Featuring ACE2 binding SARS-CoV and SARS-CoV-2 through a conserved evolutionary pattern of amino acid residues

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Coronaviruses attach to host cell surface receptors via their spike (S) proteins to mediate the entry into the host cell. The S1 coronavirus subunit of S-proteins contains the receptor-binding domain (RBD) that is able to recognize different host receptors, highlighting its remarkable capacity to adapt to their hosts along the viral evolution. While RBD in spike proteins is determinant for the virus-receptor interaction, the active residues lie at the receptor-binding motif (RBM), which is part of RBD and plays a fundamental role binding the outer surface of their receptors. Here, we address the hypothesis that SARS-CoV and SARS-CoV-2 strains able to use angiotensin-converting enzyme 2 (ACE2) proteins have adapted their RBM along the viral evolution to explore specific conformational topology driven by the amino acid residues YGF to infect host cells. We also speculate that this YGF-based mechanism can act as a protein signature located at the RBM to distinguish coronaviruses able to use ACE2 as a cell entry receptor.

INTRODUCTION

Viruses are the most numerous type of biological entity on Earth and the identification of novel viruses continues to enlarge the known viral biosphere \[1\] [2]. This collection of all viruses presents enormous morphological and genomic diversity as a result of continuous exchange of genetic material with the host cells \[3\] [4]. Moreover, this well succeeded long-term virus-host interaction indicates that viruses are more than simple genomic parasites in all cellular life forms \[5\]. A number of evidences has led to the proposal that viruses play an astonishing role as agents of evolution because of their capacity in propagating between biomes \[6\] and in gene transfer between species \[7\] [10]. For this purpose, viruses have developed large number of genome replication and protein expression strategies to benefit from the host translational machinery over time \[11\].

Despite all of such enormous diversity in gene sequence, it is not possible to achieve huge number of highly distinct protein structures mainly because of stereochemical constraints on the possible protein folds \[12\]. In fact, it has been observed common secondary structures throughout different virus families while the sequences are not fully conserved \[12\] [13]. This may result in evolutionary efficiency once viruses can exploit already well designed motifs from similar cellular functions \[11\].

Currently, the world population is confronting a new coronavirus disease (COVID-19), a highly infectious disease to humans. This disease is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and is affecting human health worldwide. Coronaviruses (CoVs) belong to the large and diverse family \textit{Coronaviridae}, within the order \textit{Nidovirales} and suborder \textit{Coronavirinae} \[14\]. Their subfamily \textit{Orthocoronavirinae} contains four genera based on phylogeny and termed as \textit{α}, \textit{β}, \textit{γ}, and \textit{δ}-coronavirus.

SARS-CoV-2 belongs to the \textit{β}-coronavirus genus as well as SARS-CoV, middle east respiratory syndrome coronavirus (MERS-CoV), and hCoV-HKU1, to cite a few \[15\]. Other important representative viruses as human hCoV-NL63 and hCoV-229E belong to \textit{α}-coronavirus. Phylogenetic relationships among the known members of this subfamily indicate that \textit{α} and \textit{β}-coronavirus infect mammals, while \textit{γ} and \textit{δ}-coronavirus infect both mammalians and avians.

Members of \textit{Coronaviridae} family are enveloped, positive single-stranded RNA (+ssRNA) viruses and render the largest genomes among all known RNA viruses \[16\] [19]. The +ssRNA genomes undergo rapid mutational changes \[20\], leading to faster adaptation to new hosts, though also contain conserved sequence motifs as observed, for example, in multiple alignments do CoV strains \[13\] [21] [22].

Coronaviruses attach to host cell surface receptors via their spike (S) glycoproteins, located on the viral envelope, to mediate the entry into the host cell. Each monomer of trimeric S-proteins comprises two subunits S1 and S2, responsible for the viral attachment and for the membrane fusion, respectively \[23\] [25]. The S1 coronavirus subunit contains the receptor-binding domain (RBD) that is able to recognize different host receptors, highlighting its remarkable capacity to adapt to their hosts along the viral evolution. Thus, it is not unexpected to observe in this domain high sequence divergence even for the same coronavirus identified in different host species. In contrast, the S2 subunit presents the most conserved region in the S-protein.

The binding of RBD spike proteins to the receptor on the host cell is the first step in virus infection. This initial step is followed by an entry mechanism of enveloped viruses into target cells. Usually, most viruses enter cells through endocytic pathways with the fusion occurring in the endosomes, although a direct entry into cells can

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occur by fusion of their envelopes with the cell membrane.

A number of CoVs utilizes angiotensin-converting enzyme 2 (ACE2) as the entry receptor into cells, exemplified by β-genus human respiratory SARS-CoV, SARS-CoV-2, and α-genus hCoV-NL63 [15, 27, 28]. In particular, SARS-CoV, as well as SARS-CoV-2, enter the cell via endocytosis induced by RBD complexed with human ACE2 (hACE2) receptor [30–34]. In contrast, the β-genus MERS-CoV and its genetically related bat CoV-HKU4 utilize dipeptidyl peptidase 4 (DPP4) as the viral receptor [35]. Other viral receptor is aminopeptidase N (APN), recognized for example by the α-genus hCoV-229E [36].

The human coronaviruses hCoV-HKU1, hCoV-229E, hCoV-NL63, and hCoV-OC43, cause mild to moderate upper respiratory tract infections [37], while SARS-CoV and SARS-CoV-2 cause severe respiratory diseases, with SARS-CoV-2 being far more lethal than SARS-CoV. SARS-CoV strains vary enormously in infectivity, which can be connected to their binding affinities to hACE2 [38]. This binding affinity, in turn, can be correlated with disease severity in humans [39].

While RBD in spike proteins is determinant for the virus-receptor interaction, the active residues lie at the receptor-binding motif (RBM), which is part of RBD and plays a fundamental role binding the outer surface of their receptors [27, 28, 38, 40, 41]. The importance of the RBM is further explored here in relation to its structural topology. Thus, instead of analysing specific residues that make contacts with ACE2 after binding, we followed the molecular origin that drives the viral attachment to this cell receptor. This investigation has revealed a highly conserved amino acid residue sequence Tyr-Gly-Phe (YGF) in coronavirus variants that employ this receptor. Thus, we hypothesize that the short sequence YGF is vital for RBD-ACE2 interaction because of the formation of the hydrophobic pocket proper to the receptor specificity [40, 42, 43], as exposed next. It is likely that SARS-CoV and SARS-CoV-2 strains able to use ACE2 proteins have adapted their RBD along the viral evolution to explore this YGF-based mechanism to infect host cells.

### The conserved XGF loop in UBA-ubiquitin interaction

Amino acid sequences of type XGF, where the residue X is frequently the residue Met, form a highly conserved loop characteristic of ubiquitin-associated (UBA) domain that occurs in a variety of proteins. The UBA domain is a conserved motif through eukaryotic evolution and is found in many proteins related to the ubiquitin metabolism and in particular, associated with ubiquitin-mediated proteolysis [15, 16]. The MGF loop in the UBA domain is typical of a hydrophobic pocket that is critical for recognition and binding affinity to ubiquitin through a hydrophobic surface patch located in the vicinity of this loop [17, 54].

NMR analyses of UBA-ubiquitin interactions identify hydrophobic surface patches formed by the conserved MGF sequence as the main determinants for the dimerization interface. A number of alignments of p62/SQSTM1 UBA domain with other UBA-domain proteins has revealed single-point mutation in the MGF sequence, mainly M→I, or F→Y, maintaining its overall hydrophobic characteristic [55].

### RESULTS AND DISCUSSION

**Spike receptor-binding motifs in human CoVs**

Here, we examine the occurrence and importance of the specific amino acid residue sequence YGF for SARS-CoV and SARS-CoV-2 strains able to use ACE2 proteins as receptors. It is displayed in Fig 1 the interface of SARS-CoV RBD spike-protein (magenta and green color) complexed with hACE2 (blue color) to gain insight about the importance of this kind of conformational mechanism in creating a shape complementarity between receptor and ligand. The RBM is in magenta color, with the yellow color displaying the YGFY sequence in that pocket, which establishes the proper relative position for favorable binding to ACE2 exposed residues. The YGFY sequence seems strongly conserved for many SARS-CoV. It is located at residues 481–484 in RBM. Noteworthy, no other GF sequence occurs in this region, neither in its RBD. As a consequence of this hydrophobic pocket, amino acid residues responsible for binding interaction are located close to this conformational structure. For instance, the residues N479 and T487, which have been identified to be essential for receptor binding [39, 41, 56]. The residue N479 in SARS-CoV is located near K31 of hACE2 and makes a salt bridge with E35, a residue buried in that hydrophobic environment. The residue T487 is located close to K353 on hACE2, and in turn makes a salt-bridge with D38, also buried in that pocket (Fig 2). Other important residues for this attachment are Y442, L472, and D480 [40].

Figure 1 displays the interface of SARS-CoV-2 RBM spike-protein complexed with hACE2, also in blue color. Now, the sequence YGFY observed in SARS-CoV is replaced by YGFQ (Fig 3). The single-point mutation Y484→Q498 replaces a hydrophobic residue in SARS-CoV by a hydrophilic one in SARS-CoV-2.

Figure 3 compares amino acid sequences of human SARS-CoV and SARS-CoV-2 strains aligned with RBM of SARS-CoV Tor2, an epidemic strain isolated from humans during the SARS epidemic in 2002-2003. The human Tor2 strain has high affinity for hACE2 [38]. We highlight in Fig 3 in medium purple color the hydrophobic sequence YGFY typical of SARS-CoV, occurring at positions 481–484 in the spike protein. The corresponding mutated sequence occurs now at positions 495–498 in
The important residues for the interface interaction found in SARS-CoV are mutated in SARS-CoV-2. The sequence alignments show the mapping: Y442→L455, L472→F486, N479→Q493, D480→S494, and T487→N501. These mutations do not present a drastic change in their hydrophobic character [57], thus preserving the overall receptor-binding topological structure for these viruses. In particular, residues L455 and Q493 in SARS-CoV-2 preserve the noted favourable interactions with the residues E35 and K31 in hACE2 [58] (Fig 2b). Interestingly, a new GF sequence appears in the RBM of SARS-CoV-2 strains as a consequence of the mutation L472→F486, producing a small hydrophobic surface, but does not seem to disrupt the proposed topological formation mechanism for ACE2.
binding. No other GF sequence appears in their RBD.

Details of protein-protein binding interfaces can be quite different among strains, likely related to their infectivity degree. It has been noted that mutations in RBD residue T487 have an important role in the human-to-human and animal-to-human transmission of SARS-CoV [36, 38, 56].

Spike receptor-binding motifs in bats

It is known that not all SARS-CoV strains isolated from bat hosts have exploited ACE2 as a cellular attachment. Therefore, the set of amino acid sequences displayed in Fig 4 may exemplify the successful relation between virus evolution and the binding mechanism. This set highlights in medium purple color the preserved amino acid residues in the sequence YGFY, characteristics of human SARS-CoV. For comparison, we also display CoV strains with mutations in that SARS-CoV pattern to explore the relation between the hypothesized mechanism and the cell receptor recognition.

It has been demonstrated that LYRa11 [28], Rs3367 [59], Rs4874 [60], WIV1, and WIV16 [28, 61], have the capacity to use ACE2 for cell entry, in line with our hypothesis. Also, the near single-point mutation Y→F in

Figure 3. Sequence alignments of human CoVs restricted to RBM residues. The medium purple color highlights the YGFY pattern followed by the mutation Y498Q in the RBM of SARS-CoV-2 strains.

Figure 4. Sequence alignment of bat CoVs restricted to RBD residues of SARS-CoV Tor2. The residues of YGFY pattern are in medium purple color. Last three alignments are placed together for direct amino acid sequence comparison.

SARS-CoV Tor2

Rhinolophus affinis CoV LYRa3
Rhinolophus affinis CoV LYRa11
Bat SARS-like CoV Rs3367
Bat SARS-like CoV Rs4874
Bat SARS-like CoV WIV1
SARS-like CoV MHV16
Bat CoV RatG13
Bat CoV RRatG27
Bat SARS-like CoV Rs9401
CoV Btks-BetaCoVYN2018B
Bat SARS-like CoV Rs934014
Bat SARS-like CoV Rs4323
SARS-CoV BM12
SARS-like CoV B990448/B008
Bat CoV BM48-31/B008
CoV BnH-BetaCoVYN2018
Btks-BetaCoVYN2013
Bat SARS-CoV HKU3-1
Bat SARS-like CoV bat-SL-CoVZC45
Bat SARS-like CoV bat-SL-CoVZC21
Bat SARS-CoV R1/2004
Bat SARS-CoV R1/2004
Bat CoV Anlong-103
Bat CoV Anlong-112
Bat SARS-CoV R3/2004
Bat SARS-CoV R3/2004
Btks-BetaCoVYN2018C
Bat CoV R7/2011
Bat SARS-CoV Am1/2004
Bat SARS-CoV Tor2
Bat CoV RatG13
SARS-CoV-2 Wuhan-Hu-1
the next six strains does not interfere, as expected, in the attachment mechanism. This remark is supported by cell entry studies for Rs7327 \[28, 60\], Rs9401, RsSHC014, Rs4084, and Rs4231 \[60\], because they are in a group that is likely to use the ACE2 receptor. Moreover, this mutation replaces a hydrophobic residue by another one with higher hydrophobicity, reinforcing the conformational topology for binding with the receptor.

The next group corresponds to the mutation Y → T, decreasing the initial hydrophobicity of the expected pocket. It seems unlikely that this mutation and amino acid residue deletions in the RBM associated to Tor2 sequence affect the YGF-based attachment mechanism for BtKY72 and BB9904/BGR. However, there is no available experimental data concerning their receptors. It is important to remark that the residue F492 in BM48-31/BGR produces another hydrophobic sequence IGF at residues 490-492 (Fig 4). We speculate that this double occurrence may disrupt the aforementioned mechanism because of indications that BM48-31/BGR does not interact, at least with human ACE2 \[28\]. No other GF sequence occurs for these strains in the RBM nor in their RBD.

Next CoV strains do not contain such GF sequences of residues in the RBM neither in their RBD, except Rf1/2004, which is located in RBD and with GF surrounded by hydrophilic residues. Although we have considered only part of their sequences that better align with RBM of Tor2, it has been demonstrated that the spikes of HuB2013, HKU3, CoVZC45, CoVZXC21, Rf1, Rf4092, and Shaanxi2011 do not use hACE2, a result that is not just a consequence of deletions at the RBD \[28\]. Further support has been presented against HKU3 in using hACE2 \[62\]. It seems unlikely that Rm1/2004 infects hACE2 because its unfavourable binding free energy \[63\]. Another result concludes that Rp3 is unable of infect hACE2 or even bat ACE2 \[64\].

We have placed together the alignments involving Tor2, RaTG13, and SARS-CoV-2 at the end of Fig 1 for further comparison. The whole genome of RaTG13 shares 96% amino acid sequence identity with SARS-CoV-2, and it is considered the most closely related genome to this CoV \[65\]. Considering its spike protein, and RBM, RaTG13 shares respectively 97% and 76% amino acid identity with SARS-CoV-2. For comparison, RaTG13 shares 79%, 77%, and 53% identity, respectively, for the whole genome, spike protein, and RBM with SARS-CoV Tor2. Therefore, SARS-CoV-2 is mostly similar to RaTG13 than SARS-CoV strains in all regions.

**Spike receptor-binding motifs in palm civets and pangolins**

To explore further the role of YGF-based attachment mechanism, we exhibit comparative residue sequences for civet and pangolins, again aligned with RBM of SARS-CoV Tor2 (Fig 5). This figure shows that the pattern YGFY characteristic of human SARS-CoV is maintained for the collected data, but with a single-point mutation Y → H for pangolin hosts PCoV. We have included SARS-CoV-2 on the last line of Fig 5 for a direct comparison. PCoV GX-P2V shares 79%, 77%, and 50% amino acid identity with Tor2, respectively for whole genome, spike protein, and RBM aligned with Tor2. In relation to SARS-CoV-2, PCoV GX-P2V shares 85%, 92%, and 75% amino acid identity, respectively, for the whole genome, spike protein, and RBM. It is believed that human SARS-CoV passed from palm civets to humans in the 2002-2003 epidemic because their genome sequences are highly similar \[38, 56, 66\]. The amino acid alignments show an almost identical RBM
between human SARS-CoV, represented by Tor2 strain, and collected data from palm civet strains. This identification also includes the YGF-based mechanism able to use ACE2 proteins. Nevertheless, these alignments display high similarity between pangolins and SARS-CoV-2, which also support previous conclusions on pangolins being the probable origin of SARS-CoV-2 [65, 67]. However, based on our data related to host receptor binding and their RBM and S-protein alignments, we can not discard bat RaTG13-like strain as also the possible origin of SARS-CoV-2.

SARS-CoV and hCoV-NL63: only functionally related

Although there is no many available experimental data identifying the viral receptor-binding protein for CoVs, it is well established that human SARS-CoV and hCoV-NL63 both employ ACE2 as the cell receptor to infect host cells [68, 69]. Interestingly, SARS-CoV and hCoV-NL63 domains do not present high sequence similarity. For example, their spike-S1 subunits share only 10% in similarity. Other features separate SARS-CoV and hCoV-NL63 [70]. SARS-CoVs are classified as β-coronavirus with subgenus sarbecovirus, while hCoV-NL63 is in genus α-coronavirus and subgenus setra-covirus. Although hCoV-NL63 also enters the cell via endocytosis, its functional receptor requires heparan sulfate proteoglycans for the initial attachment, representing an important extra factor for ACE2 act as a functional receptor [33, 70]. Moreover, the spike-S1 glycoprotein of SARS-CoV binds more efficiently ACE2 than the corresponding spike-S1 of NL63 (NL63-S) [71]. This may be linked to the fact that SARS-CoV and NL63-S contact hACE2 differently, a conclusion based upon the experimental results that NL63-S does not bind to hACE2 through a single and large domain [69, 72]. Actually, different RBD regions have been identified within NL63-S. One of these regions was positioned at residues 476-616 and comprising three discontinuous RBM regions, RBM1 (residues 497-501), RBM2 (residues 530-540), and RBM3 (residues 575-594) [73–75]. A slightly different RBD has been identified for this CoV [72]. It would be located at residues 482-602, also with three discontinuous RBM regions, which surround a shallow cavity at hCoV-NL63-ACE2 binding interface. Curiously, its spike protein alignment with Tor2 does not show the expected residue pattern in the corresponding RBM of Tor2 nor in the aforementioned RBD regions of NL63-S. This may help to explain the unusual pathway of binding to ACE2 for this CoV.

METHODS

All sequences were analysed with ClustalW and Jalview. The list of GenBank accession codes for the spike proteins analysed in this work is available in supplementary Table S1.

CONCLUSION

We have analysed a number of CoV strains to support the hypothesis that SARS-CoV and SARS-CoV-2 strains share a common evolutionary mechanism for the initial attachment to ACE2. Moreover, we speculate that the YGF-based mechanism can act as a protein signature to distinguish CoVs able to use ACE2 as a cell entry receptor whenever this residue sequence is located at the CoV RBM region. For example, SARSr-CoV ZXC21 and ZC45, the closely related spike sequences to SARS-CoV-2, can be promptly put under suspicious in their ACE2 binding affinity. Of course, as exemplified by hCoV-NL63, we can not discard that another mechanism can act helping such binding. It must be accentuated that the occurrence of other XGF sequences, mainly with X being a hydrophobic residue, in the RBM, or even in the RBD region, is likely to disrupt the proposed topological mechanism for ACE2 binding. This because it might introduce hydrophobic loops promoting a new ligand-substrate recognition.

SUPPORTING INFORMATION

Table S1 GenBank accession numbers for the coronavirus sequences used in this study.

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| Name                  | Spike Glycoprotein GenBank | Name                  | Spike Glycoprotein GenBank | Name                  | Spike Glycoprotein GenBank |
|-----------------------|---------------------------|-----------------------|---------------------------|-----------------------|---------------------------|
| SARS-CoV-Tor2         | AAP41037.1                | SARS-CoV PC4-115      | AAV49719.1                | Rhinolophus affinis CoV LYRa3 (SARS-like) | AHX37669.1 |
| SARS-CoV BJ01         | AAP30030.1                | SARS-CoV PC4-145      | AAV49721.1                | Rhinolophus affinis CoV LYRa11 (SARS-like) | AHX37558.1 |
| SARS-CoV CUHK-W1      | AAD13567.1                | SARS-CoV PC4-241      | AAV49723.1                | Bat SARS-like CoV Rs3367 | AG248818.1 |
| SARS-CoV Urbani       | AAD13441.1                | SARS-CoV civet014     | AAV49661.1                | Bat SARS-like CoV R4674 | AT096205.1 |
| SARS-CoV Frankfurt-1  | AAD33697.1                | SARS-CoV PC4-137      | AAV49720.1                | Bat SARS-like CoV WTV1  | AG248828.1 |
| SARS-CoV CUHK-AG01    | AAD14737.1                | SARS-CoV civet020     | AAV49664.1                | SARS-like CoV WTV16    | ALX02457.1 |
| SARS-CoV TW6          | AAD187567.1               | SARS-CoV PC4-127      | AAV493318.1               | Bat SARS-CoV RfTG13    | QH63300.2 |
| SARS-CoV TW11         | AAD87512.1                | SARS-CoV B039         | AAV79931.1                | Bat SARS-like CoV Rs7327 | AT096218.1 |
| SARS-CoV HGU-39649    | AAD35463.1                | SARS-CoV PC4-205      | AAV493310.1               | Bat SARS-like CoV Rs9401 | AT096231.1 |
| SARS-CoV-2 Wuhan-Hu-1 | AMD43416.1                | SARS-CoV PC4-199      | AAV49722.1                | CoV Btr-BetaCoV/WH2018B | QDF43825.1 |
| SARS-CoV-2 CA-CDC-0139| QJV86828.1                | SARS-CoV civet010     | AAV49669.1                | Bat SARS-like CoV RsSHC014 | AG248866.1 |
| SARS-CoV-2 France     | QJ772690.1                | SARS-CoV civet019     | AAV49662.1                | Bat SARS-like CoV Rs8048 | AT096132.1 |
| SARS-CoV-2 WHUH/WH011 | QIU82034.1                | SARS-CoV A022         | AAV51631.1                | Bat SARS-like CoV Rs4231 | AT096157.1 |
| SARS-CoV-2 CA-CZB-1248| QKE42668.1                | civet SARS-CoV 0072004| AAV49646.1               | SARS-CoV strain BK72   | APO40579.1 |
| SARS-CoV-2 CA-CZB-1033| QJ83756.1                 | SARS-CoV A001         | AAV79841.1                | SARS-like CoV BatCoV/BB9004/BGR2008 | AL30436.1 |
| SARS-CoV-2 France/10070SK | QJ771010.1            | PCoV GX-P2V           | QJ54048.1                 | Bat CoV BM48-31/BGR2008 (SARS-like) | ADX66441.1 |
| SARS-CoV-2 CA-CZB0103 | QJE83301.1                | PCoV GX-P4L           | QA48614.1                 | CoV Btr-BetaCoV/SC2018 | QDF43815.1 |
| SARS-CoV-2 BetaCoV/Wh/WH05 | QHU36864.1            | PCoV GX-P5L           | QA48632.1                 | Btr-BetaCoV/Wh/2013   | AIA62301.1 |
| SARS-CoV-2 CruiseA-18 | QHS7278.1                | PCoV GX-P4L           | QA48632.1                 | Bat SARS-CoV HKU3-1    | AAY88866.1 |
| SARS-CoV-2 CA-CZB-1105 | QJHS4754.1               | PCoV BX-2C45          | AAP78031.1                | Bat SARS-like CoV bat-SL-CoV2C45 | ALP78031.1 |
|                      |                          | PCoV BX-2C21          | AAP78042.1                | Bat SARS-like CoV bat-SL-CoV2C21 | ALP78042.1 |
|                      |                          | PCoV BX-2C45          | AAP78033.1                | Bat SARS-CoV R132004   | ABQ75333.1 |
|                      |                          | PCoV BX-2C45          | ATO71549.1                | Bat SARS-like CoV RM1   | ABQ75333.1 |
|                      |                          | PCoV BX-2C45          | ATO71549.1                | Bat SARS-CoV RM1       | ABQ75333.1 |

Table S1: GenBank accession numbers for the coronavirus sequences used in this study.