Clues to the Virulence of H5N1 Viruses in Humans

Robert M. Krug

H5N1 strains of avian influenza A virus have already caused the deaths of more than 90 people since the outbreak of infection in Southeast Asia in 1997, corresponding to a death rate of ~50% for known infections. These viruses, which have now spread from Asia to Europe and Africa, are strong candidates for causing the next flu pandemic if they acquire the ability for efficient human-to-human transmission. A major research goal has been to identify the molecular basis of the virulence of H5N1 viruses in humans (1, 2). Several virus-encoded proteins will likely contribute to virulence in humans, because previous studies have shown that the virulence of influenza A virus of different organisms is caused by multiple genes (2). The study by Obenauer et al. (3) on page 1576 of this issue, presents evidence suggesting that the virulence of H5N1 viruses may be caused at least in part by the function of a previously unnoticed amino acid sequence motif in the virus-encoded nonstructural protein called NS1 (see the figure).

The NS1 protein is synthesized in infected cells but not incorporated into virus particles. Rather, this small, multifunctional protein participates in both protein-RNA and protein-protein interactions during infection. Its amino-terminal RNA-binding domain binds to double-stranded RNA (dsRNA) with low affinity (4), but the significance of this activity during viral infection is controversial (5, 6). The NS1 protein also binds and inhibits the function of a number of cellular proteins or dsRNA are not part of the putative new virulence determinant in NS1.

Ten other proteins are encoded by influenza A virus, whose genome consists of eight single-stranded RNAs (8). Three proteins (PB1, PB2, and PA) comprise the polymerase that is associated with each of the viral genomic RNAs in the virus particle. The polymerase copies these genomic RNAs into viral mRNAs and also catalyzes the replication of the genomic RNAs in infected cells. Investigators have identified the amino acid sequences of the PB1, PB2, and PA proteins that function in specific steps of virus-specific RNA synthesis or in mediating interactions between the three proteins (2). The amino acid at position 627 in PB2, which has been implicated in human virulence of H5N1 viruses, does not participate in these known functions.

Variation locations. Strains of the H5N1 influenza A virus that are virulent in mammals, including mice and humans, have alterations in the sequences of any of three viral proteins hemagglutinin (HA), the viral polymerase protein PB2, and the nonstructural protein NS1. Influenza A virus has 8 genomic RNA strands and 10 proteins, as shown.

H5N1 viruses that are virulent in mice encode lysine at this position in PB2, whereas H5N1 viruses that are not virulent in mice, as well as other avian influenza A virus strains, encode glutamic acid at this position (9). It is thought that this change from glutamic acid to lysine represents an adaptation of H5N1 viruses for efficient replication in mammalian cells (10).

Another virulence determinant for the H5N1 virus in mammals has previously been identified in the hemagglutinin, the major surface protein of the virus (8). Hemagglutinin, which binds to sialic acid–containing receptors on host cells, is the protein against which neutralizing antibodies are produced. Because the H5 type of hemagglutinin in avian influenza A viruses has not been found in previously circulating human influenza A virus strains, humans are potentially susceptible to infection by these viruses. Cleavage of hemagglutinin into two disulfide-linked sub-

References and Notes
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Why is H5N1 avian influenza so virulent? Genomic analysis of various isolates suggests that, in addition to two known surface proteins, a third previously unnoticed sequence in a small viral protein may contribute to virulence.
units is a prerequisite for initiating infection (8). H5N1 viruses that are highly pathogenic in mice contain a stretch of basic residues adjacent to the hemagglutinin cleavage site, enabling these hemagglutinins to be cleaved by ubiquitous intracellular proteases, including furin. Recombinant H5N1 viruses lacking these basic amino acids are no longer virulent in mice (9), demonstrating that the presence of these amino acids, and the consequent cleavage by intracellular proteases, are required for the virulence of these viruses.

To further understand the molecular basis of virulence, Obenauer et al. first sequenced the genes of a large number of H5N1 viruses isolated from wild birds and poultry, providing an invaluable resource for many investigators. This analysis revealed not only the expected variability in the sequences of the two major surface proteins of the virus, hemagglutinin and neuraminidase, but also variability in the sequence of the NS1 protein. Despite variability in the latter, it was noted that the carboxyl terminus of the NS1 proteins of the vast majority of avian H5N1 viruses contains a sequence motif, Glu-Ser-Glu-Val (ESEV). These residues are predicted to mediate binding to proteins bearing a region called a PDZ domain. The multitude of human proteins that contain a PDZ domain function in diverse cellular signaling pathways including those that regulate protein traffic within the cell and those that maintain cell morphology and organization. Another PDZ-binding sequence, Glu-Pro-Glu-Val (EPEV), was identified at the carboxyl terminus of the NS1 proteins of all the virulent H5N1 viruses isolated from humans. In contrast, the carboxyl terminus of the NS1 proteins of low-virulence human influenza A usually contains a different sequence, Arg-Ser-Lys-Val (RSKV), which is not a PDZ-binding motif. Further, Obenauer et al. verified that the carboxyl-terminal ESEV and EPEV sequences indeed bind to PDZ domains. Consequently, the presence of a functional carboxyl-terminal PDZ-binding domain in the NS1 protein of H5N1 viruses correlates with human virulence. This supports the authors’ hypothesis that the carboxyl-terminal domain of the NS1 proteins of avian H5N1 viruses acts as a virulence factor by binding cellular PDZ-containing proteins and disrupting their participation in important cellular processes.

This is an intriguing hypothesis that, however, needs to be evaluated in animal experiments with H5N1 viruses that have been altered to express a NS1 protein lacking the carboxyl-terminal ESEV/EPEV sequence. Such experiments are critical because it has already been established that this carboxyl-terminal sequence is not required for the virulence of previously isolated H5N1 viruses in ferrets (11). An analysis of the virulence of H5N1 viruses isolated in 2004 identified the human isolate A/Vietnam/1203/04 as the most pathogenic isolate. The NS1 protein encoded by this virus is truncated and consequently lacks the suspect carboxyl-terminal ESEV/EPEV motif. Future experiments will establish whether eliminating the carboxyl-terminal ESEV/EPEV sequence of the NS1 protein of other H5N1 viruses has any effect on their virulence in animal models. In addition, the search for other molecular determinants of the virulence of H5N1 viruses in humans will undoubtedly continue.

References

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Seamless Proteins Tie Up Their Loose Ends

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In the early 1970s, tribeswomen in the Congo were reported to drink a medicinal tea made from a local plant to induce labor and facilitate childbirth (1). Twenty-five years later, it was discovered that the active ingredient, robust enough to withstand boiling and ingestion, is a small protein with a circular shape (2). It turns out that the protein, kalata B1, was not a one-off example. Many other naturally occurring circular and stable proteins have since been found in bacteria, plants, and animals from Africa, South America, Australia, and Europe (3). What makes them so interesting? The exceptional stability and wide range of activities of these circular proteins, from insecticidal and antimicrobial to thwarting cellular infection by HIV (4), may guide the development of more effective and stable drugs.

The discovery of proteins bearing two ends that are linked together, producing a circular topology (2), is a new and mysterious twist in protein synthesis. Most proteins are synthesized as linear chains of amino acids in which the amino terminus of one residue is linked to the carboxyl terminus of the next. Whether assembled in the cell by nature’s ribosomal machinery that translates a genomic blueprint, or by the synthetic methodology of peptide chemists, a newly formed chain folds into a complex three-dimensional shape that determines the protein’s function. Circular proteins have no beginning or end, and deciphering their mode of construction presents some interesting challenges. So far, we know very little about how cyclization occurs. Circular proteins appear to derive from larger precursor proteins (see the figure), but we have little knowledge of the enzymes or other processes that cleave the mature peptide from its precursor and facilitate formation of a cyclic backbone.

The diversity of sequence of the nearly 100 circular proteins known to date across species suggests that cyclization has evolved independently in vastly different organisms as a way of tiding up the loose ends of conventional proteins. Microorganisms appear to have seized upon the advantages of cyclizing peptides long ago, as has the pharmaceutical industry. For example, in the course of making the cyclic peptide cyclosporin for their own defense, fungi have provided humankind with a drug that has now revolutionized transplant therapy because of its potent immunosuppressive activity. But cyclosporin and other previously known cyclic peptides are typically small rings of fewer than a dozen amino acids and are produced not by direct gene translation but by multidomain enzymes called peptide synthetases. The excite-
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