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The Role of CD4 in HIV Binding and Entry

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The role of CD4 in HIV binding and entry

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SUMMARY

The primary cellular receptor for the human and simian immunodeficiency viruses HIV-1, HIV-2 and SIV is the CD4 antigen (Sattentau *et al.* 1988; Sattentau & Weiss 1988). HIV infection of $CD4^+$ cells is initiated by binding of the virus to the cell surface, via a high-affinity interaction between the first domain of CD4 and the HIV outer envelope glycoprotein, gp120. The use of a soluble recombinant form of CD4 (sCD4) as a receptor mimic has simplified the analysis of receptor binding and post-binding events which result in virus–cell membrane fusion. With cell-line adapted isolates of HIV-1, sCD4 binding induces conformational changes in gp120, leading to the complete dissociation of gp120 from the transmembrane glycoprotein, gp41, and exposing cryptic epitopes of gp41. Similar observations have been made with cell-anchored CD4: recruitment of CD4 molecules leads to exposure of cryptic gp41 epitopes at the fusion interface between clusters of CD4 expressing and HIV-infected cells. It has therefore been proposed that CD4 binding induces exposure of fusogenic components of gp41 which mediate virus–cell membrane coalescence, a process termed receptor-mediated activation of fusion. With the related lentiviruses HIV-2 and SIV, the CD4 induced molecular rearrangements in gp120 are more subtle, implying that there is a spectrum of responses to sCD4 binding.

1. INTRODUCTION

The CD4 molecule has been adopted by the human and simian immunodeficiency viruses HIV-1, HIV-2 and SIV as their primary cellular receptor, determining the viral tropism for T cells and macrophages. The binding of HIV to CD4 has been extensively studied in recent years, and by a combination of site-directed mutagenesis and X-ray crystallography it has been established that the high-affinity binding site on CD4 consists of a ridge centered around the CDR-2-like loop, which projects from one face of the first extracellular domain of CD4 (Sweet *et al.* 1991). CD4 binds with high affinity to a region of the virus outer (SU) envelope glycoprotein predicted to be a cleft.

The first step in the infection of permissive cells by enveloped viruses is the binding of virions to specific cellular receptors. Subsequent entry of the virus capsid into the cell cytoplasm is achieved by fusion of the virion and cell membranes. There are two well-defined pathways leading to virus–cell membrane fusion: fusion within endosomes, typified by the alpha, flavi, rhabdo and orthomyxovirus families, and direct fusion at the plasma membrane which is the route of entry for certain members of the paramyxo, herpes and retrovirus families (Marsh & Helenius 1989; Moore *et al.* 1993; Weiss 1993). Electron microscopic and biochemical studies on the human and simian immunodeficiency viruses HIV-1, HIV-2 and SIV indicate that the major route of virus entry is by direct fusion at the cell membrane (Grewe *et al.* 1990) in a pH-independent manner (Stein *et al.* 1980; McClure *et al.* 1988). Further evidence supporting fusion at the plasma membrane is the ability of HIV-infected cells to fuse directly with $CD4^+$ cells to form syncytia (Dalgleish *et al.* 1984).

As with enveloped viruses from other families, the HIV glycoproteins are derived from a single polypeptide precursor which is cleaved by a cellular protease to yield the two mature forms, the transmembrane (TM) and SU subunits. Cleavage creates a new N-terminus in the TM subunit containing a stretch of highly hydrophobic residues which is structurally homologous within several virus families (Gallagher 1987; Gonzalez-Scarano *et al.* 1987). This peptide segment, termed the fusion domain, is thought to be directly involved in the induction of membrane fusion in HIV (Freed *et al.* 1990; Bergeron *et al.* 1992) and other enveloped viruses (Marsh & Helenius 1989).

It is clear that for viruses which undergo pH-dependent fusion such as influenza, that it is the reduction in pH which induces conformational changes in the envelope glycoproteins (Skehel *et al.* 1982; White & Wilson 1987), leading to exposure of the fusion peptide, disruption of the lipid bilayers, and coalescence of the virus and cell membranes. Like influenza, it seems likely that HIV also requires exposure of the fusion peptide for membrane fusion to occur, but such conformational changes must be triggered by an event other than acidification. Evidence is presented in this paper that HIV-induced fusion of the virus and cell membranes is activated by

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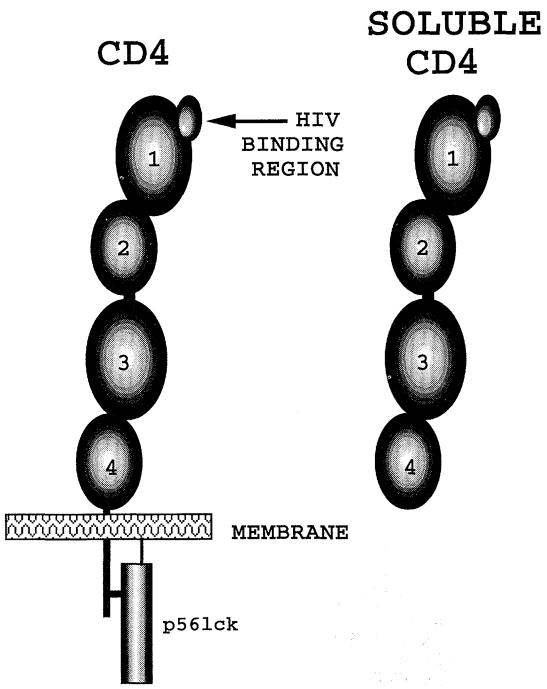

Figure 1. The CD4 molecule. CD4 is a membrane-anchored molecule found predominantly on a subset of T lymphocytes and macrophages. There are four extracellular domains with strong sequence and structural homology to immunoglobulin domains. The extracellular portion is anchored to the cell by a transmembrane domain, and there is a short cytoplasmic tail which associates with the tyrosine kinase, p56lck. The HIV binding site has been localized to the CDR-2-like loop in domain 1. The insertion of a stop codon in the cDNA has allowed the expression of a truncated, soluble form of CD4 (sCD4). This molecule retains a native conformation and its HIV binding properties.

binding of the SU glycoprotein to the CD4 molecule. This is termed receptor-mediated activation of viral fusion (Allen 1991; Moore *et al.* 1991*a*).

2. RESULTS

(*a*) *Soluble CD4 as a receptor mimic*

The expression and purification of soluble, recombinant forms of CD4 known as sCD4 has been achieved by the introduction of a stop codon into the CD4 cDNA before the segment coding for the transmembrane domain (Sweet *et al.* 1991). This glycoprotein contains the four extracellular domains (figure 1), binds to the viral SU glycoprotein with an affinity comparable to that observed for wild-type, cell-anchored CD4, and is conformationally correct as determined by the binding of a large panel of monoclonal antibodies (mAbs) raised against the native, membrane anchored molecule (Sattentau *et al.* 1989). We have used this protein to investigate the interactions occurring between CD4 and the HIV envelope glycoproteins which lead to membrane fusion.

(*b*) *SCD4 binding induces SU–TM glycoprotein subunit dissociation*

Initial studies focused on the consequences of the interaction of sCD4 with virions of cell-line adapted HIV-1 isolates. To investigate the hypothesis that CD4 binding to the HIV-1 SU subunit (gp120) might lead to exposure of the TM subunit (gp41) fusion peptide by inducing dissociation of the two glycoprotein subunits, a system of gel filtration was used to separate HIV virions from soluble protein complexes. Measurement of the gp120 content in the different fractions recovered from the column revealed that prior to sCD4 treatment, virtually all was associated with the virions. Subsequent to treatment with sCD4 at 37°C, however, approximately 80% of the gp120 was recovered in the fractions corresponding to soluble protein (Moore *et al.* 1990), indicating that sCD4 binding at 37°C has catalysed the release of gp120 from gp41. This dissociation was only detected when sCD4 was bound to virions, could not be induced with mAbs to gp120, and did not occur efficiently at temperatures below about 18°C (Moore *et al.* 1990, 1991*b*; Sattentau & Moore 1991). Similar results have been obtained by others using electron microscopy: treatment of HIV-infected cells with sCD4 resulted in the loss of visible surface spikes, which represent oligomers of gp41–gp120 complexes on the surface of budding or mature virions (Hart *et al.* 1991).

(*c*) *SU/TM dissociation requires multimeric interactions*

Using the gel-filtration strategy, kinetic analysis of the sCD4-viron interaction provided some interesting insights. Firstly, binding of sCD4 to virions did not precisely parallel gp120 dissociation: there was a differential between the binding of sCD4 and the detection of soluble complexes (Moore *et al.* 1991*b*). A statistical analysis revealed that this probably represented the requirement for a dimer of gp120 molecules to bind two sCD4 molecules before dissociation could take place, implying cooperativity between gp120 molecules in an oligomer. This is in agreement with another recent study: a mathematical analysis of the neutralisation kinetics of HIV-1 by sCD4 lead to the conclusion that multiple CD4 binding interactions are required for HIV infection (Layne *et al.* 1990).

(*d*) *SCD4 binding catalyses TM glycoprotein exposure*

The dissociation of gp120 from gp41 implies, but is not evidence for, the exposure of the gp41 fusion peptide on the surface of virions or HIV-infected cells. A system was therefore established for measuring the expression of gp41 on the surface of HIV-infected cells with or without prior sCD4 treatment. The T cell line H9 chronically infected with HIV expresses a large amount of functional HIV envelope glycoprotein, as demonstrated by the ability of these cells to form syncytia when cocultivated with $CD4^+$ cells, and by cell surface staining with anti-gp120 antibodies. The

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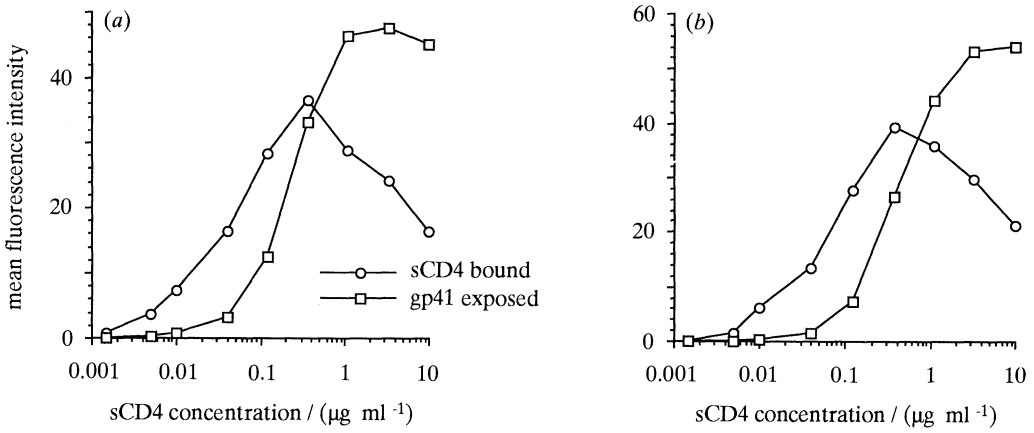

Figure 2. Soluble CD4 binding to HIV-1-infected cells induces conformational changes in the viral envelope glycoproteins. The binding of sCD4 and the exposure of a cryptic gp41 epitope at 37°C on the surface of cells infected with HIV-1 isolates RF (*a*) or SF-2 (*b*) was monitored by the use of a mAb which binds to the fourth domain of CD4, or to the immunodominant domain of gp41 respectively. MAb binding was detected by indirect immunofluorescence and flow cytometric analysis. Each point represents the mean fluorescence intensity of the sCD4-treated test sample minus the background staining with the non-sCD4-treated sample. Ten thousand events were acquired and analysed for each point. The sCD4 binding curve is bell shaped because the binding of high concentrations of sCD4 induces gp120 dissociation from the cell surface, reducing the signal.

binding of sCD4 to infected cells and modulation of gp41 epitope exposure can be detected by indirect immunofluorescent staining with mAb reactive with epitopes of gp41, followed by flow cytometric analysis. Human mAbs reactive with two distinct epitopes on gp41 (579–613 and 644–663 as defined by peptide binding) stain HIV-1-infected cells very poorly, implying masked epitopes. Treatment with sCD4 greatly increases binding of these mAbs (figure 2; Sattentau & Moore 1991). This brighter staining probably represents an increased number of available epitopes rather than the same number of epitopes with a higher affinity for the antibody, as the mAbs were used at a saturating concentration. Neither of these mAbs reacts with the region corresponding to the fusion peptide of gp41 (attempts by several laboratories to prepare such reagents have been unsuccessful), thus we have not directly demonstrated exposure of this precise region: this can only be inferred from unmasking of adjacent epitopes. Therefore although the mAb binding we observe demonstrates gross gp41 exposure, we are unable at present to detect more subtle changes which may be occurring at the N-terminus of gp41. The dissociation of gp120 from gp41 is related but not precisely correlated with gp41 exposure, as some gp41 unmasking occurs in the absence of gp120 shedding. Thus complete gp120 dissociation is not required for gp41 exposure, implying a range of conformational changes which can take place following receptor binding.

(*e*) *Modulation of antibody epitopes on gp120 as a consequence of sCD4 binding*

The interaction of sCD4 with gp120 leads to efficient dissociation from gp41 only at temperatures above about 18°C, indicating the necessity of overcoming an energy threshold before this process is efficiently catalysed. We have taken advantage of this to analyse the modulation of mAb epitopes in gp120 oligomers on HIV-1-infected cells. As expected, mAb binding to regions of gp120 which overlap the CD4 binding site is competed by sCD4 binding. By contrast, mAb reactivity with certain other epitopes is enhanced by the formation of a complex between gp120 and sCD4: anti-V2 and V3 mAbs bind with increased affinity in the presence of sCD4 (figure 3; Sattentau *et al.*, unpublished results). One explanation of such an observation is that multi-site binding of sCD4 to oligomeric HIV envelope glycoprotein forces the gp120–gp41 monomers apart, facilitating the exposure of gp41 and the dissociation of gp120.

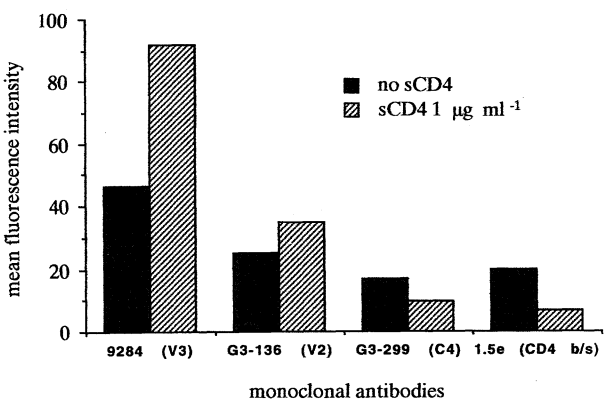

Figure 3. Modulation of gp120 epitopes by sCD4 binding. The binding of mAbs to various epitopes of gp120 on HIV-1-infected cells was analysed in the presence or absence of 1 μg ml^{-1} of sCD4 as described above. The bars in the histogram represents the mean fluorescence intensity of the test sample minus the background. The mAb specificity is as follows: 9284, gp120 variable loop 3 (V3 loop); G3-136, gp120 variable loop 2 (V2 loop); G3-299, constant domain 4 (C4); 1.5c, a complex, discontinuous epitope containing regions overlapping the CD4 binding site (CD4 b/s).

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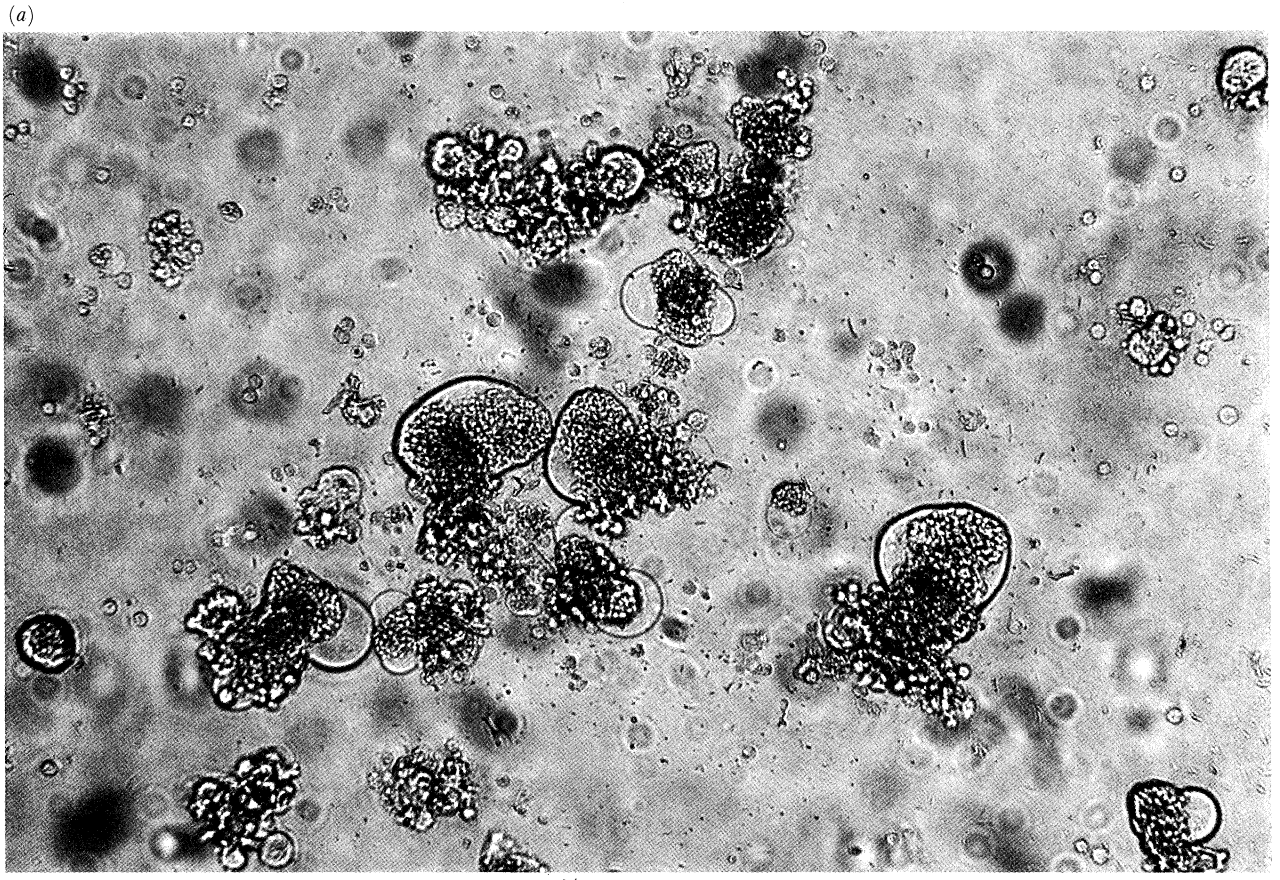

(*b*)

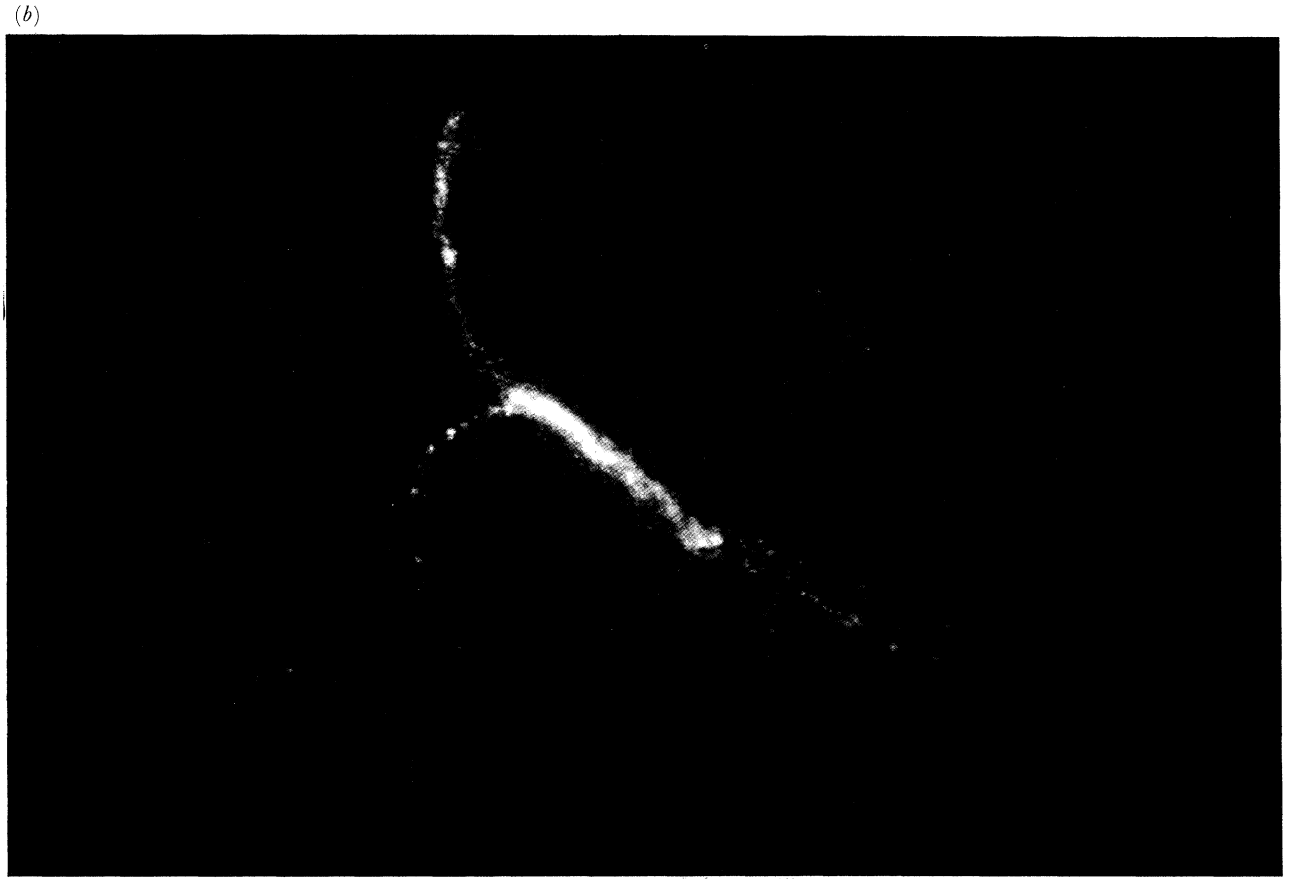

Figure 4. Cell–cell fusion of HIV-infected and CD4$^+$ cells. (*a*) Syncytium formation in cultures of HIV-1-infected and CD4$^+$ cells (HPB-ALL) after overnight cocultivation at 37°C. (*b*) Exposure of a cryptic gp41 epitope at the interface between fusing cells. HIV-1-infected cells were mixed with CD4$^+$ cells for 20 min at 37°C, then stained with an antibody to the immunodominant region of gp41. Fluorescence can only be seen on the infected cell where it contacts the CD4$^+$ cell, indicating the exposure of a previously occluded epitope.

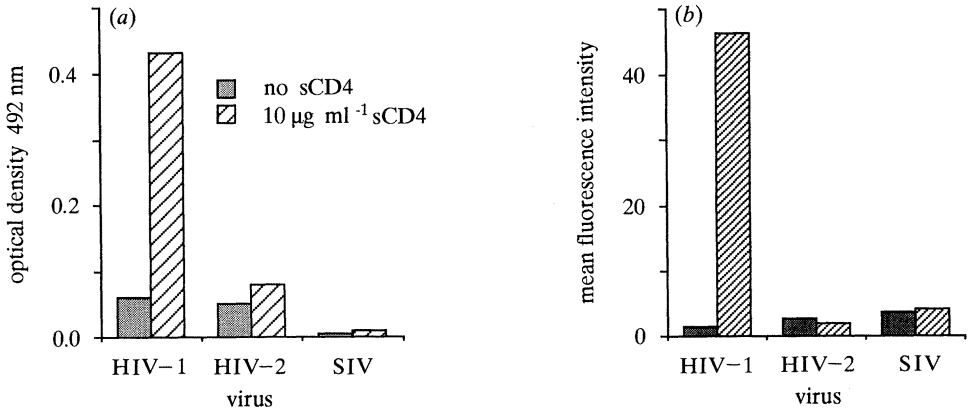

Figure 5. Soluble CD4 binding does not induce dissociation of the HIV-2 or SIV SU–TM glycoprotein subunits. (*a*) Ten µg ml^{-1} of sCD4 was added to HIV-1, HIV-2 or SIV-infected cells for 2 h at 37°C and the supernatants were tested for soluble SU glycoprotein–sCD4 complexes by ELISA. Values are expressed as the optical density at 492 nm. (*b*) The cells from which the supernatants were analysed were stained for TM glycoprotein expression with a mAb reactive with the immunodominant domain of the SIV TM glycoprotein as described above (figure 2). Each point represents the mean fluorescence intensity minus the background for 10 000 events acquired.

(*f*) *Soluble CD4 is a good mimic of membrane CD4*

All of the studies described above have used sCD4 as a mimic of the native, membrane form of the molecule, leading to the concern that the events that we have observed might be artefacts of this model system. We have therefore developed a method for analysis of the conformational changes induced by receptor binding at the interface between HIV-infected and CD4$^+$ cells in the process of fusing. HIV-infected cells expressing large amounts of envelope glycoprotein in their membranes fuse with CD4$^+$ cells to form syncytia (figure 4*a*). HIV-infected and CD4$^+$ cells are mixed on ice, heated for various times to initiate fusion, chilled on ice to arrest fusion, and stained by two colour indirect immunofluorescence with mAbs to CD4, gp41 and gp120. These 'fusion clusters' are then fixed and analysed by confocal microscopy. We find that within a few minutes of incubation at 37°C, small clusters of CD4$^+$ and gp120 expressing cells have formed, and CD4 molecules are recruited to the fusion interface. After 20 min at 37°C mixing of CD4 and gp120 is occurring as the membranes of the two cell types fuse. Staining with a combination of gp41 and CD4 mAbs reveals that gp41 epitopes are exposed at the fusion interface, and are colocalised with aggregated CD4 molecules (figure 4*b*). Thus membrane anchored CD4 appears to behave similarly to sCD4 in the induction of gp41 exposure as a consequence of CD4–gp120 binding.

Taken together, the results described above support the idea that receptor binding drives molecular rearrangement of the HIV envelope glycoproteins leading to exposure of the gp41 fusion peptide resulting in virus–cell membrane fusion. These studies, however, have all been carried out on cell-line adapted isolates of HIV-1: the interaction of sCD4 with primary isolates of HIV-1 and with the related immunodeficiency viruses HIV-2 and SIV yields somewhat different results, allowing us to refine this model further.

(*g*) *The interaction of sCD4 with HIV-2, SIV and primary isolates of HIV-1*

It has recently become evident that the adaptation of HIV-1 to growth in immortalized cell-lines selects for virus species which replicate efficiently in tissue culture, but which may not be representative of most of the viral clones present *in vivo*. Thus HIV isolated directly from peripheral blood mononuclear cells into PHA activated T cell blasts or primary macrophage cultures ('primary isolates') have different biological properties from cell line-cultured isolates. In particular, the susceptibility of primary isolates to sCD4 neutralization is substantially lower than that of cell-line adapted isolates (Daar *et al.* 1990). The explanation for this resistance appears to lie in the reduced ability of the envelope glycoproteins of primary isolates to bind sCD4 and undergo the consequent conformational changes. Although sCD4 binds to soluble gp120 cloned or solubilized from primary isolates with an affinity similar to that of gp120 from cell line-adapted isolates (Moore *et al.* 1992), gp120 expressed in a native, oligomeric form on the surface of virions has an affinity which is at least an order of magnitude lower (Moore *et al.* 1992). Moreover, sCD4 is very inefficient at inducing gp120 dissociation in primary isolates. A biological property of HIV-1 which appears to segregate with low-sCD4 binding and resistance to gp120 shedding is low cytopathicity in culture. It may be, therefore, that adaptation to efficient growth in cell lines (in the absence of immune selection pressures) allows the emergence of rapidly replicating, highly cytopathic clones of HIV which bind CD4 with high affinity and readily dissociate gp120. This implies that the opposite may well be true *in vivo*; that immune selection favours non-cytopathic, slow-replicating clones of virus, as has been proposed by other workers (Miedema *et al.* 1991).

With the recent availability of mAbs specific for or cross-reactive with the HIV-2 and SIV envelope glycoproteins, it has become possible to do similar

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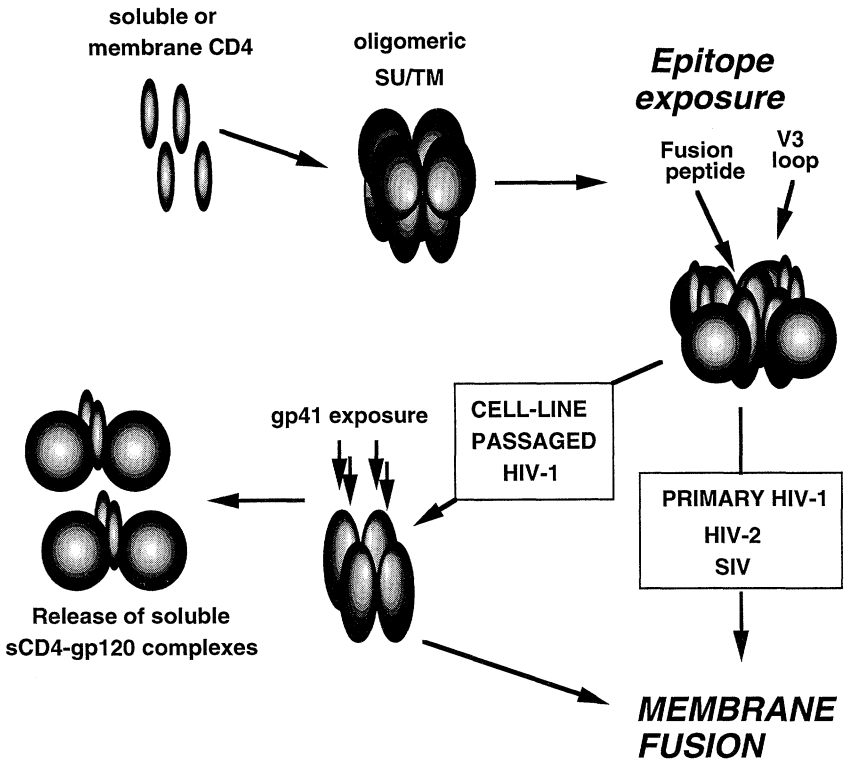

Figure 6. A model for receptor-mediated activation of HIV/SIV fusion. Two or more CD4 molecules (or sCD4 molecules in the model system) bind in a multimeric interaction to the oligomeric SU glycoprotein complex on the surface of virions or infected cells. This induces conformational changes in the SU glycoprotein, modulating mAb epitope exposure on this subunit. Following this, HIV-1 primary isolates, HIV-2 and SIV fuse with the $CD4^+$ target cells without dissociation of their glycoprotein subunits. The SU and TM glycoproteins of laboratory-adapted HIV-1 isolates, however, may dissociate in response to sCD4 binding prior to membrane fusion.

experiments to those described above with HIV-1. Using the test of binding sCD4 to HIV-2 or SIV-infected cells, we have noted a maximum difference of eight-fold between HIV-1 cell line-adapted isolates and HIV-2 or SIV isolates (Sattentau *et al.* 1993). It is currently unclear whether this decrease in affinity may be mechanistically related to the differences between HIV-1 and HIV-2/SIV described below, but is in accord with another study which demonstrated decreased gp120 dissociation in mutant HIV-1 virions with a lower affinity for sCD4 (Thali *et al.* 1992). Dissociation of the SU and TM subunits was not observed with any of the HIV-2 or SIV isolates when sCD4 was reacted with infected cells over a period of 2 h, whether this was done at 4°C or 37°C (figure 5). Moreover, there was no detectable TM glycoprotein exposure before or after sCD4 treatment. One interpretation of these data is that the SU–TM subunits of HIV-2 and SIV are held together by bonds stronger than those in the cell-line-adapted HIV-1 isolates. We have confirmed this by demonstrating that this SU–TM association of HIV-2 and SIV is resistant to non-ionic detergent solubilization, whereas the HIV-1 counterpart is susceptible and is disrupted in 1% NP40 (data not shown).

If the envelope glycoproteins of primary isolates of HIV-1, HIV-2 and SIV do not rearrange in response to sCD4 binding in such a way as to result in SU–TM dissociation, how can exposure of the fusogenic N-terminus of gp41 take place to mediate membrane fusion? One clue comes from studies of the analysis of epitope modulation of SU glycoprotein epitopes of HIV-2 and SIV in response to sCD4 binding: as with HIV-1, the V3 loops of HIV-2 and SIV become more available to mAb binding after exposure to sCD4 (Sattentau *et al.* 1993). We therefore propose that similar conformational changes take place in the glycoprotein oligomers of HIV-2, SIV and probably primary isolates of HIV-1, but that they are more subtle than in those observed with cell-line-adapted isolated of HIV-1. We propose a model which incorporates the results described above (figure 6). In this model, receptor binding induces rearrangement of the glycoprotein monomers which results in the exposure of only the region containing the fusion peptide of the TM subunit, and not in the complete dissociation of SU and TM subunits. An extension of this idea is that complete dissociation of the glycoprotein subunits is a consequence of passage in HIV-1 immortalized cell lines, and is not a prerequisite for fusion to occur.

3. CONCLUSIONS

Over the last few years the mechanism of HIV entry into $CD4^+$ cells has been studied intensely. Most of the findings can be summarized as follows: (i) after binding, CD4 induces conformational changes in gp120 which activate the fusogenic potential of HIV, a process termed receptor-mediator activation of fusion; (ii) culture conditions affect the fusogenicity of

virus isolates, and their ability to undergo CD4-induced conformational changes; and (iii) by contrast with cell-line-passaged isolates of HIV-1, HIV-2 and SIV behave like primary isolates of HIV-1 in that their envelope glycoproteins undergo conformational changes more subtle than those observed with cell-line-adapted isolates of HIV-1 in response to receptor binding.

A number of important questions remain to be answered. Some interesting correlations have become evident between the behaviour in culture in terms of fusogenicity and tropism of certain types and isolates of HIV, and their ability to undergo CD4-induced conformational changes. Elucidation of the relationship between these phenomena will help to understand the mechanism of fusion and the molecular basis of aspects of viral pathogenesis. Direct detection of exposure of the fusion peptide as a consequence of receptor binding would be important evidence to support the current models of HIV fusion. Preparation of reagents which recognize this region of the TM protein is therefore a priority. Such reagents may have the additional benefit of inhibiting fusion, and may therefore have theraputic potential. Despite intense investigation, the functional significance of a region of gp120 known as the V3 loop (the major neutralizing domain of HIV-1) remains obscure, despite the fact that it plays a key role in the fusion process. Understanding the function of this domain will be important not only for increasing our knowledge of the mechanisms of HIV neutralization, but also in the design of novel inhibitors of HIV infection. Finally, there is a considerable body of evidence suggesting that there are membrane components additional to CD4 required for HIV entry (Clapham *et al.*, this symposium). Although this was originally proposed many years ago, little progress has been made in the identification of such factors. Identification of such molecules would open up new areas in HIV research with possibilities for designing therapies aimed at inhibiting viral replication at a stage prior to entry.

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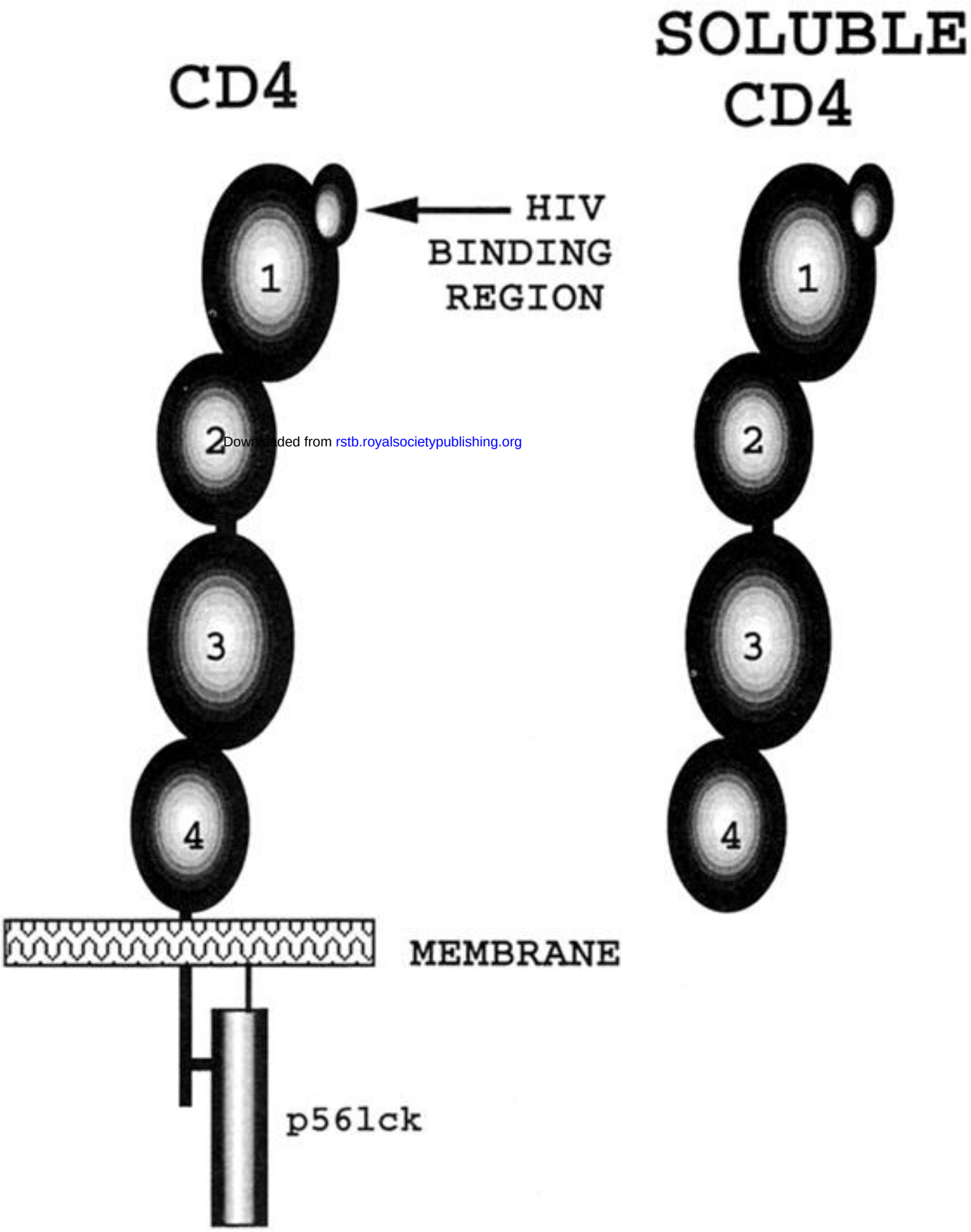
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



igure 1. The CD4 molecule. CD4 is a membrane-anchored olecule found predominantly on a subset of T lymphocytes nd macrophages. There are four extracellular domains ith strong sequence and structural homology to immuno-obulin domains. The extracellular portion is anchored to ie cell by a transmembrane domain, and there is a short toplasmic tail which associates with the tyrosine kinase, 56lck. The HIV binding site has been localized to the DR-2-like loop in domain 1. The insertion of a stop codon the cDNA has allowed the expression of a truncated, oluble form of CD4 (sCD4). This molecule retains a native onformation and its HIV binding properties.

(a)

(b)

Figure 4. Cell–cell fusion of HIV-infected and CD4$^+$ cells. (*a*) Syncytium formation in cultures of HIV-1-infected and CD4$^+$ cells (HPB-ALL) after overnight cocultivation at 37°C. (*b*) Exposure of a cryptic gp41 epitope at the interface between fusing cells. HIV-1-infected cells were mixed with CD4$^+$ cells for 20 min at 37°C, then stained with an antibody to the immunodominant region of gp41. Fluorescence can only be seen on the infected cell where it contacts the CD4$^+$ cell, indicating the exposure of a previously occluded epitope.

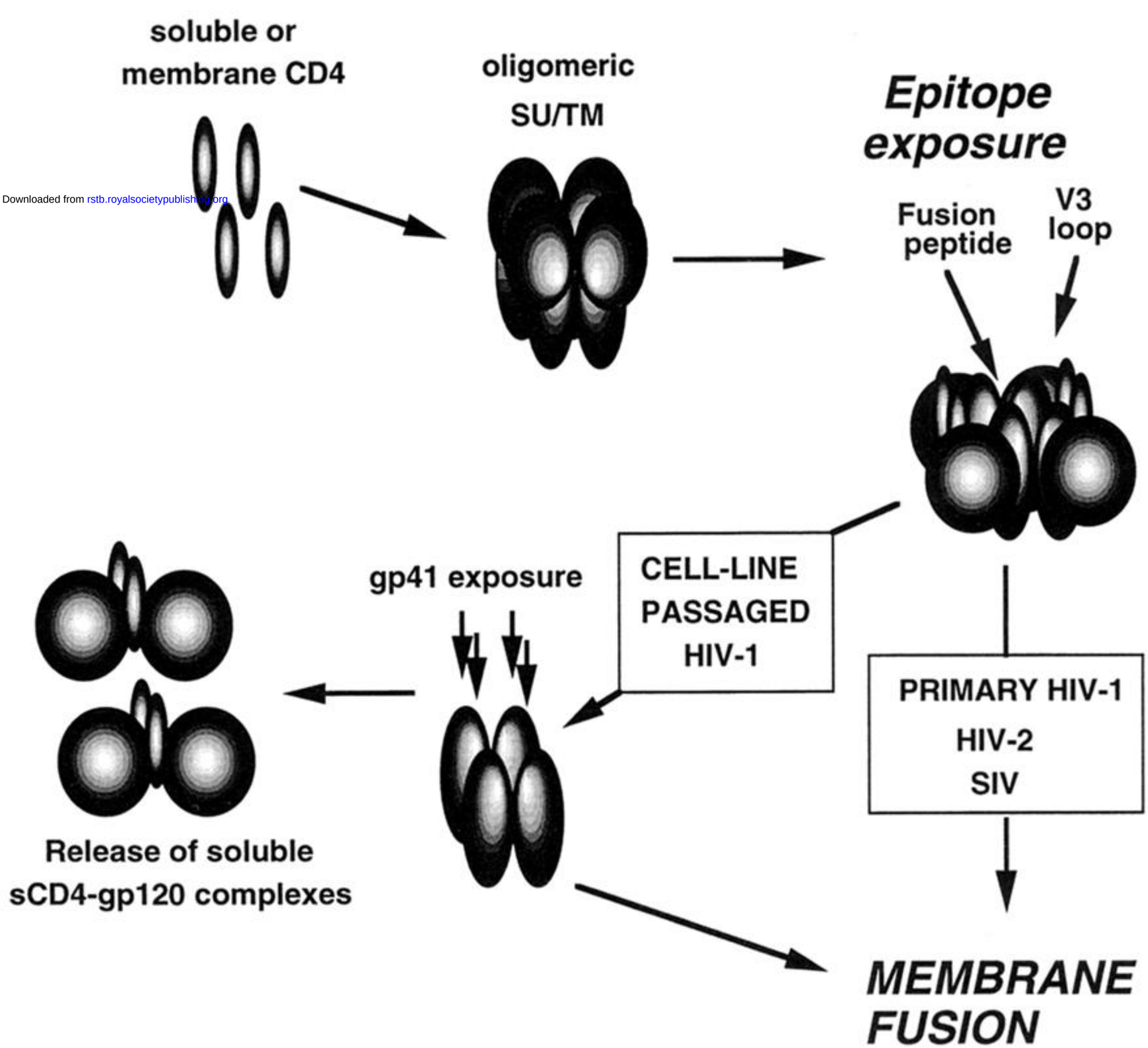


Figure 6. A model for receptor-mediated activation of HIV/SIV fusion. Two or more CD4 molecules (or sCD4 molecules in the model system) bind in a multimeric interaction to the oligomeric SU glycoprotein complex on the surface of virions or infected cells. This induces conformational changes in the SU glycoprotein, modulating mAb epitope exposure on this subunit. Following this, HIV-1 primary isolates, HIV-2 and SIV fuse with the $CD4^+$ target cells without dissociation of their glycoprotein subunits. The SU and TM glycoproteins of laboratory-adapted HIV-1 isolates, however, may dissociate in response to sCD4 binding prior to membrane fusion.