Stabilizing Mutations Allow Subsequent Immune Escape during Flu Evolution

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Influenza causes roughly 500,000 deaths per year in the human population. New strains of flu virus emerge constantly due to extremely high rates of viral mutation and the possibility that viral genes will re-assort when two subtypes of influenza infect the same host. The names of different influenza subtypes have become all too familiar in the news due to outbreaks of human infections with swine and avian flu viruses, most recently H1N1 in 2009 and H7N9 in 2013. The names of these Influenza A viruses are based on the sequence differences in hemagglutinin (H-) and neuraminidase (N-) proteins appearing on the outer viral surface.

Often springing from East and Southeast Asia, seasonal flu viruses spread around the globe before tapering off or evolving into next year's flu. Perennial reformulations of flu vaccines invariably contain antigens corresponding to different forms of H3N2, one of which caused the Hong Kong Flu in 1968 and 1969. In a study published by the journal *eLife* on May 14th, Ian Gong of the Bloom Lab, Dr. Marc Suchard (Biomathematics, UCLA) and Dr. Jesse Bloom (Basic Sciences Division) investigated the effects of past amino acid substitutions in the virus's internal nucleoprotein (NP) that occurred during the evolution of the Hong Kong flu virus from 1968 to 2007. By reconstructing this trajectory of NP evolution, they revealed instances in which a stabilizing protein mutation permitted a subsequent mutation in the same protein (NP) that enhanced the virus's ability to evade the human immune system.

The authors framed their study around John Maynard Smith's concept of an evolutionary 'protein space'. Maynard Smith (1970) compared protein evolution to a game in which one word is converted to another one letter (or amino acid) at a time, subject to the constraint that all intermediate steps also involve meaningful words (*i.e.*, functional proteins): for instance, SICK → SILK → SILL → WILL → WELL. This analogy illustrates the phenomenon of within-protein epistasis, in which the effect of an amino acid substitution depends profoundly on the identities of other amino acids. That is, without the first substitution in this sequence, the second substitution would be maladaptive, as 'SICL' is not a word in English.

To test whether epistasis has constrained flu evolution in this manner, Gong *et al.* first reconstructed many of the intermediate NP sequences that arose during the 39-year history of H3N2 evolution. The authors focused on NP because its function is authentically maintained in tissue-culture assays, and this protein is targeted by cytotoxic T lymphocytes (CTLs) of the human immune system. Moreover, NP evolves in a nearly clock-like manner, accumulating approximately one amino acid substitution per year. Though there are more than 10^{46} possible orderings of the 39 most probable mutational steps for NP, the authors were able to confidently identify 25 of the 39 intermediate sequences (see figure).

The authors then used site-directed mutagenesis to recreate the high-confidence NP intermediates along this evolutionary trajectory. They also introduced each mutation individually into the parent strain that caused the Hong Kong Flu pandemic. The authors reconstructed viruses containing these historical versions of NP, and they tested the replication of these viruses in a tissue culture assay. All 25 intermediate viruses replicated well. However, three of the mutations (L259S, R384G and V280A) were deleterious to viral growth and the thermodynamic stability of NP when they were introduced into the parental NP from 1968.

The team of researchers wanted to know why these mutations were not deleterious during the actual evolutionary trajectory. Two of the mutations were permitted because they were preceded or accompanied by protein-stabilizing mutations that alleviated the negative effects of the amino acid substitutions on viral fitness. In the third case (R384G), the authors' findings suggest that a potential negative impact on viral fitness was alleviated by a compensatory mutation (E375G) that affected the electrostatic charge on the surface of NP.

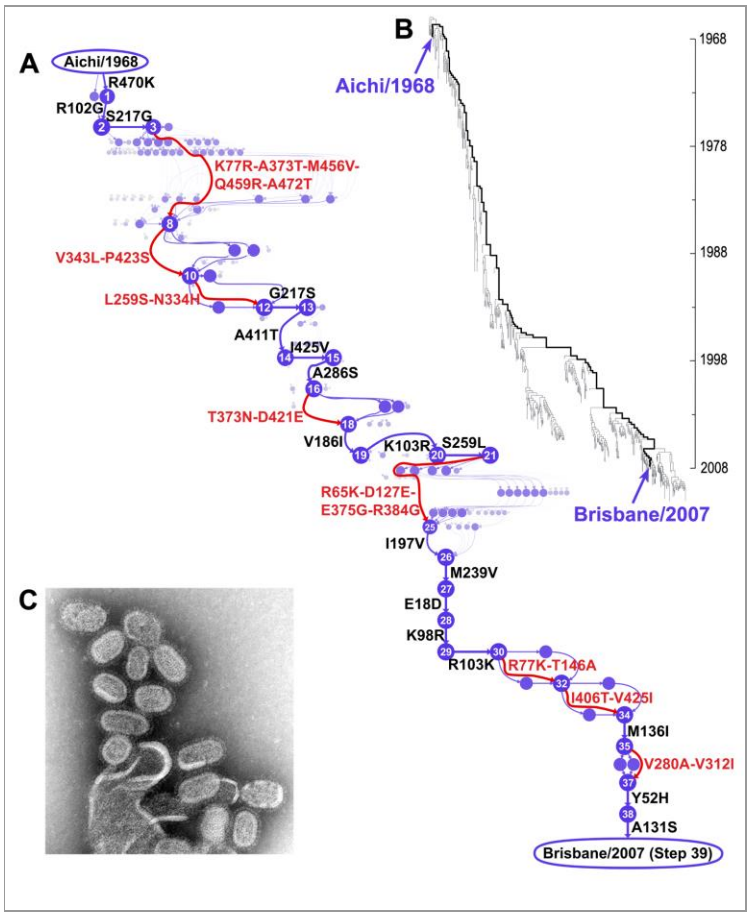
Finally, Gong and co-authors wondered what selective force might have driven the fixation of these three epistatically-constrained substitutions in NP. One possibility was that mutations to regions of the protein acting as CTL epitopes (*i.e.*, regions which enable the immune system to recognize the virus as a threat) are beneficial to the spread of influenza because they help the viruses evade immune memory in the human population. Indeed, the researchers found that the three destabilizing mutations represented a much greater share of literature-characterized or computationally predicted CTL epitopes than predicted by chance alone. These mutations were apparently favored during H3N2 evolution because they enhanced the virus's ability to escape detection by the immune system, but they were only permitted when compensatory mutations opened the door to these possibilities in viral protein space.

Complementing the recent discovery of stabilizing hemagglutinin mutations that enhance H5N1 transmissibility (Imai *et al.*, 2012), the work of Gong *et al.* establishes a central role for epistatic constraints during flu evolution. Additional studies like these may one day make it possible to identify novel flu viruses with the largest capacity for devastating evolutionary changes due to enabling mutations. According to Dr. Bloom, "if we could identify such viruses, they would be excellent candidates for targeting with public health efforts, such as surveillance and vaccination."

Gong LI, Suchard MA, Bloom JD. 2013. Stability-mediated epistasis constrains the evolution of an influenza protein. *eLIFE*2:e00631.

Also see: Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, Zhong G, Hanson A, Katsura H, Watanabe S, Li C, Kawakami E, Yamada S, Kiso M, Suzuki Y, Maher EA, Neumann G, Kawaoka Y. 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. *Nature* 486:420-428.

Maynard Smith J. 1970. Natural selection and the concept of a protein space. *Nature* 225:563-64.



Panels A and B provided by Ian Gong and Jesse Bloom; C downloaded, with permission, from Wikimedia Commons.

Reconstructed trajectory of nucleoprotein (NP) evolution for the H3N2 subtype of influenza. (A) Trajectory through 'protein space', beginning with a sample isolated in 1968 from the Aichi Prefecture of Japan, and ending with a sample isolated in 2007 from Brisbane, Australia. Each purple circle represents a unique inferred sequence; the area and intensity of each circle is proportional to the posterior probability that the true evolutionary trajectory passed through that sequence. Clearly resolved mutations are shown in black, whereas mutations occurring in an unknown order are shown in red. For example, L259S indicates the substitution of a leucine at amino acid position 259 by a novel serine residue at that position. (B) Maximum clade credibility tree summarizing the results of a Bayesian phylogenetic analysis of NP nucleotide sequence divergence using BEAST software. Branch tips correspond to different H3N2 strains. Dates of strain branching and isolation are indicated with the calibrated time-scale at the right. (C) Transmission electron micrograph of influenza virions (ca. 100,000 X).