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TITLE:
Receptor-binding domains of spike proteins of emerging or re-emerging viruses as targets for development of antiviral vaccines

ABSTRACT:
A number of emerging and re-emerging viruses have caused epidemics or pandemics of infectious diseases leading to major devastations throughout human history. Therefore, developing effective and safe vaccines against these viruses is clearly important for the protection of at-risk populations. Our previous studies have shown that the receptor-binding domain (RBD) in the spike protein of severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) is a key target for the development of SARS vaccines. In this review, we highlight some key advances in the development of antiviral vaccines targeting the RBDs of spike proteins of emerging and re-emerging viruses, using SARS-CoV, influenza virus, Hendra virus (HeV) and Nipah virus (NiV) as examples.

Introduction:
Epidemics and pandemics of emerging and re-emerging viral infectious diseases, such as severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV),1,2,3 influenza virus,4 Hendra virus (HeV) and Nipah virus (NiV),5,6 have posed a great threat to public health, human safety and economic stability worldwide. Therefore, development of effective and safe vaccines to prevent these diseases is urgently needed.

Safety concerns have arisen over the use of whole inactivated or live-attenuated viruses for the development of antiviral vaccines;7 therefore, scientists have been attempting to design vaccines based on the spike proteins of emerging and re-emerging viruses. Indeed, spike protein-based vaccines are safer and more effective than vaccines based on inactivated or live-attenuated viruses.8 However, new concerns have been raised over the use of full-length viral spike proteins for developing subunit vaccines because some of the non-neutralizing, or immunodominant, epitopes in the spike proteins may induce antibodies that enhance, rather than neutralize, viral infection, or otherwise cause harmful inflammatory responses in vaccinated hosts.9,10 Therefore, selection of the key functional domains that contain neutralizing and/or T-cell epitopes as the immunogens appears to be an important strategy in developing antiviral vaccines.

Infection is initiated upon binding of the viral spike protein with the receptor or coreceptor on the target cell surface, as mediated by the spike protein's receptor-binding domain (RBD) (Figure 1). Therefore, the RBD plays an important role in viral attachment, fusion and entry and may serve as an attractive target for designing antiviral vaccines. Our previous studies have demonstrated that the vaccines based on the RBD in the spike protein of SARS-CoV (Figure 2A) induced the most potent neutralizing antibody responses and protective immunity in vaccinated animals,11,12,13,14,15,16,17 suggesting that the RBDs in the spike proteins of other viruses that cause emerging and re-emerging infectious diseases, such as influenza virus (Figure 2B), HeV and NiV (Figure 2C), may also serve as targets for developing vaccines to prevent corresponding infectious diseases.18

Here, we reviewed important advances in the development of antiviral vaccines that mainly target the RBD of spike proteins of some viruses which have caused serious emerging and re-emerging infectious diseases during the past three decades, and assess the feasibility of applying similar strategies in the design and development of vaccines against other emerging and re-emerging viruses.

The S protein and its RBD of SARS-CoV ::: Development of SARS vaccines targeting the RBD of SARS-CoV spike protein:
SARS-CoV is an enveloped virus containing a single and positive-stranded RNA. The genome RNA encodes nonstructural replicase polyprotein and structural proteins, including spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins.2,25 SARS-CoV infection can trigger a series of humoral and cellular immune responses, mainly targeting the S protein.26,27
The S protein of SARS-CoV is a type I transmembrane glycoprotein with its N terminus on the surface of the infected cell or the virus particle. Its precursor, consisting of 1255 amino acids (aa), can be cleaved by proteases into the surface subunit S1 (aa 17–680) and the transmembrane unit

S2 (aa 681–1195)28,29 (Figure 2A). S1 recognizes and binds to host cell receptors, leading to the conformational changes of S2 that mediate viral fusion between the viral envelope and the host cell membrane.30

The minimal RBD of SARS-CoV S protein is located in the S1 subunit (aa 318–510) and is responsible for viral binding to host cell receptors31,32 (Figure 2A). Besides the main receptor, angiotensin-converting enzyme 2, there are several alternative receptors, such as dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin and/or liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin.33 Two residues (aa 479 and aa 487) in RBD determine SARS progression and tropism, and their mutations may enhance animal-to-human or human-to-human transmission.29 Some residues (aa 109, 118, 119, 158, 227, 589 and 699) in S protein are critical for dendritic cell/lymph node-specific intercellular adhesion molecule-3-grabbing integrin-mediated virus entry.34

Vaccines based on the RBD of SARS-CoV S protein ::: Development of SARS vaccines targeting the RBD of SARS-CoV spike protein:

The roles of S protein in receptor binding and membrane fusion make it a perfect target for vaccine and antiviral development. Several vaccine candidates based on the full-length S protein of SARS-CoV have shown high immunogenicity in inducing neutralizing antibody responses and protection against SARS-CoV challenge.8,9 However, these vaccines may also induce harmful immune responses that cause liver damage in the vaccinated animals10 and/or enhance SARS-CoV infection through an antibody-dependent mechanism.9 All of these have raised significant concerns about the safety and ultimate protective efficacy of vaccines based on the entire S protein.

Previous studies indicated that RBDs of S protein of coronaviruses (mouse hepatitis virus and human coronavirus 229E contain major antigenic determinants that can induce neutralizing antibody responses.35,36 We therefore postulated that RBD of SARS-CoV may also be the target of neutralizing antibodies. Indeed, we found that a majority of the neutralizing antibodies in the sera of SARS patients and animals immunized with the inactivated SARS-CoV strongly reacted with the RBD of SARS-CoV S protein since depletion of the RBD-specific antibodies resulted in elimination of the neutralizing activity.12,37 Chen et al.38 also demonstrated that recombinant RBD could absorb and remove most neutralizing antibodies from the antisera of mice, rabbits and monkeys immunized with a live-attenuated modified vaccinia Ankara virus expressing the full-length S protein. We have constructed several subunit SARS vaccine candidates using recombinant RBD expressed in Escherichia coli, insect and mammalian cells and used them to immunize mice. We found that all of them could induce highly potent and broad cross-reactive neutralizing antibody responses11,17 and protective immunity against challenge with live SARS-CoV. We also demonstrated that intramuscular and mucosal immunization of animals with the adeno-associated virus-based RBD vaccine could elicit strong neutralizing antibody responses and protection against SARS-CoV challenge.15,16,17 These findings suggest that this RBD-based vaccine has considerable potential for development into a safe and effective SARS vaccine to prevent future SARS epidemics.

Therefore, our previous studies on SARS vaccines have proven the concept of RBD-based vaccines and have shown that this strategy could also be applied to the development of vaccines against other infectious diseases.

IAV hemagglutinin and its RBD ::: Rational design of influenza vaccines based on neutralizing epitopes in the RBD in the hemagglutinin of influenza A viruses:

IAVs belong to the family of Orthomyxoviridae. The genome of the virus consists of eight single-stranded, negative-sense RNA segments, encoding several important proteins, including surface proteins hemagglutinin (HA), neuraminidase (NA) and matrix 2 protein (M2), nucleoprotein (NP), matrix protein 1 (M1), non-structural proteins (NS1, NS2), as well as polymerase proteins PB1, PB2 and PA.44 HA and NA are two important envelope glycoproteins of the virus, and all IAVs are classified into 16 antigenically distinct HAs (H1–H16) and 9 different NAs (N1–N9), according to the serotypes of HA and NA.

The HA of IAVs is a homotrimer and plays important roles in virus infection. Each of the single-chain monomers is initially synthesized as a precursor polypeptide, HA0, which is then cleaved by host proteases into two subunits, HA1 and HA2. The HA1 subunit is mainly responsible for receptor binding. It mediates viral attachment to the cellular receptors of the target cells known as sialic acid of glycoproteins and glycolipids.45 Different HAs of IAVs recognize different sialic acid receptors. For example, the HAs from humans bind preferentially to sialic acids in the α (2,6)

linkage, while those from avians and equines recognize α (2,3) linkages. It appears that the HAs from swine may recognize both α (2,3) and α (2,6) linkages.46 After viral attachment to the target cells, the HA2 subunit located at the membrane-proximal helix-rich stem region mediates virus entry into the target cell by fusion of the viral envelope and the cellular membrane via the fusion peptide at the N-terminus of HA2.45 The HA2 N-terminal fusion peptide contains the highly conserved sequence among all IAVs, and neutralizing epitopes targeting this region have been identified.47

As the main region for binding cellular receptors, RBD is located at the membrane-distal end (HA1) of each HA monomer, spanning less than 300 aa (from residues around 105 to residues around 319, depending on different HAs). The receptor-binding site is composed of three structural elements, including the 190-helix (HA1 188–190), the 130-loop (HA1 134–138) and the 220-loop (HA1 221–228), as well as several conserved residues at the W153, H183 and L194 positions.45 The RBD of HA is the primary target of neutralizing antibodies, based on which anti-IAV vaccines can be designed and developed.48

Current influenza vaccines and disadvantages ::: Rational design of influenza vaccines based on neutralizing epitopes in the RBD in the hemagglutinin of influenza A viruses:
The latest influenza pandemic caused by 2009 swine-origin IAV H1N1, coupled with the continuing threat of the highly pathogenic avian influenza A virus H5N1, makes it essential to develop efficacious vaccines to prevent future influenza pandemics and epidemics. Conventionally, influenza vaccines have been prepared on the basis of inactivated virus, in which viruses are grown in embryonated chicken eggs and then inactivated with formalin and purified for use with appropriate adjuvants. However, these vaccines are mainly administered parenterally, limiting their ability to induce high immunity and efficacy in preventing heterologous virus infection.49 Therefore, live-attenuated, cold-adapted influenza vaccines have been produced to increase immunogenicity. When injected intramuscularly into mice, these live influenza virus-based vaccines induced greater protection than those based on the inactivated virus.50 Unfortunately, a safety concern arose from the possibility of recovering virulence or the potential re-assortment of the viruses with circulating influenza viruses. Although other available influenza vaccines, including those based on DNA, protein and viral vectors, as well as virus-like particles, may induce immune responses and/or protection against IAV infection,51,52 their capacity to elicit highly potent and broad immune responses and protection is greatly restricted.
These disadvantages have made it essential to explore alternative novel approaches for future vaccine development. Preference will be given to those vaccines that are capable of stimulating broad neutralizing antibody responses and cross-protective immunity against divergent IAVs, including the pandemic strains of 2009 H1N1 and highly pathogenic strains of H5N1.

Discovery of the neutralizing epitopes in the RBD of IAV ::: Rational design of influenza vaccines based on neutralizing epitopes in the RBD in the hemagglutinin of influenza A viruses:
Neutralizing antibodies play critical roles in protecting against IAV infection. The neutralizing antibodies could be induced by virus infections or raised by vaccination with specific antigens. The produced antibodies may neutralize various viruses by first recognizing and targeting specific positions (epitopes) of viral antigens. Currently, a number of neutralizing epitopes have been identified by binding of the neutralizing antibodies to the exposed loops surrounding the receptor binding site of HA protein and interfering with virus attachment, or selecting antibody escape mutants.53
Using genome-fragment phage display libraries, a group from the US Food and Drug Administration was able to map the epitopes of the neutralizing monoclonal antibody (mAb) FLD21.140 into two aa clusters in the RBD, 121-SWS-123 and 164-YNNT-167, the highly conserved sequences among clades 1, 2, 5, 6 and 8 of H5N1 viruses.48 Using yeast surface display of neutralizing mAb NR2728 with a full-length H5 HA, researchers identified a conformation-dependent neutralizing epitope in the interface overlapping the receptor binding site of HA1. Another conformational epitope adjacent to the RBD was identified by mapping mAb S139/1 with HA. This mAb showed cross-reactive neutralization and HA inhibition against particular strains of H1, H2, H3 and H13 subtypes of IAVs.54
Conformational neutralizing epitopes covering residues 125–129 and 157–169 at the apex of the RBD were identified by co-crystal structure of the 1918 HA with 2D1 mAb from a survivor of the 1918 Spanish flu. These epitopes were conserved in pandemic viruses causing the 1918 Spanish flu and 2009 swine-origin IAV.55 Studies on the HA of Qinghai-type influenza H5N1 virus revealed other antigenic epitopes at the globular head region of H5 HA containing residues 113, 117, 118,

120 and 123.56 Neutralizing epitopes at residues 260 and 261 of RBD were also identified using H5N1-specific neutralizing monoclonal Ab 9F4. These residues are well-conserved across different clades of H5N1 strains.57

In addition to the above neutralizing epitopes identified by antibody binding assays, several immunodominant epitopes were identified by selection of escape mutants using neutralizing mAbs. These neutralizing epitopes cover residues at positions 138, 140, 155, 159, 189, 194, and 218 of RBD.58 Other conserved neutralizing epitopes in the head region of HA1 were identified from naturally occurring antibodies F045-092 and F026-427.59 The neutralizing epitopes at residues 178–181 and 227–239 of the receptor binding site of HA globular region were identified by two human neutralizing antibodies, B-1 and D-1, to H3N2 derived from different donors. These neutralizing epitopes are proven to be highly conserved in human H1N1, swine-origin pandemic H1N1, human H5N1 and avian H5N1 strains.60 It was also reported that a neutralizing mAb, CH65, was capable of recognizing the receptor-binding pocket of IAV H1N1 HA, indicating that broad neutralization of IAV could be achieved by antibodies targeting the RBD pocket.61

Prospective development of novel influenza vaccines based on the neutralizing epitopes in RBD of the IAV HA1 ::: Rational design of influenza vaccines based on neutralizing epitopes in the RBD in the hemagglutinin of influenza A viruses:

Identification of the above neutralizing epitopes in the RBD region brings hope for the development of innovative vaccines because the identified epitope regions may be applied as novel vaccine targets. Vaccines based on these neutralizing epitopes could induce neutralizing antibodies against a broad spectrum of IAV strains. Studies on the development of such vaccines have been initiated, and some of them have shown promising results.62

It is reported that an E. coli-expressed recombinant protein, HA63-286-RBD, containing HA-RBD of the A/H1N1/2009 influenza virus, is immunogenic with the capacity to trigger neutralizing antibodies and induce protective activity in the tested ferret model.63 Studies have shown that recombinant vaccines targeting the globular head RBD region fused to flagellin of Salmonella typhimurium fljB gene induced protective immunity to H1N1 and H5N1 infections in challenged mice.62 It was demonstrated that a recombinant oligomeric protein covering H5N1 (A/Vietnam/1203/2004) HA1-RBD neutralizing epitopes elicited potent neutralizing antibodies in the vaccinated mice against homologous and heterologous H5N1 strains and protected ferrets from homologous and heterologous H5N1 challenge.48 Previous studies by the same group also demonstrated the protective immunity of vaccines containing H1N1 (A/California/07/2009) HA1-RBD region against challenge of H1N1 pandemic influenza virus. A report by Prabakaran and colleagues indicated that a recombinant baculovirus-expressed trivalent vaccine covering the major neutralizing epitopes in the RBD of H5N1 effectively neutralized viruses from clades 1, 2.1, 2.2, 4, 7 and 8 of H5N1 and protected 100% of the mice against challenge with three different clades (clade 1.0, clade 2.1 and clade 7.0) of H5N1 strains. Our recent studies also revealed that a recombinant HA1-Fdc vaccine containing RBD neutralizing region induced high titers of antibodies that cross-neutralized clades 0, 1, 2.2 and 2.3.4 of H5N1 strains and completely protected vaccinated mice against high-dose lethal challenge of different strains of H5N1 covering clades 0, 1 and 2.3.4.64

The above findings further support our hypothesis that the neutralizing epitopes in the RBD of IAV HA are attractive targets for development of universal influenza vaccines, which can be used to prevent future influenza pandemics or epidemics caused by emerging or re-emerging IAV strains.

RBDs in the G proteins of HeV and NiV as targets for vaccine development:

HeV and NiV are emerging zoonotic viruses discovered during outbreaks in Australia in 1994 and in Malaysia in 1998, respectively. As newly defined viruses, HeV and NiV belong to the family Paramyxoviridae and genus Henipavirus.5 The broad species tropism and the ability to cause severe diseases in both humans and animals distinguish HeV and NiV from other zoonotic viruses. Symptoms of HeV and NiV infection of humans can be respiratory, including hemorrhage and edema of the lungs, or encephalitic, resulting in meningitis. The principal natural reservoirs for HeV and NiV are pteropid fruit bats; however, recent evidence of henipavirus infection has been found in a wider range of species, including flying foxes, humans, cats, horses, rabbits and laboratory rodents6 (Figure 4). Because HeV and NiV can potentially cause significant morbidity and mortality in humans, with consequent major economic and public health impact, they are classified as biological safety level-4 pathogens.65

Both HeV and NiV have non-segmented, negative-stranded RNA genosomes consisting of transcription units encoding six major structural proteins, including nucleocapsid protein (N),

phosphorprotein (P), matrix protein (M), fusion protein (F), glycoprotein (G) and large protein (L).66 The G protein, which contains a length of 602 aa, is responsible for virus binding to the receptor and entry into the host cell.

While the G protein has neither HA nor neuraminidase activities, it plays an important role in HeV and NiV binding to ephrin-B2 and ephrin-B3, which are members of a large family of important signaling proteins involved in cell–cell interactions.67 Binding of RBD in the G protein with its receptor facilitates F protein-mediated viral fusion with the target cell. Mutations of some residues in the RBD of the G protein significantly impaired viral binding and fusion activities.68 The G protein of HeV or NiV is comprised of a short cytoplasmic tail, a transmembrane domain, a stalk region and a globular head domain68 (Figure 2C). The globular head region of the G protein contains the minimal RBD (aa 439–468)68 (Figure 2C). The G protein of HeV or NiV appears to be the dominant target antigen for neutralizing antibodies.69 While no vaccines are available for the prevention of henipavirus disease in humans, several G protein-based henipavirus vaccine candidates have been tested in three different animal models.70 Two of them using ALVAC canarypox as vector showed protection against NiV infection in pigs. A recombinant soluble G protein-based vaccine adjuvanted with CpG was shown to induce strong immune responses in a ferret NiV infection model, which could protect cats from NiV infection.71

The first human neutralizing mAb, m101, with exceptional potency against HeV and NiV, was identified in 2006.72 A panel of 17 alanine-scanning mutants of the G protein of HeV was tested for binding ability to the human neutralizing antibodies m101 and m102.4. It was found that the binding of HeV G mutants D260A, G439A, K443A and K465A to both m101 and m102.4 was almost completely eliminated, while the binding activity of mutants G449A and D468A was significantly decreased.73 These results suggest that the epitopes of the neutralizing antibodies overlap the RBD of HeV or NiV G proteins and that some discontinuous residues located in the RBD are critical for HeV or NiV binding to its receptors ephrin-B2 and ephrin-B3.74 Based on these findings, the design of an RBD-based henipavirus vaccine to prevent HeV or NiV infection is very feasible.

Conclusion and prospects:

The use of RBD of spike proteins for developing antiviral vaccines to prevent epidemics of emerging or re-emerging viral infectious diseases is attracting more research interest. The common features of RBDs of spike proteins of SARS-CoV, influenza virus and Hendra virus include: (i) being located in the spike proteins; (ii) mediating binding of the virus to the target cell via the interaction between viral spike protein and the receptor on the target cell; (iii) having a length ranging from 30 to 215 aa; and (iv) containing discontinuous neutralizing epitope(s).

With its relatively conserved sequences, RBD plays a critical role in the elucidation of antiviral immune response and protective immunity. The best example is the RBD-based SARS vaccine. It is a safe vaccine candidate and has been shown to induce the most effective immune response and protective immunity against SARS-CoV. Thus, it is expected that an RBD-based SARS vaccine will be one of the first SARS vaccines going to clinical trials.

To design vaccines with the ability to induce highly potent and cross-reactive neutralizing antibodies with high binding affinity, we recommend the following strategies: (i) construct an immunogen containing the discontinuous sequences of bonding set and/or induced epitopes; (ii) induce conformational changes by mutation or glycan shield of the non-neutralizing immunodominant epitopes from the immunogen in order to reduce the immunogenicity of these epitopes; and (iii) add an immunopotentiation motif to the immunogen in order to enhance the immunogenicity of the RBD-based vaccine.

Similar to the RBD of SARS-CoV spike protein, the IAV HA protein RBD spans about 215 aa (aa 105–319) with three conserved structural elements45 and contains primary neutralizing antibody epitopes that serve as important targets for influenza vaccine development.48 A number of broadly cross-reactive neutralizing mAbs, such as NR2728 (146), S139/1,54 B-1 and D-1,61 were identified to specifically bind to the conformation-dependent neutralizing epitopes in the interface overlapping with, or adjacent to, the RBD in HA1. Our recent studies have shown that vaccination of mice with the fusion protein HA1-Fdc, which consists of H5N1 HA1, a trimeric motif foldon and a human immunoglobulin G Fc fragment, resulted in induction of potent neutralizing antibody responses and complete cross-clade protection against H5N1 infection,64 including elucidation of neutralizing mAbs whose epitopes were mapped to RBD (unpublished data). All these findings support the feasibility of developing RBD-based influenza vaccines. It is anticipated that these RBD-based influenza vaccines could be effective against divergent IAV strains, including those that may cause future influenza pandemics or epidemics.

HeV and NiV, which belong to the family Paramyxoviridae and genus Henipavirus,5 are emerging zoonotic viruses that caused outbreaks in humans in Australia and resulted in high mortality. The G protein of HeV or NiV contains the minimal RBD overlapping about 30 aa (aa 439–468) in its globular head domain.68 The first human neutralizing antibody, m101, is highly potent in neutralizing both HeV and NiV,71 and its epitope overlaps the minimal RBD in the G protein, suggesting the potential of developing RBD-based henipavirus vaccine to prevent HeV or NiV infection.

Besides the viruses mentioned above, the strategy of designing RBD-based vaccines can also be applied to the development of vaccines against other emerging or re-emerging viruses with identified receptor(s) and RBDs, such as Zaire Ebolavirus and Lake Victoria Marburgvirus.75 Although the receptors for some viruses have not been clearly defined, the sites in the spike proteins of these viruses for binding to the cellular cofactors that facilitate viral entry may also be used to design vaccines able to induce antibodies to block viral entry or neutralize viral infectivity. For example, entry of hepatitis C virus (HCV) into the hepatocyte requires at least four cellular cofactors, including CD81,76 the tight junction proteins claudin-1 and occludin,77 and scavenger receptor BI.78 It is expected that the sites in the E1 and E2 proteins of HCV for binding to the above cofactors or putative HCV coreceptors may serve as targets for development of preventive vaccines against HCV in the near future.