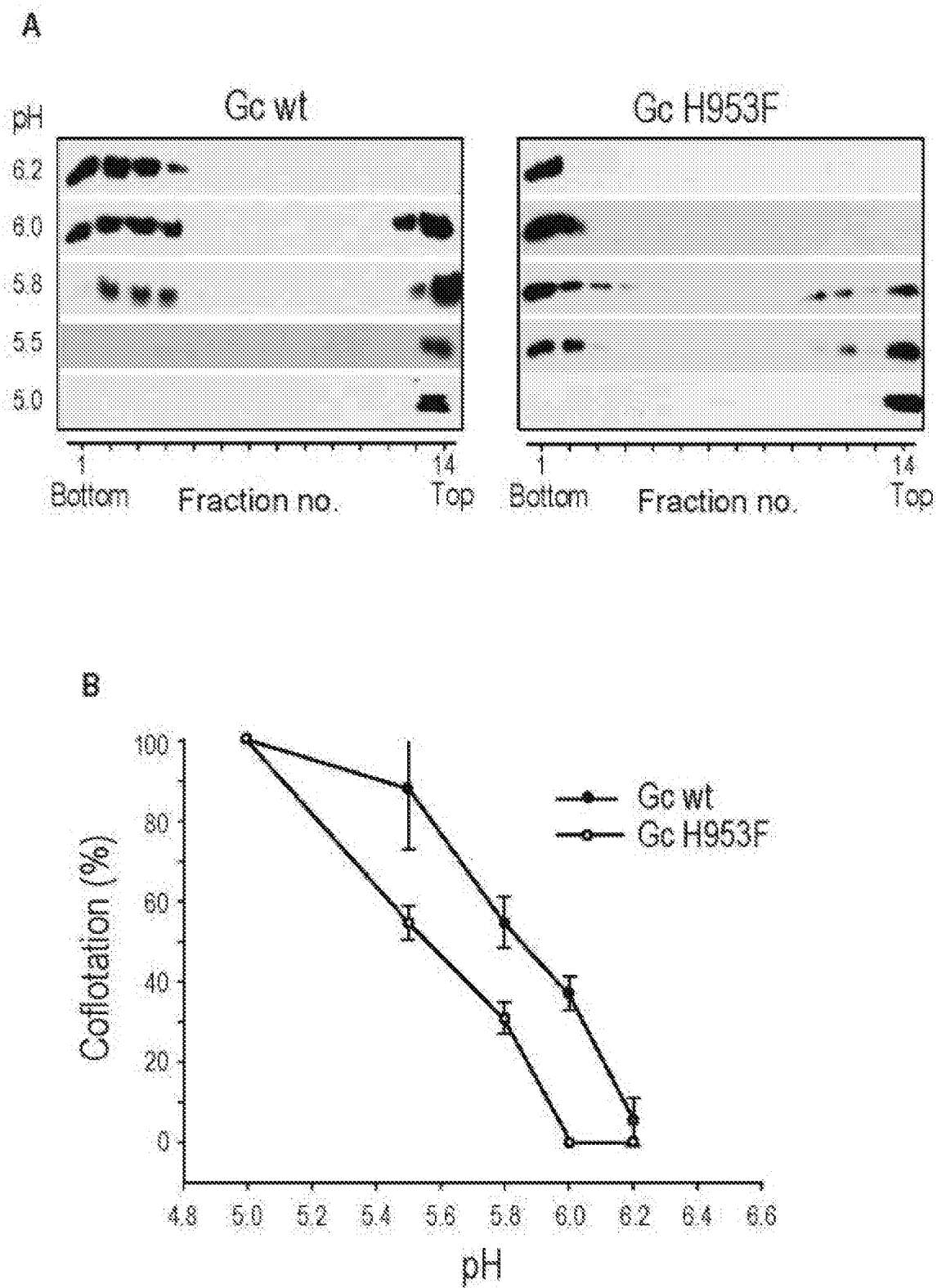


FIG. 6

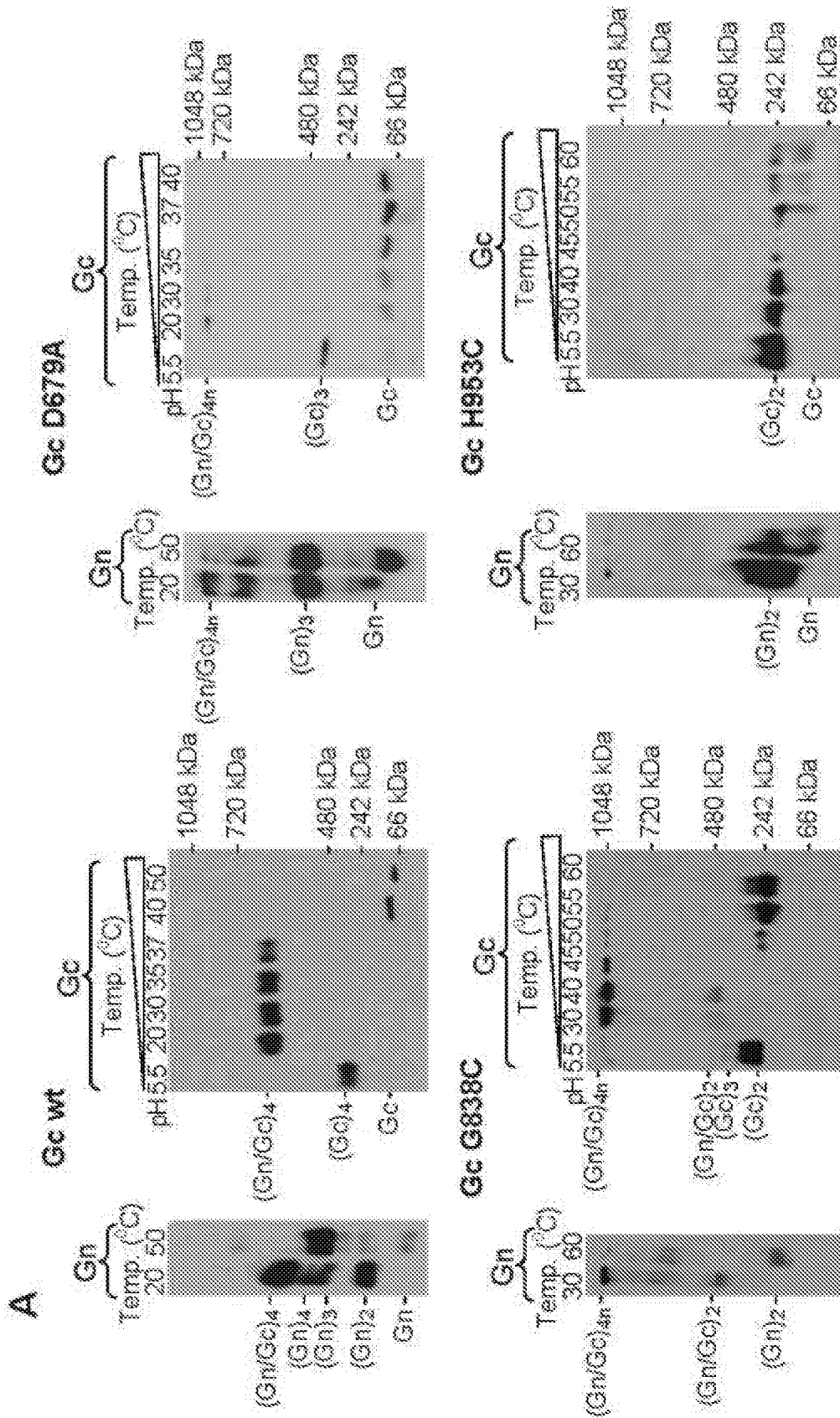


FIG. 7

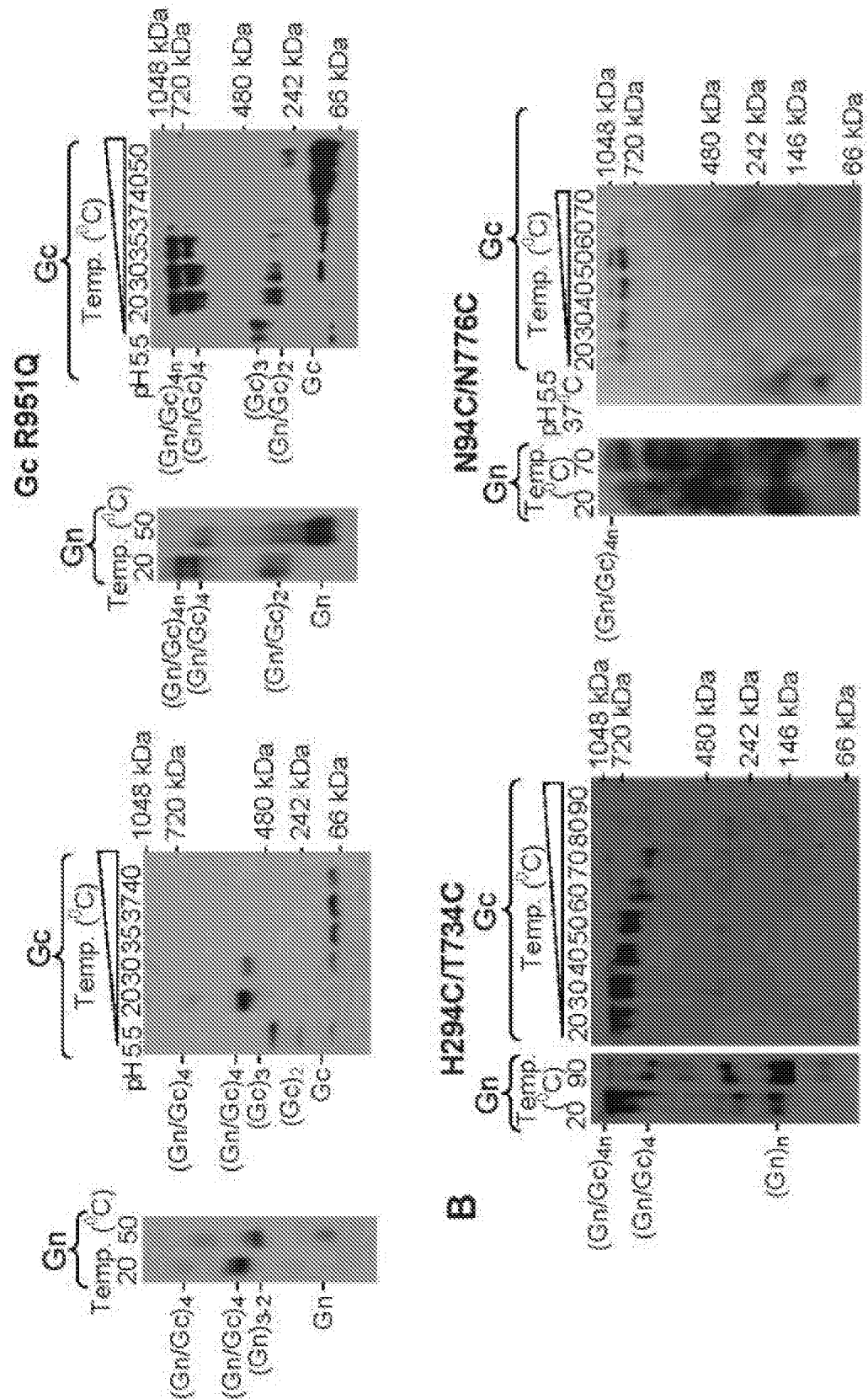


FIG. 7 (cont.)

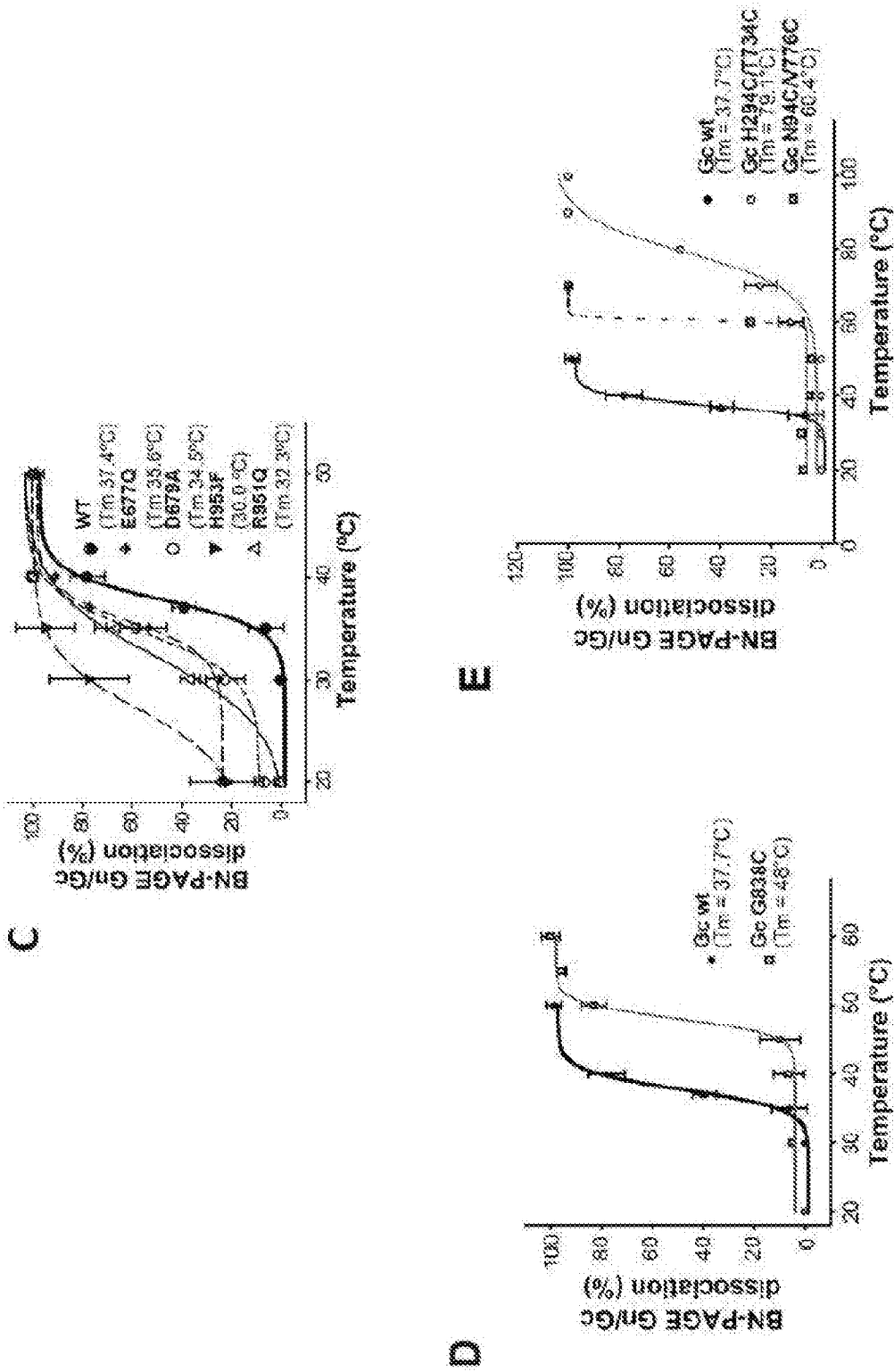


FIG. 7 (cont.)

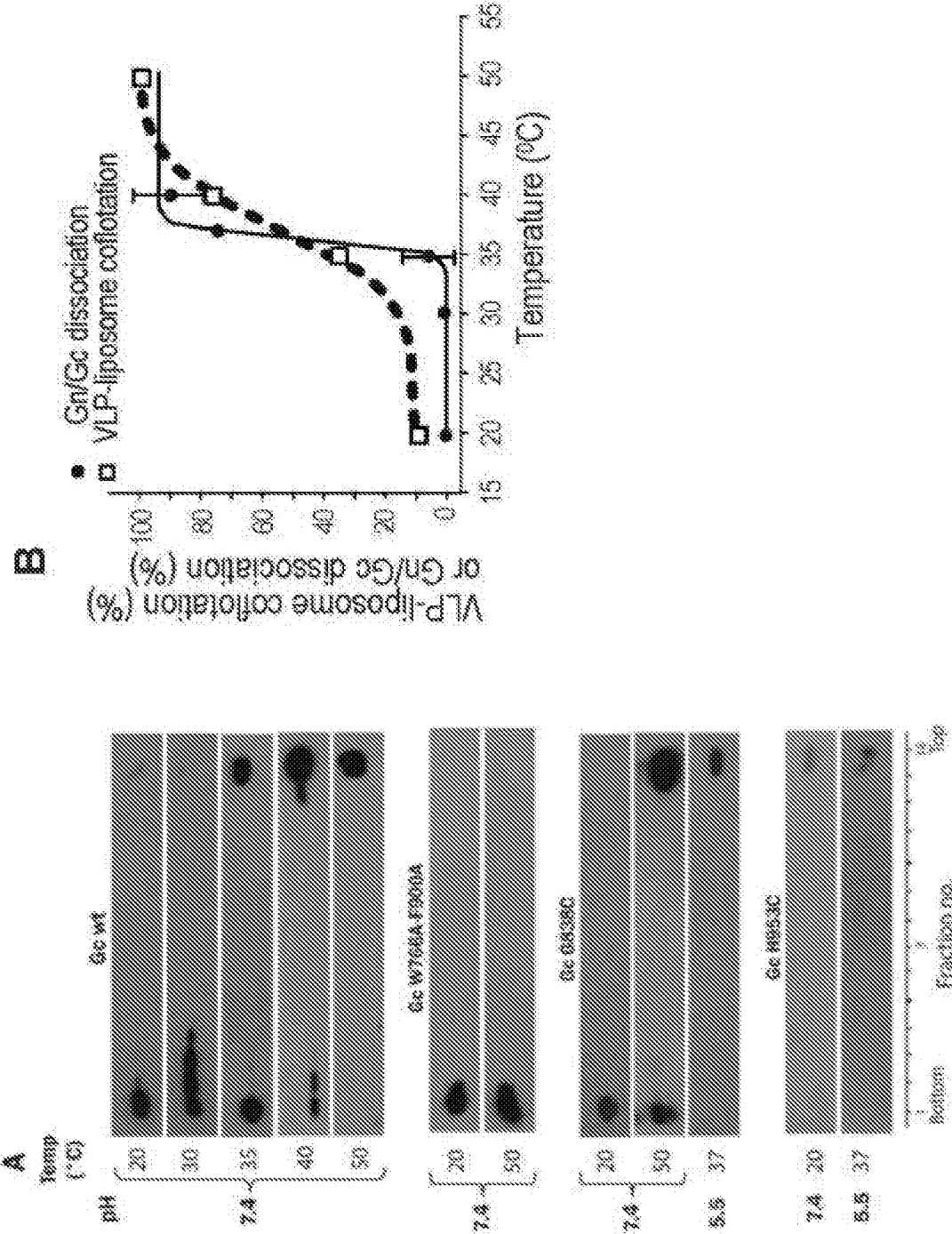


FIG. 8

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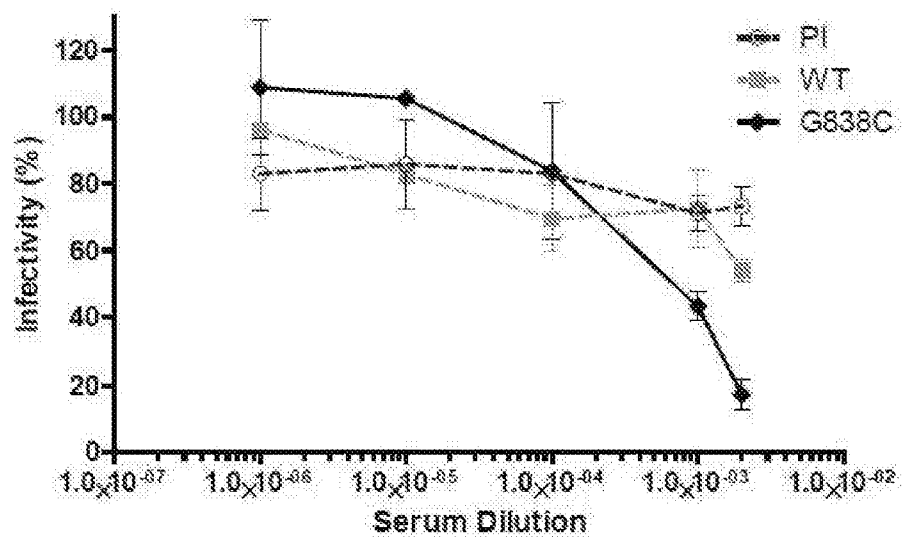
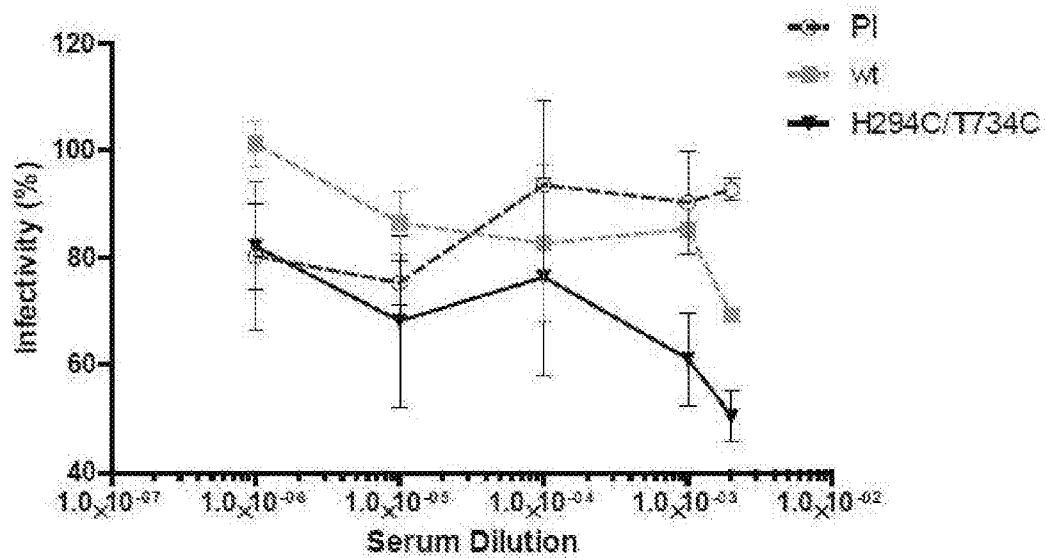
A**B**

FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/22134

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 39/12 (2019.01)

CPC - C07K 14/005, C07K 14/705, A61K 39/00, C12N 2760/12122

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SEN et al. "Degrons at the C Terminus of the Pathogenic but Not the Nonpathogenic Hantavirus G1 Tail Direct Proteasomal Degradation" Journal of Virology, April 2007, Vol 81, No 8, p. 4323-4330. Especially pg 4323, col 2, para 1, pg 4324, col 1, para 2, pg 4327, col 1, para 1, Figs. 6, 7A	1, 19/1
Y	GUARDADO-CALVO et al. "Mechanistic Insight into Bunyavirus-Induced Membrane Fusion from Structure-Function Analyses of the Hantavirus Envelope Glycoprotein Gc" PLoS Pathog, October 2016, Vol 12, No 10, page e1005813. Especially pg 15, para 2, pg 20, para 5	1, 19/1

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 June 2019

Date of mailing of the international search report

09 JUL 2019

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-8300

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Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/22134

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

- a. ☐ forming part of the international application as filed:
 - ☐ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
- b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
- c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

The USPTO will not be able to provide a sequence search for this application for the following reason:
ISA/225 mailed on 26 March 2019. No approved electronic sequence listing was submitted in response to the ISA/225. Accordingly, the international search is carried out only to the extent possible.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 19/22134

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 2-18, 19/(2-18)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The USPTO will not be able to provide a sequence search for this application for the following reason:
ISA/225 mailed on 26 March 2019. No approved electronic sequence listing was submitted in response to the ISA/225. Accordingly, no international search report has been established for claims 2-18, 19/(2-18), because a meaningful opinion could not be formed without the sequence listing.

3. ☒ Claims Nos.: 20-28
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.