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Comprehensive Invited Review

Cell Entry by Enveloped Viruses: Redox Considerations for HIV and SARS-Coronavirus

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ABSTRACT

For enveloped viruses, genome entry into the target cell involves two major steps: virion binding to the cell-surface receptor and fusion of the virion and cell membranes. Virus–cell membrane fusion is mediated by the virus envelope complex, and its fusogenicity is the result of an active virus–cell interaction process that induces conformation changes within the envelope. For some viruses, such as influenza, exposure to an acidic milieu within the cell during the early steps of infection triggers the necessary structural changes. However, for other pathogens which are not exposed to such environmental stress, activation of fusogenicity can result from precise thiol/disulfide rearrangements mediated by either an endogenous redox autocatalytic isomerase or a cell-associated oxidoreductase. Study of the activation of HIV envelope fusogenicity has revealed new knowledge about how redox changes within a viral envelope trigger fusion. We discuss these findings and their implication for anti-HIV therapy. In addition, to compare and contrast the situation outlined for HIV with an enveloped virus that can fuse with the cell plasma membrane independent of the redox status of its envelope protein, we review parallel data obtained on SARS coronavirus entry. *Antioxid. Redox Signal.* 9, 1009–1034.

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Human thioredoxin
mvkqieskta fqealdaagd klvvvdfsat wcgpckmikp ffhslsekys
nviflevdvd dcqdvasece vkcmptfqff kkgqkvgefs gankekleat
inelv

Human protein disulfide isomerase
mrlrrlalfp gvalllaaar laaasdvlel tddnfesris dtgsaglmlv
effapwcghc krlapeyeaa atrlkgivpl akvdctantn tcnkygvsgy
ptlkifrdge eagaydgprt adgivshlkk gagpasvplr teeefkkfis
dkdasivgff ddsfseahse flkaasnlrd nyrfahtnve slvneyddng
egiilfrpsh ltnkfedktv ayteqkmtsg kikkfiqeni fgicphmted
nkdliqgkdl liayydvdye knakgsnywr nrvmmvakkf ldaghklnfa
vasrktfshe lsdfglesta geipvvairt akgekfvmqe efsrdgkale
rflqdyfdgn lkrylkscpi pesndgpvkv vvaenfdeiv nnenkdvlie
fyapwcghck nlepkykelg eklskdpniv iakmdatand vpspyevrgf
ptiyfspank klnpkkyegg relsdfisyl qreatnppvi qeekpkkkkk
aqedl

Mo-Murine Leukemia Virus Env
marstlskpl knkvnprgpl iplillmlrg vstaspgssp hqvynitwev
tngdretvwa tsgnhplwtw wpdltpdlcm lahhgpsywg leyqspfssp
pgppccsggs spgcsrdcee pltsltprcn tawnrlkldq tthksnegfy
vcpgphrpre skscggpdsf ycaywgcett graywkpsss wdfitvnnnl
tsdqavqvck dnkwcnplvi rftdagrrvt swttghywgl rlyvsgqdpg
ltfgirlryq nlgprvpigp npvladqqpl skpkpvksps vtkppsgtpl
sptqlppagt enrllnlvdg ayqalnltsp dktqecwlcl vagppyyegv
avlgtysnht sapancsvas qhkltlsevt gqglcigavp kthqalcntt
qtssrgsyyl vaptgtmwac stgltpcist tilnlttdyc vlvelwprvt
yhspsyvygl fersnrhkre pvsltlalll ggltmggiaa gigtgttalm
atqqfqqlqa avqddlreve ksisnleksl tslsevvlqn rrgldllflk
eggleaalke eccfyadhtg lvrdsmaklr erlnqrqklf estqgwfegl
fnrspwfttl istimgpliv llmillfgpc ilnrlvqfvk drisvvqalv
ltqqyhqlkp ieyep

FIG. 1. Sequences and thiol reactive motifs in thioredoxin, PDI, and MLV Env. The potential thiol reactive motifs are boxed (*grey*). For PDI, the two thiol-reactive (*underlined*) and the two thiol-inactive domains are indicated (*square brackets*). For MLV Env, the sequence of the transmembrane subunit engaged in a disulfide link with the CWLC thiol-reactive motif on the surface subunit is indicated (*boxed*).

I. PREAMBLE: PROTEIN DISULFIDE ISOMERASE AND ACTIVITY AT THE CELL SURFACE

DISULFIDE BOND FORMATION, the coupling by oxidation of adjacent cysteine residues, is a posttranslational modification that affects most proteins destined for the cell surface or secretion into the cell medium. The reaction is traditionally considered to occur in the endoplasmic reticulum (11, 52, 93), although disulfide bonds have been increasingly found in cytosolic proteins (*e.g.*, 184) and a complete cytosolic pathway of disulfide bond assembly, virus encoded, has recently been reported (175).

The primary function of disulfide bonds is in folding but also in stabilizing proteins through the introduction of inter- and intramolecular covalent bonds, which ensure the appropriate secondary and tertiary protein structure required for biologic activity and protection against proteolysis and denaturation (11, 40, 93). Disulfide bond formation on nascent proteins is facilitated by a family of enzymes located in the endoplasmic reticulum, the thiol isomerases. These catalysts are capable of reduction, oxidation, and rearrangement of the disulfide bond network, and two of the main representatives of the family are thioredoxin and protein disulfide isomerase (PDI) (59, 85).

Thioredoxin is a small ubiquitous protein of ~12 kDa that acts as a protein disulfide reductase and as a hydrogen donor for various enzymes (67, 85). It contains a canonic -Cys-Gly-Pro-Cys- motif, the thioredoxin box (Fig. 1), and after reduction of its substrate, the enzyme is recycled by the thioredoxin reductase/NADPH complex in the cytosol, nucleus, or mitochondria compartment (59, 67).

PDI is a highly abundant protein found in the lumen of the endoplasmic reticulum, where it can represent up to 1% of the total cellular protein and reach millimolar concentrations (52, 59, 162). It is expressed as a soluble homodimer of 510 amino acid residues with a carboxy-terminal KDEL motif, which serves as both an endoplasmic reticulum retention signal and a retrieval signal to recycle the enzyme back to the endoplasmic reticulum from the late Golgi through interaction with the KDEL-receptor pathway (59, 198). The redox exchange catalyzed by PDI in the cell involves both the enzyme and a regenerating complex that includes Ero1, a molecular partner with oxidant ability, which is itself recycled through the FADH system and subjected to a variety of controls (15, 24, 77, 113, 148, 154, 160).

The main structural feature of the protein family represented by PDI is the presence of one or more domains of ~100 amino acid residues related to thioredoxin (see Fig. 1). Such domains either contain or lack a reactive dithiol sequence corresponding to the consensus tetrapeptide -Cys-X-X-Cys- (50, 59, 99).

In the case of PDI, catalytic activity is due to the reactivity of the N-terminal Cys residue in two of its thioredoxin-like boxes, each of which can function independently (59, 126). Catalysis can be either disulfide bond formation or reduction, and, if more than one disulfide bond is present, a disulfide isomerization can also occur (Fig. 2). The reaction involves a transient covalent linkage between the enzyme and the substrate: the N-terminal Cys in the active site of PDI attacks a disulfide bond in the protein substrate to form a mixed disulfide between PDI and the substrate protein. The N-terminal Cys residues in each -Cys-Gly-His-Cys- at the active sites act as strong nucleophiles when unprotonated, and the disulfide between PDI and

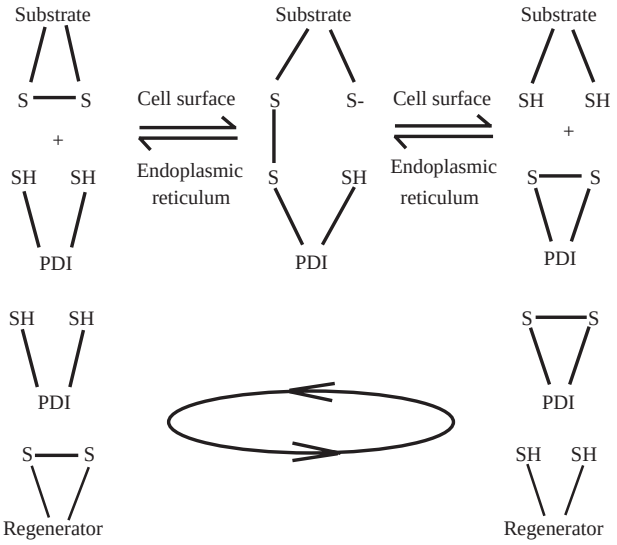


FIG. 2. The enzymatic redox activity of PDI, including known intermediates and regeneration. Catalysis can be either disulfide bond formation or reduction and, if more than one disulfide bond is present, a disulfide isomerization also can occur. For reduction, the N-terminal Cys in the active site of PDI attacks a disulfide bond in the protein substrate to form a mixed disulfide, which is unstable. Resolution is *via* oxidization of the vicinal Cys residues in the PDI active site, formation of a disulfide within PDI, and release of the protein substrate with a reduced Cys (the reverse pathway is for oxidization). The latter is free to reform another disulfide bridge with an adjacent Cys residue present on the protein, eventually completing the isomerization reaction. The oxidized/reduced form of the catalyst is reduced/oxidized by its regeneration system to complete further rounds of reduction. See the text for more details.

the substrate is unstable as a result of the proximity of the C-terminal Cys of the PDI active site. Resolution is *via* oxidization of the vicinal Cys residues in the PDI active site, formation of a disulfide within PDI, and release of the protein substrate with a reduced Cys that is free to reform another disulfide bridge with an adjacent Cys residue, eventually completing the isomerization reaction. Finally, the now-oxidized form of the catalyst is reduced by its regenerating system to complete further rounds of reduction (see Fig. 2).

Besides its capacity to exert redox reactions, PDI has other functions, with an important role in the processing of secretory proteins in the endoplasmic reticulum (59, 111, 122). At high concentrations, it behaves as a chaperone that binds unfolded or incorrectly folded proteins and helps them attain their native conformation by preventing aggregation and facilitating pathways of refolding to the correct conformation, whereas at low concentration, it facilitates aggregation (25, 41, 101, 106, 160, 161). Both the chaperone and the thiol-dependent activity of PDI depend on the ability of the catalyst to interact with polypeptides. Two peptide binding sites—one in the thiol-reactive domain and one in the nonredox-active domain (see Fig. 1)—have been described (59, 62, 152). The binding affinity is weak and is proportional to the length of the substrate sequence and the presence of Cys residues (59). It is inferred that as PDI activity is modulated through recognition of the peptide sequence, uncontrolled disulfide bond formation or isomerization of protein substrates is avoided. Thus, PDI can act as part of the quality control system for the production of correctly folded proteins.

The action of PDI differs from that of thioredoxin in that its redox potential is higher (59, 67, 148), and the presence of domains devoid of the active sequence uniquely confers peptide binding and chaperone capacity on PDI. This may explain also why PDI is more efficient at redox reactions than thioredoxin, because it interacts better with its substrates through the peptide-binding domain (25, 101, 148).

Despite possessing an endoplasmic reticulum retention signal (59, 198), PDI has also been detected in association with the cell surface of various secretory cells [*e.g.*, rat exocrine pancreatic cells (2), platelets (23, 53), lymphoid cells (103, 172) (Fig. 3), hepatocytes (186), and thyrocytes (39)]. Thioredoxin does not contain a consensus secretory-signal sequence, but it is constitutively present in the biosynthetic pathway, associated with the plasma membrane and secreted (78). The export from the endoplasmic reticulum compartment of PDI and its association with the cell surface may be explained by the fact that endoplasmic reticulum vesicles can fuse with the cell surface, as observed in macrophages, and so deliver their cargo, such as PDI, to the exterior (83). PDI has no transmembrane domain, so its association with the plasma membrane may occur through noncovalent interactions with integral membrane proteins, lipids, or glycans (83, 187). The mechanisms by which other endoplasmic reticulum redox catalysts are associated at the cell surface remains to be determined but may involve disturbance of the normal retrieval pathway or expression of a routing signal. Both hypotheses imply either secretion of the catalyst into the cell medium and subsequent capture by the cell membrane or stable association during the biosynthesis with a partner eventually associated with the cell surface. It also is possible that cell-surface PDI remains associated with the cell surface simply through electrostatic interactions (59, 83, 187, 198).

Whereas the redox system of catalysis inside the cell involves the enzyme and its known regenerating system, the possible counterparts outside the cell remain unknown (15, 148). The direction of the reaction catalyzed by PDI is determined by its substrate and product concentrations, redox potential, and the redox conditions of the medium (59, 148). Because the PDI active-site redox couples are mildly oxidizing, PDI in the endoplasmic reticulum acts as both an oxidant and a disulfide isomerase, whereas when at the plasma membrane, PDI acts solely as a reductant (59, 67, 148) (see Fig. 2).

Inhibition of the catalytic activity of PDI at the cell surface can be achieved through the use of several inhibitors with various modes of action (Table 1). Membrane-impermeant PDI inhibitors include the antibiotic bacitracin (130), thiol reagents such as 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) (9), reagents of the CXXC vicinal thiols of the active site (64), such as *para*-amino-phenylarsine oxide (aPAO) and its glutathione conjugate (GSAO), the acetylated form of tri-iodothyronine (AT3) (64), and anti-PDI monoclonal antibodies (9, 169). The mode of action of bacitracin has not been clearly established, but AT3 inhibits PDI, presumably through an allosteric mechanism and, like aPAO, it is not strictly membrane impermeant.

The detection of redox catalysts at the cell surface arose concurrent with observations that disulfide bonding is a way of

Table 1. PDI Inhibitors and Supposed Mode of Action

Reaction with sulfhydryls
DTNB, 5,5′-dithiobis(2-nitrobenzoic acid) (169)
PAO, phenylarsine oxide (64)
aPAO, para-amino PAI (64)
GSAO, 4-(N-(*S*-glutathionylacetyl)aminophenylarsenoxide (139)
Steric hindrance of either the catalytic site or the peptide recognition domain
Bacitracin (130)
T3, triiodothyronine (64)
AT3, N-acetylated triiodothyronine (64)
Anti-PDI antibodies (9)

Numbers in parentheses refer to references.

controlling how and when exofacial proteins exert their biologic activity (9, 39, 59, 88, 169, 170). A number of reports have shown that cell-surface PDI catalyzes thiol disulfide interchange reactions that control the functions of extracellular proteins through the induction of structural modifications (39, 64, 94, 183, 187). However, the first demonstration of activation of exofacial protein function by cell surface–associated PDI activity was the cleavage of a disulfide containing poly(D-lysine) conjugate interacting nonspecifically with the surface of fibroblasts (55). The reaction was inhibited by the membrane-impermeant thiol reagent DTNB, as well as by anti-PDI antibodies known to inhibit catalytic activity (9, 169). Another early example of protein activation by surface PDI is the diphtheria toxin model. Diphtheria toxin is a heterodimeric molecule composed of catalytic and receptor-binding subunits linked by disulfide bonds. PDI mediates the cleavage of disulfides required for activation of its toxicity through a thiol disulfide interchange reaction, and its specific inhibition with bacitracin prevents cytotoxicity (170). Reduction of the disulfides between the heterodimer after its binding to the cell-surface receptor triggers cytotoxicity by enabling the catalytic subunit to be translocated through endosomal membranes so that the membrane-binding subunit can insert into the membrane (170, 171).

Further evidence for PDI-mediated reduction in various cell–cell interaction processes is also apparent throughout the literature. Platelet activation increases the level of thiols associated with the cell surface and, notably, those associated with the active sites of cell-surface PDI. PDI activity is required for the integrin-mediated platelet adhesion mechanism through activation of an integrin conformation *via* reduction or reshuffling of disulfide bonds or both, as shown by the use of PDI inhibitors that inhibit the adhesion process (82, 108). Among the events regulated by PDI, a disulfide isomerization process takes place within thrombospondin, a multifunctional protein secreted by platelets and involved in cell–cell and cell–matrix interactions, leading to unmasking the RGD tripeptide, which is bound by integrins on platelet or endothelial cell surfaces during platelet activation (82, 86, 108). PDI also associates with the receptor glycoprotein GP1b on platelets, an antigen that binds the serum von Willebrandt factor and is involved in hemostasis through interaction with collagen and aggregation (82, 108). Thus, a growing body of evidence suggests that the disulfide bond pattern of mature proteins can be modified by PDI and that such modification can trigger protein function at the cell surface, especially as part of a process of cell interaction.

Most viruses encode few proteins, and the range of biochemical reactions they can perform is limited. The completion of the life cycle must therefore make use of host enzymes whose activity is co-opted into participation in virus replication. Nowhere is this more clearly demonstrated than in the processes of virus entry, in which a number of viruses have shown evidence of thiol exposure and sensitivity to PDI inhibitors (1, 3, 91, 102, 134) and in which some viruses, notably the human immunodeficiency virus (HIV), have had such sensitivity explored in detail.

II. REDOX AND HIV ENTRY

Viruses can be divided into two groups based on the organization of their outer surfaces. The genomes of nonenveloped viruses are simply encapsidated by a protein coat, the capsid, whereas in enveloped viruses, the capsid is further cloaked by a lipid membrane derived from the host cell. To fulfil the first step of their infectious cycle, the injection of their genome into

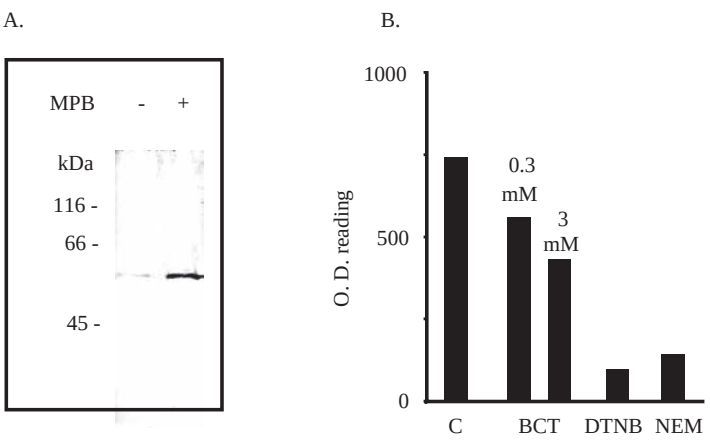


FIG. 3. Presence of active PDI at the human lymphocyte surface. (**A**) Human CEM lymphoid cells were incubated with MPB (+), a biotin-coupled thiol reagent, or mock-treated (−) and washed. After lysis, samples were incubated with streptavidin-agarose before separation by using SDS-PAGE, membrane transfer, and Western blotting with anti-PDI antibodies. The band corresponding to PDI was significantly enriched by the MPB labeling and pull-down when compared with the mock-treated cells, showing that PDI is accessible to exogenous MPB at the cell surface. (**B**) Cells were treated by using the various compounds (BCT, bacitracin; DTNB or NEM, *N*-ethylmaleimide) and then similarly incubated with MPB. Samples were washed and incubated with streptavidin-peroxidase before addition of a chromogenic substrate and spectrophotometric assessment. Treatment with bacitracin reduced cell-surface thiol labeling, indicating that active PDI is present at the lymphocyte surface. For specificity, cell preincubation with thiol reagents dramatically reduced MPB reactivity.

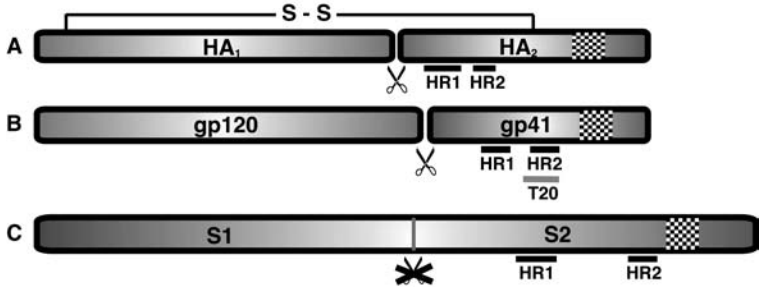


FIG. 4. Cartoon representation of three viral envelope proteins: HA, HIV Env, and SARS-CoV S. (**A**) The hemagglutinin protein of influenza virus, the paradigm for type I fusion proteins, is cleaved into two domains, HA1 and HA2, that remain covalently attached *via* a single intermolecular disulfide bond. (**B**) The envelope protein of HIV is also cleaved into two domains, gp120 and gp41, which remain attached only by noncovalent interactions. (**C**) The spike protein of SARS-CoV is not cleaved but can be described as two domains (S1 and S2) by virtue of alignment with related proteins that do undergo cleavage. In all three proteins, the heptad repeats (HRs) characteristic of a similar fusion mechanism, are shown. The *hatched area* represents the transmembrane domain in all cases. T20 marked the site of action of the HR-competing peptide drug that blocks fusion between HIV and the host cell surface.

a susceptible cell, the genome of enveloped viruses must traverse both viral and cellular membranes. Accordingly, genome entry into the target cell by enveloped viruses involves two major steps (46, 47, 49). First, the virion binds to its receptor, a molecule associated with the surface of the target cell; and second, the viral and cell membranes fuse together. Membrane fusion is not the simple consequence of cell-surface binding but is an active process, and many enveloped viruses (*e.g.*, influenza virus, baculovirus, hepatitis C virus) require special conditions such as acidic pH or processing by catalysts to allow penetration into target cells (33, 49, 146, 159, 193). Although the conditions used to enter target cells vary, they have in common that they enable a conformational change within the viral envelope proteins needed to trigger fusion activity. Without this, the membrane fusion process between the surface of the pathogen and its host cell, the initial step of the infection cycle, cannot take place (47, 49).

A much-investigated model of this process is that of the hemagglutinin protein (HA) of influenza virus, a disulfide-linked two-subunit protein consisting of HA1, the receptor-binding domain, and HA2, the membrane fusion domain (33, 177) (Fig. 4). The mature protein can be considered a spring-loaded device in which the disulfide linkage stabilizes the conformation of the protein and the association of the two subunits (28, 132). Binding to the cell surface occurs *via* interaction of the HA1 domain with sialic acids present on cell-surface glycoproteins. The tethered virus is then internalized by endocytosis, and conformational change is initiated by the acidic pH that occurs during the internalization process within the maturing intracellular vesicle, exposing the HA2 domain. The release of HA2, and particularly a hydrophobic sequence of amino acids, the fusion peptide, triggers the fusion of viral and vesicle membranes, and the infectious cycle begins (177). Essentially the same acid-dependent route, possibly using similar amino acid motifs, is followed by many enveloped viruses despite considerable differences in the structures of their envelope proteins (96, 201).

For several viruses, however, the viral envelope is not exposed to an acidic environment during the early steps of the infection, and for those viruses, an alternate form of activation must take place. A variety of evidence suggests that a number of viral envelope glycoproteins depend on a precise thiol/disulfide balance at the viral surface to mediate virus/cell-membrane fusion. Such evidence is twofold: on the one hand, manipulation of the native disulfide network of mature virus envelopes at the virus surface by using reducing or alkylating reagents has demonstrable consequences for their subsequent ability to carry out virus/cell fusion (1, 3, 9, 57, 62, 169, 190, 191). On the other hand, specific thiol/disulfide rearrangements are known to occur within several viral envelopes during the conformational change that results in insertion of the fusion peptide into the cell surface, leading to virus–cell-membrane fusion and virus entry (1, 9, 63, 64, 102, 135, 173, 190). Thus, reduction of the structure stabilizing disulfide bonds in envelope proteins by either cell-associated oxidoreductase activity or endogenous redox autocatalytic isomerase activity may represent a mechanism for triggering the profound envelope conformational change needed to activate fusogenicity (168, 173). Several exemplars exist. Thiol/disulfide interchange triggers aggregation of the baculovirus fusion protein, gp64, into a fusogenic form, hence enabling membrane fusion with the target-cell surface (134). Similarly, vaccinia virus entry also depends on the reduction of critical disulfides in the virus core to enable disassembly of the virus and entry of the core into the target cell (124). The E1 protein of the togavirus rubella (69) and the envelope of bovine viral diarrhea virus (102), a pestivirus, also exhibit reactive thiols, the latter appearing to destabilize the envelope during endocytosis to become fusogenic at endosomal acidic pH, although the precise mechanism of disulfide reduction remains unknown. In Sindbis virus, intact disulfide bonds within the envelope glycoproteins have been shown to be important for the stability and function of the envelope (1, 3), the addition of DTNB alters fusion, and treatment with reducing reagents indicates that the cleavage of critical disulfides probably plays a role during entry (1). A further case of disulfide reduction within a viral envelope during the acquisition of fusogenicity was observed very recently in the case of the paramyxovirus Newcastle disease virus (91). During entry, the F envelope protein undergoes conformation changes that necessitate and follow PDI reduction of some of its disulfides, as inhibitors of the catalyst prevent virus–cell fusion but not virus binding. Several retroviruses have also been shown to use redox changes to activate envelope fusion. For MLV- and HTLV-Env, disulfide-bond reshuffling is a determinant of virus entry (157), and fusion requires that the disulfide bond that links the transmembrane subunit with a CXXC thiol-reactive motif on the surface antigen be isomerized within the CXXC sequence by an endogenous oxidoreductase activity supported by the mo-

tif (see Fig. 1). These examples suggest that redox-catalyzed envelope changes may emerge as a common feature of enveloped virus cell entry, and for one virus, HIV, a substantial literature already exists for the role of oxido-reduction in entry. A dedicated review of these results, with their obvious implication for therapeutic intervention, is therefore warranted.

A. Env, CD4, CXCR4/CCR5

HIV is a lentivirus belonging to the family *Retroviridae*. The viruses are enveloped, and cell entry is mediated by fusion of their envelope with the cell membrane (47, 89, 168). Somewhat similar to the situation with HA discussed earlier, the lentivirus envelope protein complex is a homotrimer composed of three units, each of which comprises a transmembrane and an outer subunit (see Fig. 4). As for the HA, the outer domain mediates cell surface–receptor binding, whereas the transmembrane domain is responsible for fusion activity (47, 65). In sharp contrast to the HA, however, fusion does not require endocytosis and acidification but occurs at the cell surface after virus binding. During Env biosynthesis and before insertion into the lentivirus envelope, the transmembrane subunit is trapped within an uncleaved, nonfusogenic, precursor molecule, which is primed for fusion by cleavage to its mature form late in its biosynthetic cycle. After binding to the target-cell surface, the outer subunit is released from the virion surface or exhibits conformational change (or both), while the transmembrane fusion protein achieves a fusogenic state and triggers virus–cell fusion (47, 51, 125, 168) (Fig. 5).

The outer domain of the mature Env molecule of HIV is a heavily glycosylated and disulfide-bonded molecule, gp120, whereas the transmembrane domain, gp41, is less posttranslationally modified (Fig. 6). The precursor molecule, gp160, from which both derive, is cleaved into its component parts by a cellular endoprotease activity such as furin in the trans-Golgi compartment before virus assembly (51). The gp120 and gp41 subunits remain noncovalently associated, and trimers of the gp120–gp41 complex are incorporated into the viral envelope (47, 51). Gp41 is associated with the virus surface through its C-terminal domain, whereas gp120 remains bound to the viral surface through electrostatic interactions with gp41 (51) (see Fig. 5). On the virion surface, the viral envelope is arranged in clusters of spikelike structures formed by trimeric complexes of gp120 atop gp41 "legs," which appear separate (205). Gp120-mediated attachment of the virus to the target-cell surface first occurs with the primary receptor, glycoprotein CD4, an extended four-domain molecule on the surface of T-helper and other immune cells. CD4 binding is insufficient for virus entry but triggers contact with a coreceptor such as CCR5 or CXCR4, both members of the seven-transmembrane family of proteins that normally act as receptors for the action of chemokines (14, 35, 47). CD4 binding causes conformation change in gp120 (47, 65, 125) and reveals the coreceptor-binding domain, a highly conserved structure that exists as a cryptic site in the original envelope, unveiling conserved functional epitopes only on gp120-CD4 interaction (47, 107, 125) (see Fig. 5).

The stepwise mode of interaction of the HIV envelope complex with its cell-surface receptors to gain virus entry to the cell provides an efficient mechanism for hiding functional, highly conserved, neutralization domains from the induction of, and recognition by, neutralizing antibodies (65, 125). Moreover, because these events occur close to the cell surface and over a brief time, neutralizing epitopes that are transiently revealed remain essentially beyond the reach of the immune system. Some exceptions exist, as a number of reports have described primary strains of HIV-1 capable of infecting coreceptor-expressing cells without the requirement for CD4 binding (44, 125). The fact that the coreceptor-binding region is able to induce a neutralizing response is illustrated by the observation of infected individuals who possess high titers of antibodies directed against this domain (44). These are probably induced by the shedding of monomeric gp120 associated with cell-surface CD4 and may act to counter the emergence of CD4-independent variants (125). In addition, although largely a cryptic site, some epitopes of the coreceptor-binding surface are partially accessible in the native, CD4-unbound envelope (107), suggesting that this region could be manipulated to produce a vaccine immunogen capable of producing broadly neutralizing antibodies (125). Despite these exceptions, the gross conformational change within gp120 on CD4 contact is supported by the finding of major structural differences between the native and the CD4-bound conformations of gp120 (125) and provides a structural explanation for the lack of coreceptor-binding competence by CD4-unbound gp120.

As noted, chemokine receptors (although principally CCR5 and CXCR4) serve as coreceptors for HIV entry, and the major determinants of this specificity have been mapped within the third variable (V3) loop of gp120, which makes direct contact with the coreceptor during virus entry (14, 35, 37, 125). In addition, the coreceptor-binding surface also includes a notable structural feature, the "bridging sheet" found in gp120 (165). The bridging sheet is a highly conserved sequence that probably constitutes the common determinant of coreceptor recognition. In contrast, the structure and charge of the highly variable V3 sequence dictate the specificity for either CCR5 or CXCR4 (14, 47, 125). The V3 loop is also an immunodominant sequence and varies considerably as a result of antibody pressure. Changes in the sequence of the loop are used by the virus to deceive the immune system, but they also enable the virus to explore the use of different chemokine receptors for entry, and this evolution of the virus is apparent in clinical infection. Long before the discovery of the viral coreceptors, different biologic phenotypes of HIV-1 were recognized in relation to the clinical stage of disease (38, 174). The discovery of the chemokine receptor–binding step has resulted in an established relation between use of the two major coreceptors, CCR5 and CXCR4, and the clinical stage (14, 38, 125, 174). The HIV-1 strains that are most commonly responsible for transmission and predominate during the asymptomatic phase are generally restricted to CCR5 use (47, 114, 125). They replicate efficiently in macrophages, dendritic cells, and activated/memory cells, all of which express high levels of CCR5. During the course of the disease, HIV-1 strains that use CXCR4, a homeostatic chemokine receptor expressed on a broader range of cells, including naive/resting T cells and thymic precursors, is observed. The emergence of CXCR4-using virus is associated with higher virulence and is usually considered to be the underlying cause of accelerated immune depletion that occurs late in the course of the disease (125, 174).

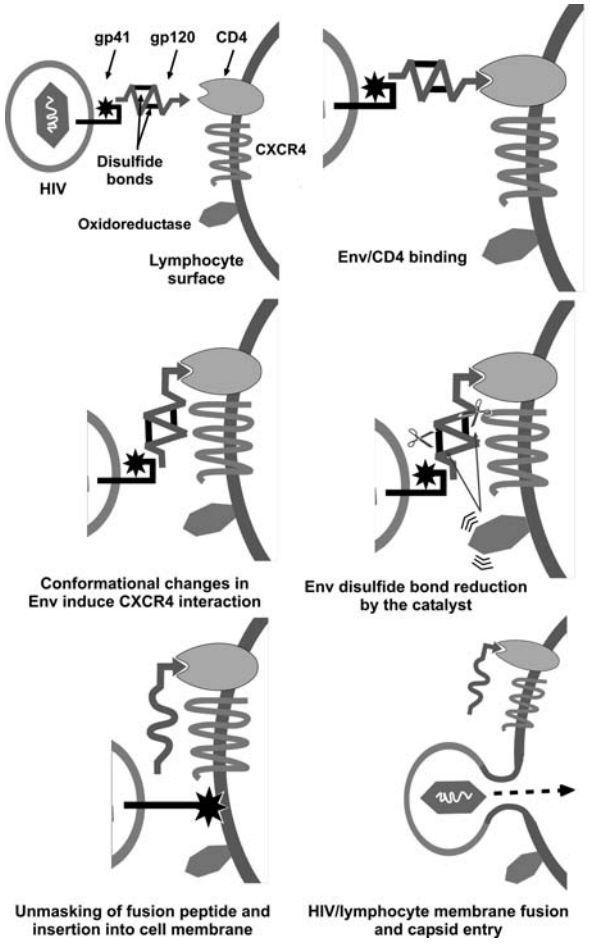


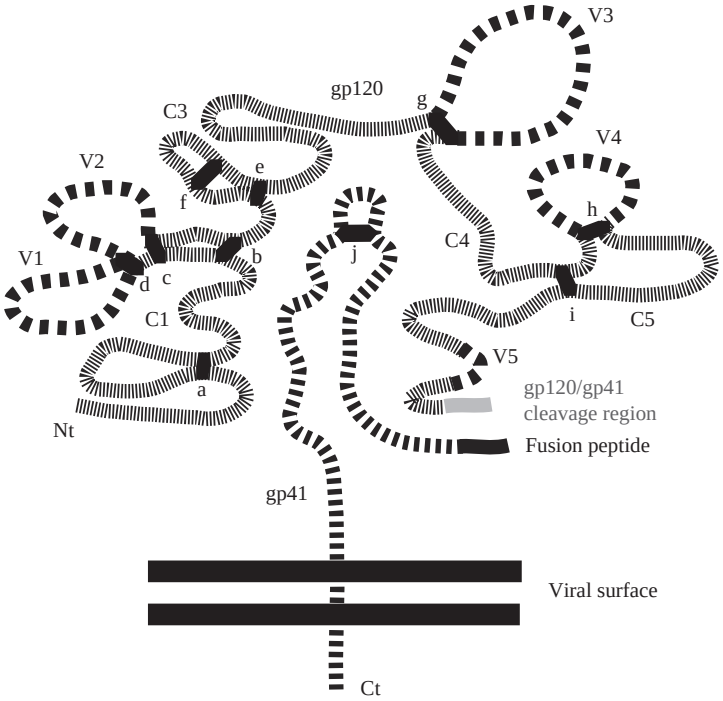
FIG. 5. A model of the molecular events occurring as part of the HIV–lymphocyte surface interaction. The outer subunit gp120 binds to CD4 on CD4+ lymphocytes and other cells of the monocyte/macrophage lineage. This binding event triggers interaction between gp120 and cellular coreceptors, which leads to conformational change within the envelope complex. Reduction of disulfides takes place during this interaction. The transmembrane subunit gp41 then acquires a "fusion-active" conformation and can subsequently mediate fusion between the cellular and viral membranes. See text for further details.

B. PDI and HIV entry

The conformational changes in Env that accompany HIV cell entry are complex, and despite the elucidation of the roles of primary and secondary receptors, the precise mechanism of the receptor-induced conformational change still remains to be defined. For instance, no cell-mediated trigger comparable to the acid pH–induced conformational change that drives the entry of influenza virus (177 and see earlier) has been identified in HIV. However, in addition to the requirement for the primary receptor CD4 and a chemokine coreceptor (CCR5 or CXCR4) for entry into human lymphoid cells (14, 47), a third group of cell-surface proteins has been hypothesized to assist in the structural changes that trigger virus–cell-surface fusion: cell-surface enzymes. Indeed, although the structural changes within Env after interaction with the cellular receptors have been attributed to the intrinsic properties of the viral envelope, it is important to note that they occur in the context of proteolytic and other catalytic activities at the cell surface whose activities may be co-opted by Env to allow both its coreceptor binding and fusogenic competent conformation. Cooperativity between receptor binding and catalytic processing of HIV Env has been outlined in some reports: binding of gpl20 to soluble CD4 increases the susceptibility of V3 to proteolytic cleavage by thrombin (36) or by certain endogenous proteases such as the serine esterase TL2 (100), cathepsin E-like, cathepsin G, and thrombin-like proteinases (4, 36). Gp120 may serve also as a substrate for dipeptidyl peptidase type IV (27).

Besides protease activities, lymphocyte surfaces also possess, as do other cell types, a reductive function (103, 172). This function can cleave disulfide bonds in membrane-bound peptides, as illustrated by the release of [^{125}I]tyramine-SH from the cationic conjugate [^{125}I]tyramine-SS-poly(D-lysine) after its association with the lymphocyte surface (64). The release of radioactivity during incubation can be inhibited in a dose-dependent manner by three PDI inhibitors (DTNB, PAO, and AT3), which act by different mechanisms (see Table 1). In addition, in experiments in which incubation of lymphocytes with the biotinylated thiol reagent MPB was followed by lysis and pull-down with streptavidin, PDI was identified by using specific antibodies (9, 57) (see Fig. 3). This indicates that PDI is present at the lymphocyte surface, that it is accessible to the exogenous reagent, and that it is capable of disulfide reduction. When cells were treated with bacitracin, MPB labeling was reduced by 50% (9), consistent with inhibition of surface-associated PDI (see Fig. 3) and similar to the reduction in the thiol pool of the cell surface obtained after knockdown of PDI expression by using anti-PDI antisense phosphorothioates (200). Thus, PDI is present at the lymphocyte surface and is demonstrably the catalyst responsible for a proportion of the reductive processes that occur there. Given that disulfide-bond rearrangement can obviously induce a formidable structural change and that, similarly, drastic conformation change accompanies the binding of Env to its receptors, it follows that disulfide-bond exchange could form part of the Env remodelling during virus entry and that PDI could play a role in that process.

The first evidence suggesting this was the case was in 1994, when Ryser and colleagues (169) reported that the reductive catalytic function of cell-surface PDI was required for HIV infection *in vitro*. The primary observation was that low doses of membrane-impermeant thiol reagents such as DTNB inhibited virus production by five orders of magnitude compared with controls and that this effect was independent of cell type, being observed in lymphoid cells, macrophages, and human peripheral blood mononuclear cells (169). The mode of action was not immediately clear, however, as the presence of PDI inhibitors during virus–cell interaction in these conditions may have altered HIV infection at several stages of the viral cycle. Inhibition of HIV entry could have occurred at any one of three different steps (for example, CD4 binding, the gp41-mediated membrane fusion process, or final uncoating). It was also possible that HIV expression or budding events may have been modified by cell metabolism changes or cell-membrane alterations resulting either from entry of these "nonpermeant" inhibitors into cells by endocytosis or after cell-surface contact



a	b	c	d	e	f	g	h	i	j
24	89	96	101	188	198	266	348	355	603
44	175	166	127	217	209	301	415	388	608

FIG. 6. The domains organization of HIV Env and the positions of the intramolecular disulfide bonds. V, variable domain; C, conserved domain; *black bars*, disulfide bond (numbering is indicated below). The vicinity of the different domains, as it appears on the cartoon, is not necessarily representative of their spatial juxtaposition within the three-dimensional structure of the envelope.

with these chemical reagents. The early stage of infection was most likely, however, as CD4 binding could have been impaired by PDI inhibition inasmuch as this interaction is known to be strongly dependent on the correct folding of both gp120 and CD4. PDI inhibition may preclude unfolding of gp120 subsequent to CD4 binding and before virion fusion with the membrane of the host cell as part of its interaction with the various HIV receptors (*e.g.,* CXCR4 on CD4+ lymphocytes), and the gp41-mediated membrane-fusion event could have been altered by DTNB, bacitracin, or anti-PDI antibodies because it is known to require a stepwise reorganization of the conformation of Env to trigger the ability of gp41 to mediate fusion (47, 125). Interference with subsequent steps of the infectious pathway, although plausible, was considered less likely in the original study (169).

That the cell surface–associated pool of PDI was the likely target of the inhibitory process was indicated by the fact that the IC_{50} of the DTNB-induced inhibition was close to that obtained for DTNB-induced inhibition of soluble PDI activity and far less than the dose required for cytotoxicity (57, 169, 170, 171) (Table 2). Moreover, treatment of the virus stock by DTNB before infection had no significant effect on the infectious dose, leading Ryser and colleagues (169) to infer that cell-surface PDI was the likely target of the reagent rather than any virion-associated molecule. Later studies confirmed and extended these observations. When cells expressing HIV Env were incubated with DTNB before incubation with target cells expressing CD4 and CXCR4 (CD+/CXCR4+ cells), no inhibition of fusion between Env-positive and receptor-positive cells was observed, whereas significant inhibition was observed

TABLE 2. EFFECT OF VARIOUS PDI REAGENTS AND ENTRY INHIBITORS ON HIV ENTRY AND ENV REDUCTION

Inhibition of	*HIV entry*	*Env reduction*
DTNB	1 m*M* (56), 2.5 m*M* (166), 5 m*M* (134)	5 m*M* (134)
PAO	50 μM (ref. 63)	50 μM (63)
Bacitracin	300 μM (ref. 63), 3 m*M* (56, 166), 5 m*M* (134)	1 m*M* (9)
AT3	100 μM (63)	100 μM (9, 63)
anti-PDI	(56, 134, 166)	(56, 134, 166)
SDF1-α	1 μM (19)	No effect (63), 2 μM (9)
Heparin	20 μg/ml (8)	50 μg/ml (8)
Heparan sulfate	20 μg/ml (8)	50 μg/ml (8)

Numbers in parentheses refer to references.

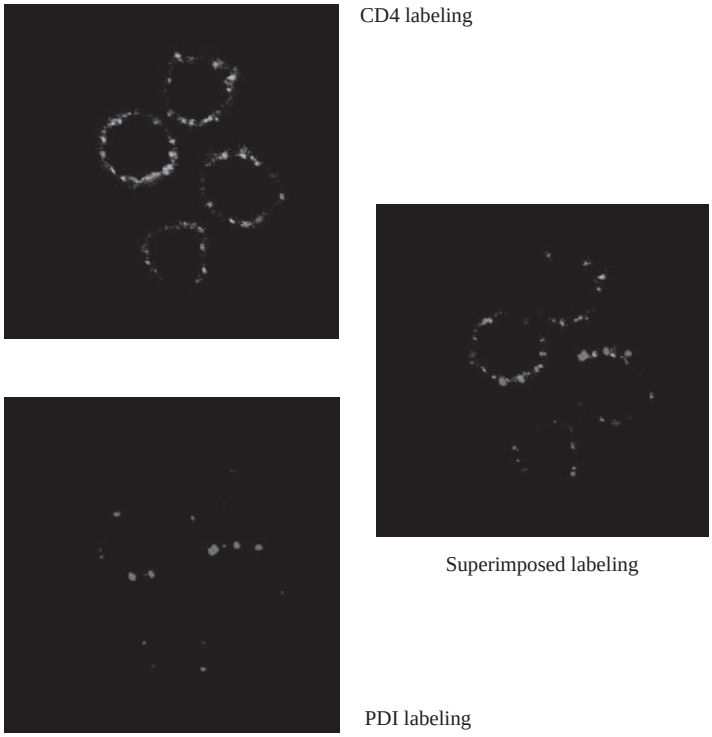


FIG. 7. CD4 and PDI are present within the same regions of the surface of human lymphocyte. Human CEM lymphoid cells were incubated with either a mouse anti-CD4 monoclonal antibody or rabbit anti-PDI polyclonal antibody. Secondary antibodies coupled to fluorescent dyes were used for detection. Panels show each single labeling and the overlay, as indicated.

when the receptor-expressing cells were pretreated with the PDI inhibitor, indicating that fusion depends on the presence of a functional PDI on the CD4/CXCR4-expressing fusion-cell surface (9). These observations agreed with the early conclusions by Ryser *et al.* (169), who stated, "DTNB must act at the time of initial virus–cell interaction" and "by blocking cell surface sulfhydryls, DTNB prevents cleavage of viral disulfides required for the normal sequence of HIV infection" (169). The cell-surface location of the PDI activity involved was also supported by the observation that anti-human PDI or anti-rat PDI antibodies, as well as the antibiotic bacitracin, which is known to inhibit both the reductive and the oxidative functions of PDI (130) and enters cells only poorly, also inhibited HIV infection of lymphoid cells (169). Further, these data indicated that PDI was the primary enzyme involved and that two other cellular oxidoreductases capable of cleaving intracellular protein disulfide bonds, glutaredoxin and thioredoxin, were unlikely components, as neither is affected by anti-PDI monoclonal antibodies. Although thioredoxin has the same dithiol motif as PDI at its active site, it is not blocked by bacitracin (169, 170). Other data supported this conclusion as well. First, the presence of either bacitracin or DTNB in the culture medium exerted a dose-dependent inhibition of cell-to-cell fusion when Env-expressing cells were added to native human lymphocytes, inhibition being observed at a concentration of 3×10^{-4} *M* DTNB, and syncytium formation, the consequence of cell fusion, was wholly abolished at 10^{-3} *M* (9). Direct evidence of the involvement of PDI in the fusion process also was demonstrated by the use of anti-PDI antibodies, which specifically inhibited cell-to-cell fusion (9).

Second, the effect of PDI inhibitors on the accumulation of minus-strand strong-stop DNA, the first product of reverse transcription synthesized after virus entry, was examined by using polymerase chain reaction (64). PDI inhibitors DTNB, aPAO, and AT3 caused a dose-dependent inhibition of strong-stop DNA accumulation in SupT1 cells when added before or during infection (or both) but not when added after infection. Infection by a second isolate of HIV, the CCR5-tropic HIV-1JR-FL, in both PM1 and primary monocyte-derived macrophages, also was impaired in the presence of PDI inhibitors (64). These results indicate that infection requires that cell-surface PDI must be active at the time of virus–cell interaction and that the PDI requirement is independent of the viral tropism.

Third, when added before the virus–cell contact step, PDI inhibitors AT3 and aPAO inhibited virus-induced β-galactosidase activity in a dose-dependent fashion when P4 cells were infected by HIV-1 NL4-3 in a reporter gene assay that monitored transactivation by the incoming virus of an LTR-driven LacZ reporter gene (64).

This body of data confirms a key role for PDI in the activation of HIV fusion. The possibility that such reduction occurs *via* a different oxidoreductase or that the target molecule is not gp120 is raised by two studies that reported a role for thioredoxin in viral entry (139, 153). The observation, that thioredoxin activates T lymphocytes by reducing a disulfide bond of the D2 region of CD4, was supported by data indicating that blocking reduction by thioredoxin through the use of an arsenic compound prevented HIV-1 entry. It was therefore concluded that thioredoxin-mediated CD4 reduction was a requirement for HIV entry (138, 139). However, arsenic compounds are known to block the vicinal thiols of the CXXC motifs at the active sites of PDI as well as thioredoxin, leading to the possibility that the inhibition of HIV entry observed was the result of interference with gp120 reduction rather than CD4 (58, 64). The results obtained contrast those obtained by three independent groups describing HIV Env reduction and also raise the possibility that

redox changes observed within CD4 (139) may be a consequence of thiol/disulfide interchange occurring within the CD4/CXCR4/Env complex. After Env reduction by PDI, we must consider which proteins at the target-cell surface could form a disulfide reservoir for the thiol-disulfide exchange reaction occurring within the PDI–receptor complex. Gp120 contacts the N-terminal D1 domain of CD4, but the D2 domain, although not playing an active function in Env binding, could play this role and act in a donor/acceptor capacity to allow gp120 reduction (58, 64). Another study also implicated thioredoxin in HIV–cell fusion: by using siRNA to downregulate PDI or overexpression of wild-type or variant forms of PDI, Ou and Silver (153) observed only small effects on the cell-fusion process mediated by NL4-3 or AD8 strains of HIV-1. Thus, in these conditions, cell-surface thiol-containing molecules have a greater effect than PDI on fusion. Based on the capacity of thioredoxin to reduce gp120 disulfides *in vitro* (153), these authors then proposed that thioredoxin is the catalyst involved in gp120 reduction. It is plausible, however, that inhibition of PDI expression with siRNA leads to overexpression of thioredoxin as part of an SOS mechanism, and, consequently, it substitutes for use in achieving Env reduction, as both catalysts exhibit close redox activities. As discussed herein, that the natural HIV infection process implicates PDI is strongly supported by the studies that show that the specific PDI inhibitor bacitracin blocks HIV entry (57, 169).

C. PDI inhibitors block HIV entry after CD4 binding

Ryser and colleagues (169) originally postulated a functional association between CD4 and PDI, arguing that HIV binding to a CD4 receptor that is not spatially associated with PDI would not be sufficient for infection. However, direct evidence for an association between CD4 and PDI was not available until revealed by confocal microscopy (57). Incubation of lymphoid cells with an anti-CD4 antibody and fluorescent conjugate showed a distribution of CD4 that was rather homogeneous, covering ~50–70% of the lymphocyte surface. In contrast, the labeling obtained with anti-PDI antibodies was organized in small clusters on the cell surface (Fig. 7). These distributions were shown to be independent of the state of activation of the lymphocytes (57). Additionally, PDI labeling was generally in regions of the cell surface where CD4 labeling was most dense, although strict colocalization of the two antigens was not unambiguously demonstrated (57) (see Fig. 7).

Co-immunoprecipitation experiments also confirmed the capacity of CD4 and PDI to interact both *in vitro* and at the lymphocyte surface. First, when soluble PDI was biotinylated on its thiols, and soluble CD4 was added before precipitation with immobilized avidin, CD4 was found in the pellet (64). Second, a PDI–CD4 complex could be affinity-isolated from lymphoid cell lysates expressing CD4-Spep by using S-protein-agarose (64). Third, when U937 cells were treated with the thiol-specific biotinylating agent MPDOD, which essentially labels PDI thiols on the cell surface, and tagged proteins subsequently isolated from cell lysate with immobilized avidin, both CD4 and PDI were identified in the pellet, although only a small amount of cellular CD4 was coprecipitated by this rather harsh procedure (64). The low amount of CD4 isolated by using this protocol is consistent with the weak colocalization of PDI and CD4 observed by using confocal microscopy (57). Last, an association between PDI, gp120, CD4, and CXCR4 was observed at the cell surface (135). In this analysis, done to determine whether PDI colocalizes with CD4 and CXCR4 at the cell surface and whether the addition of gp120 induces remodeling of their distribution, PDI localization at the plasma membrane was analyzed in the presence and absence of gp120 by using scanning confocal microscopy. CD4 and CXCR4 exhibited significant colocalization. PDI showed no colocalization with CD4 or CXCR4 in the absence of gp120, whereas, in the presence of gp120, PDI was distributed predominantly in the CD4/CXCR4-dense regions (135), indicating considerable colocalization under these conditions. Such data indicate that PDI distribution at the cell surface is modified after gp120 binding to its receptors and that, although PDI is not a transmembrane protein, it can relocate to specific cell-surface regions depending on the composition of the cell surface. Triple colocalization of PDI, CXCR4 and CD4 was shown (135), and the association of PDI, CD4, and CXCR4 within the same molecular complex was also observed in pulldowns by using a CXCR4-specific antibody that precipitated complexes containing PDI from CD4+/CXCR4+ cells in the presence of gp120 (135).

Together, these results indicate that gp120, CD4, CXCR4, and PDI form a transient tetramolecular protein complex induced by the interaction of gp120 with the cell surface. This strongly suggests that this complex is used as a portal for HIV entry. Such complexes could also include a larger range of molecules, such as those recruited to the points of interaction of myeloid cells with T cells, in what has been termed the fusion synapse (95). The corollary of these observations is that, be-

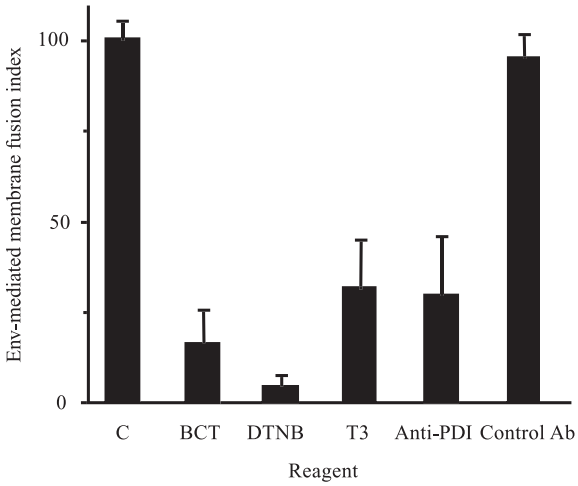


FIG. 8. The relative efficacy of various compounds in preventing HIV-Env–mediated fusion. HIV Env expression at the cell surface was enabled by using recombinant vaccinia virus infection before incubation of Env-expressing cells with fusion partner CD4+ human lymphocytes. The effects on Env-mediated cell-to-cell fusion of bacitracin (1 m*M*), DTNB (1 m*M*), 3,3′,5-triiodothyronine (T3; 200 μM), anti-PDI polyclonal antibodies (Abs; SPA-890; 1:50), and control antibodies are shown. The control fusion reaction in the absence of reagents (C) corresponds to 100% cell-to-cell fusion.

cause CD4 labeling also appears in membrane regions where PDI is lacking, only a subset of the CD4 receptors present in PDI-enriched surface regions is involved in HIV entry into the cell.

These data provide direct evidence to show that the inhibition of PDI-mediated thiol/disulfide changes within Env is correlated with abrogation of fusion at a step that occurs after CD4 binding but before the membrane-fusion step. Further details of the step at which PDI acts have been obtained by using a system involving CD4+/CXCR4+ lymphoid cell plasma membranes interacting with HIV Env expressed at the surface of cells encoding the corresponding gene. Cell-aggregation and cell-to-cell fusion assays have been used to allow discrimination between the CD4/gp120 binding step, which results only in cell aggregation, and the coreceptor- and gp41-mediated membrane-fusion step, which leads to syncytium formation. The data show that the presence of either DTNB or anti-PDI antibodies during the coincubation of Env-expressing cells and a $CD4^+$ fusion partner significantly affect cell-to-cell fusion (57) (Fig. 8 and Table 2). Moreover, a substantial inhibition of fusion was obtained when the fusion-partner cells were pretreated with DTNB to block surface PDI before DTNB removal by washing and subsequent incubation with Env-expressing cells. In contrast, DTNB presence during coincubation with fusion-partner cells had no effect on cell aggregation. Similar data were obtained by using anti-PDI antibodies with anticatalytic activity (57). The lack of a potent effect of PDI inhibitors on the cell-aggregation process is consistent with an inhibitory effect that occurs after CD4 binding. *In vitro* evidence also was obtained by using a molecular CD4/gp120-binding assay in which Env-expressing cells were fully able to bind soluble CD4 in the presence of DTNB (57).

Together, these data demonstrate that the inhibition by specific PDI inhibitors acts at a post–CD4-binding step in the Env-mediated membrane-fusion process. It is not restricted to any one cell system and includes data obtained with both recombinant and viral envelope protein interaction with cell-surface CD4 in a variety of *in vivo* fusion assays.

D. PDI mediates reduction of gp120

Based on the observation that PDI is found within CD4–CXCR4–gp120 complexes that occur at the cell surface after gp120 interaction with the target cell and that PDI inhibition affects fusion at a post–CD4-binding step (57, 64, 135), evidence for a direct enzymatic consequence of PDI and gp120 interaction was sought. Direct evidence for reduction of cell-bound gp120 and its inhibition by PDI inhibitors has been obtained by three groups, and its functional relevance in terms of HIV entry has also been demonstrated (see Table 2).

Incubation of HIV target cells with recombinant soluble Env in the presence of biotinylated thiol reagents such as Biotin-BMCC or MPB, followed by Env purification, has shown that Env becomes biotinylated (9, 64). Because native soluble Env does not exhibit free sulfhydryls (9), this observation demonstrates that interaction of Env with its target-cell surface results in reduction of some of its disulfides; hence the production of free thiols. This conclusion was obtained by using soluble monomeric Env (9, 64) as well as soluble oligomeric antigens from various CXCR4 and CCR5 with HIV isolates with different tropisms (9, 64, 135). Furthermore, the increase in the thiol content of monomeric soluble gp120 after its interaction with the cell surface was similar to that observed for oligomeric gp120 associated with the surface of cells involved in syncytium formation (9, 64, 135), leading to the conclusion that changes in redox state do not depend on Env oligomeric status or on the presence of gp41. The ability to detect an increase in the thiol content of Env associated with the surface of cells engaged in syncytium formation (9) is consistent with the finding that the surface of fusing cells is heavily enriched in fusion-competent Env species, as well as with the idea that the half-life of such species (60) is sufficiently long to allow their labeling with MPB.

When incubation was performed in the presence of increasing concentrations of PDI inhibitors such as PAO, T3, or bacitracin, the biotinylation of free thiols resulting from Env reduction was reduced in a dose-dependent manner, whereas CD4 binding remained unchanged (9, 64, 135). In these experiments, retrieval of biotinylated gp120 from the cell surface by using immobilized avidin led to the detection of small amounts of a gp120–D4–PDI complex, confirming the data obtained by using confocal microscopy (57, 64). The concentrations of PDI inhibitors that inhibited Env reduction were in the same range as those that inhibited HIV infection (57, 169). The PDI-inhibitor concentrations that inhibit reduction of gp120 are also within the range used to inhibit the cleavage of [^{125}I]tyramine-SS-conjugate by soluble PDI or by the cell surface, further indicating that PDI is the active catalyst involved during HIV entry (55, 64) (see Fig. 8 and Table 2).

When CCR5-tropic gp120JR-FL was incubated with either U87.CD4 cells or U87.CD4.CCR5 cells, Env reduction was comparable (64), and inhibitors reduced biotinylation equivalently in both cell lines, indicating that PDI is involved in Env reduction, irrespective of the presence of a coreceptor. Env was also reduced when CXCR4-tropic gp120IIIB was bound to cells whose CXCR4 coreceptor had been blocked after incubation with the natural CXCR4 ligand SDF-1 or by bicyclam (64). This indicates that reduction does not require gp120 binding to a functional coreceptor. An independent study (135) led to a similar conclusion: to determine whether gp120 undergoes disulfide rearrangement independent of coreceptor interaction, soluble recombinant gp120 IIIB was added to HOS-CD4+/CXCR4+, HOS-CD4−/CXCR4+, or HOS-CD4−/CXCR4− cells concomitant with MPB. The level of gp120 biotinylation with HOS-CD4+/CXCR4− cells was comparable to that obtained by using HOS-CD4+/CXCR4+ cells, indicating again that CD4 is sufficient to allow PDI to reduce disulfide bonds within Env. Incubation of HOS-CD4−/CXCR4− cells devoid of both CD4 and CXCR4 with Env did not lead to labeling with MPB, as expected of a CD4-dependent interaction. These observations suggested that Env reduction occurs after interaction with the CD4 receptor in a coreceptor-independent manner, although this conclusion is at variance with data obtained by using incubation of Env with authentic lymphocyte surfaces (9). By using a quantitative thiol measurement microassay that quantified the thiol content of an antigen within a picomolar sensitivity range, the thiol content of Env after its interaction with either CD4 or both CD4 and CXCR4 in various conditions was assessed (9). By using high concentrations of SDF1-α (the natural ligand for CXCR4; 19) or CD4 or both, which specifically impaired Env binding to one or both surface antigens, thiol

labeling of the cell surface was performed by using MPB after the interaction of Env with lymphocytes. Labeled Env was then specifically recovered from the cell surface by immune affinity, and the thiol content was measured. The thiol content of gp120 associated with the lymphocyte surface in conditions in which both CD4 and CXCR4 were accessible was found to be four free thiols (9), leading to the conclusion that two disulfides out of the total of nine in the complete molecule (115) of native gp120 are reduced on binding. Comparison of the data obtained when cells were preincubated or not with SDF1-α showed that changes in the redox status of gp120 required CXCR4 (9) (see Table 2). This observation would suggest that Env reduction occurs after CXCR4 binding, consistent with the observation that reduction of a single disulfide bond was sufficient to impair the ability of gp120 to bind CXCR4 at the lymphocyte surface (9). Thus, in this system, the conclusion reached was that the presence of free thiols on Env before CXCR4 binding was inconsistent with the process leading to fusion and that Env reduction more probably occurs at a post-CXCR4 binding step.

The different conclusions reached by three independent groups on this one point might be explained by the origin of the envelope used in these experiments (9, 64, 135) or by the fact that Barbouche and colleagues (9) used SDF1-α to block CXCR4, whereas Markovic and colleagues (135) and Gallina and colleagues (64) used cells wholly devoid of the coreceptor. It may also relate to the nature of the cell-surface partner used to reduce Env, as it is conceivable that recombinant expression of a coreceptor on the surface of a non-natural cell type may induce a cell-surface organization of the CD4–CXCR4–PDI complex that fails to engage the natural interaction. In these non-natural conditions (64, 135), PDI may be expressed in regions of the cell surface not rich in CD4 and CXCR4, and its capacity to reduce Env may be the same irrespective of complex formation with CD4, as also suggested by the capacity of soluble recombinant PDI to reduce Env in solution in the absence of both CD4 and CXCR4 (8, 64).

Some results have indicated how PDI and Env may come into contact with each other through their interaction with CD4. The PDI–CD4 interaction does not prevent the binding of CD4 to gp120 at the cell surface, suggesting that the ectodomain of CD4 has separate binding sites for the two proteins. As gp120 binds to the outermost CD4 domain [*i.e.*, D1 (125, 168)], it appears probable that PDI binds to the innermost domains (D3 or D4). Despite being distinct on CD4, the proximity of these binding sites enables CD4 to bring PDI close to gp120. The distal binding of gp120 to CD4 represents a difficulty for subsequent contact with the coreceptor as the seven transmembrane-domain proteins CCR5 or CXCR4 are close to the membrane surface. However, the CD4 molecule is thought to flex about the hinge that is the junction of D2 and D3, so enabling gp120 to come into contact with the chemokine receptor. This would be an effective mechanism to bring gp120 into close contact with PDI and, additionally, as the peptide-binding domains of PDI do not overlap with its active site (62, 152), the latter would be free to interact with gp120 as it was brought close.

Although the increased concentration of PDI in CD4-enriched regions (57, 64, 135) is probably used to advantage by the virus to carry out Env conformation changes to reach the fusion state, gp120 reduction is not the sole driver for that particular receptor-induced conformation to progress, despite this being the norm during a natural infection. In addition to reduction of gp120 *in vivo,* reduction also occurs *in vitro* by using soluble partners, and in either condition, the effects on the disulfide network of Env are similar (8, 9). That reduction is detected only after receptor binding may therefore be a consequence of the increased probability of contact between Env and PDI within the CD4–Env–PDI complex as part of the process leading to fusion. This would also explain the discrepant observations that gp120 reduction was observed after CD4 binding in $CXCR4^-$ cells yet after CXCR4 interaction by using native lymphocytes (9, 64, 135): the tighter the Env/cell-surface interaction, the stronger the reduction.

As the oxidoreductive function of PDI in the endoplasmic reticulum may be assisted by chaperones, an interpretation of the PDI–CD4–Env complex on the target lymphocyte surface is that CD4 provides a chaperone-like function to enable PDI to reach its Env substrate. Nonetheless, PDI-mediated Env reduction can be obtained *in vitro* in the absence of HIV receptors and the cellular environment (8, 64). The capacity of soluble PDI to reduce gp120 adequately is supported by the observation that high concentrations of exogenous PDI can rescue cell–cell fusion previously blocked by DTNB, after washout of the inhibitor (135). In this situation, exogenous PDI must function either by direct redox activity on HIV Env, irrespective of its presence in complex with CD4 and the coreceptor in a way similar to that observed in solution, or after reassociation with the cell–membrane complex.

The observation that soluble PDI can trigger the virus–cell fusion process is also consistent with the report that certain CD4-independent HIV-1 strains are infectious and that CCR5+ CD4− cells, when mixed with Env-expressing cells, undergo limited fusion in the presence of soluble CD4 (16). In such systems, the soluble form of the catalyst present in the cell medium may be enough to reduce Env despite the lack of association with membrane CD4, as large amounts of PDI are secreted by cells (see Introduction) and are therefore available to interact in solution with added soluble CD4 to activate refolding of the Env complex.

E. Targeting Env reduction to treat HIV infection

The first anti-HIV agents discovered and used to control the disease were nucleoside analogues such as zidovudine, dideoxycytidine, or didanosine, which target the viral reverse transcriptase (6). Non-nucleoside reverse transcriptase inhibitors such as nevirapine and efavirenz have since been discovered, as well as agents inhibiting the protease (42), another essential viral enzyme, and all were rapidly used in therapy. Routinely, AIDS patients are now treated with a cocktail of drugs (6) to counter a major problem with HIV monotherapy, the occurrence of drug resistance, ultimately the result of the reverse transcriptase ability to mutate the viral genome. New anti-HIV agents are continuously required to control the emergence of multi–drug-resistant isolates and, in this perspective, blockade of virus entry is of paramount importance because it blocks infection at the first step of the viral cycle (*i.e.*, cell entry), and accordingly, a number of agents targeting various steps of the virus–cell-membrane fusion process have been developed (54, 143).

The use of entry inhibitors to fight HIV infection is possible because of the kinetics of the entry process. Whereas influenza virus enters cells after endocytosis and a subsequent pH-dependent fusion step between the viral and the endosome membranes takes only milliseconds (28, 177), effectively preventing its inhibition by drugs, the time scale of the corresponding HIV entry process is ~20 min (65, 66), allowing a finite time window for competitive or structural inhibition at a number of points in the process. In addition, as HIV fusion occurs at the cell surface, it is accessible to relatively large molecules, such as peptide inhibitors, which may not efficiently penetrate the cell membrane.

Three classes of anti-HIV agents effective at the stage of cell entry can be described from a chronologic point of view. The first class of inhibitor prevents gp120 binding to CD4. These agents were designed early in the history of the disease after the discovery of CD4 as the primary virus receptor, and several compounds targeting the binding of HIV to CD4 were developed (121, 136, 192). However, no therapeutic advantage was found for these agents, partly because the CD4-binding pocket on gp120 is largely recessed and is difficult to access (125). Antibodies against the region of CD4 involved in Env binding have side effects such as interference with the function of the immune system, but a recombinant CD4–IgG_2 fusion protein [PRO-542 (204) and BMS-488043 (79)], effectively presents decoy receptors that prevent HIV attachment and do offer some therapeutic prospect. The second class of compounds affecting entry inhibits the subsequent interaction of gp120 with the cell-surface coreceptors CCR5 or CXCR4 (37, 73, 74, 179). A small percentage of the population encodes a natural deletion in the CCR5 gene and is highly resistant to HIV infection, yet apparently healthy (123), suggesting that drugs designed to block the gp120-CCR5 interaction may be clinically beneficial. Several such compounds have been isolated (141) and progressed to clinical trials, although some side effects such as liver toxicity, which may limit their use, have been observed (151). The third class interferes with the mechanistic device of fusion, the formation of the six-helix bundle structure of gp41 (22, 30, 43, 131, 140), and although relatively recently developed, these compounds, which represent a new class of drug, have progressed rapidly to a therapeutic compound approved by the FDA in 2003 for the treatment of HIV infection: T20 (22, 43).

T20 is a 36-amino-acid peptide inhibitor corresponding to a segment of gp41, the C-terminal heptad repeat (C-HR). Based on the similar characteristics of the fusogenic state of the envelope of the influenza virus (28, 33, 49) and that of HIV Env, the six-helix-bundle conformation of gp41 (trimer-of-hairpins; 30, 131, 140) was proposed to be the fusogenic form of the molecule that draws the viral and cellular membrane together, leading to pore formation, which allows viral core entry into the cytoplasm (65, 128). The two gp41 heptad repeats (HRs) are separated by a sequence that forms a small conserved disulfide loop, the integrity of which is required for gp41 interaction with gp120 and infection (65). The N-terminal and C-terminal sequences of either side of the loop compose the NHR and CHR, respectively. The two halves of gp41 bend upon each other to form a hairpin that brings the two heptad sequences close together (128), whereupon the three NHR helices form a central coiled coil, and the three CHR helices wrap themselves around the central coil such that the overall structure forms a six-helix bundle (30, 65, 128, 131, 140). The T20 peptide can access the gp120 molecule and bind to the NHR sequence of gp41, so preventing the formation of the six-helix bundle and blocking further conformational change and membrane fusion (22, 43). The stage of inhibition is crucial as, later in the process, the highly stable interaction between the CHR and the NHR regions of gp41 drives the membrane-fusion reaction to completion, and T20 can no longer act on the fusion process (76, 140, 176). T20 fulfils the main criteria of an effective inhibitor of therapeutic relevance, as it blocks a step that is essential for the viral life cycle (*i.e.*, membrane fusion), and it has low cytotoxicity.

The data reported here on gp120 reduction by PDI as a prerequisite for virus entry offer an additional and novel target for anti–HIV-1 agents (9, 64, 135). Moreover, because a major problem in anti-HIV compounds targeting an HIV protein is the generation of drug-resistant mutants, developing agents that can block PDI-mediated gp120 reduction, a host-specified reaction (57, 135, 169), appears at first sight a useful way of avoiding the high reverse transcriptase mutation rate. Indeed, resistance to this class of agent would imply a multisite reorganization of the disulfide network of gp120, which appears intrinsically unlikely. On the basis of these considerations, three additional target events in relation with Env reduction have been proposed to allow entry blocking (133, 168): cell-surface PDI, formation of the PDI–Env–receptor complex, and recognition by PDI of Env (see Fig. 5). In addition, the conformational changes triggered by disulfide-bond reduction may uncover novel PDI-induced gp120 epitopes of potential use in vaccine development. These aspects of the topic have been discussed in great detail in recent reviews by Fluckiger and Ryser (168) and Markovic and Clouse (133) and so will be only briefly discussed here.

1. Interfering with the enzymatic activity of PDI. As discussed, PDI, including-cell surface PDI, has several functions required for the maintenance of cell physiology and, even with nonpermeant agents (Table 1) that interfere with solely cell-surface activity, it appears unlikely that successful development can occur of an inhibitor that can both block HIV infection and have acceptable side effects.

2. Interfering with the PDI–CD4 complex. Agents of therapeutic interest might inhibit CD4–PDI interaction (57, 64, 135) and so block Env reduction, as disulfide cleavage is the result of the delivery of the Env substrate to the catalytic domain of PDI *via* a complex formed between CD4 and gp120. PDI is thought to bind the D3 domain of CD4, and an antibody directed against D3 could block the PDI–CD4 interaction (64, 168). However, Env reduction by PDI *in vitro* is independent of the presence of CD4 in the incubation medium (8, 64), which questions the capacity of a compound that blocks PDI–CD4 interaction to inhibit Env reduction in the context of the authentic viral infection pathway.

3. Recognition of Env by PDI. Commenting on a therapeutic approach aimed at blocking Env reduction by interfering with the Env–PDI interaction, Fluckiger and Ryser

(168) proposed the use of BMS-806, which blocks CD4-induced conformational changes in gp120, as a lead compound, in that it has some characteristics indicative of an agent that interferes with the PDI–gp120 substrate relation. Their rationale is based on the observation that Phe43 is a critical amino acid residue in the CD4 domain that interacts with gp120, and that the cavity within gp120 that accommodates Phe43 overlaps the BMS 806 binding site (127). These authors argue that the presence of four disulfide bonds in this region suggests that BMS 806 binding might prevent presentation of critical gp120 disulfides to PDI, prevent Env refolding, and so HIV entry. The success of T20 (22, 43), which acts on the similar short-lived phase of gp41 activation, indicates that even transient targets deserve investigation from a therapeutic perspective. Recent work has also suggested that glycosaminoglycans may be of interest to interfere with the Env–PDI interaction. During its interaction with the cell surface, gp120 interacts with glycosaminoglycans (142, 188), although it is now considered that these components do not play a major role in mediating viral entry (202). Lymphotropic gp120 binding to glycosaminoglycans is thought to proceed from an initial high-affinity association with the positively charged V3 domain to a second, CD4-induced conformational region, likely to be the revealed CXCR4 binding site (10, 72, 147, 166). Reports describing the potent anti-HIV effect of heparins and dextran sulfate (90, 164) indicate that, despite their weak concentration on the CD4+ lymphocyte surface, Env interaction with glycosaminoglycans may have therapeutic relevance for HIV treatment, and soluble sulfated polyanions were used early in the 1990s in clinical trials and are still under consideration as potential anti-HIV therapeutic agents. Heparin and related compounds such as heparan sulfate are reported to neutralize HIV, not through interference with gp120-CD4 binding, but more probably through interference with CXCR4 interaction (72, 147, 164, 166). The effect of soluble glycosaminoglycans on Env reduction by PDI has been examined to study whether their anti-HIV effect can also involve interference with the Env reduction process. By using an *in vitro* assay in which three to four disulfides within soluble gp120 are specifically reduced on addition of soluble PDI in the presence of GSH (8, 64), it was found that the presence of CD4 only marginally influences disulfide cleavage (8). In these conditions, it was observed that a 3 *M* excess of heparin and heparin sulfate in the incubation medium decreased the extent of Env reduction by 80% when gp120 was pre- or coincubated with CD4 (8) (see Table 2). A weaker inhibition was observed in the absence of CD4. The effect of CD4 interaction with Env on the subsequent capacity of glycosaminoglycans to block its reduction by PDI is consistent with the induction of an additional glycosaminoglycan binding domain on Env, besides V3, after CD4 binding (72, 147). The inhibition in reduction by glycosaminoglycans of CXCR4-utilizing Envs with highly positively charged V3 loops and of the CCR5-utilizing Envs with lesser charged V3 loops (*e.g.*, the C clade isolate CN54) was similar in the presence of CD4 (8). Thus, glycosaminoglycans, besides their capacity to protect proteins from degradation by proteases (26, 71), can also provide a level of protection through inhibition of their denaturation by reduction. Importantly, this study shows that soluble glycosaminoglycans can inhibit infection by isolates of various tropisms through interference with Env reduction by PDI, in addition to the direct effect on the interaction of Env with cell-surface components such as coreceptors. Thus, inhibition of PDI-mediated gp120 reduction could constitute part of the anti-HIV therapeutic effect of soluble glycosaminoglycans. Virus escape from such compounds would be expected to be restricted, as mutations that avoid the inhibition of coreceptor binding (147, 164, 166) are likely not to be able to complete the entry process. Interference by glycosaminoglycans of Env reduction could be plausibly considered either by bridging, hence stabilizing, distant disulfide-bonded domains within Env or by steric hindrance after Env binding, so preventing PDI access to the susceptible bonds within the molecule.

F. Structure changes and redox

The level of disulfide bonding in gp120 is unusually high and consists of nine bridges, the positions of which are highly conserved among HIV isolates despite otherwise extensive variation (115). It is probable that this complexity relates to the complex functions of gp120 as it interacts with at least four different ligand surfaces to trigger HIV entry: CD4, the primary receptor; a chemokine receptor as the secondary receptor; PDI, a required catalyst to trigger HIV entry; and gp41, the fusogenic partner (14, 65, 168). Immunologic studies have demonstrated that new gp120 epitopes recognized by human neutralizing antibodies are exposed at each stage of the Env–target cell interaction process (125), and so a diverse range of conformations occurs during CD4-binding, CCR binding, and the acquisition of fusogenicity. It follows that this heavily glycosylated protein (56) must be sufficiently pliable to fulfil the conformational changes required for the eventual virus–cell fusion. A plausible illustration of the relation between Env, CD4, and PDI can be obtained from the structural data gained from a number of studies on the interaction between Env and CD4, as well as on docking studies that have examined the PDI–CD4–Env interplay (168).

1. Disulfide reduction and changes within the gp120 domain. Triggering conformational changes within Env before fusion requires a reduction of the activation energy of the molecule to induce a low-energy state compatible with such changes. The cleavage of disulfide bonds is able to cause significant conformational changes within proteins, and the data reviewed above give indications that such consequences follow Env reduction by PDI (9, 64, 135). If PDI can act at the cell surface (94, 183, 187) as it does in the endoplasmic reticulum (11, 59, 93) [*i.e.*, as a redox-driven chaperone (25, 41, 101, 160, 161) to unfold proteins after reduction], it may also directly catalyze some of the conformational changes within Env required to unmask ultimately the gp41 fusion peptide.

The first crystal structure of a gp120 core interacting with CD4 in the presence of an anti-gp120 FAb identified the amino acid residues of gp120 that interact with CD4, the Phe 43 pocket (105) previously noted to be the binding site for BMS-806, which results in blockade of the CD4 interaction (127). This pocket forms as a result of the docking of two distinct subdomains of gp120, an inner and outer domain, that are joined by a flexible bridging sheet (Fig. 9). The docking juxtaposes sequences that are distal in the linear sequence, and their bringing together has a high energy cost that, in part, explains why

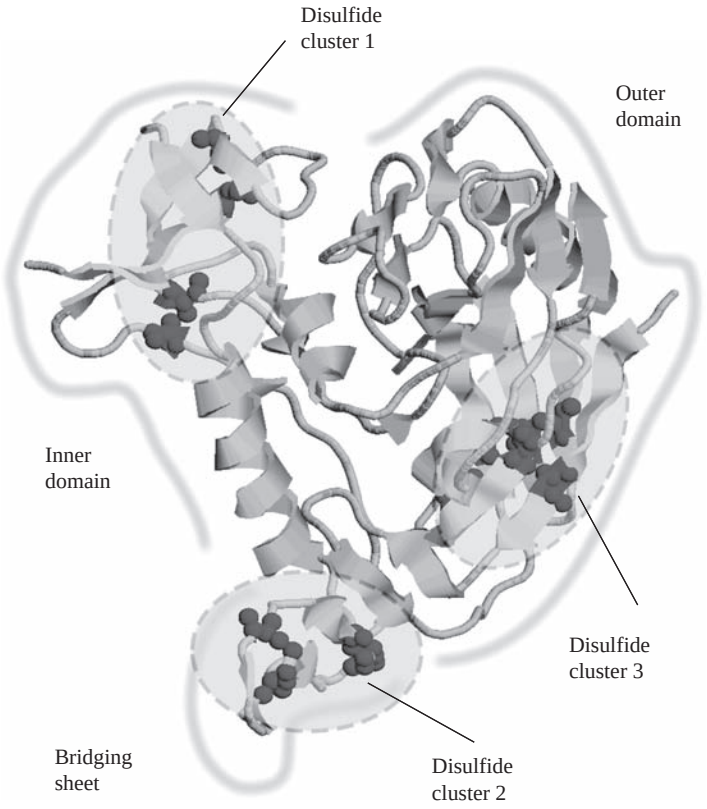


FIG. 9. The three-dimensional structure of the CD4-bound form of HIV gp120. The structure has been orientated to reveal the domain organization of the molecule, and each domain identified. The residues involved in disulfide bonds are highlighted. Note the clustering of bonds in the inner and outer domains and bridging sheet, any of which could be the focus of PDI-mediated reduction at the cell surface.

neutralizing antibodies that block CD4 are so rarely generated (199). A subsequent structure of the gp120 core unliganded with CD4 (31) reveals considerable structural differences in the inner domain when compared with the CD4-bound state, indicating that the innate flexibility of the inner domain is lost by the adoption of a fixed conformation on CD4 binding. It is reasonable to suppose that, to move on from the rigid, CD4-bound conformation to allow progression to fusion, gp120 must be freed from this structural restriction and that considerable energy must be used. As noted, one possibility suggested is that the Phe-43 pocket displays substrate disulfides to PDI (168) (see Fig. 9, cluster 1), and the energy released from bond breaking is used to power the required conformational change. However, this would require that PDI acted on CD4 binding and before CCR binding, which, as discussed, is a current point of contention (9, 64, 135). Treatment of gp120 with PDI has been shown recently to reduce binding by a monoclonal antibody, 17b, whose binding depends on CD4 interaction, further supporting the idea that PDI acts after CD4 binding and that the disulfides directly involved in the post-CD4 binding structure are the target of PDI (17).

Besides the CD4-bound conformation, functional and experimental data suggest that other regions may be PDI substrates, a case in point being the third variable loop or V3 domain. The loop is prominent in structures of gp120 that include it, explaining both its immune dominance and involvement in contacting the second receptor (87, 165). Its prominence also explains its peculiar sensitivity to cleavage, which can be mimicked *in vitro* by thrombin treatment, which induces a single proteolytic nick near the tip of the loop (36). Cleavage is easily obtained biochemically, and 70- and 50-kDa fragments are detected after 2-mercaptoethanol reduction of preparations of gp120. Gallina *et al*. (64) examined the effect of Env reduction on such V3 cleavage and showed that reducing reagents or PDI resulted in a similar pattern of fragmentation, indicating that PDI can recognize and cleave the disulfide bond that exists at the base of V3 (64). In the same experiment, by using a biotin-coupled thiol reagent, it was observed that disulfide bonds other than the bridge at the base of the V3 loop (see Fig. 9, cluster 2) also can be labeled and hence cleaved (64). In addition to the capacity of PDI to cleave the disulfide of V3, these experiments also suggested that the reduction of gp120 by PDI before incubation with thrombin leads to more global conformational changes within gp120, as several additional fragments other than those at 50 and 70 kDa were detected. These additional fragments result from the unmasking, after Env reduction by PDI, of thrombin-cleavable sites in Env other than within the V3 sequence (64). Results were independent of the presence of CD4 in the incubation medium, an observation consistent with that reported by Barbouche *et al*. (8), who showed that CD4 binding to Env is not necessary for the reduction of the latter by PDI *in vitro*.

Of particular note, the highly basic nature of the V3 region of gp120 (36) has physicochemical characteristics of a PDI substrate, allowing interaction with the C-terminus of PDI, which is rich in acidic amino acids (59). Direct evidence that the disulfides of V3 may be substrates for PDI has recently been obtained during characterization of a stable trimeric gp140 molecule thought to resemble the virion spike, although the artificial character of this molecular species may obfuscate the *in vivo* relevance of this observation (17). Finally, the basic character of the region encompassing the disulfides at 378-445 and 385-

418, which are close to CD4-binding sites in gp120 domains C3 and C4 (see Fig. 9, cluster 3), also suggests that these gp120 disulfides display features consistent with being potential PDI substrates (105).

2. Disulfide reduction and changes within the gp41 domain. How conformational changes in gp120 activate the fusogenicity of gp41 is unknown, but a relation between reduction of gp120 by PDI, the acquisition of the active six-helix bundle conformation of gp41, and the subsequent triggering of gp41-mediated fusion has been demonstrated (30, 131, 135, 140). A role for the disulfide bond within gp41 and its association with gp120 has also been described (129), and changes in the gp120 redox state may have an indirect consequence for gp41 disulfide bond, leading to its reduction and the loss of contact, manifest as shedding (144) of the gp120 subunit.

Some experiments have focused on the effect of DTNB on the activation of gp41 fusogenicity, measured by using a monoclonal antibody specific for the six-helix bundle conformation of gp41 (131, 135, 140). The failure of this antibody to bind gp41 in the presence of DTNB suggests that PDI-induced redox shuffling in gp120 is a necessary component that allows formation of the fusogenic conformation of gp41, and further, that Env reduction occurs before the activation of gp41 (135). The data provide a direct link between the enzymatic action of cell surface–associated PDI and gp41 fusogenicity and strongly suggest that the PDI-induced reduction of gp120 disulfide bonds is the key molecular event that triggers HIV-1 entry. They further suggest that PDI-induced refolding of gp120 is transmitted, directly or indirectly, to the gp41 subunit.

3. Disulfide reduction and gp120 shedding. Shedding of the gp120 subunit occurs after Env binding to the (co)receptors and before fusion (144). The gp120 molecule is primarily responsible for receptor binding and, once completed, must disengage from gp41 to allow its action (144). Proteolytic processing of gp120, such as that described for the V3 loop, may act to facilitate gp120 separation from gp41. Cleavage of gp120 has been observed by a variety of proteases: serine esterase TL2, cathepsin E-like and thrombin-like proteinases, and dipeptidyl peptidase type IV (27, 36, 100). Both the V3 loop and the C-terminal domain of gp120 (7, 51) show unambiguous cleavage sites for processing by endoproteases such as thrombin and cathepsin (27, 36, 100). The role of gp120 reduction may be similar, in that the unfolding of Env that follows reduction may facilitate endoproteolytic cleavage by exposure of sensitive sequences, as has been demonstrated for V3 and the C-terminus of gp120.

Such a hypothesis is consistent with a model drawn for another cell-surface glycoprotein, L-selectin, an adhesion-receptor protein associated with the surface of vascular cells that regulates leukocytes adhesion (13, 108). L-selectin is associated with the cell membrane of leukocytes in a particular conformation, which renders it impervious to proteolytic degradation. The maintenance of this conformation requires the reducing activity of PDI. On activation of leukocytes, L-selectin displays conformational changes that lead to cleavage of the extracellular moiety of the protein and a loss of the adhesive properties of the cell. The observed changes in conformation and L-selectin cleavage can be also induced by inhibitors of PDI (13, 108). Thus, maintenance of the proteolytic-resistant conformation requires the reducing activity of PDI, and L-selectin degradation is triggered by redox changes within the surface antigen (13, 108). Reduction by cell-surface PDI has also been found to be positively associated with the shedding of the thyroid-stimulating hormone receptor from the thyrocyte surface, so regulating the physiologic expression of the receptor. In this case, cleavage of disulfide bonds between the transmembrane beta subunit of the receptor and the external alpha subunit releases the latter in the extracellular medium or in the bloodstream (39) in a manner that parallels the gp120 shedding process.

In conclusion, the literature shows that a lymphocyte surface–associated PDI-related reductase activity assists the cleavage of disulfide bonds within gp120 after CD4 binding and is obligatory for triggering the membrane-fusion process. This strongly suggests that disruption of the Env disulfide network serves as the basis for post–receptor-binding conformational changes that are known to be required for the acquisition of fusion competence. The current challenge is to determine which clusters of bonds are reduced to bring about such conformational changes.

III. REDOX AND SARS-CoV

That cell surface–associated PDI can be recruited into viral fusion junctions and that the energy released on reduction can be beneficially used to drive the fusion mechanism suggests that it may be widely associated with virus entry. It is of interest, therefore, to compare and contrast the situation outlined for HIV with that of other enveloped viruses that must also fuse viral and plasma surface membranes to begin the formal replication cycle.

Coronaviruses are large, enveloped, positive-strand RNA viruses that infect a variety of mammalian and avian species and can cause upper respiratory, gastrointestinal, and central nervous system diseases (109, 110). Among them, SARS-CoV is the agent responsible for the severe acute respiratory syndrome (SARS), a major pulmonary infection characterized by an atypical pneumonia that results in progressive respiratory failure and death in ~10% of infected individuals (48, 61, 84, 104, 155, 167). The virus isolated from patients during the 2002–2003 epidemic, and also from milder sporadic cases in 2003 to 2004, appears to derive from a nearly identical virus circulating in palm civets and raccoon dogs in Asia (70, 178). These are unlikely to be the primary sources, however, and although the precise natural reservoir of the virus is unclear, the horseshoe bat has been suggested to be the main agent of dissemination to wildlife (163). The virus spread worldwide between 2002 and 2003, although most cases were restricted to China because of the containment put in place at other infected regions. The pathogen has the potential to reappear, however, if one considers the cyclic pattern displayed by other human-infective coronaviruses that attack in winter, sometimes skipping years (109, 110).

The conformational rearrangements of viral envelope glycoproteins during cell interaction and before virus entry, in part by redox reshuffling (see earlier), also affect some coronaviruses. For instance, DTNB blocks mouse hepatitis virus (MHV) entry by reaction with a free sulfhydryl in its S2 sub-

unit, whereas chemical reduction was found to enhance structural transitions associated with induction of the fusion process (63). Accordingly, the redox status of the SARS-CoV envelope protein and its evolution during the course of the virus-entry process were examined early after the discovery of the virus to assess whether the same was true.

A. The SARS-CoV S-glycoprotein

The SARS-CoV spike (S) protein is another type I viral fusion protein, and in the same way that the HIV envelope complex initiates virus entry into cells by binding to cell-surface receptors followed by membrane fusion and delivery of the genome to the cytoplasm, the S glycoprotein mediates virus binding to host cells and membrane fusion. However, whereas HIV entry occurs at the cell surface, the viral entry of coronavirus into cells could involve fusion of the viral envelope with either the plasma membrane or endosomal membranes, and the mode used by SARS-CoV remains unclear (109, 110, 180).

The spike protein is a major target for neutralizing antibodies, and the entry of SARS-CoV into cells can be inhibited by antibodies against S (18, 75, 182, 189). Sera from convalescent SARS patients neutralize infection and anti-S antibodies play an important role in virus neutralization *in vivo* (80, 185). Protective antibody responses can be elicited *in vivo* by using the full-length S glycoprotein, soluble fragments, including the whole ectodomain, and fragments containing only the receptor-binding domain (18, 197). Immunization of mice with S also elicits neutralizing antibodies that protect animals from infection (18), and a human monoclonal antibody that competes with the major SARS-CoV cell-surface receptor for binding to the S glycoprotein potently inhibits membrane fusion at nanomolar concentrations (181). These observations suggest that, in sharp contrast to the situation in HIV, a subunit vaccine that includes the S protein receptor-binding domain may protect from a new epidemic.

The S glycoprotein forms the characteristic corona of large, distinctive spikes in the viral envelope, 20-nm surface projections around the periphery of the virions (195, 196). The protein has been directly visualized by cryoelectron microscopy, revealing a wedge-shaped trimer with the receptor-binding domain slightly offset at the tip of the projection (12, 149). The S protein migrates at ~180–200 kDa on SDS gels, consistent with posttranslational modifications such as glycosylation (18, 195). According to the functional structure of both Env and HA mature proteins, the S molecule can be split in two component parts, S1 and S2, although it is not cleaved into two polypeptide chains after biosynthesis (see Fig. 4). Rather, S1 and S2 designation is on the basis of sequence alignment with other coronavirus spike proteins, some of which are cleaved into their component parts.

In common with other enveloped viruses, SARS-CoV entry into target cells involves two major steps [*i.e.*, binding to a receptor associated with the surface of target cells and fusion of the virus and cell membranes (195, 196)]. The S1 domain represents the globular region of the protein distal to the virus membrane, whereas the S2 domain forms the stalk.

S1 has been shown to mediate high-affinity (nanomolar) binding with the primary receptor, angiotensin-converting enzyme 2, ACE2 (29, 116, 117, 118, 145, 181), and the affinity of S binding to different species of ACE2, human or other species, may correlate with the adaptation of the virus to humans (118, 119). Similar to the interaction between HIV-1 and its primary receptor, CD4, binding of S to ACE2 anchors the virus some distance from the cell surface, and it is likely that ACE2 docking induces conformational changes required for membrane fusion. Additional membrane receptors at the target cell surface are L-SIGN (92) and L-SECtin (68), although these are thought to provide localization of the virus to the cell surface but not necessarily cell entry.

The S2 region of S includes a transmembrane domain and functions to anchor the protein in the viral membrane (see Fig. 4). It also contains the heptad repeats that mark S as a member of the type I fusion proteins and function similarly to those of gp41 and HA2 to trigger the virus–cell membrane fusion process (20, 196, 203). SARS-CoV exhibits sequence originality because the level of overall sequence similarity between the predicted amino acid sequence of the SARS-CoV S glycoprotein and the spike proteins of other coronaviruses is low (<30% pairwise amino acid identity) except for regions of the S2 subunit, including the heptad repeats (196). It exhibits additional particular characteristics compared with other members of the three previously defined genetic and serologic coronavirus groups, and it does not use any previously identified coronavirus receptors, although the newly described human coronavirus NL63 also uses ACE2 as receptor (81). Whereas the S proteins of several avian and mammalian coronaviruses, such as that of the mouse hepatitis virus, are cleaved by furin or a related protease into S1 and S2 during their biosynthetic pathway (109), the surface glycoproteins of coronaviruses, such as the human coronavirus 229E and SARS-CoV, are not endoproteolytically processed (195, 196). By alignment, however, the deduced S1 and S2 regions of the SARS-CoV spike contain 666 and 583 amino acid residues, respectively. Because cleavage of class I fusion proteins such as those of HIV, influenza virus, and Ebola virus is required to expose an N-terminal fusogenic sequence composing the fusion peptide (51, 177) (see Fig. 4), the lack of cleavage of the S protein of some coronaviruses is remarkable (109, 110). Despite this difference, however, the location of the two heptad repeat peptides from the N- and C-terminal regions of S2 (20, 203) suggests that the formation of the typical six-helix bundle structures important for the fusion process and that the postfusion conformation of S is likely to have the trimer of hairpins organization characteristic of B class 1 fusion proteins (21).

B. S and disulfides

Cells expressing recombinant SARS-CoV S fuse with receptor-expressing cells at neutral pH (145, 195), suggesting that the recombinant glycoprotein is functional, that its membrane fusogenic activity does not require other viral proteins, and that low pH is not required for triggering membrane fusion. Large fragments of the S sequence can be removed without effect on the binding capacity, and polypeptide fragments containing the N-terminal amino acid residues 17–537 and 272–537, but not 17–276, specifically bind Vero E6 cells (5, 195). Further mapping has delineated the binding domain to an independently functional fragment of 193 residues (194) through sequential deletion of the S1 sequence at the N and C termini to yield a construct expressing only residues 318–510 that retains efficient binding to soluble ACE2 (Fig. 10). Slightly smaller frag-

mfifllfltl tsgsdldrCt tfddvqapny tqhtssmrgv yypdeifrsd
tlyltqdlfl pfysnvtgfh tinhtfgnpv ipfkdgiyfa ateksnvvrg
wvfgstmnnk sqsviiinns tnvviraCnf elCdnpffav skpmgtqtht
mifdnafnCt feyisdafsl dvseksgnfk hlrefvfknk dgflyvykgy
qpidvvrdlp sgfntlkpif klplginitn frailtafsp aqdiwgtsaa
ayfvgylkpt tfmlkydeng titdavdCsq nplaelkCsv ksfeidkgiy
qtsnfrvvps gdvvrfpnit nlCpfgevfn atkfpsvyaw erkkisnCva
dysvlynstf fstfkCygvs atklndlCfs nvyadsfvvk gddvrqiapg
qtgviadyny klpddfmgCv lawntrnida tstgnynyky rylrhgklrp
ferdisnvpf spdgkpCtpp alnCywplnd ygfytttgig yqpyrvvvls
fellnapatv Cgpklstdli knqCvnfnfn gltgtgvltp sskrfqpfqq
fgrdvsdftd svrdpktsei ldispCsfgg vsvitpgtna ssevavlyqd
vnCtdvstai hadqltpawr iystgnnvfq tqagCligae hvdtsyeCdi
pigagiCasy htvsllrsts qk

C Cys residue

C Cys residue required for ACE-2 binding

C Cys residue required for expression

C Cys residue not required for ACE-2 binding or expression

Bold sequence: minimal ACE-2 receptor binding domain

FIG. 10. Salient features of the SARS-CoV S1 sequence. Shown are the positions of the Cys residues and their importance for protein expression or ACE2 binding, as assessed by mutagenesis studies (29, 191). The region of S1 responsible for ACE2 binding is highlighted.

ments did not bind ACE2 (194). Interestingly, the 318–510 residue variant of S1 binds ACE2 at least twice as efficiently as the full-length S1 domain. It was also able to block S protein–mediated infection with an IC_{50} that was fivefold lower than S1 (194). The higher affinity of the 193-residue fragment raises the possibility either that other regions of the S protein partially mask or destabilize the receptor-binding domain or that the short receptor-binding domain may fold more efficiently than the membrane-bound native S presented within the trimeric complex. Because the 193-amino acid receptor-binding region quite efficiently blocked S protein–mediated infection of ACE2–expressing cells, this fragment may represent a defined fragment for development of therapeutics that block SARS-CoV infection and a substrate for functional studies. Additional delineation of the binding domain using the expression of a number of variants with single point mutations around Asp 454 showed altered binding to ACE2, suggesting that ACE2 interacts with the SARS-CoV S domain in the vicinity of this residue (194). This result is consistent with the data obtained from S gene sequencing from civet and human specimens, which has shown that their receptor-binding domains differ at only four positions (97), two of which (residues 479 and 487) are in the minimal binding fragment. Human SARS-CoV viral spike protein binds the human receptor three orders of magnitude more tightly than does its civet spike counterpart (70, 116, 118, 178), and it is probable that changes in just a few residues in this domain lead to efficient cross-species transmission.

The importance of the cysteine residues within the short 193-amino-acid fragment has been examined (see Fig. 10). Because it contains an odd number of cysteine residues (194), it is likely that at least one Cys residue in S is not involved in disulfide bonding. A series of point mutations was made in which each of the seven cysteines within 318–510 was altered to alanine. The variant in which cysteine 323 was mutated bound ACE2 as efficiently as the 318–510 fragment itself. Alteration of cysteine 378 also had only marginal effects on binding (194). Complementation of these two Cys residues might occur, however, because combination of mutations at residues 323 and 378 altered ACE2 binding. Other cysteine residues were shown to be required either for S folding or ACE2 binding: alteration of cysteine 366 or 419 inhibited expression of the antigen, and alterations of cysteines 348, 467, and 474 prevented recognition by ACE2 without alteration of expression. Thus, the minimum binding domain of S, the 193-amino-acid fragment that retains receptor binding, includes seven cysteines, five of which are essential for expression or ACE2 association (194).

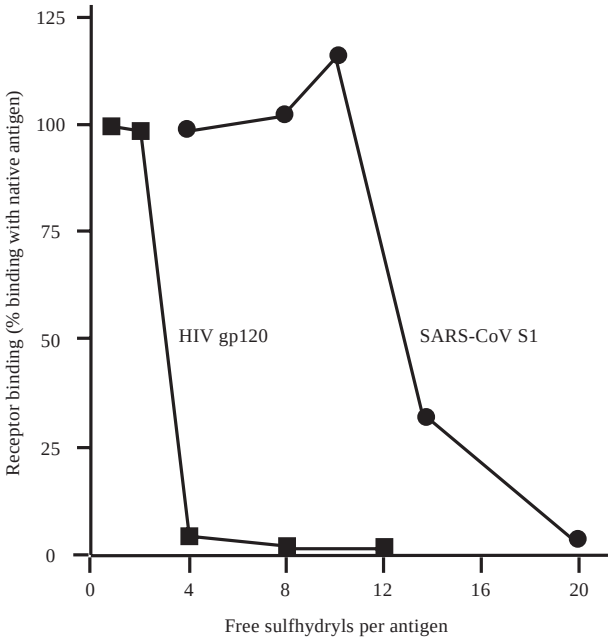


FIG. 11. Relation between the thiol content of HIV gp120 or SARS-CoV S1 and their capacity to bind their primary receptors. Each protein domain was reduced by using increasing concentrations of β-mercaptoethanol, and the thiol content was then determined, as described (8). Each sample of SARS-CoV S1 was assessed for ACE2 binding by using an enzyme-linked immunosorbent assay (ELISA) based on immobilized ACE2, whereas each gp120 sample was assessed for CD4 binding by using a solid-phase assay based on [^{125}I]-sCD4 binding.

As another type I fusion protein, the resistance to chemical reduction and the dependency of SARS-CoV entry on S reduction by either an endogenous oxidoreductase activity supported by an S motif like the HTLV model (190) or an exogenous activity such as PDI as in the HIV model (9, 169) were investigated. First, the involvement in disulfide bond formation of the 20 cysteine residues present on a mature, recombinant form of S1 was examined. A recombinant glycoprotein consisting of the S1 domain fused with the Fc domain of IgG was shown to possess an average of ~4 of its 20 cysteine residues as unpaired cysteines (112). The sensitivity of S1 to chemical reduction by β-mercaptoethanol was studied and shown to be weak. A reduced-carboxymethylated form of S1 displaying about half of its cysteine residues as unpaired residues (*i.e.,* a total of ~10 SH per antigen) could bind ACE2, whereas a specific interaction remained detectable even when three fourths of its cysteine residues were present as unpaired residues (112) (Fig. 11). These data, obtained *in vitro* by using purified recombinant protein, were confirmed by using infection assays addressing the S-mediated entry into ACE2-expressing VeroE6 cells of an MLV virus pseudotyped with S and carrying the gene encoding the green fluorescent protein. As the retrovirus integrates the marker gene into the genome of the cell after successful cell entry, the number of cells expressing green fluorescent protein provides an accurate and facile measure of the efficiency of S-mediated membrane fusion. With this system, an S antigen exhibiting half of its cysteine residues as unpaired residues was shown to mediate efficiently the entry process, whereas an S antigen exhibiting three fourths of its cysteine residues as unpaired residues failed to enter (112). Together, the data obtained from *in vitro* and *in vivo* experimentation led to the conclusion that both S binding to ACE2 and the fusion capacity of the spike complex are significantly insensitive to reduction, a singular contrast to the situation described for HIV and suggested for a number of other enveloped viruses.

The finding that four unpaired cysteine residues are present on native S1 and three further disulfides are dispensable for ACE2 binding (Figs. 10 and 11) may indicate that these residues have no role in the function of mature S1, other than their role in the folding of S during its normal biosynthetic pathway. Alternatively, this finding may indicate that the four unpaired cysteines and the three dispensable disulfides act as donor/acceptor as part of redox reactions that may occur within the spike complex or between the complex and cell-surface proteins to activate fusogenicity. In this sense, the S protein would be "preactivated," and free sulfhydryls would contribute to entry, as in the case of HTLV entry. To address this question, the effect of the presence of DTNB and bacitracin in the cell environment during the interaction between the S-pseudotyped viruses and VeroE6 cells was examined. Neither the thiol reagent nor the PDI inhibitor affected infection, indicating that unpaired cysteines and redox shuffling within S or cell proteins or both have no role during entry (112). The capacity of the S1–S2 complex to fulfil the conformation changes inferred by the presence of the heptad repeats and trigger fusion competence independent of cell-surface catalysts or endogenous redox isomerase confirms the innate instability of the S disulfides, as shown by the high tolerance to chemical reduction of the disulfide bonds of S (112) when compared with those of HIV Env (9). Such independence toward redox changes may be also part of the reason for the wide host range of the virus and could have contributed to the propensity of the virus for the zoonosis that led to human infection (34, 70, 97, 137, 158, 178).

The lack of effect of blocking the free sulfhydryls of the SARS-CoV envelope protein by using DTNB raises the question of the functions of the unpaired cysteine residues and indicates that they are unlikely to have a functional role as part of a virus–cell interaction process, at least not as donor or acceptor during redox shuffling reactions involving an endogenous redox catalytic motif, such as in the HTLV model. However, it is conceivable that such unpaired cysteine residues have been available for the creation of additional bonding at some stage of envelope evolution and that such plasticity may have contributed to the capacity of this coronavirus to infect many species and adapt to human cell-surface ligands (34, 70, 97, 137, 158, 178).

Together, these results further highlight the differences that exist between the characteristics of the envelopes of SARS-CoV and other enveloped viruses (see Fig. 4). In particular, they illustrate the unusual incomplete complement of cysteine residues of mature S in disulfides and the lack of redox changes within SARS CoV-S and cell-surface molecules during the virus-entry process.

Interestingly, it appears that cysteine residues other than those of the outer membrane domain of S are involved in fusion. In a study by Petit and colleagues (156), the four cysteine-rich amino acid clusters of the cytoplasmic portion of the S glycoprotein have been individually mutated, and transport to cell-surface and fusogenicity studied. The two Cys clusters located proximal to the predicted transmembrane were shown to

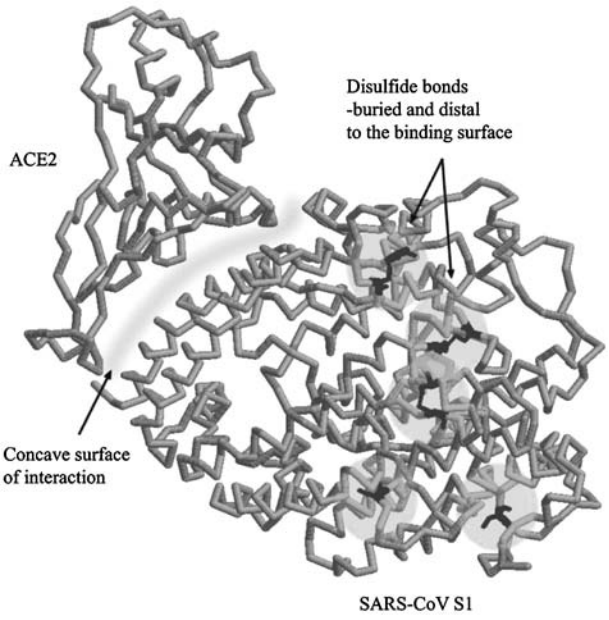


FIG. 12. The SARS-CoV receptor interaction as revealed by the solved structure of human ACE2 residues 323-502 and SARS S1 residues 19-615 (116). The disulfide bonds within the S1 fragment are indicated, as is the broad concave surface of interaction between the molecules. Note that the region of interaction within S1 is a distinct helix not directly affected by any of the disulfides highlighted.

be involved in fusion, probably *via* their palmitoylation, as suggested by their experimental data.

A possible rationale for the low sensitivity of the outer-membrane portion of S toward chemical reduction (112), hence the independence of ACE2 binding on disulfide bond integrity, and the finding that mutagenesis of several amino-acid residues only marginally affects receptor binding (194) can be obtained from the crystallography studies of the spike-receptor complex (116). The latter indicates that the SARS-CoV S protein contacts the tip of one lobe of ACE2 distal to the peptidase active site. The receptor-binding domain contains two subdomains: a core and an extended loop. In the core, disulfide bonds connect cysteines 323 to 348, 366 to 419, and 467 to 474 (116). The remaining cysteines are disordered, but two of them (C378 and C511) are in the same neighborhood and could form a disulfide in the recombinant fragment, even if they have other partners in the native S protein. The extended loop subdomain lies at one edge of the core and presents a concave outer surface. It comprises the Cys467–Cys474 disulfide bridge and contacts ACE2 directly. Crystallographic data also indicate that residues 424 to 494 belong to the receptor-binding loop that makes all the contacts with the complementary domain on ACE2, 18 residues of the receptor contacting 14 residues of the viral spike protein (116) (Fig. 12). Thus, the key residues involved in the contacts between the concave S1 and the convex ACE2 surfaces are very few compared with the overall size of the molecules involved and contain only two cysteine residues (C467 and C474) within the receptor-binding motif. Although one can consider that most of the Cys residues of the receptor-binding domain are probably required for intracellular folding, it is possible that only the C467 and C474 residues are of paramount importance on the mature, folded antigen for receptor binding.

C. Treatment

Because both the binding and the fusion steps are crucial for SARS-CoV infection, their characteristics were examined in detail with regard to possible therapy. The number of antiviral drugs, directed mainly against HIV (see earlier), is steadily growing, but, in addition to this long-term development, the SARS epidemic emphasized the need to be able to develop drugs quickly against emerging viral infections.

Inhibitors of three viral enzymes of SARS-CoV [*i.e.*, the helicase (98), the RNA-dependent RNA polymerase (32) and the main 3C like protease (120)] are under development. Fighting the virus by using a vaccine approach directed against S has also been investigated (18) and appears promising, because an efficient immune response develops after human infection and seems to be able to protect from reinfection (45). Treatment of infection by using inhibitors of SARS-CoV entry has also been considered. Binding to the viral receptors is the first target step. Approaches to date have included the use of a soluble form of ACE2 as a receptor decoy (117) and an antibody recognizing S (181, 182, 189). It has been shown that the 193-amino-acid fragment that corresponds to the receptor-binding domain and efficiently binds ACE2, acts against entry, with an IC_{50} in the nanomolar range (194). Studies of the disulfide requirement of S also have a role to play, in that they indicate that engineering of cysteine residues, for example, to improve immune presentation or S stability, may not adversely affect the biologic conformation of the protein (112, 116), enabling a further restriction of the length of a peptide sequence that can efficiently interfere with S binding to the target-cell surface. The remaining second target step of therapeutic attack is represented by the fusion step, which occurs within 15 min of receptor binding (150). This window of time is sufficient for the use of fusion inhibitors, and the similarity of the NHR and CHR region in the SARS-CoV S2 domain with those found in gp41 of HIV suggests that peptides corresponding to the C-HR of S2 may act similar to T20 in HIV (20, 203). A fuller description of the coronavirus-fusion reaction, including the structures of larger S–ACE-2 complexes, will inform the development of such compounds in the future and may also provide a molecular explanation for the remarkable insensitivity of S to reduction.

IV. CONCLUSIONS

The iconic disulfide bond has been a fundamental subject of biochemistry for many years and has been shown to be the key to the biologic activity of a number of molecules (antibodies, for example) that we take for granted. In recent years, however, the range of molecules shown to exhibit plasticity in their disulfide bond pattern has grown, with an increasing number of observations that the enzymes that control bond formation during biosynthesis, such as PDI, are more widely distributed than was originally thought. It follows that the range of biologic activities associated with bond formation and reduction has also grown and that the flexibility afforded by bond breaking is made use of by processes that were hitherto thought to function alone. Viral envelope glycoproteins, which must necessarily undergo considerable conformational change as part of their function, have provided a particular example of where disulfide bond reduction is recruited by the virus to gain cell entry. The findings are not universal, however, and whereas ample evidence exists for the involvement of oxidoreductase activities in the entry of some viruses, others seem relatively immune to redox change or even the integrity of a large part of their disulfide network. It is particularly intriguing that these differences can be found among viruses that appear to fuse viral and cellular membranes by essentially similar mechanisms. It may be that the size, innate flexibility, or evolutionary histories of viral envelopes distinguish those that make use of oxidoreductase activities and those that do not. What is clear, however, is that the number of examples of redox being involved in fusion by enveloped viruses continues to grow and that the interest it generates will inform a deeper level of understanding of the fusion mechanisms and a greater range of possibilities for therapeutic intervention, chemical or antibody based, to block this most crucial of steps in the infectious cycle, entry into the susceptible cell.

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ABBREVIATIONS

ACE2, angiotensin-converting enzyme 2; aPAO, *para*-aminophenylarsine oxide; AT3, the acetylated form of triiodothyronine; biotin-BMCC, (1-biotinamido)-4-[4′-(maleimidomethyl) cyclohexanecarboxamido]hexane; CCR5, chemokine CC motif receptor 5; CD4, cluster of differentiation number 4; CXCR4, chemokine CXC motif receptor 4; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); Env, envelope glycoprotein; gp120, surface glycoprotein of HIV-1; gp41, transmembrane glycoprotein of HIV-1; GSAO, glutathione arsenoxide; HA, hemagglutinin protein; HA1, the receptor-binding domain of HA; HA2, the membrane-fusion domain of HA; HIV, human immunodeficiency virus; HR, heptad repeats; HTLV, human T-leukemia virus; L-SECtin, lymph node sinusoidal endothelial cell C-type lectin; L-SIGN, liver/lymph node–specific intercellular adhesion molecule-3-grabbing integrin; LTR, long terminal repeat; MLV, murine leukemia virus; MHV, mouse hepatitis virus; MPB, 3-(*N*-maleimidylpropionyl)biocytin; MPDOD, biotinyl 3-maleimido propionamidyl-3-6-dioxaoctane diamine; PDI, protein disulfide isomerase; S1, receptor-binding domain of the SARS-CoV envelope glycoprotein; S2, fusion domain of the SARS-CoV envelope glycoprotein; SARS-CoV, severe acute respiratory syndrome coronavirus; SDF-1, stromal cell–derived factor 1; V3, third highly variable domain of gp120.

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