Ancient viruses in the fight against HIV

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In the past few years, AIDS has become a disease that can be treated effectively even though it cannot yet be cured. Therapies directed against the replication of HIV, the lentivirus which causes AIDS, that involve a combination of drugs that inhibit viral enzymes can reduce the amount of HIV in the blood to barely detectable levels, restoring normal levels of immune function in patients.

Unfortunately, there are limitations to the current approaches used to treat HIV. The drugs that patients take are directed towards only two steps of the retroviral replication cycle: those catalyzed by the enzymes reverse transcriptase and the viral protease. Resistance to the drugs can arise, especially if there is a lack of adherence to the rigid drug regimens. There has been considerable progress in making the schedule of medicine intake less difficult, but problems with the side effects of the drugs remain. Therefore there is an ongoing endeavor to find other pharmaceutical agents to add to the anti-HIV armory.

Recently, efforts have been focused upon peptide inhibitors of the membrane-fusion event that leads to the entry of HIV. Retroviral envelope (Env) glycoproteins consist of two polypeptides: the surface (SU) protein, which binds to the receptor present on target cells, and the transmembrane (TM) protein, which possesses the fusion peptide whose insertion into the target cell membrane leads to the fusion of the viral and cellular membranes. Enfuvirtide (T-20 or DP178), a peptide that possesses the sequence of part of the C-terminal helix of a six-helix bundle, the center three helices of which form the core of the HIV TM (gp41) homotrimeric coiled coil, which is characteristic of a large class of viral proteins that promote membrane fusion [1], inhibits the conformational changes that lead to membrane fusion following HIV Env binding to cellular receptors [2]. Enfuvirtide has demonstrated safety and efficacy in clinical trials [3]. Some viruses resistant to inhibition by the peptide have arisen: these have amino acid substitutions in the TM protein [4].

Another method for inhibiting membrane fusion is to block cellular receptors chemically so that they no longer interact with the envelope protein on HIV particles. Alternatively, the receptor-binding site of the HIV Env could be filled with something that resembles the cellular receptor or that fits the site. Here we examine other potential approaches for inhibiting HIV entry based on findings concerning ancient retroviruses that infect nonhuman hosts.

The major strides that have been taken in developing an understanding of HIV and anti-HIV therapies would not have been possible without the groundwork laid by studies of retroviruses that infected nonhuman mammals at a time when no human retroviruses were known. In particular, investigations of mouse retroviruses, the murine leukemia viruses (MuLVs), have made crucial contributions to our understanding of the viral life cycle. Indeed, one of the virions where reverse transcriptase activity was first identified was that of a MuLV. The biochemical and structural basis of entry by the MuLVs [5,6] is one of the best-understood aspects of these viruses and should help in designing anti-HIV strategies.

Target: thiol-disulfide exchange

MuLV Envs are synthesized as single polypeptide precursors that form a trimer during progress through the secretory system. Env is subsequently proteolytically processed into two subunits, SU and TM, which are linked through a disulfide bond [7–10]. SU is on the outside of the retrovirus particle, whereas TM possesses an extraparticle domain, with a fusion peptide (whose insertion into the target membrane is believed to promote the process of fusion of the viral and cellular membranes) at its N-terminus, a membrane-spanning domain, and a 35-amino acid domain that resides within the particle (Fig. 1) [11].

Examination of the available data on MuLV Envs and of the related bovine leukemia and human T-cell leukemia viruses indicates that disulfide-bond rearrangement plays a crucial role in the induction of Env-mediated membrane fusion by receptors [8,10]. The SU subunit possesses a CxxC motif (where x represents any amino acid) that is reminiscent of the active site of thiol–disulfide exchange enzymes. The biochemical data support a model in which one of the cysteines in the SU subunit CxxC motif forms a disulfide bond with the last of the cysteines in a conserved TM subunit Cx_6CC motif and the two other TM cysteines form an intramolecular cystine bridge [5,12]. The intermolecular disulfide rearranges to form an intramolecular bond (i.e. between the two cysteines of the SU subunit CxxC motif) upon receptor binding. Extrusion of the TM fusion

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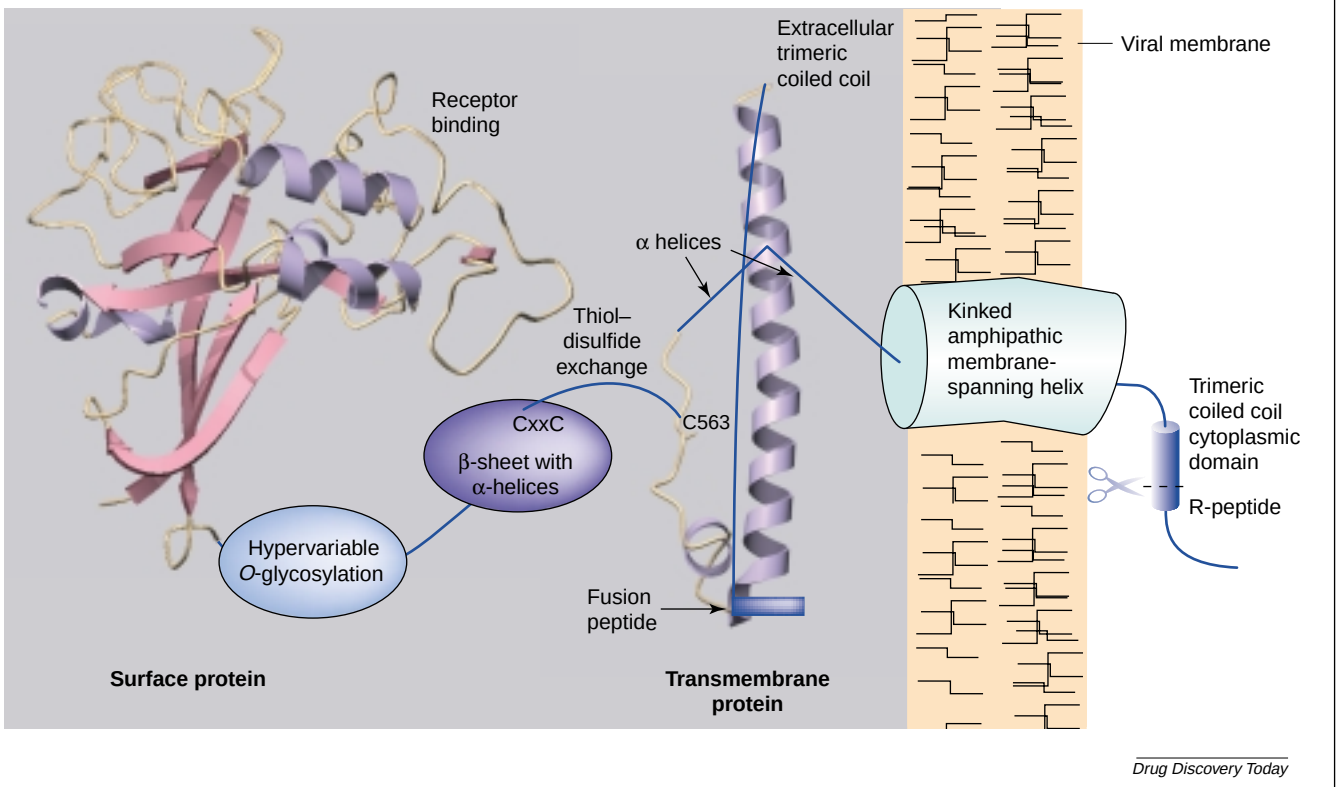

Figure 1. A model of the Moloney murine leukemia virus (Mo-MuLV) envelope (Env) domain and secondary structure. The model is a depiction of a monomer within the envelope-protein complex trimer. Mo-MuLV surface protein (SU) and transmembrane protein (TM) are shown as separate entities although they are originally synthesized as a single polypeptide that is cleaved during transit of the protein through the secretory system. The 3D structure of the N-terminal receptor-binding domain of a MuLV SU is shown on the left [6]. The central SU region, which is heavily *O*-glycosylated and the C-terminal domain of SU, which possesses a thiol–disulfide exchange motif, are shown as ovals because their structure is unknown. The site of the SU–TM disulfide bond is indicated by a curved blue line and the fusion peptide at the N-terminus of TM is depicted as a blue rectangle. The TM residue that forms the cystine bridge with SU, C563, is indicated. The TM extracellular coiled coil is depicted in the conformation observed in the crystal structure [5], which might not represent the conformation when SU and TM are linked. The membrane-spanning domain, which is predicted to form a kinked, amphipathic α-helix lies in the viral membrane. A predicted trimeric coiled coil in the TM cytoplasmic domain is also shown. The R-peptide is removed by the viral protease upon incorporation of Env into a budding retroviral particle.

peptide occurs via an irreversible spring-loaded mechanism analogous to that proposed for the influenza virus hemagglutinin protein, and membrane fusion ensues (Fig. 2). [5,10,13]

The thiol–disulfide exchange mechanism provides an opportunity for an antiviral strategy. Treatment of MuLVs with reagents that react with free sulfhydryls prevents the rearrangement of the Env disulfide bonds through a modification of one of the SU cysteine residues (Fig. 2) [7,8,14,15]. Synthesis of a reagent that cannot cross membranes and that is specific to the SU free cysteine residue could lead to the development of a treatment for this class of viruses, which includes the closely related feline leukemia viruses and the more distantly related human T-cell leukemia virus. Free thiols are fairly rare in the oxidizing extracellular environment so it could be possible to design a pharmaceutical that can specifically react with the SU cysteine.

Based upon the MuLV findings, it has been proposed that the entry of other viruses is dependent on cellular thiol–disulfide exchange activities, and therefore inhibiting these enzymes might interfere with viral entry [10]. Indeed, the replication of HIV-1 can be prevented by inhibitors of cell-surface protein disulfide isomerase (PDI) activity [16]. Although PDI is normally considered an endoplasmic reticulum-localized protein [17], cell-surface PDI has been detected by, for example, flow cytometric analysis using anti-protein disulfide isomerase monoclonal antibodies [16]. It was thought that a cell-surface protein disulfide isomerase is involved in promoting HIV-1 entry, presumably by reducing a crucial disulfide bond upon receptor binding [16]. Recently it has been shown that cellular PDI reduces disulfide bonds in HIV gp120 (SU) after receptor binding and that this reduction is required for membrane fusion and viral entry

[18,19]. Thiol–disulfide exchange reactions therefore provide an attractive target for future antiviral therapies.

Target: the membrane-spanning domain

The role of the membrane-spanning domain (MSD) of viral transmembrane proteins has only recently been examined. Experiments in several laboratories have examined the effects of replacing all or part of the MSD of a viral fusion protein with sequences that encode signals for glycosylphosphatidylinositol linkage attachment [20–23]. In each case, a defect in membrane fusion was observed. Similarly, engineered viral fusion proteins that are not membrane bound do not fuse membranes. These findings suggest that a protein MSD is crucial for the function of the fusion proteins.

One major issue raised by these data is whether any protein MSD can suffice for promoting membrane fusion or whether the amino acid sequence of the MSD is crucial. It is noteworthy that the sequences of the MSDs of the MuLV TM proteins are conserved. It has been shown that the MuLV TM MSDs play important sequence-specific roles in the promotion of membrane fusion [24]. The TM MSD sequence is predicted to form a kinked amphipathic α-helix similar to that of pore-forming toxins, such as bee mellitin; elimination of the TM MSD kink through mutation inhibits the membrane-fusion function without affecting protein expression, incorporation into viral particles or receptor binding [24]. Evidence for a sequence-specific role for the MSDs of other viral glycoproteins has also been described [25–27].

The MuLV TM MSD studies demonstrate that the question of the functional significance of the HIV TM membrane-spanning domain sequence should be re-examined [28]. Positive results would indicate that an anti-HIV strategy that targets the TM membrane-spanning domain might be successful. Sequence-specific reagents could be designed that bind to this domain and disrupt the completion of membrane fusion and consequently the entry of HIV.

Lessons from mouse retroviral resistance

Investigations into how mice have acquired natural resistance to retroviruses have led to important insights that might provide a guide for anti-HIV therapies. There are several genetic loci in mice that render them resistant to retroviral infection. These are referred to as Fv^r (Friend MuLV virus resistance) loci. The mouse *Fv4* gene controls susceptibility to infection by MuLVs that are only capable of entering rodent cells but does not affect susceptibility to other MuLV subgroups [29–32]. The *Fv4* locus is an endogenous defective provirus that contains a complete *env* gene [33] whose sequence is highly similar to those of the MuLVs [6,34] that bind to a receptor present on rodent cells [35]. The *Fv4 env* protein is expressed, processed and incorporated into recombinant Moloney MuLV particles at normal levels [34]. However, a recombinant virus bearing the *Fv4 env* protein is not infectious [34].

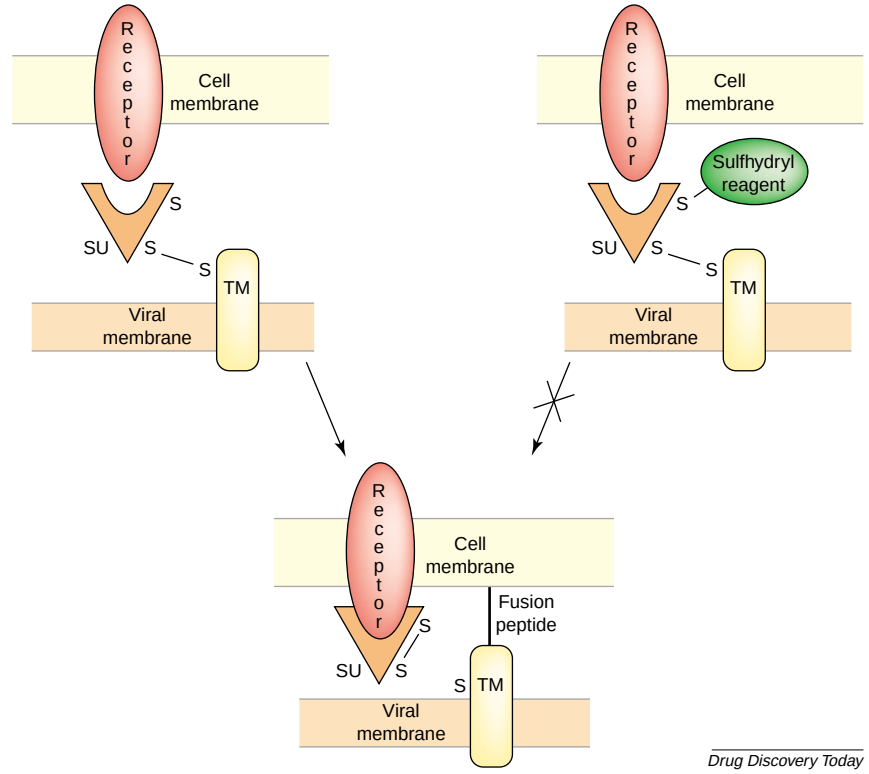

Figure 2. Model of murine leukemia virus (MuLV) thiol–disulfide exchange and receptor induction of membrane fusion. One of the cysteines in the surface protein (SU) (orange) CxxC thiol–disulfide exchange motif initially forms a disulfide with one of the cysteines in the transmembrane protein (TM) (yellow), which spans the viral membrane. Exposure of the fusion peptide is inhibited by the binding of SU to TM. There is a free thiol in SU. The intermolecular disulfide bond rearranges to an intramolecular bond (between the two cysteines on SU) upon binding to the receptor (red) in the cell membrane. Extrusion of the fusion peptide and membrane fusion ensue. If the virus is treated with a reagent (green oval) that reacts with sulfhydryls [such as a maleimide, a mercurial, or 5,5′-dithiobis-(2-nitrobenzoic acid)] the free thiol on SU is blocked and the disulfide-bond rearrangement cannot occur.

The basis of the resistance to infection by ecotropic viruses conferred by the *Fv4* gene had been the subject of a variety of proposals. The immune response had been suggested to play a subsidiary [36] or, more recently, a dominant role in resistance [32,37], although the mechanism was incompletely defined. Overall, the data are most consistent with the lack of susceptibility of *Fv4*^{r} mice resulting from viral interference [36,38–40]. A cell productively infected by a retrovirus is infected much less efficiently by the same retrovirus or by a retrovirus that uses the same receptor for entry, although it can be infected readily by retroviruses that employ a different receptor [41]. This retroviral interference phenomenon is caused by the endogenous retroviral Env binding to the cellular receptor so that it is unavailable for interaction with the envelope protein on an exogenous virus that uses the same receptor to enter cells. The binding of the endogenously expressed MuLV SU and the cellular receptor probably occurs intracellularly [42].

It was recently demonstrated that the genetic alteration in the Fv4 Env that made it defective for entry interferes with the membrane-fusion step and is a single substitution in the fusion peptide [40]. Furthermore, expression of an Fv4-like Env in a cell not only reduces virus entry into it through interference but also diminishes the infectiousness of a virus resulting from DNA transfection of recombinant viral progenomes into the cell [40]. The reduction of virus infection of mouse cells is dependent on the level of *Fv4* locus expression and is not absolute. However, the results indicate that the titer of virus produced by the rare infected cell is reduced through incorporation of the Fv4 Env into progeny virions leading to inhibition of the function of the wild-type envelope protein. In a recent gene-therapy experiment, resistance to retroviral disease was conveyed upon immunocompromised mice by introducing the *Fv4*^{r} gene into bone marrow cells [43].

The experiments discussed suggest a way of introducing resistance to a retrovirus such as the HIV into an organism. Transfer of a gene encoding a normally processed but fusion-defective retroviral envelope protein into susceptible cells would interfere with viral entry and potentially reduce the infectiousness of virus emerging from the cell. In the case of HIV, it might be additionally desirable if the envelope protein could bind to the chemokine receptor(s) without needing to bind to CD4. This would ensure that all the chemokine receptors could be blocked without interfering with the important function of CD4.

Another naturally occurring locus in mice that restricts MuLV infection is referred to as *Fv1*. This gene encodes a protein that resembles a retroviral polyprotein (Gag) that contains the components that form the core of a retroviral particle [44]. The gene has two principal dominant alleles; *Fv1*^{N} restricts the replication of certain strains of MuLV (called B-tropic) and *Fv1*^{B} restricts the replication of other strains of MuLV (called N-tropic). These strain differences in susceptibility to *Fv1* restriction result from sequence variations at one residue in the MuLV capsid (CA) protein [45]. Inhibition appears to act at a stage following membrane fusion but before DNA provirus integration. Interestingly, the entry of N-tropic MuLV into many nonmurine cells is restricted, and it is the same CA sequence variation that is responsible for the tropism [46]. This is true even though there is no close homolog to the *Fv1* gene in nonmurine cells [44]. It has been shown recently that primate cells express an activity that restricts lentiviral tropism [47,48]. The inhibition resembles that mediated by Fv1 in several aspects, including the dependence of restriction upon sequences in the CA (and/or adjacent p2) region of the lentiviral Gag protein. If the biochemical basis of *Fv1* restriction can be determined or if the cellular factors that restrict lentiviral tropism can be identified, developing novel approaches to anti-HIV therapies should be possible.

Conclusions

Our knowledge of the initial steps in infection by murine leukemia viruses is a promising resource for the mining of anti-HIV strategies. Laboratory experience with these viruses demonstrates that thiol–disulfide exchange, the activity of the viral envelope protein membrane-spanning domain, interference with receptor binding, and viral capsid-dependent steps following membrane fusion are all potential points of intervention that can be exploited for disrupting the HIV life cycle. Without the recent investigations of the entry mechanisms of the murine leukemia viruses it is unlikely that these targets would have been fully appreciated. Nevertheless, demonstration of the efficacy of these anti-HIV strategies will require extensive direct experimentation with HIV.

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