Chapter 13.

Sulfhydryl Involvement in Fusion Mechanisms

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1. PROTEIN THIOLS—AN INTRODUCTION

1.1. Cysteine—A Special Residue

At first one might wonder about the wisdom of focusing on the role of a single type of amino-acid residue in such a central biological process as protein-mediated membrane fusion. Indeed, an initial response might consist of the words of the daughters of Jerusalem, "What is thy beloved more than another beloved? [Song of Songs 5:91" There are excellent reasons, however, for acknowledging the special roles that cysteine residues play in protein-mediated membrane fusion. One important distinguishing feature is their sensitivity to the oxidation/reduction potential of their environment. It would not be inaccurate to state that the major difference between proteins that are within the cytoplasm and those that are topologically outside of the cell (this includes proteins and domains of proteins that are located in the lumens of eukaryotic secretory organelles or the periplasms of Gram-negative eubacteria) is that the cytoplasmic cysteines

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possess thiols (except in the few cases when the thiol is posttranslationally modified), whereas the extracellular cysteines are found predominantly in cystine bridges.

Another important characteristic of cysteine is that its sulfhydryl group (a term that will be used interchangeably with thiol) is commonly the most reactive protein functional group.The sulfur atom is quite nucleophilic and therefore can be modified by a number of reagents (Lundblad, 1991). It is therefore relatively straightforward to probe the roles of cysteine residues in the function of a given protein or process. In addition, cysteine residues are highly conserved in protein sequences virtually exclusively because of the reactive sulfur. The interpretation of mutagenesis experiments in which conserved cysteine residues have been replaced is greatly simplified by the knowledge that it is unlikely that the cysteine is participating in some unique way in secondary-structure or hydrophobic-core formation. Finally, our interest is justified by existing data on the special roles of thiols and disulfides and their interchange in protein-mediated membrane fusion.

1.2. Oxidation and Reduction—Environment and Enzymatic Catalysis

From the start one must recognize that an evaluation of the roles of cysteine residues in membrane fusion requires a distinction between two separate problems, membrane fusion in a reducing environment and membrane fusion in an oxidizing environment. Proteins that are likely to promote membrane fusion during intracellular vesicular transport in eukaryotic cells reside in the reducing environment of the cytoplasm, whereas those participating in the promotion of membrane fusion between enveloped viruses and cells or between two or more cells (sperm-egg fusion, syncytium formation resulting from fusion of myoblasts into myotubes, etc.) are exposed to an oxidizing milieu. Therefore, the functions played by cysteine residues under the two conditions and the analytical techniques used to investigate them may be quite different.

A clear example of this principle is that the formation, rearrangement, and reduction of disulfide bonds are the most common events involving cysteine residues in extracellular proteins or protein domains, whereas they are uncommon in cytoplasmic proteins. Sequence analyses and residue-substitution experiments with membrane-bound or extracellular proteins must therefore focus on pairs of cysteine residues.The cystine bridges formed from these pairs are frequently critical to the integrity of the folded protein in the extracellular and secretory organelle lumenal environment. Incompletely folded or unstable proteins tend to be processed inefficiently or are retained intracellularly and degraded by the secretory system (Hammond

and Helenius, 1995). Determination of the specific role of a particular disulfide linkage may therefore not be amenable to mutational analysis.

In addition, the elimination of just one of a pair of cysteine residues that form a disulfide in the wild-type protein would leave a free thiol. Exposed free thiols in an extracellular protein domain generally result in retention of the protein in the endoplasmic reticulum (Fra *et al.,* 1993; Isidoro *et al.,* 1996; Lodish and Kong, 1993; Reddy *et al.,* 1996). This is an important aspect of the protein-folding quality control that the secretory system imposes on proteins that enter it. The molecular logic is the following: Virtually all cysteine residues in extracellular domains are present in disulfide bonds. Disulfide-bond formation does not, however, dictate the folded conformation of a protein. The cystine bridges present in the mature protein form between cysteine residues that are brought into juxtaposition by the same thermodynamic forces that promote the proper folding of cytoplasmic proteins. Therefore, the formation of disulfide bonds and the consequent removal of free thiols from the protein surface is an excellent indication that the protein has reached its final folded state and is thus prepared to proceed to its ultimate destination.

There are three points relating to the foregoing that are worth addressing and that are relevant to the discussion of the roles of thiols in protein-mediated membrane fusion. First, although disulfide-bond formation between two cysteine residues can occur spontaneously under oxidizing conditions, thiol-disulfide exchange enzymes catalyze the formation and reduction of cystine bridges in proteins that are topologically extracellular. These enzymes are referred to as protein disulfide isomerases in eukaryotes and Dsb proteins in prokaryotes (Bardwell, 1994; Freedman *et al.,* 1994). Whereas catalysis of disulfide-bond formation occurs during the process of protein exit from a cell, disulfide-bond reduction appears to be required during the entrance of certain proteins into a cell.

A consideration of some examples of disulfide-bond reduction during entry possesses relevance for our treatment of the role of disulfides in membrane fusion. Evidence has been presented for the reduction of protein disulfides during processing of antigens, such as insulin, that are taken up into cells and presented by MHC class II molecules (Jensen, 1991). Studies of the entry of toxins into cells provide additional examples. Cholera toxin consists of two subunits (A and B). The A subunit is cleaved into two fragments (A1 and A2) linked by disulfide bonds. Cholera toxin is trafficked by retrograde transport through the secretory system to the endoplasmic reticulum where the reduction of the disulfide bonds takes place. As a result the A1 fragment, which possesses the ADP-ribosyltransferase activity of the toxin, is released (Majoul *et al.,* 1996). Ricin and diphtheria toxin consist of two subunits that are linked by interchain disulfide bonds. Reduction of the

disulfide bond appears to be a rate-limiting step in membrane penetration by both toxins (Lewis and Youle, 1986; Papini *et al.,* 1993)). The cytotoxic effects of exposing cells to diphtheria-toxin, but not ricin, can be prevented through incubation of the cells with reagents, such as 5,5′-dithiobis-(2-nitrobenzoic) acid and p-chloromercuriphenylsulfonic acid, that inhibit cell-surface protein-disulfide-isomerase activity (Ryser *et al.,* 1991). It is thus evident that such enzymatic activities need to be taken into consideration when evaluating the oxidation state of cysteines in membrane-fusion-promoting proteins.

The second point is that disulfide-bond formation between cysteine residues that are not linked in the final folded structure can occur during the process of protein folding. Normally, this would result both in the temporary stabilization of a partially folded or misfolded state of the protein and in the presence on the protein surface of free thiols pertaining to the two cysteine residues whose partners in the final folded state are for the moment linked in an unsanctioned union. A eukaryotic protein bearing aberrant disulfide bond(s) would tend to be retained in the endoplasmic reticulum where protein-disulfide-isomerase could reduce the cystine bridge. Refolding of the proteins could then take place, and the appropriate disulfide linkages could consequently be made. The probable reasons that there is not an infinite cycle of oxidation and reduction for the correct disulfide bonds are that the folded proteins rapidly exit from the compartment where the protein disulfide isomerase is localized and that a disulfide bond is more hydrophobic than are a pair of thiols, so once it has formed, and the tertiary and quaternary structure of the protein are stabilized, the cystine bridge is unlikely to remain exposed to the protein disulfide isomerase.

Finally, unpaired cysteines in extracellular protein domains are rare but they do exist (Reddy *et al.,* 1996). In almost all cases they are not absolutely conserved among homologous proteins and virtually all of those that have been investigated are not exposed on the surface. One of the rare exceptions to the rule about the absence of conservation of an odd number of cysteine residues in the extracellular domains of a homologous family of proteins will be considered below. It is the very rarity of such an anomaly that contributed to the recognition of its importance in a class of proteins that promotes membrane fusion.

1.3. Thiol and Disulfide Modification Reagents

Part of our appreciation of the roles of thiols in membrane fusion arises from studies involving the chemical modification of protein cysteine residues. A thorough comprehension of the implications of such studies can

only be achieved through an in-depth understanding of the reactions involved. It is the goal of the following brief discussion to familiarize the reader with the chemistry of the commonly used reagents with an emphasis on the nature of the reactive cysteine-containing species, the product of the reaction, and the reversibility of the modification. Technical aspects of the use of the reagents will not be considered.

Cysteine residues differ from most functional groups on a protein in that they can be readily oxidized, alkylated, acylated, cyanylated, or arylated (Lundblad, 1991). It should be noted that the acid dissociation constant (pKa) for an average cysteine residue in a polypeptide is approximately 8.5 and that the thiolate anion is more reactive with modifying reagents than is the free thiol. Cysteine residues in active sites often have lowered pKas and, therefore, increased concentration as the thiolate anion. Consequently, they are frequently more rapidly modified than other cysteine residues.

Among the reagents that have achieved the most widespread use for modification of protein thiols are the α-haloacetic acids, such as iodoacetic acid and chloroacetic acid, and α-haloacetamides, such as iodoacetamide and chloroacetamide (Lundblad, 1991). The product of the reaction of a cysteine residue with iodoacetic acid or iodoacetamide is a stable carboxymethylated or carboxamidomethylated cysteine. It is important to keep in mind that histidine residues can also be carboxymethylated by iodoacetic acid.

Monobromobimane and its derivatives are very useful reagents for fluorescent labeling of proteins containing thiols (Kosower and Kosower, 199%). Nucleophilic attack by the thiolate on the nonfluorescent bromobimane displaces the bromide ion and results in a fluorescently labeled cysteine residue.

Other popular reagents are *N*-ethylmaleimide and its derivatives. Conditions can normally be found where they react specifically with cysteine residues (Lundblad, 1991). Upon acid hydrolysis of a thiol-containing protein modified with *N*-ethylmaleimide, *S*-succinyl cysteine is obtained. Important characteristics distinguishing modifications by the α-haloacetic acids, α-haloacetamides, *N*-ethylmaleimide and maleimide derivatives and those resulting from reactions with some other thiol reagents are that the former modifications are essentially irreversible and that the reagents cannot catalyze disulfide-bond formation.

In contrast, the modification of a cysteine residue with 5,5′-dithiobis-(2-nitrobenzoic) acid, which leads to the formation of a mixed disulfide in which the cysteine is linked to a 2-mercapto-5-nitrobenzoic acid, can be readily reversed by addition of a reducing reagent such as dithiothreitol and can lead to formation of a protein cystine (Lundblad, 1991).*N*-succinimidyl

3-(2-pyridyldithio)propionate (SPDP) is a heterobifunctional reagent, whose hydroxysuccinimide ester reacts with amino groups to form an amide bond and whose 2-pyridyldisulfide group reacts with thiols to form a disulfide (Jou *et al.*, 1983). Through use of SPDP one can link a molecule that contains an amino group to a protein thiol.

Other useful reagents include 2-nitro-5-thiocyanobenzoic acid, which can cyanylate cysteine residues, and organic mercurials, such as mersalyl acid (*o*-[(3-Hydroxymercuri-2-methoxypropyl)carbamoyl]phenoxyacetic acid), *p*-chloromercuribenzoic acid and *p*-chloromercuriphenylsulfonic acid, which can bond to the cysteine sulfur atom through the mercury atom (Lundblad, 1991). Of particular interest are the facts that peptides can be cleaved at *S*-cyano residues under alkaline conditions and that the organomercurials can be displaced by thiol-containing reagents. It is also worth noting that *p*-chloromercuribenzoic acid and *p*-chloromercuriphenylsulfonic acid are converted to the active reagents *p*-hydroxymercuribenzoic acid and *p*-hydroxymercuriphenylsulfonic acid when the solids are dissolved in water.

Aspergillus japonicus produces a potent inhibitor specific for cysteine proteases, E-64, which was determined to be an L-*trans*-*epoxysuc*cinylleucylagmatine derivative (Katunuma and Kominami, 1995). It and other subsequently synthesized L-*trans*-epoxysuccinylpeptides have been shown to form covalent thioether bonds with the active-site cysteines of the proteases. Compounds specific for particular cysteine proteases have been synthesized.

A number of reagents have been used to examine the effects of oxidizing cysteines to form cystine bridges. Sodium periodate has been reported to be a mild agent that oxidizes only vicinal cysteines to disulfides (Rippa *et al.*, 1981). Diamide (diazenedicarboxylic acid bis(*N*,*N*-dimethylamide)) reacts with thiols in two stages (Kosower and Kosower, 1995a). In the first, the thiolate adds to the diazene double bond to form a sulfenylhydrazine, which, in the second step reacts with a second thiolate to yield a disulfide and a hydrazine. Molecular iodine and Cu(II)(1,10-phenanthroline)$_3$ are both used to oxidize cysteine residues to disulfides but have different properties. Iodine is a reactant that promotes rapid oxidation and is consumed during the process, whereas Cu(II)(1,10-phenanthroline)$_3$ is a catalyst that promotes a slower oxidation (Hughson *et al.*, 1997). Sodium tetrathionate is also commonly used to promote the formation of cystine bridges from protein thiols.

Protein disulfides can be reduced by a number of reagents, the most common of which are 2-mercaptoethanol, dithiothreitol, and dithioerythritol. The reagents are not completely interchangeable. Dithiols, such as dithiothreitol, and monothiols, such as 2-mercaptoethanol, are of

comparable effectiveness for reduction of an intermolecular disulfide. However, a dithiol is kinetically superior as a reductant for intramolecular cystine bridges when compared to a monothiol (Gilbert, 1990). This fact may underlie the very different *in vivo* effects of dithiothreitol treatments versus 2-mercaptoethanol treatments upon the secretion from mammalian cells of proteins containing cysteine residues and/or cystine bridges (Valetti and Sitia, 1994).

2. PROTEIN THIOLS IN CELLULAR MEMBRANE FUSION

2.1. Identified Thiol-Reagent-Modified Proteins

2.1.1. *N*-ethylmaleimide-Sensitive Factor-NSF

The most prominent protein bearing a thiol whose modification interferes with cellular membrane-fusion processes is the *N*-ethylmaleimide-sensitive factor (NSF). A complete treatment of the roles of NSF in secretory vesicular transport and its interactions with soluble NSF attachment proteins (SNAPs) and SNAP receptors (SNAREs) is beyond the scope of this review. Numerous recent reviews of the function of these proteins are available (Clague, 1998; Hay and Scheller, 1997; Nichols and Pelham, 1998; Robinson and Martin, 1998; Woodman, 1997). However, consideration of a number of points concerning the effects of *N*-ethylmaleimide treatment upon its function will be instructive. It is also important to be familiar with some of the characteristics of NSF, because in publications that will be discussed below the properties of other membrane-fusion factors are explicitly contrasted with those of NSF.

Biosynthetic transport between Golgi cisternae in cell-free systems is inhibited by treatment with 1 mM *N*-ethylmaleimide and restored by addition of untreated NSF, which appears to be the inactivation target in the membranes. NSF is a hexameric ATPase that plays a central role in many vesicular transport pathways including endoplasmic reticulum-Golgi apparatus transport, transport between Golgi complex cisternae, endosomal vesicle fusion, and transport from the Golgi to specialized membranes in polarized cells (Woodman, 1997). The membrane-fusion-promotion and ATPase activities of purified NSF are inhibited by treatment with 2 mM *N*-ethylmaleimide (Block *et al.,* 1988; Tagaya *et al.,* 1993). NSF forms an ATP-dependent complex with soluble NSF attachment proteins (SNAPs) that in turn interact with SNAP receptors (SNAREs) and, it is believed, thereby induce conformational changes that affect SNARE function (Woodman, 1997). It has been recently suggested that NSF may also act as a molecular

chaperone for other proteins besides those involved in membrane fusion (Haas, 1998). There are two ATPase domains in NSF (Tagaya *et al.,* 1993), referred to as D1 and D2, which differ in affinity for ATP (Matveeva *et al.,* 1997).The low-affinity sites, which are on the D1 domains, appear to be critical for NSF to associate with the SNAP-SNARE complex (Matveeva *et al.,* 1997; Whiteheart *et al.,* 1994).

It has been suggested that a cysteine residue in the β-phosphate binding loop of D1 contains the thiol that reacts with *N*-ethylmaleimide (Tagaya *et al.,* 1993). Consistent with this hypothesis is the fact that the yeast homologue of NSF, Sec18p, has a threonine residue at the corresponding position and is resistant to inactivation by *N*-ethylmaleimide (Steel *et al.,* 1999). However, it would seem prudent if the site of modification were determined directly; *N*-ethylmaleimide treatments that abolish ATPase activity do not appear to inhibit ATP binding, as might have been expected if the modification were in the β-phosphate binding loop (Matveeva et *al.,* 1997).

2.1.2. Calpains

It would appear that the most prominent example of the effects of thiol-reagent treatments upon membrane fusion is a protein whose presumed modified cysteine is not, in fact, essential for function; it is the modification that is inhibitory. Potential targets of thiol-modifying reagents that do possess active-site cysteine residues are the calpains (intracellular, Ca^{2+}-dependent thiol proteases). The possibility of the participation of calpains in various membrane-fusion processes has been investigated. The membrane mobility agent 2-(2-methoxyethoxy)ethyl-*cis*-8-(2-octylcyclopropyl)octanoate(A_2C) promotes fusion of rat erythrocytes in the presence of Ca^{2+}. Cell fusion and the Ca^{2+}-dependent membrane-protein degradation that appears to precede it are prevented by pretreatment of the erythrocytes with *N*-ethylmaleimide or monobromobimane (Glaser and Kosower, 1986). In contrast, there was no effect when the cells were preincubated with pepstatin, phenylmethylsulphonyl fluoride, or 1,10-phenanthroline, which are inhibitors, respectively, of aspartyl proteases, serine proteases, and metalloproteases. Erythrocyte ghosts undergo A_2C and Ca^{2+}-dependent cell fusion and membrane-protein degradation solely when they contain hemolysate. Cell fusion and membrane-protein degradation are not detected with erythrocyte ghosts reconstituted with hemolysate treated with *N*-ethylmaleimide or monobromobimane (Glaser and Kosower, 1986). A partially purified proteolytic activity, that possessed the biochemical and chromatographic characteristics of calpain I, when loaded with Ca^{2+} into the

erythrocyte ghosts could restore cell fusion in the presence of A_2C (Glaser and Kosower, 1986).

The same laboratory has probed the role of calpain in myoblast fusion into multinucleated myotubes. It was demonstrated that whereas the level of calpain polypeptide does not undergo major changes during rat L8 myoblast differentiation and fusion, the level of calpastatin, the endogenous inhibitor of calpain, decreased dramatically during the stages before and during myoblast fusion (Barnoy *et al.,* 1996). Calpain activity was stimulated during this period of time. Again, when calpain activity was inhibited either directly, by inhibitors such as calpeptin or E-64d, or indirectly, through incubation of the myoblasts with TGF-β (which appears to prevent the diminution of calpastatin levels that accompanies myoblast differentiation), protein degradation within the myoblasts and myoblast fusion are inhibited (Barnoy *et al.,* 1997; Barnoy *et al.,* 1998).

2.2. Experimental Systems with Thiol-Reagent- or Disulfide-Reagent-Modified Proteins of Unknown Identity

2.2.1. Frog Neuromuscular Junction

There are numerous systems where protein thiols have been implicated in cellular membrane-fusion events, but the targets of modification have not been identified. In the amphibian neuromuscular junction the thiol-oxidizing agent diamide stimulates the frequency and amplitude size of miniature endplate potentials (transmitter release) in a temperature-dependent fashion (Publicover and Duncan, 1981). The stimulation is reversed by incubation of the frog cutaneous pectoris nerve-muscle preparation with dithioerythritol and does not appear to be acting through effects on mitochondria. The authors conclude that oxidation of protein thiols promotes vesicle-plasma membrane fusion (Publicover and Duncan, 1981).

2.2.2. Mammalian Sperm-Egg Fusion

Mammalian fertilization culminates with the fusion of a spermatazoon that has penetrated the zona pellucida with the plasma membrane of an egg. The effects of the reactive oxygen species, superoxide and hydrogen peroxide, on mouse sperm motility and sperm-egg fusion have been investigated. The reactive oxygen species were generated by addition of xanthine oxidase at various concentrations to hypoxanthine; the higher the concentration of xanthine oxidase the higher the level of the reactive oxygen species. At high levels of these species sperm motility and sperm-egg fusion

were decreased, and lipid peroxidation was increased (Mammoto *et al.,* 1996). Sperm motility was not restored by incubation with 1mM dithiothreitol. At lower levels of reactive oxygen species, sperm-egg fusion was still greatly inhibited, although sperm motility and lipid peroxidation were not perturbed. Preincubation of the sperm with both superoxide dismutase and catalase prevented the inhibition of sperm-egg fusion by the oxygen species, whereas incubation with only one or the other of the reagents had only a partial protective effect. Most significantly, 250 mM dithiothreitiol completely reversed the effects of the xanthine oxidase treatment on sperm-egg fusion (Mammoto *et al.,* 1996).

The effects of treatments with *N*-ethylmaleimide, sodium tetrathionate and 5,5′-dithiobis-(2-nitrobenzoic) acid on sperm-egg fusion have also been investigated. These reagents inhibited fertilization without affecting sperm motility (Mammoto *et al.,* 1997). It was demonstrated that the effect of *N*-ethylmaleimide treatment was not upon penetration of the zona pellucida by the sperm, nor was it upon either sperm-egg binding or the sperm acrosome reaction. Sperm nuclear incorporation into eggs was reduced by the thiol-modifying reagents, so it was concluded that sperm-egg fusion was specifically inhibited (Mammoto *et al.,* 1997). Four additional results were described:

1. Treatment of the eggs with the thiol reagents did not inhibit sperm-egg fusion, so the targets of modification appeared to be in the sperm.
2. The effects of treatment with the oxidizing sodium tetrathionate were reversed by incubation with DTT, suggesting that oxidation of protein thiols was involved in the inhibition of sperm-egg fusion.
3. The membrane-impermeant thiol reagent, eosin-5-maleimide, failed to inhibit sperm-egg fusion, suggesting that the site of action of the other reagents was either intracellular or intramembrane.
4. Putative sperm protein targets of the thiol modifications were identified (Mammoto *et al.,* 1997).

2.2.3. Insulin and Renin Secretion

Much earlier research was directed towards investigating the effects of thiol-modifying reagents on the release of insulin resulting from the fusion of secretion granules with the pancreatic islet cell plasma membrane. Various models were proposed to explain the stimulation of insulin release by reagents such as *p*-hydroxymercuribenzoate and *N*-ethylmaleimide. The potential importance of membrane-protein thiol-disulfide exchange and nicotinamide adenine dinucleotides was emphasized. Because of the lack

of currency of the literature, I refer the interested reader to an article (from one of the laboratories in which the issues involved have been examined most extensively) that reviewed the state of the field (Cooperstein and Watkins, 1990).

More recent investigations have been conducted on the inhibition by thiol reagents of renin secretion from rabbit renal cortical slices. The poorly membrane-permeant reagents *p*-chloromercuriphenyl sulfonate (500μM) and stilbene maleimide (1 mM) stimulated renin secretion (95-fold and 15-fold respectively), whereas the membrane-permeant *N*-ethylmaleimide did not (Doh *et al.,* 1991). Incubation of the renal cortical slices with 5mM dithiothreitol had no effect upon renin secretion, but it prevented the stimulation of secretion by *p*-chloromercuriphenyl sulfonate. Incubation with 10mM dithiothreitol inhibited renin secretion by 86% (Doh *et al.,* 1991). Inhibition by stilbene maleimide was not reversible. Peculiarly, it was found that the thiols that reacted with *p*-chloromercuriphenyl sulfonate were not accessible to *N*-ethylmaleimide (Doh *et al.,* 1991), although they would appear to have reacted with the stilbene maleimide. Perhaps the membrane-permeant *N*-ethylmaleimide was consumed through reaction with cytoplasmic glutathione (Doh *et al.,* 1991). Stimulation of renin secretion (50-fold) by 1 mM mersalyl was also detected; stimulation was reversed by incubation with 5 mM dithiothreitol (Park *et al.,* 1991). It was concluded that thiol-disulfide exchange in proteins on the extracellular side of the membrane played an important role in renin secretion (Doh *et al.,* 1991).

2.2.4. Sea Urchin Pronuclear Fusion during Fertilization

Studies of sea urchin eggs yielded evidence for effects of thiol-disulfide-modifying reagents on a number of different membrane-fusion steps. When sea urchin eggs are treated with 1 mM dithiothreitol three minutes after fertilization, pronuclear fusion does not take place, although pronuclear migration and chromosome condensation and decondensation are not affected (Schatten, 1994). Mitosis continues in a fashion, but proper karyomere membrane fusion and division-furrow construction are not achieved. It was concluded that the maintenance of some protein disulfide-bond(s) is necessary for the three membrane-fusion events to transpire normally. The roles of thiols and disulfides in fertilization and cell division are also reviewed (Schatten, 1994).

2.2.5. Sea Urchin Egg Cortical Granule Exocytosis

Sea urchin egg cortical granule exocytosis is a regulated response triggered by the rise in Ca^{2+} concentrations that accompanies fertilization. The

cortical vesicles that fuse with the plasma membrane are tightly associated with its cytoplasmic face in the mature egg before fertilization. Fragments of egg cortex that contain the cortical vesicles and the plasma membrane can be utilized as a cell-free system for studying Ca^{2+} -induced exocytosis (Vogel *et al.,* 1991). The cortical vesicles and plasma membranes can also be isolated from one another and then recombined to reconstitute Ca^{2+}-riggered exocytosis (Vogel *et al.,* 1991). *N*-ethylmaleimide treatments inhibit the fusion step in both the egg-cortex-fragment and reconstituted systems (Jackson and Modern, 1990). The *N*-ethylmaleimide-sensitive proteins appeared to be firmly associated with the membranes (Jackson and Modern, 1990).

Interestingly, reconstitution of exocytosis could be achieved by recombination of *N*-ethylmaleimide-treated plasma membrane with untreated cortical vesicles or of *N*-ethylmaleimide-treated cortical vesicles with untreated plasma membrane, whereas the combination of *N*-ethylmaleimide-treated plasma membrane and *N*-ethylmaleimide-treated cortical vesicles was inactive (Jackson and Modern, 1990). These data suggested that the *N*-ethylmaleimide-sensitive protein(s) resided in both the plasma membrane and cortical vesicles and could be supplied by either one (Jackson and Modern, 1990).

It has been demonstrated that the cortical granules will themselves fuse in the presence of Ca^{2+} in a reaction that is inhibited by *N*-ethylmaleimide (Vogel *et al.,* 1992; Vogel and Zimmerberg, 1992). Untreated vesicles will fuse with *N*-ethylmaleimide-treated vesicles, thereby demonstrating that the *N*-ethylmaleimide-sensitive proteins that promote fusion need be on only one of the fusing membranes (Vogel *et al.,* 1992). There appears to be no requirement for cytoplasmic proteins in the fusion reaction. In addition, the cortical granules fuse with liposomes in an *N*-ethylmaleimide-inhibitable fashion (Vogel *et al.,* 1992).

Additionally, it has been demonstrated that treatment of the membranes with 3-(2-pyridyldithio)propionate coupled to dextran (dextran-PDP) inhibited granule exocytosis in a reaction that was reversible by subsequent addition of dithiothreitol (Whalley and Sokoloff, 1994). The dextran-PDP appeared to react with the same thiols as the *N*-ethylmaleimide, because it prevented the irreversible inhibition of fusion by *N*-ethylmaleimide. These data suggested that the functionally significant thiols are readily exposed on the membranes.

2.2.6. Microsome Fusion

Homotypic fusion of microsomes derived from disrupted rat liver endoplasmic reticular membranes is dependent on GTP and inhibited by

incubation of the membranes with *N*-ethylmaleimide. By a number of criteria the *N*-ethylmaleimide-sensitive component was demonstrated to be distinct from NSF (Sokoloff *et al.,* 1995). Subjecting the untreated membranes to a procedure known to extract NSF from membranes did not affect microsome fusion, whereas addition of cytosol that had not been treated with *N*-ethylmaleimide did not restore fusion between *N*-ethylmaleimide–treated microsomes. NSF was not detected immunologically in microsomes that retained fusion capacity.

Treatment of the membranes with 3-(2-pyridyldithio)propionate coupled to bovine serum albumin (BSA-PDP) inhibited microsome fusion in a reaction that was reversible by subsequent addition of dithiothreitol (Sokoloff *et al.,* 1995).The BSA-PDP appeared to react with the same thiols as the *N*-ethylmaleimide, because it prevented the irreversible inhibition of fusion by *N*-ethylmaleimide. These data suggested that the functionally significant thiols are highly accessible on the membranes. Inhibition of fusion could also be achieved through treatments with sodium periodate in a reaction that was also reversible by subsequent addition of dithiothreitol. Incubation of the membranes with 1 mM Mg^{2+}-GTP did not protect the fusion activity of the membranes from inhibition, thereby indicating that the reactive thiol was unlikely to be in a GTP-binding site (Sokoloff *et al.,* 1995).

2.2.7. Endocytosis

N-ethylmaleimide-sensitive factors that differ from NSF have been reported to participate in vesicular transport and membrane fusion during endocytosis. In a cell-free system that examines the transport of mannose 6-phosphate receptors from late endosomes to the trans–Golgi network, it has been demonstrated that transport was 80% inhibited when mixtures of cell extracts and Golgi membranes were treated with 0.2 mM *N*-ethylmaleimide (Goda and Pfeffer, 1991). Transport was largely restored when untreated, but not *N*-ethylmaleimide-treated, cytosol was added to the mixture. Glycerol-gradient sedimentation analysis of the active component of the cytosol, dubbed ETF-1, indicated that it had a molecular mass of 50–100 kDa, whereas NSF is a hexamer of 76-kDa subunits (Fleming *et al.,* 1998). Furthermore, the ETF-1 activity was still present in cytosol depleted of NSF by passage over columns containing bound anti-NSF antibodies. In addition, levels of *N*-ethylmaleimide that inhibited endosome → trans-Golgi network transport did not inhibit NSF-dependent intra-Golgi transport indicating that NSF is not the target of *N*-ethylmaleimide in the endosome → trans-Golgi network transport system (Goda and Pfeffer, 1991). ETF-1 activity was found to be required for an early stage in transport but has not been further characterized.

Fusion between endosomal vesicles was inhibited 75% when 1 mM *N*-ethylmaleimide was incubated with a mixture of membrane and cytosol fractions (Rodriguez *et al.,* 1994). When the fractions were incubated separately with 1 mM *N*-ethylmaleimide and then combined, fusion was not diminished substantially. When the fractions were incubated with 3 mM *N*-ethylmaleimide either together or separately and then combined. vesicular fusion was almost completely eliminated. Addition of untreated cytosol, but not untreated membranes, could restore fusion in mixtures treated with either 1 mM or 3 mM *N*-ethylmaleimide. Immunological analysis of the fractions indicated that NSF was found mainly in the membrane fraction\, and was therefore unlikely to be the active cytosolic component (Rodriguez et *al.,* 1994). Recombinant NSF that is active in intra-Golgi transport assays restored fusion only slightly. On the other hand, when treatments known to deplete NSF activity from the cytosol (incubation for 30 minutes at 37°C after desalting in the absence of ATP) were conducted, the fusion restoration activity of the cytosol was not substantially reduced. It was concluded that there is a cytosolic *N*-ethylmaleimide-sensitive factor involved in eddosomal vesicle fusion that differs from NSF, although NSF itself is also required. It is also proposed that the thiol on the second factor is exuosed only upon interaction of the protein with the membrane (Rodriguez *et al.,* 1994).

3. PROTEIN THIOLS IN VIRAL-GLY COPROTEIN-MEDIATED MEMBRANE FUSION AND VIRUS ENTRY

3.1. Human Immunodeficiency Virus

Much of the best evidence for the role of thiols and, specifically, thiol-disulfide exchange in membrane fusion comes from studies of the entry of enveloped viruses and viral glycoproteins. However, the solidity of the support for the participation of thiol-disulfide exchange varies considerably. The largest body of data buttressing the role of cell surface thiol-disulfide exchange proteins in promoting the entry of an enveloped virus concerns the abrogation of human immunodeficiency virus type 1 (HIV-1) replication by inhibitors of cell-surface protein disulfide isomerase activity. Although protein disulfide isomerase is normally considered an endoplasmic reticulum-localized protein (Freedman, 1989), cell-surface protein disulfide isomerase has been detected by, for example, flow cytometric analysis utilizing anti-protein disulfide isomerase monocolonal antibodies (Ryser *et al.,* 1994). These antibodies also substantially inhibited HIV-1 infection of human T-cells, H9 (HUT-78). In addition, treatment of the cells

prior to infection with 2.5 mM 5,5′-dithiobis-(2-nitrobenzoic) acid, 1 mM monobromotrimethylammoniobimane, or 3 mM bacitracin (an inhibitor of protein disulfide isomerase (Mandel *et al.,* 1993)), dramatically inhibited HIV-1 infection (Ryser *et al.,* 1994). Effects of these three reagents on cell-surface expression of the HIV-1 coreceptor CD4 and on cell viability were negligible. Treatment of the cells with 2.5 mM 5,5′-dithiobis-(2-nitrobenzoic) acid following initial HIV-1 infection but before transfer to regular growth medium did not reduce viral replication. Pretreatment of the virus with the reagents prior to binding did not inactivate the virus (the treated virus was diluted so that the concentration of the inhibitors was much lower than their effective concentrations when the virus was incubated with the cells). In addition, exposure of HIV-1 to dithiothreitol decreased HIV infectivity in a dose-dependent manner. It was therefore concluded that a cell-surface protein disulfide isomerase was involved in promoting HIV-1 entry, presumably by reducing a critical disulfide bond upon receptor binding (Ryser *et al.,* 1994).

3.2. Coronaviruses

Murine hepatitis virus, a coronavirus, possesses mature glycoprotein spikes that consist of two subunits, S1 and S2, which are responsible for both the binding of virions to cell membranes and virion-cell membrane fusion. S2 is a transmembrane protein, whereas S1 is associated with the viral envelope through binding to S2. It was demonstrated that incubation of the JHM strain of MHV with 1 mM 5,5′-dithiobis-(2-nitrobenzoic) acid at 37°C for one hour reduced infectivity by 99%, whereas incubation at 21°C or incubation of the A59 strain with 1 mM 5,5′-dithiobis-(2-nitrobenzoic) acid did not affect infectivity (Gallagher, 1996). The effect on the JHM strain resulted from reduced viral-cell fusion and not from diminished binding of the virus to the cell. It was noted that the S2 of the JHM strain possesses 13 cysteine residues and the S2 of strain A59 contains 12, presumably paired in six disulfides. The 13th S2 cysteine in JHM is not involved in covalent intermolecular associations, so it is likely to exist as a thiol. This residue (cysteine-1163) was altered to tyrosine, the residue in the equivalent position in A59, and the recombinant S protein was assayed in a cell-cell fusion assay. The mutation greatly reduced fusion potency, but it also reduced sensitivity to 1 mM 5,5′-dithiobis-(2-nitrobenzoic) acid (Gallagher, 1996). Introducing the cysteine residue into the A59 strain S2 increased sensitivity to the reagent. It was concluded that cysteine-1163 is exposed at higher temperatures, and that its modification can lead to an inhibition of fusion (Gallagher, 1996). The fact that the cysteine residue is not required for fusion activity reduces the likelihood that it is involved in

thioldisulfide-exchange-induced conformational changes that promote membrane fusion. It had previously been suggested that such exchange might be important for coronavirus glycoprotein-induced membrane fusion because of the enhancement of S1 release and virus aggregation by dithiothreitol treatment of **A59** strain virions and because of the possibility that these conformational changes were related to those that accompany fusion (Sturman *et al.,* 1990). In conclusion, it is unlikely that the 5,5′-dithiobis-(2-nitrobenzoic) acid-sensitivity of JHM strain virions is related to the effects of dithiothreitol treatment of the A59 strain virions. Further studies are required to determine whether thiol-disulfide exchange plays a role in coronavirus entry.

3.3. Alphaviruses

A series of investigations into the roles of disulfide bonds and thiol-disulfide exchange in the structure of alphavirus glycoproteins and their promotion of membrane fusion have been conducted. The glycoproteins of the alphaviruses are synthesized as a polyprotein (E3-E2-6K-E1), which is processed into the individual subunits (Strauss and Strauss, 1994). The amino-terminal section of the polyprotein functions as a signal sequence to direct the translocation of the subsequent polypeptide region into the endoplasmic reticulum. A hydrophobic sequence approximately 400 residues after the signal sequence acts as a stop-transfer signal and as the membrane anchor for E2. The following segment of approximately 30 residues transiently functions as a signal sequence for the carboxy-terminal half of the molecule. Proteolytic cleavage following this signal sequence results in the release of E3-E2 (referred to as proE2 or PE2), which is anchored in the membrane. Other processing events lead to the formation of the transmembrane El protein.

PE2-E1 heterodimers form in the endoplasmic reticulum and are transported to the Golgi apparatus where PE2 is cleaved into E3 and E2 at a sequence recognized by the furin class of protein convertases (Strauss and Strauss, 1994). Cleavage of PE2 to E3 and E2 appears to be required for normal infectivity of alphaviruses in mammalian cells (Strauss and Strauss, 1994). The mature E2-E1 glycoprotein complex from the Sindbis virus envelope is stable in detergent but is disrupted by treatment with dithiothreitol or 2-mercaptoethanol (Anthony and Brown, 1991), although there is no evidence for stable intermolecular E1-E2 disulfide bonds. Incubation of Sindbis virus with 5 mM dithiothreitol induces the exposure of epitopes that are also recognized by certain monoclonal antibodies upon attachment of the virus to cells (Meyer *et al.,* 1992). Prolonged exposure of the virus to 5 mM dithiothreitol (6 hours) results in a dramatic decrease in

infectivity, an increase in the susceptibility of the glycoproteins to digestion with trypsin, and morphological changes (Anthony *et al.,* 1992). It was inferred that disulfide bonds are responsible for maintaining the structural integrity of the Sindbis virus envelope protein lattice (Anthony *et al.,* 1992).

Subsequent experiments on the role of thiol-disulfide exchange in Sindbis virus entry have figured into a controversy on the cellular site of entry of alphaviruses. Most of the data are consistent with the concept that Semliki Forest and Sindbis viruses enter cells through a clathrin-dependent pathway that involves acidified endosomes (DeTulleo and Kirchhausen, 1998; Glomb-Reinmund and Kielian, 1998; Marsh and Helenius, 1989; Strauss and Strauss, 1994). It has been suggested, however, that the reduction in infection by Sindbis virus resulting from incubation of cells with lysosomotropic weak bases is mediated through an inhibition of viral replication rather than through an abrogation of entry resulting from the inhibition of the acidification of endosomes (Cassell *et al.,* 1984).

There is an alternative hypothesis to the proposal that alphavirus entry occurs through the endocytic pathway. It postulates that thiol-disulfide exchange reactions at the cell surface lead to Sindbis virus-mediated fusion (Abell and Brown, 1993). The chain of reasoning begins with experiments that appear to support a role for acidic pH, that is, the endocytic pathway, for Sindbis virus-promoted membrane fusion. Exposure of Sindbis virus to pH 5.3 is necessary for its mediation of cell-cell fusion that occurs when the treated virus is present at high concentrations. However, fusion itself occurs efficiently only when the pH is raised towards neutrality. This was interpreted to mean that fusion could normally only take place at a pH where protein thiolates would be present. Based upon this hypothesis, it was demonstrated that if the cells were incubated with submicromolar concentrations of 2-mercaptoethanol, then fusion could occur efficiently at pH 5.3 (Abell and Brown, 1993). In addition, if cells to which virus had been adsorbed were incubated with 1 mM 5,5′-dithiobis-(2-nitrobenzoic) and thereafter warmed to allow virus penetration, then virus infectivity is inhibited 50–40%. By contrast, treatment of the virus and cells before or after the process of penetration had little effect. It was hypothesized that certain thiols and/or disulfides become accessible because of the conformational changes in the glycoproteins that occur during penetration (Abell and Brown, 1993; Meyer *et al.,* 1992). Blockage by 5,5′-dithiobis-(2-nitrobenzoic) acid of the thiols or those on a cellular protein that binds to the glycoproteins is proposed to be the mechanism by which membrane fusion is inhibited (Abell and Brown, 1993).

Additional studies of the nature of the disulfide bonds within the Sindbis virus glycoproteins possess potential relevance for understanding

the role of thiol-disulfide exchange in virus entry. Processing of the Sindbis E1 protein was investigated utilizing pulse-chase experiments and analysis of the migration of E1 in SDS-polyacrylamide gel electrophoresis under nonreducing and reducing conditions (Mulvey and Brown, 1994). Cells and virus were lysed in the presence of 20 mM *N*-ethylmaleimide in order to inhibit artifactual disulfide bond formation or rearrangement. Mobility of endoglycosidase H-treated E1 during SDS-polyacrylamide gel electrophoresis was also determined to examine whether a particular E1 species had left the endoplasmic reticulum and thereby acquired endoglycosidase H-resistant oligosaccharides. It was established that E1 in the endoplasmic reticulum exists in a number of species that differ in the number and/or arrangement of their disulfides. One oxidized form of E1, referred to as E1ε, appeared to be competent for export from the endoplasmic reticulum. By a number of criteria, including analysis of the effects of brefeldin A treatments, which induces the return of Golgi components to the endoplasmic reticulum, E1ε was demonstrated to be a stable entity during progress through the Golgi apparatus (Mulvey and Brown, 1994). Additional experiments indicated that Ele was resistant to 5–minute exposure to 5mM dithiothreitol *in vivo,* indicating that it had acquired a mature conformation (Carleton and Brown, 1996). However, late in the secretory pathway, Ele was found to be unstable with regard to its disulfide bonds. This instability was also found during analysis of E1 from Sindbis virions (Mulvey and Brown, 1994).

As noted above, a precursor to E2, PE2, associates with E1 in the endoplasmic reticulum. No disulfide rearrangements of PE2 or E2 were detected during an analysis of their processing (Mulvey and Brown, 1994). Association of PE2 with E1 in the endoplasmic reticulum can be disrupted by *in vivo* dithiothreitol treatments, but while the two subunits are associated, PE2 appears to protect E1 itself from reduction, possibly by promoting the export of the complex from the endoplasmic reticulum (Carleton et *al.,* 1997). It may be significant that expression of a temperature-sensitive E1 mutant at the restrictive temperature results in the retention in the endoplasmic reticulum of E1 and PE2 in disulfide-stabilized aggregates (Carleton and Brown, 1997). Most interestingly, the acquisition by E1ε of its final metastable character appears to correlate with the processing of PE2 to E2 (Mulvey and Brown, 1994).

This entire line of investigation has been questioned (Glomb-Reinmund and Kielian, 1998). The weak base ammonium chloride and inhibitors of the vacuolar proton ATPase, bafilomycin A1 and concanamycin, prevent endosomal acidification. Incubation of cells with any of these reagents during the process of entry of either Semliki Forest virus or Sindbis virus inhibited infection. No inhibition was detected if the cells were

incubated with the reagents after entry. It was concluded that entry of both viruses requires uptake into endosomes and exposure to low pH. The authors were able to confirm the earlier finding that treatment of the virus and cells with 5,5′-dithiobis-(2-nitrobenzoic) acid during entry inhibited both Sindbis and Semliki Forest virus infection. However, incubation with 1 mM monobromotrimethylammoniobimane did not reduce infection. In addition, treatment with 0.1 mM *p*-chloromercuriphenylsulfonic acid had only modest inhibitory effects on infection by both viruses. These results were contrasted with the more dramatic effects these reagents have on the reduction of HIV-1 infection (Ryser *et al.,* 1994) or diphtheria toxin intoxication (Ryser *et al.,* 1991). The authors concluded that entry occurs through the endocytic pathway and that blocking cell-surface sulhydryls did not substantially or specifically inhibit infection.They asserted that they had no evidence for a specific role for thiol-disulfide exchange reactions in Semliki Forest or Sindbis virus infection.

These experiments rule out the participation in alphaviral entry of the same cellular enzymes that play roles in HIV-1 infection and diphtheria toxin infection. The experiments do not, however, exclude the possibility of viral protein-mediated thiol-disulfide-exchange reactions. A model for alphaviral entry that incorporates both acidified endosomes and thiol-disulfide exchange will be presented following a discussion of the system in which there is the most detailed and well-supported proposed mechanism for the role of thiol-disulfide exchange in viral-glycoprotein-mediated membrane fusion.

3.4. Murine Leukemia Viruses

Examination of the available data on murine leukemia virus (MuLV) envelope proteins (Envs) indicates that disulfide-bond rearrangement plays a critical role in the induction by receptors of Env-mediated membrane fusion. The MuLV Envs are synthesized as single polypeptide precursors with cleaved signal sequences (Henderson *et al.,* 1984; Shinnick *et al.,* 1981). During its progress through the secretory system the Env protein forms a trimer (Kamps *et al.,* 1991) and is subsequently proteolytically processed into two subunits (Henderson *et al.,* 1984; Witte *et al.,* 1977), SU and TM, which are linked through a labile disulfide bond (Pinter *et al.,* 1978). SU is on the outside of the retrovirus particle, whereas TM possesses an extraparticle domain, a membrane-spanning domain, and a 35-amino-acid domain that resides within the particle (Pinter and Honnen, 1983).

The sequence of the MuLV Envs predicts that each SU protein will possess an even number of cysteine residues, whereas the extracellular

domain of TM will include just three, which are present in a sequence CX_6CC that lies at the end of a putative coiled-coil forming region. The cysteine residues are absolutely conserved among members of an MuLV group and many are conserved across group boundaries. Extracellular cysteines are normally found in disulfide bonds, so it is intriguing that there is an odd number of highly conserved cysteines in the Env extracellular domain. The TM cysteine residues and a CXXC motif present at the amino-terminal portion of the carboxy-terminal domain of the SU protein are conserved in sequence and placement even among the Envs of more distantly related retroviruses such as human T-cell leukemia virus type 1 (HTLV-1), bovine leukemia virus (BLV), and Mason-Pfizer monkey virus (M-PMV) (Figure 1A).

CXXC motifs are relatively rare, and are conserved virtually exclusively in two other classes of polypeptides, transition-metal binding proteins (Holden *et al.,* 1994; Sahlman and Skarfstad, 1983) and thiol-disulfide-exchange enzymes, such as DsbA, DsbB, thioredoxin and protein disulfide isomerase (PDI), where the two-cysteine motifs are the active sites of the enzymes (Freedman et *al.,* 1994; Holmgren, 1985). In the handful of other proteins where a CXXC motif is conserved at least one of the cysteines is modified (by palmitylation, in the case of H-ras (Hancock *et al.,* 1989)) or the cysteines are each disulfide-linked to other cysteines in the protein (ovomucoid (Weber *et al.,* 1981) and surfactant protein B (Johansson *et al.,* 1991)).

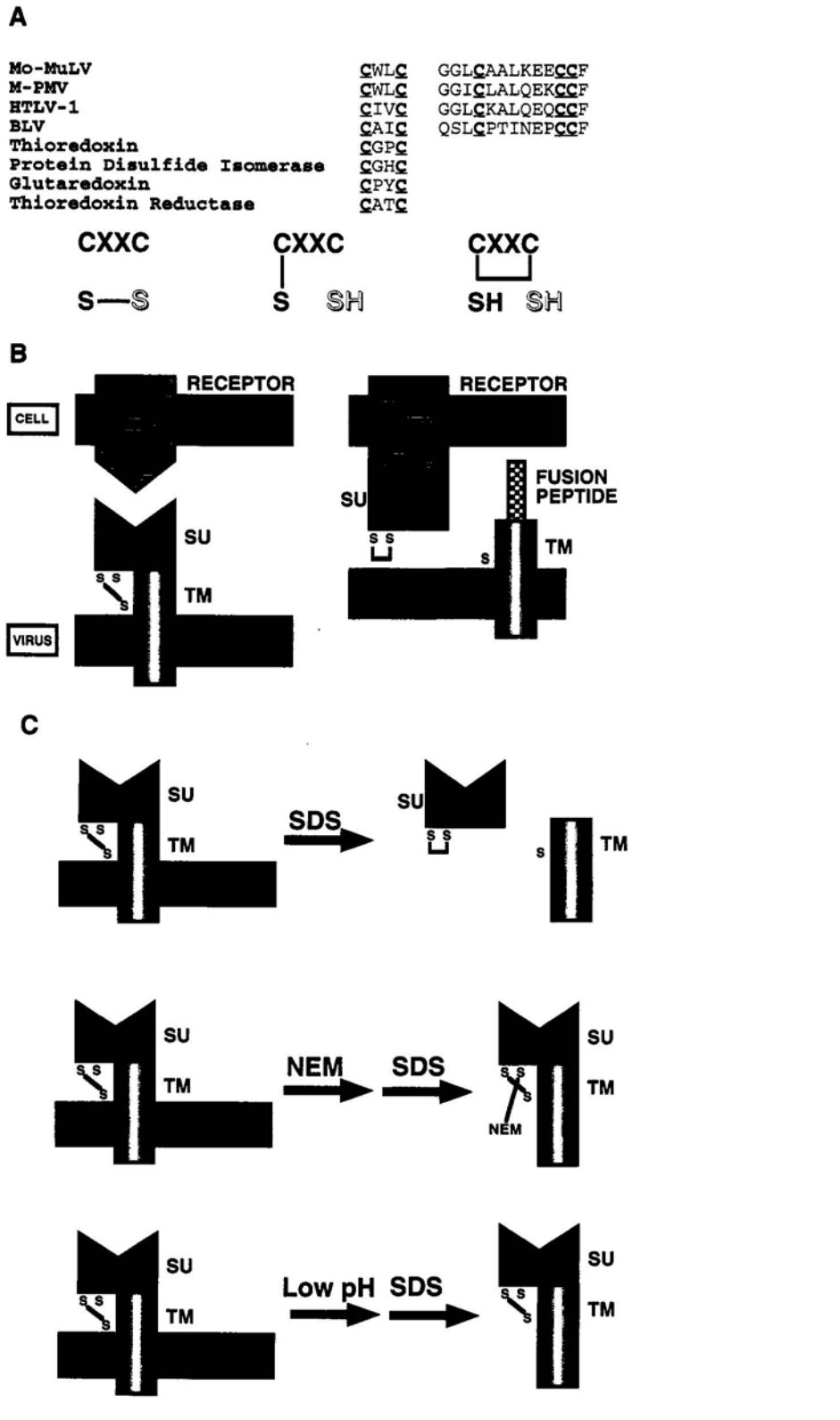
The SU subunit CXXC motif is thus reminiscent of the active site of thiol-disulfide-exchange enzymes (Figure 1A), which function by the following mechanism: When the reduced exchange protein encounters a substrate disulfide, a mixed disulfide is formed between the first cysteine of the enzyme active-site CXXC and one sulfur atom of the substrate; the second substrate sulfur atom is reduced to a sulfhydryl. Next an intramolecular cystine bridge forms between the first and second cysteine of the active site of the enzyme, resulting in the reduction of the substrate disulfide to two sulfhydryls (Holmgren, 1985).

Analysis of the roles of the envelope-protein sequences in the context of the mechanism of the thiol-disulfide-exchange enzymes suggests an attractive model. In this model, the first cysteine in the SU subunit CXXC motif forms a disulfide with the last of the cysteines in the TM subunit CX_6CC motif, whereas the two other TM cysteines form an intramolecular cystine bridge (Fass *et al.,* 1996; Fass and Kim, 1995). The intermolecular disulfide rearranges to an intramolecular bond, Le., between the two cysteines of the SU subunit CXXC motif, upon receptor binding. Extrusion of the TM fusion peptide occurs via an irreversible spring-loaded mechanism

analogous to that proposed for the influenza virus hemagglutinin protein, and membrane fusion ensues (Figure 1B).

There are three lines of evidence suggesting that such a scenario may be correct. The first is that the SU-TM disulfide is labile in the presence of detergents, but it is stabilized by *N*-ethylmabhide treatment prior to solubilization (Gliniak *et al.,* 1991; Pinter and Fleissner, 1977; Pinter *et al.,* 1997; Pinter *et al.,* 1978) or by reducing the pH during solubilization (Opstelten *et al.,* 1998).This suggests that the blocking of a free sulfhydryl,presumably the second cysteine of the CXXC motif, stabilizes the preexistent intermolecular cystine bridge (Figure 1C). The second is that the products of protease digestion of the disulfide-linked Friend MuLV SU and TM indicates that the bond is between a half-cystine in the disulfide exchange motif and one in the CX_6CC motif (Pinter *et al.,* 1997).The third is that investigations of the disulfide bonds in the isolated Friend MuLV and Friend Mink-Cell Focus-inducing virus SU proteins indicate that the two cysteines in the CXXC motif are linked in a cystine bridge (Linder *et al.,* 1992;Linder *et al.,* 1994). Such cystine bridges are known to be highly strained and are found heretofore only in proteins with thiol-disulfide exchange activity (Darby and Creighton, 1995; Zapun *et al.,* 1993). The idea that thiol-disulfide exchange accompanies receptor binding is as yet unsupported by direct experimental data. However, it is plausible considering the conservation and placement of the motifs and the analogies with ricin and diphtheria toxin: Interchain disulfide bond reduction appears to be a rate-limiting step in membrane penetration by both toxins (Lewis and Youle, 1986; Papini *et al.,* 1993). In contrast to the HIV glycoproteins, which appear to be dependent on exogenous enzymatic activities for the catalysis of disulfide-bond rearrangements (Ryser *et al.,* 1994), the MuLV and other homologous Envs would possess a thiol-disulfide-exchange enzyme active site as part of their structures. Other than the regions of the two cysteine-containing motifs and a GXDP motif (Gallaher *et al.,* 1995), the MuLV envelope proteins share essentially no sequence identity with those of the more distant HTLV-1, BLV, and M-PMV, suggesting that these conserved motifs have coevolved and are likely to play similar roles in all cases. Although a covalent interaction between SU and TM has not been found for some of the more distantly related viruses (Brody *et al.,* 1994), these studies have not utilized conditions capable of stabilizing a labile disulfide bond.

It has been suggested that the SU-TM disulfide bond is not “labile” to reduction but merely appears to rearrange as an artifact of solubilization (Opstelten *et al.,* 1998). It is therefore, in fact, a “stable” disulfide when present in intact glycoprotein complexes on the surface of cells or virions. Nevertheless, the SU-TM linkage is “labile” in the sense that extra care is


A
Mo-MuLV
M-PMV
HTLV-1
BLV
Thioredoxin
Protein Disulfide Isomerase
Glutaredoxin
Thioredoxin Reductase
CWLC
CWLC
CIVC
CAIC
CGPC
CGHC
CPYC
CATC
GGLCAALKEECCF
GGICLALQEKCCF
GGLCKALQEQCCF
QSLCPTINEPCCF
CXXC
S—S
CXXC
S SH
CXXC
SH SH
B
CELL
RECEPTOR
SU
TM
VIRUS
RECEPTOR
SU
FUSION PEPTIDE
TM
C
SU
TM
SDS
SU
TM
NEM
SDS
NEM
Low pH
SDS

required to maintain it during analysis of the proteins (Figure IC) and that severance of the linkage may ensue during the conformational changes associated with membrane fusion.

The proposed thiol-disulfide-exchange mechanism suggests that there is a conformational coupling between receptor binding and the relative stability of the intermolecular versus intramolecular cystine bridge. It is possible that there is an equilibrium between the two disulfides and that when the SU protein binds to the receptor, the mobility of the receptor in the

FIGURE 1. Envelope-protein thiol-disulfide exchange motifs and receptor induction of membrane fusion.

(A) Comparison of the retroviral envelope protein sequences and those of the thiol-disulfide exchange enzymes and the catalytic mechanism of the enzymes. The conserved thiol-disulfide exchange motifs in the Envs of Moloney MuLV (AA 336–339), Mason-Pfizer monkey virus (AA 247–250), human T-cell leukemia virus type I isolate MT-2 (AA 225–228), and bovine leukemia virus (AA 212–215) and in the active sites of *E. coli* thioredoxin, rat protein disulfide isomerase, *E. coli* glutaredoxin (Fahey and Sundquist, 1991), and *E. coli* thioredoxin reductase (Russel and Model, 1988) are compared. Also shown are the three-cysteine motifs in the envelope TM subunits–Mo-MuLV AA 552–564 (Shinnick *et al.,* 1981), M-PMV AA 472–484 (Sonigo *et al.,* 1986), HTLV-1 MT-2 isolate AA 390–402 (Gray *et al.,* 1990), and bovine leukemia virus AA 380–392(Sagata et *al.,* 1985).

The catalytic mechanism of thioredoxin (Holmgren, 1985) is depicted below. When the reduced thioredoxin (represented by its active site motif) encounters a disulfide, a mixed disulfide is formed between the first cysteine of the enzyme active site and one of the sulfurs of the substrate. An intramolecular cystine bridge then forms between the first and second cysteine of the active site of the enzyme, resulting in the reduction of the substrate disulfide to two sulfhydryls. It is the last two steps (ignoring the sulfhydryl in outline) that are similar to the proposed disulfide shuffling in Env.

(B) Model of thiol-disulfide exchange and receptor induction of membrane fusion. The first cysteine in the SU (black) CXXC motif initially forms a disulfide with one of the cysteines in the TM (shaded figure in viral membrane) CX_6CC motif. In this conformation the binding of SU to TM prevents exposure of the fusion peptide. There is a free thiol in SU.

The intermolecular disulfide rearranges to an intramolecular bond upon receptor (striped figure in cell membrane) binding. Extrusion of the TM fusion peptide (the checked rectangle at the end of the TM protein) and membrane fusion follow. Complete dissociation of the SU and TM subunits may not be necessary for membrane fusion; the model requires only that substantial subunit reorganization results from intermolecular cystine-bond cleavage.

(C) Fate of the Mo-MuLV Env intermolecular disulfide bond under different experimental conditions. When the Moloney murine leukemia virus is disrupted in SDS the disulfide bond between the two MuLV Env subunits rearranges in an experimental artifact during the process of denaturation. If the virus is pretreated with *N*-ethylmaleimide (NEM) before disruption (Pinter and Fleissner, 1977; Pinter *et al.,* 1997; Pinter *et al.,* 1978) or if the pH is lowered to pH 6.0 (Opstelten *et al.,* 1998), then the disulfide bond linking SU and TM is preserved. In the former case rearrangement is prevented by alkylation of the free cysteine (Pinter *et al.,* 1997), whereas in the latter case it is inhibited by reducing the concentration of the thiolate, which is the nucleophile that participates in the exchange reaction (Opstelten *et al.,* 1998).

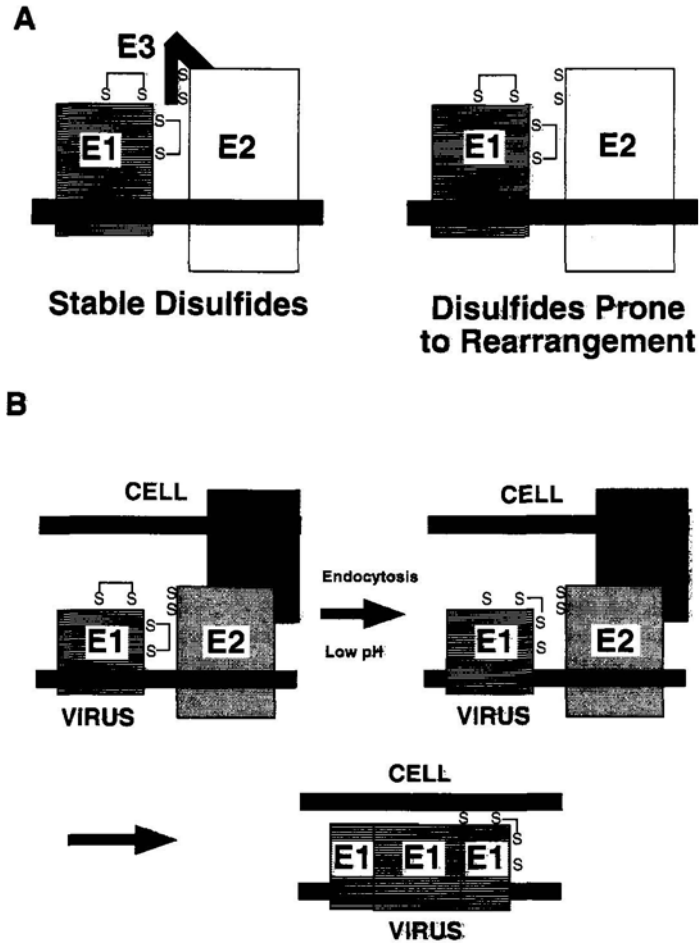


FIGURE 2. A model for alphavirus entry into cells.

(A) Alphaviral PE2 processing and activation of thiol-disulfide exchange. During the progress of the alphaviral glycoproteins PE2 and El (striped rectangle) associate to form a trimer of heterodimers. Only one heterodimer is illustxated for the sake of simplicity. PE2 *con*-sists of E3 (black) covalently linked to E2 (light-gray rectangle). The predicted E3 alpha helices (David A. Sanders, unpublished observations) overlie the E2 amino-terminal thiol-disulfide (CXXC) motif inhibiting its activity. The glycoproteins are transported to the Golgi apparatus where PE2 is cleaved into E3 and E2 at a sequence recognized by the furin class of protein convertases (Straws and Strauss, 1994). The cleavage is postulated to expose the thiol-disulfide exchange motif. This contributes to the instability of El with respect to its disulfide bonds during denaturation (Mulvey and Brown, 1994). There is no intention of implying that evidence for any particular configuration of oxidized or reduced cysteine residues exists within El and E2; one possibility is illustrated.

cellular membrane results in the stripping of the SU protein from the virus envelope-protein complex during the otherwise brief periods when the intramolecular disulfide is present. The irreversibility of the proposed TM conformational change leads to exposure of the fusion peptide and membrane fusion. It appears more likely that binding of the cellular receptor directly affects the conformation of protein segments that follow the receptor-binding domain sequence in the SU amino-terminal domain and that these in turn stabilize the intramolecular form of the disulfide relative to the intermolecular form.

3.5. Other Retroviruses and Filoviruses

Some retroviruses, such as the avian sarcoma and leukosis viruses (ASLVs), and the filoviruses contain a CX_6CC motif in their TM subunits in an equivalent position (at the end of a predicted coiled-coil forming region) to that of the MuLVs. However, they do not contain the CXXC motif in their SU subunits and have a stable disulfide linkage between their subunits rather than a labile one. It is noteworthy that both the ASLVs and the filoviruses are predicted to have internal fusion peptides within a cystine-bridged loop of TM rather than at the amino-terminus of TM, as is the case for the MuLVs (Gallaher, 1996). It is probable that the first two cysteine residues in the ASLV and filovirus TM CX_6CC motifs form an intramolecular disulfide bond while the third cysteine residue participates in an intermolecular cystine bridge with a residue from the SU subunits. In the case of the filoviruses, it appears possible that, the acidic environment of the endosome induces a conformational change in the GP during viral entry, which makes the filovirus SU-TM disulfide linkage more readily reduced by cellular factors, which in turn leads to dissociation of the subunits and membrane fusion. The filoviruses, and potentially other viruses, would thus be dependent during entry upon cellular factors for elimination

(B) Activation of thiol-disulfide exchange on alphaviral glycoproteins in endosomes. An alphavirus binds to a cellular receptor (dark-gray polygon) through E2. Conformational changes in the glycoprotein complex transpire and the virus is taken into the cell by endocytosis. The acidity in the endosomes produce structural changes in the glycoproteins that alter the accessibility of El disulfides to the E2 thiol-disulfide exchange motif. This leads to reduction of particular cystine bridges and/or formation of novel disulfide linkages. Again, there is no intention of implying that evidence for any particular configuration of oxidized or reduced cysteine residues exists within El and E2.The disulfide rearrangements accompany or lead to the fusion-promoting conformation of El, which probably involves dissociation of E2 from El and formation of an E1 homotrimer (Wahlberg *et al.,* 1992). The pKa of one or both of the cysteines in the CXXC motif could be altered so that thiol-disulfide exchange is promoted even in acidic endosomes (Wells *et al.,* 1993).

of the covalent bond, whereas the retroviruses whose Envs are most closely related to those of the MuLVs would encode their own enzymatic activity. One possible alternative scenario for the filovirus GPs is that reduction of the disulfide bond is not required for membrane fusion but does enhance its efficiency. It has been suggested that acid-induced conformational changes may facilitate the reduction of the cystine bridge between the two subunits in cleaved influenza hemagglutinins (Roberts *et al.,* 1993).

3.6. A Reconsideration of Alphavirus Entry

Thiol-disulfide exchange may be a common biochemical step in the conformational changes that viral glycoproteins undergo during membrane fusion. Reexamining the mechanism of entry of alphaviruses in this context indicates that a hypothesis that includes a role for thiol-disulfide exchange is plausible. Three sets of facts favor this proposition. First, rubella virus, which is related to the alphaviruses (together with which it forms the togavirus family) possesses an El glycoprotein that contains a CXXC motif. Intriguingly, the two cysteines in the motif are linked in a cystine bridge (Gros *et al.,* 1997). As pointed out above, such disulfide bonds are found only in proteins with thiol-disulfide exchange activity. Second, the E2 glycoprotein of alphaviruses also possess a conserved CXXC motif. It is positioned at the amino-terminus of E2 approximately 20 amino-acid residues after the E3/E2 cleavage site. Third, the previously noted effects of 2-mercaptoethanol on Sindbis virus-mediated membrane fusion and of 5,5′-dithiobis-(2-nitrobenzoic) acid on Sindbis virus entry, and the acquisition of disulfide-bond metastability by El at the time that E3 is cleaved from E2 suggest that thiol-disulfide exchange is having an effect on glycoprotein conformation and function.

Synthesizing these data leads to the following model (Figure 2). When an alphaviral glycoprotein is progressing through the secretory system as a PE2-E1 complex, the activity of the amino-terminal PE2 CXXC peptide region is inhibited by the presence of E3. Upon cleavage of E3 the CXXC region is exposed so that it is poised to engage in the promotion of thiol-disulfide exchange during viral entry (Figure 2A). Binding of the alphavirus to its cellular receptor(s), presumably through E2, leads to some conformational changes in the glycoproteins. The virus is taken into the cell by clathrin-mediated endocytosis. Upon exposure of the receptor-bound glycoproteins to low pH, conformational changes occur that expose one or more disulfide bonds, perhaps in El, to the E2 thiol-disulfide exchange (CXXC) motif. Rearrangement of the disulfide bond(s) leads to the structural changes that result in a fusion-promoting conformation of El, which probably involves dissociation of E2 from El and formation of an El

homotrimer (Figure 2B) (Wahlberg *et al.,* 1992). It is noteworthy that cleavage of PE2 appears to be necessary for the dissociation of the heterodimer under normal conditions (Salminen *et al.,* 1992). Perhaps El and PE2 form a difficult-to-detect cystine bridge, similar to the MuLV SU-TM disulfide bond, that cannot rearrange under normal conditions, but does rearrange during solubilization of the proteins.

4. CONCLUSION

Although much of the evidence for the roles of protein thiols and thiol-disulfide exchange in membrane fusion is indirect, it appears probable that additional cellular membrane fusion-promoting proteins containing catalytically important or thiol-reagent-accessible cysteine residues, besides the *N*-ethylmaleimide-sensitive factors and cysteine proteases, will be identified. Of equal importance and probability will be the ongoing investigation of the occurrence and prevalence of thiol-disulfide exchange reactions in viral glycoproteins during the process of membrane fusion. Such studies may not only illuminate a critical and common biochemical step in membrane-fusion but may also lead to the identification of reagents that can specifically inhibit the thiol-disulfide exchange and thereby prevent viral entry.

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