Amino acid substitutions that affect receptor binding and stability of the hemagglutinin of influenza A/H7N9 virus

Eefje J.A. Schrauwen[1]*, Mathilde Richard[1]*, David F. Burke[2], Guus F. Rimmelzwaan[1], Sander Herfst[1], Ron A.M. Fouchier[1].

[1]Department of Viroscience, Postgraduate School Molecular Medicine, Erasmus MC, Rotterdam, The Netherlands.

[2]Department of Zoology, University of Cambridge, Cambridge, United Kingdom

* These authors contributed equally to this work.

Running title: Receptor binding and stability of HA of A/H7N9 viruses

A/H7N9 viruses bind to both human and avian receptors. Here, we show that a switch towards human receptor specificity caused by the G219S substitution in hemagglutinin (HA) coincided with a decrease in stability of A/Anhui/1/13 virus HA, which could be restored by several amino acid substitutions. We thus identified substitutions altering the stability and receptor binding preference of A/H7N9 viruses, properties that are associated with airborne transmission of avian influenza viruses between mammals.

Recent human infections with influenza A/H7N9 viruses in China have raised concern of a pandemic threat emerging from avian reservoirs. Until August 2014, A/H7N9 viruses caused 452 laboratory-confirmed human cases of infection, of which 124 were fatal. (19). To date, no sustained human-to-human transmission of A/H7N9 viruses has been detected, although some of these viruses possess known mammalian adaption markers in the HA and in the polymerase genes (18). Studies using the ferret model have demonstrated that the airborne transmissibility of A/H7N9 viruses was more efficient than that of other avian influenza viruses, but less efficient as compared to pandemic and seasonal human influenza viruses (1, 13, 18, 21, 25, 26). The relatively inefficient airborne transmissibility of A/H7N9 viruses could be partially due to their dual receptor specificity (15, 20). Most A/H7N9 virus isolates can recognize both avian and human receptors (15), as the result of a leucine at position 217 (H7 numbering, corresponding to position 226 in H3 numbering (2)). However, phenotypic traits beyond human receptor binding preference, such as increased acid or temperature stability, have been shown to be critical for airborne transmission of avian A/H5N1 viruses between ferrets (8, 10). Here, we describe several amino acid substitutions that change the receptor binding specificity and stability of the HA of A/H7N9 A/Anhui/1/13 virus (AN1).

We first investigated the impact of two substitutions in the receptor-binding site, L217Q and G219S. AN1 virus with amino acid substitution L217Q was previously detected in a minor virus population that emerged in ferrets upon inoculation with the AN1 virus isolate (13). The Q217L/G219S combination contributed to the emergence of the 1957 H2N2 and 1968 H3N2 pandemic viruses and is known to be responsible for the switch in binding specificity of H2 and H3 influenza viruses from avian to human receptor preference (12). Tharakaraman et al. showed that the G219S substitution resulted in increased binding of AN1 HA to the apical surface of human trachea and alveolar epithelium (17), but its impact on the receptor specificity of AN1 was not determined. Recombinant viruses containing 7 gene segments of the attenuated virus A/Puerto Rico/8/38 and HA of AN1 with or without mutations of interest were produced in 293T cells and propagated in MDCK cells by reverse genetics according to standard procedures (5). Binding properties of recombinant viruses to α2.3- and α2.6- linked sialic acids (SA), the avian- and human-type receptors respectively, were assessed using a modified turkey red blood cell (TRBC) assay (3). The latter analysis confirmed that the $AN1_{WT}$ virus possesses both avian and human

receptor binding preference as shown previously (13) (Table 1). $AN1_{L217Q}$ virus predominantly bound to avian receptors, but with residual binding to human receptors. This is in agreement with the study of Shi et al., that also showed that the L217Q substitution in AN1 HA did not completely abrogate human receptor binding, implying that other substitutions in the RBS might contribute to the α2,6-SA preference of AN1 (15). Moreover, we determined that $AN1_{G219S}$ virus bound exclusively to human receptors. This is in contrast to Yang et al., where the introduction of G219S in A/Shanghai/2/13 HA, also possessing the Q217L substitution, did not completely switch the receptor specificity as determined by glycan microarray (22).

HA-mediated cell-to-cell fusion was assessed using a syncytium formation assay in Vero cells, upon transfection of HA gene segments expressed from a pCAGGS expression plasmid and subsequent exposure to different pH (10). The pH threshold at which cell-to-cell fusion was triggered by $AN1_{WT}$ HA was 5.6, which is relatively high compared to human influenza viruses (6) (Fig 1a). For $AN1_{L217Q}$ HA, the fusion threshold decreased to pH 5.4 while $AN1_{G219S}$ HA showed a pH threshold of fusion > 6.0. The conformational change of HA from a non-fusogenic to a fusogenic state can also be triggered at neutral pH when the HA is exposed to increasing temperature. Therefore, the heat stability was assessed using a temperature sensitivity assay to further assess HA stability (10). In agreement with the results of the fusion assay, $AN1_{L217Q}$ HA showed increased temperature stability, whereas $AN1_{G219S}$ HA was less stable compared to $AN1_{WT}$ HA (Fig 1b). These results are consistent with what was recently shown for airborne-transmissible A/H5N1 virus, for which human receptor specificity coincided with decreased stability of HA in the absence of compensatory mutations (10).

We further investigated whether substitutions could increase the HA stability of $AN1_{WT}$ to levels comparable to human or airborne viruses or compensate for the impact of the G219S substitution. Two substitutions – H103Y and T315I (H5 numbering) – are known to increase the stability of A/H5N1 viruses via different mechanisms (4, 7, 8, 10, 11). Using computational modelling , we identified a N94K substitution (16), located at the trimer interface of AN1 HA, which could potentially have a similar impact as the H103Y substitution in HA of A/H5N1. Substitution N94K is predicted to interact with E74 of HA2 in the neighboring monomer (Fig 2b). However, this would come at the expense of E74 losing an interaction with Q76 in HA2 of another monomer, resulting in little overall change in stability. We further investigated

76 the A210E substitution, also located at the trimer interface although more distal to the stalk, which was
77 detected as a minor variant in ferrets upon inoculation with the AN1 virus (13). Substitution A210E is
78 predicted to yield interactions with the side chains of both T156 and S237 on the neighboring monomer,
79 thus increasing its stability (Fig 2c). These interactions are presumably affected when this mutation is
80 combined with G219S, due to their close proximity. We also investigated the K58I substitution in HA2,
81 which is known to increase the HA stability of A/H5N1 viruses (24). In AN1, amino acid K58 is predicted to
82 interact with nearby N282, causing a kink in the peptide backbone and less than optimal interaction
83 between the adjacent monomers (Fig 2d). The K58I mutation would, surprisingly, lead to a loss of this
84 interaction but presumably allows the movement of the backbone, improving interactions of the
85 neighboring charged amino acids R54 and E57 in HA2 to the adjacent monomer.
86 Substitutions N94K, K58I and A210E were assessed for their ability to alter the stability of both
87 $AN1_{WT}$ and $AN1_{G219S}$. Substitution N94K caused an increase in acid stability in both $AN1_{WT}$ and $AN1_{G219S}$
88 HA (Fig 1a). However, the temperature stability of $AN1_{N94K}$ and $AN1_{G219S\ N94K}$ viruses was not increased
89 compared to $AN1_{WT}$ and $AN1_{G219S}$ viruses (Fig 1b). The K58I substitution resulted in a marked increase in
90 acid and temperature stability of AN1 HA with and without the G219S substitution (Fig 1a-b). $AN1_{A210E}$ HA
91 presented a similar pH threshold for fusion as the $AN1_{WT}$ HA. However, the A210E substitution resulted in
92 a decrease of the pH threshold for fusion of $AN1_{G219S}$ by 0.2 pH unit (Fig 1a) and a higher HA
93 thermostability with and without the G219S substitution (Fig 1b).
94 Here, we show that a switch towards human receptor specificity coincided with a decrease in
95 stability of HA of the AN1 virus. Moreover, we demonstrated that several compensatory amino acid
96 substitutions can restore the acid and/or temperature stability of the $AN1_{G219S}$ HA and increase the
97 stability of $AN1_{WT}$ HA. Interestingly, the effect of K58I on HA stability was similar for the A/H7N9 and
98 A/H5N1 HA subtypes. In previous studies, the K58I substitution has been associated with an increase in
99 virus replication of A/H5N1 viruses in the upper respiratory tract of mice and ferrets (9, 14, 23, 24). It
100 would be interesting to study the impact of this substitution on replication and transmission in ferrets.
101 Keeping in mind that (acid) stability may only be a surrogate marker for another phenotype (e.g. stability
102 in aerosols or respiratory droplets), increased knowledge of amino acid substitutions that alter the HA

stability across HA subtypes would help to better understand stability as a biological trait for replication and transmission of influenza viruses.

This study identified amino acid substitutions that alter the HA stability and binding preference of A/H7N9 viruses, properties that have been shown critical for airborne transmission of avian influenza viruses between mammals. These data may be useful for surveillance and assessment of the pandemic potential of zoonotic viruses, upon confirmation of the virus phenotypes in appropriate animal models.

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115 Table 1. Receptor binding specificity of $AN1_{WT}$ and mutant viruses determined by a modified TRBC
116 hemagglutination assay

Virus	HA titer (HAU/50 µl)			
	TRBC	VCNA[a]-TRBC	α2,3-TRBC	α2,6-TRBC
VN1194/04(A/H5N1)	64	Neg	128	Neg
NL213/03(A/H3N2)	64	Neg	Neg	64
AN1wt	64	Neg	4	24
$AN1_{L217Q}$	64	Neg	64	2
$AN1_{G219S}$	32	Neg	Neg	8

117 [a] VCNA, *Vibrio cholerae* Neuraminidase

Figure 1. pH threshold of fusion and thermostability of $AN1_{WT}$ HA and mutant HAs

(A) Syncytium formation in Vero cells expressing wildtype or mutant AN1 HA proteins after exposure to different pH. The black boxes represent the range of pH values at which fusion was detected microscopically. (B) HA protein stability as measured by the ability of viruses to agglutinate TRBCs after incubation at 50 ºC for the indicated times (minutes). Colors indicate the HA titers upon treatment at various time-points at 50 ºC as shown in the legend.

Figure 2. Cartoon representation of a model of the trimer structure of HA of AN1 at different positions.

(A) HA of AN1 with the substitutions studied annotated. (B) Mutant N94K (cyan) is predicted to interact with E74 in HA2 of the neighboring monomer but results in the loss of an interaction between E74 and Q76. (C) Mutant A210E (shown as orange sticks), close to G219 (magenta), is predicted to interact with the side chains of both T156 and S237 on the neighboring monomer. (D) At position 58, there is a gap between the monomers due to the interaction of K58, kinking the peptide backbone. Substitution K58I could allow the backbone to straighten and form more inter-monomer interactions.

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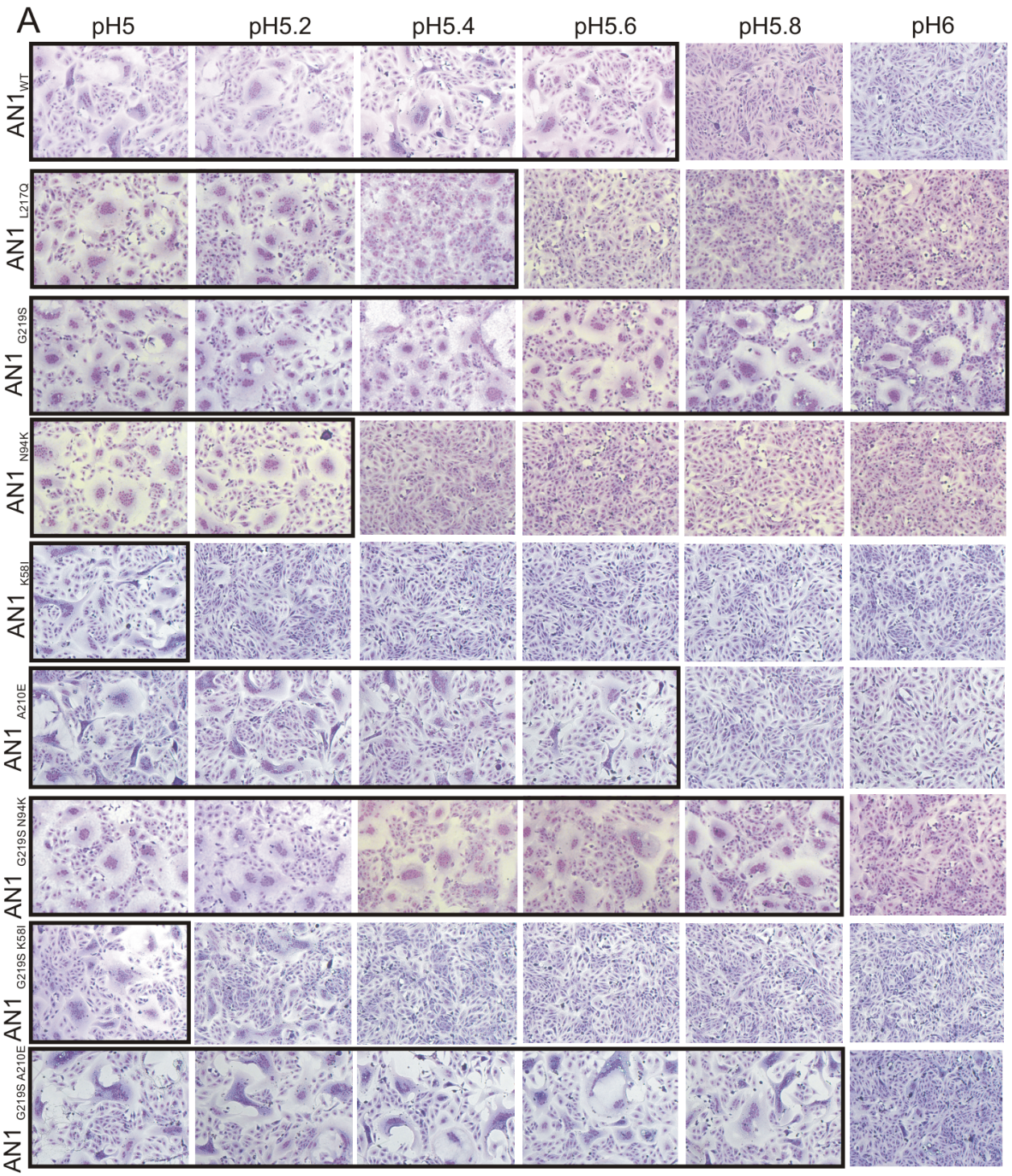
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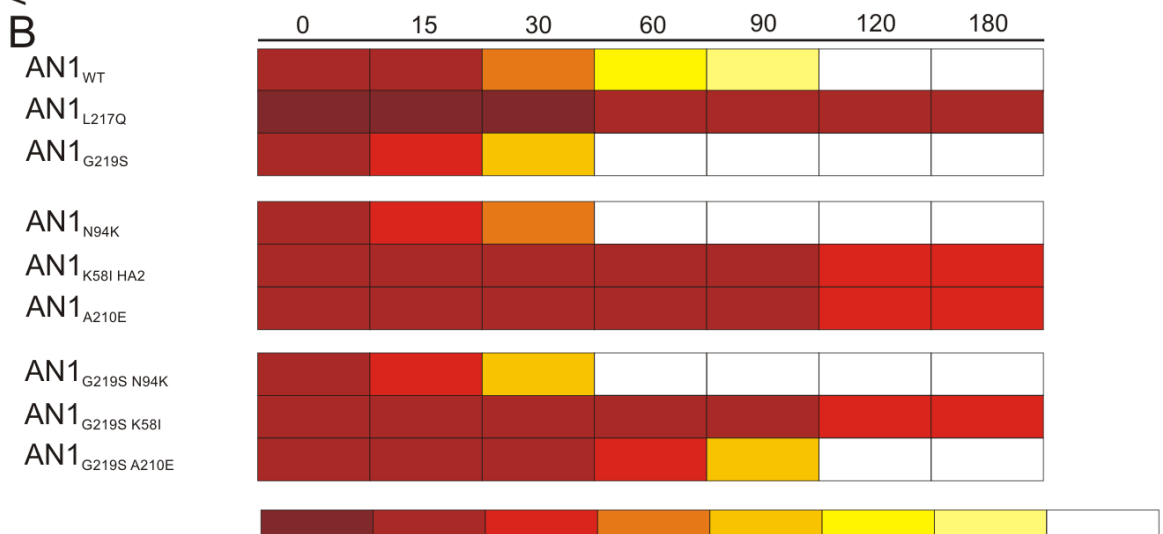
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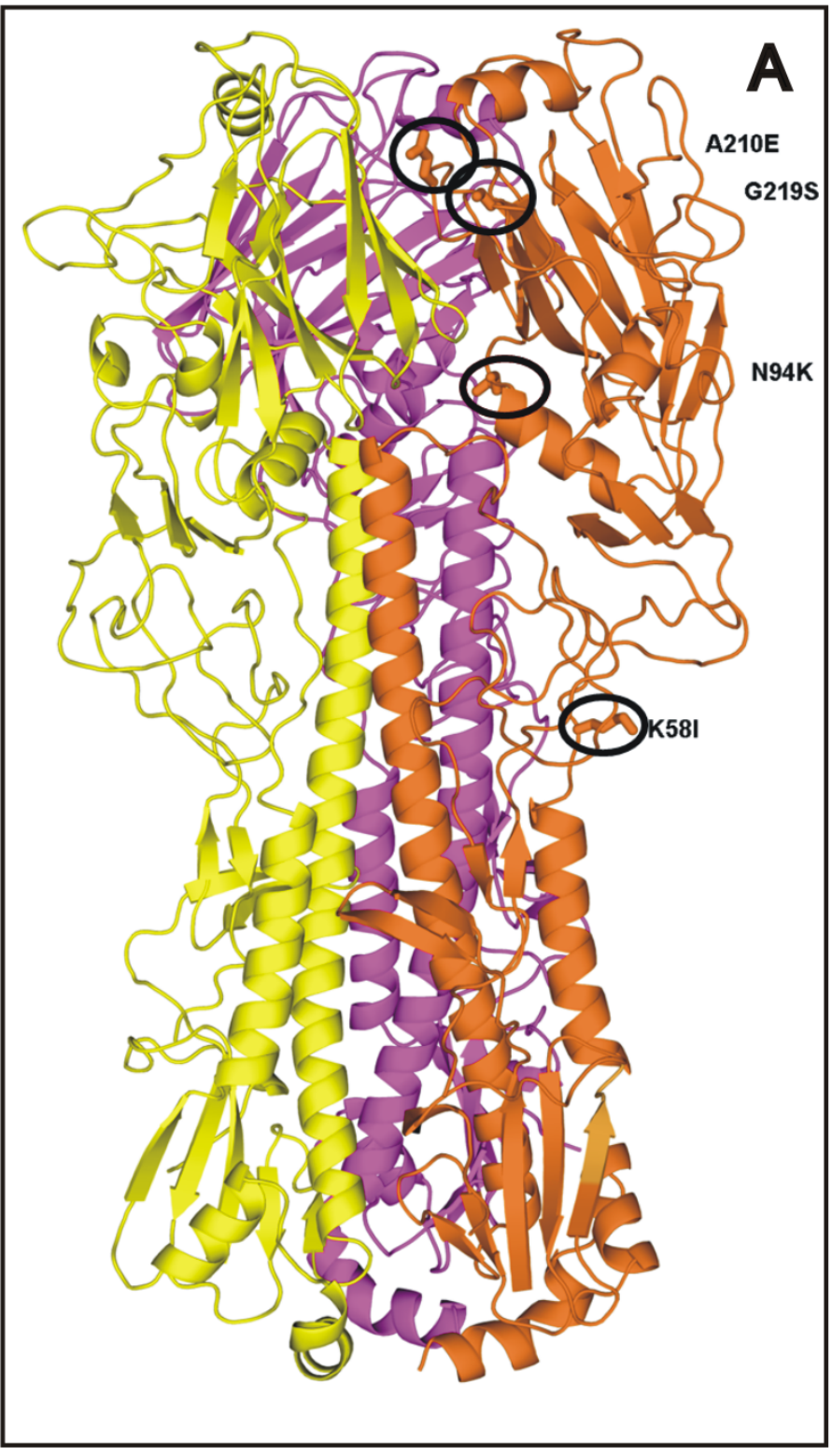
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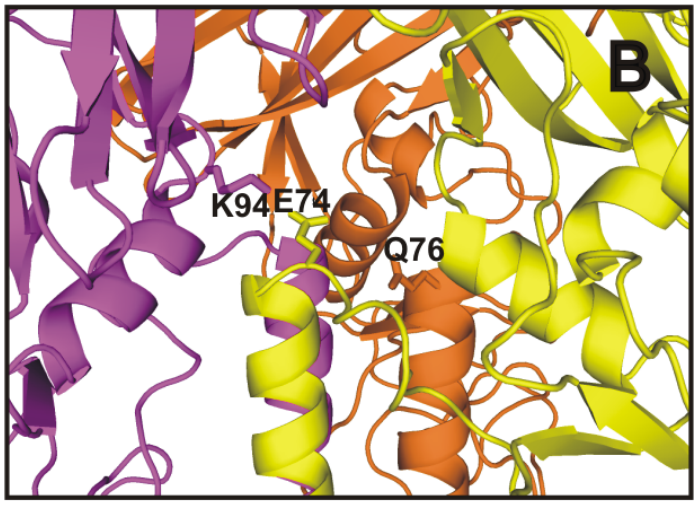
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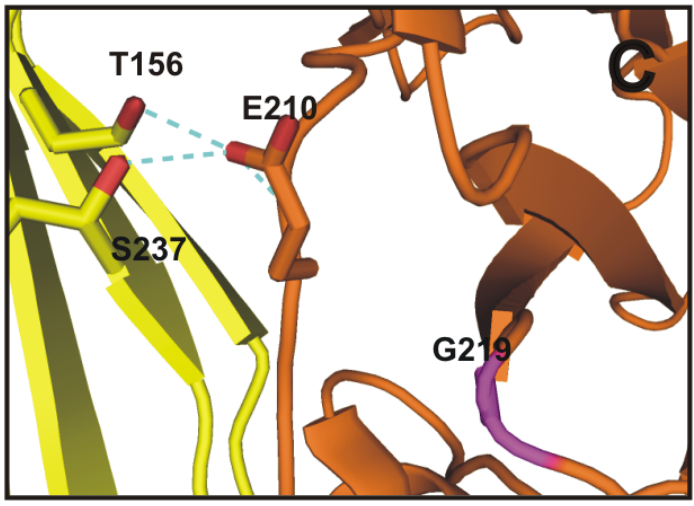
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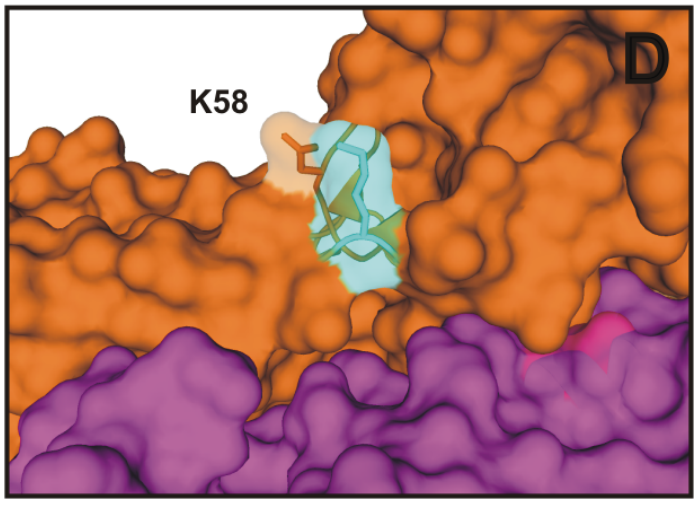
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