Avian sarcoma and leukemia virus-receptor interactions: From classical genetics to novel insights into virus–cell membrane fusion

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Abstract

For over 40 years, avian sarcoma and leukemia virus (ASLV)-receptor interactions have been employed as a useful model system to study the mechanism of retroviral entry into cells. Pioneering studies on this system focused upon the genetic basis of the differential susceptibilities of different lines of chickens to infection by distinct subgroups of ASLV. These studies led to the definition of three distinct autosomal recessive genes that were predicted to encode cellular receptors for different viral subgroups. They also led to the concept of viral interference, i.e. the mechanism by which infection by one virus can render cells resistant to reinfection by other viruses that use the same cellular receptor. Here, we review the contributions that analyses of the ASLV-receptor system have made in unraveling the mechanisms of retroviral entry into cells and focus on key findings such as identification and characterization of the ASLV receptor genes and the subsequent elucidation of an unprecedented mechanism of virus–cell fusion. Since many of the initial findings on this system were published in the early volumes of Virology, this subject is especially well suited to this special anniversary issue of the journal.

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ASLV receptors

Avian sarcoma and leukemia viruses are divided into 10 different viral subgroups (designated A–J) that are based, in part, on the specific cellular receptors that they use (Weiss, 1993).

In the 1960s and early 1970s, cross-breeding experiments performed with different chicken lines indicated that three independent genes (denoted tv for tumor virus) control susceptibility to infection by ASLV viral subgroups A–E (for a review, see Weiss, 1993). These loci were predicted to encode different subgroup-specific viral receptors. The tvA and tvC loci were genetically linked and susceptibility alleles of each locus (tvAs and tvCs) conferred susceptibility of viral subgroups A and C, respectively (Crittenden et al., 1967; Duff and Vogt, 1969; Hanafusa, 1965; Payne and Biggs, 1964; Payne and Pani, 1971; Vogt and Ishizaki, 1965). The tvb locus was predicted to encode cellular receptors for ASLV subgroups-B, -D, and -E (Crittenden et al., 1967; Payne and Biggs, 1966; Rubin, 1965; Vogt and Ishizaki, 1965). Two functional alleles of tvb were defined, tvbS1 which conferred susceptibility to infection by all three viral
subgroups and tvb\textsuperscript{S3} for viral subgroups-B and -D only (Crittenden and Motta, 1975; Crittenden et al., 1973). Moreover, other alleles were defined at each locus (designated as tva\textsuperscript{R}, tvb\textsuperscript{R}, tve\textsuperscript{R}) that were associated with host resistance to infection by the cognate viral subgroups, and each of these was recessive in nature.

The gene products of these three putative receptor loci remained unknown until the development of techniques that allowed the introduction of whole genomes into populations of transfected cultured cells. The first of the genes identified by this method was tva (Bates et al., 1993; Young et al., 1993). TVA, a low-density lipoprotein (LDL) receptor-related protein with a single extracellular LDL-A module, was found to possess the features expected of a bona fide subgroup A viral receptor. It bound specifically to the ASLV-A envelope glycoprotein, via residues in the LDL-A module, and conferred susceptibility solely to ASLV subgroup A infection when expressed in receptor naive cells. Two different isoforms of TVA are generated by alternative splicing, a transmembrane version with a single membrane anchor. Because TVC was just described, the other retroviruses, ASLV utilizes receptors with a single membrane-spanning domain, and a form with a glycosylphosphatidylinositol (GPI) anchor (Barnard and Young, 2003).

The second ASLV receptor gene to be identified by this method was tvb. The chicken tvb\textsuperscript{S3} and tvb\textsuperscript{S1} alleles were characterized and, as predicted, encoded cellular receptors for viral subgroups-B, -D, and-E, and for subgroups-B and-D, characterized and, as predicted, encoded cellular receptors for tvb. In addition, the turkey homolog of tvb (designated as tvb\textsuperscript{T}) was also isolated and was shown to encode a subgroup E-specific viral receptor (Adkins et al., 1997). TVB proteins are members of the tumor necrosis factor receptor (TNFR) family and are most likely the avian homologs of the mammalian TRAIL receptors. Subgroup-specific viral interaction determinants have been mapped in the extracellular, TNFR-related, cysteine-rich domains (Barnard and Young, 2003).

More recently, the tvc gene was identified using a positional cloning approach which took advantage of the close linkage between the tva and tvc loci. In contrast to either TVA or TVB, TVC is a member of the immunoglobulin superfamily that most closely resembles the mammalian butyrophilins (Elleder et al., 2005). The extracellular region of TVC contains two immunoglobulin-like domains while the cytoplasmic region contains a B30.2 domain. Therefore, different subgroups of ASLV have evolved to use very different types of proteins as their cellular receptor. It is interesting to note that unlike most other retroviruses, ASLV utilizes receptors with a single membrane anchor. Because TVC was just described, the viral interaction determinants of this receptor have not yet been identified.

The identification of each of these receptor genes led to the uncovering of the molecular basis of resistance associated with tva\textsuperscript{R}, tvb\textsuperscript{R}, and tve\textsuperscript{R} alleles. These alleles exhibit a variety of distinct debilitating mutations including premature stop codons, a translational frameshift, and an amino acid substitution that impacts Env binding (Elleder et al., 2004; Elleder et al., 2005; Klucking et al., 2002).

**ASLV Env and viral fusion proteins**

Like all retroviral Env proteins, those of ASLV are class I fusion proteins with an N-terminal surface (SU) subunit involved in receptor-binding and a C-terminal transmembrane (TM) subunit that directs membrane fusion. The TM subunit contains an N-terminal hydrophobic fusion peptide, a C-terminal transmembrane domain that anchors the protein to the viral membrane, and N- and C-terminal heptad repeats that juxtapose the fusion peptide and C-terminal transmembrane, respectively (for a review, see Barnard and Young, 2003). The overall architecture of ASLV Env is characteristic of other prototypical class I fusion proteins such as influenza HA, human immunodeficiency virus (HIV) Env, and Ebola Gp2 (Eckert and Kim, 2001).

Class I viral fusion proteins exist as a metastable complex on the viral surface and upon fusion activation they are converted to a more stable, lower-energy form. The fusion activities of these proteins are usually triggered either by receptor- (and in some cases also coreceptor-) induced conformational changes, as is the case with HIV, or instead by structural changes caused by the low pH environment of intracellular acidic endosomes.

The first fusion-activating changes result in exposure of the N-terminal hydrophobic fusion peptide which then inserts into the target membrane (Eckert and Kim, 2001). Further conformational changes then reorient the protein so that the fusion peptide and transmembrane domains lie in close proximity at the same end of the transmembrane protein. This step is thought to lead to the merger of the contacting lipid membranes, i.e. hemifusion (Eckert and Kim, 2001). The N- and C-terminal heptad repeats then associate into a six-helical bundle (6HB) that facilitates the completion of the fusion reaction by leading to the formation and expansion of a fusion pore (Barnard and Young, 2003; Eckert and Kim, 2001). The 6HB form of these viral glycoproteins is highly thermostable and is resistant to denaturation in SDS-containing buffers.

**ASLV entry**

Broad interest in ASLV entry was stimulated following the unexpected finding that ASLV Env utilized an unprecedented two-step fusion activation mechanism that borrowed features from both pH-independent, and pH-dependent, viruses (Mothes et al., 2000). The first step involves receptor-induced conformational changes in Env at neutral pH that lead to exposure of the fusion peptide so that it inserts into the cell surface membrane. The next step involves low pH activation resulting in completion of the fusion reaction in an acidic endosomal compartment, following virus uptake and endosomal trafficking. These events were recently reviewed in detail elsewhere (Barnard and Young, 2003).

The existence of a stable receptor-primed intermediate form of ASLV Env, with an extended conformation, is supported by studies performed using a C-terminal inhibitory heptad repeat peptide (termed R99) which can inhibit ASLV infection when added at the cell surface (Barnard et al., 2004; Earp et al., 2003; Netter et al., 2004). These results suggest that the N- and C-
terminal heptad repeats of ASLV TM are already exposed at
the plasma membrane following the receptor interaction.
Consistent with this idea, after receptor-priming, ASLV Env
becomes competent to bind to the R99 inhibitory peptide
(Netter et al., 2004). Since this peptide can access its target at
the plasma membrane, i.e. the N-terminal heptad repeat, this
suggests that the receptor-primed form of ASLV Env is likely
to be a pre-6HB intermediate form (Fig. 1, Step 1).

Unlike other low pH-dependent viruses, ASLV can remain
stable for many hours in cells that are transiently treated with
lysosomotropic agents to elevate endosomal pH (Mothes et al.,
2000; Narayan et al., 2003). This result also points toward the
remarkable stability of the receptor-primed intermediate of
ASLV Env. Thus, unlike all other class I fusion proteins, ASLV
Env seems capable of pausing for long periods of time in a pre-
6HB conformation, following insertion of the fusion peptide into
the target membrane and before acid pH triggering. Subsequent
low pH-triggering of the receptor-primed ASLV Env results in
formation of a tight SDS-resistant 6HB (Smith et al., 2004).

How far does fusion progress at the receptor-priming stage?
Studies performed with pyrene-labeled viruses suggested that
receptor-primed ASLV is capable of reaching the hemifusion
stage at neutral pH (Earp et al., 2003). However, cell–cell
fusion experiments indicated that ASLV Env-dependent hemi-
fusion and fusion occur only after receptor-priming and low pH
triggering (Melikyan et al., 2004). Consistently, the receptor-
primed form of ASLV Env can undergo acid pH-dependent
triggering at low temperatures generating a restricted hemifu-
sion intermediate (Fig. 1, Steps 2 and 3). Subsequent fusion
pore formation and expansion steps occur in a pH-independent
manner at physiological temperature (Melikyan et al., 2004)
(Fig. 1, Steps 4 and 5).

The imaging of individual retroviral fusion events has also
added support for the model that the receptor-primed, pre-6HB
form of ASLV Env is arrested at a stage prior to hemifusion.
Using a novel virus–cell fusion assay, which monitored both
lipid and content mixing of DiD/yellow fluorescent protein
(YFP) labeled virions, it was found that ASLV Env-dependent
nascent fusion pores formed only after receptor and low pH
treatment (Melikyan et al., 2005). As both hemifusion and
content mixing were inhibited by the R99 peptide, it is likely
that formation of a tight 6HB is required to drive fusion pore
formation and/or expansion. More importantly, these experi-
ments also provided direct evidence that a hemifusion
intermediate, that exists for up to several minutes, is a bona
fide intermediate in the membrane fusion reaction mediated by
ASLV Env (Melikyan et al., 2005).

Fig. 1. ASLV Env-membrane fusion driven by receptor and low pH. Following
receptor interaction at the cell surface, the fusion peptide of ASLV Env is
exposed and inserted into the target membrane (Step 1). The receptor-primed
form of ASLV Env then forms a stable pre-6HB intermediate that can bind
inhibitory C-terminal heptad repeat peptides (Steps 1 and 2). In this state,
ASLV Env can be triggered by low pH at non-physiological temperatures (Step
2). Upon addition of low pH, ASLV Env folds into its tight 6HB promoting first
a restricted hemifusion intermediate (Step 3), an unrestricted hemifusion
intermediate (Step 4), and finally formation and expansion of the fusion pore
(Step 5) that allow delivery of the viral core into the cell cytoplasm.
Concluding remarks and future perspectives

The pioneering studies on the ASLV-receptor system that were initiated over 40 years ago set the foundation for the isolation and characterization of ASLV receptor genes and for the detailed molecular and biophysical analyses of the viral entry pathway. These studies revealed that ASLV uses an unanticipated mechanism of viral entry that represents a hybrid of the more common pH-independent and pH-dependent viral entry mechanisms. These findings are probably of broader significance since we already know of other viruses that exhibit a similar two-step entry mechanism (Nurani et al., 2003; Seth et al., 2003).

The studies on the ASLV-receptor system have also revealed two novel intermediates in class I viral glycoprotein-driven fusion. The first is the highly stable receptor-primed intermediate which can exist presumably in an extended, pre-6HB, conformation for many hours. This property of ASLV Env is unique among the class I viral fusion proteins and a better understanding of the structure of this intermediate is likely to add significantly to our knowledge about the step-wise conformational changes in class I viral fusion proteins that lead to membrane fusion. Moreover, this property of ASLV was recently exploited in the development of a cell-free retroviral fusion and uncoating system that is being used to examine the ill-defined series of uncoating events that occur after viral fusion and lead to formation of an active viral reverse transcription complex (Narayan and Young, 2004). The second intermediate is arrested transiently at a stage that lies between hemifusion and fusion pore opening/expansion. The existence of this intermediate provides direct evidence that hemifusion is a bona fide intermediate in the virus–cell membrane fusion reaction. Since this intermediate stage likely exists for other class I fusion proteins, such as influenza and Ebola viruses, it may represent a viable target for future antiviral development. Future studies in the ASLV-receptor system are likely to contribute additional insights into the steps involved in viral entry as well as those that immediately follow virus–cell membrane fusion.

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