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Overexpression of Thiol/Disulfide Isomerases Enhances Membrane Fusion Directed by the Newcastle Disease Virus Fusion Protein

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Newcastle disease virus (NDV) fusion (F) protein directs membrane fusion, which is required for virus entry and cell-cell fusion. We have previously shown that free thiols are present in cell surface-expressed NDV F protein and that blocking the production of free thiols by thiol-disulfide exchange inhibitors inhibited the membrane fusion mediated by F protein (J Virol. 81:2328–2339, 2007). Extending these observations, we evaluated the role of the overexpression of two disulfide bond isomerases, protein disulfide isomerase (PDI) and ERdj5, in cell-cell fusion mediated by NDV glycoproteins. The overexpression of these isomerases resulted in significantly increased membrane fusion, as measured by syncytium formation and content mixing. The overexpression of these isomerases enhanced the production of free thiols in F protein when expressed without hemagglutination-neuraminidase (HN) protein but decreased free thiols in F protein expressed with HN protein. By evaluating the binding of conformation-sensitive antibodies, we found that the overexpression of these isomerases favored a postfusion conformation of surface-expressed F protein in the presence of HN protein. These results suggest that isomerases belonging to the PDI family catalyze the production of free thiols in F protein, and free thiols in F protein facilitate membrane fusion mediated by F protein.

A potential mechanism to facilitate these conformational changes is suggested by a number of studies of different viruses, which have shown that, during membrane fusion, fusion glycoproteins undergo thiol-disulfide isomerization, leading to the reduction of disulfide bonds and the production of free thiols in fusion glycoproteins (1, 7, 15, 16, 25, 27, 33). The production of free thiols in these glycoproteins is essential for membrane fusion and may facilitate conformational changes required for fusion. In some viruses, like murine leukemia virus (MLV), the thiol/disulfide isomerization is thought to be mediated by an isomerase motif, Cys-X-X-Cys (CXXC), in the viral Env glycoprotein sequence, and this isomerization is triggered by the binding of glycoprotein to its receptor (25, 33, 34). For viruses that do not have a CXXC motif within the glycoprotein sequence, like human immunodeficiency virus type 1 (HIV-1), the thiol/disulfide isomerization is thought to be catalyzed by host cell proteins, protein disulfide isomerase (PDI) or related proteins, that have a CXXC motif. This conclusion is based on studies showing the inhibition of HIV-1 entry and cell-cell fusion by inhibitors of the PDI family of isomerases (4, 7, 9, 16, 27).

In another study, the contribution of PDI in HIV-1 Env-mediated membrane fusion was evaluated by decreasing the expression of endogenous PDI protein using short interfering RNA (24). It was shown that the downregulation of PDI did not significantly inhibit the membrane fusion mediated by HIV-1 Env. The authors suggested that other isomerases of the PDI family also are involved in disulfide bond reduction and that this function is redundant, as many of the members of the host cell PDI family of proteins have similar catalytic domains and can catalyze the reduction of disulfide bonds (reviewed in reference 2).

PDI is a member of a family of 19 structurally related

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isomerases with a thioedoxin-like domain (reviewed in reference 2). Most of the isomerases in the PDI family have a CXXC motif that catalyzes the formation, reduction, and rearrangement of disulfide bonds in proteins (2, 5, 23, 36). These isomerases are involved primarily in the folding of proteins in the endoplasmic reticulum (ER), catalyzing the formation of disulfide bonds. Indeed, most of these proteins have ER retention signals (2). However, in recent years, isomerases from the PDI family have been shown to be present on cell surfaces, both in functional assays and biochemical assays (8). The mechanisms involved in the expression and retention of these proteins at cell surfaces are unknown, but it has been speculated that they are bound to resident host cell surface proteins (2, 8, 10, 32). Cell surface disulfide isomerases are proposed to be involved in processes such as cell adhesion, nitric oxide signaling, and the reduction of disulfide bonds in the cell entry proteins of viruses (reviewed in references 8 and 13, 32).

We recently reported that free thiols also may be detected in cell-surface-expressed NDV fusion protein, and that these free thiols are required for cell-cell membrane fusion (12). We showed that cell-cell membrane fusion as well as viral entry was inhibited by DTNB [5′,5′-dithio-bis(2-nitrobenzoic acid)], a nonspecific inhibitor of free thiols, bacitracin, an inhibitor of PDI family of isomerases, and anti-PDI antibodies. However, anti-PDI antibodies inhibited membrane fusion less efficiently than the two inhibitors. This result suggested that the reduction of disulfide bonds in the F proteins is accomplished by other members of the PDI family.

To explore the role of PDI and other PDI-like isomerases on F protein function, we studied the effects of the overexpression of two different thiol isomerases, PDI and ERdj5, on F protein-mediated fusion and on the formation of surface-expressed F protein. This approach was pursued because the inhibition of all of the PDI-like isomerases using short interfering RNA was not feasible. The results showed that the overexpression of either of the isomerases led to enhanced cell-cell fusion and favored a postfusion conformation of F protein. These results are consistent with the conclusion that free thiols have an important role in NDV membrane fusion and suggest that other thiol isomerases with a CXXC motif are involved in disulfide bond reduction in viral fusion glycoproteins and in virus entry.

**MATERIALS AND METHODS**

Cells, plasmids, and antibodies. COS-7 cells, obtained from the American Type Culture Collection, were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with nonessential amino acids, vitamins, penicillin, streptomycin, and 10% fetal calf serum.

ERdj5 and PDI genes were purchased from the American Type Culture Collection (pCMV-Sport6ccdB vector and pBluescript SK– vectors, respectively. The PDI gene was subcloned in the pCMV-Sport6ccdB vector using NotI and KpnI. The PDI gene was subcloned in the pCMV-Sport6ccdB vector using NotI and KpnI. These results are consistent with the conclusion that free thiols have an important role in NDV membrane fusion and suggest that other thiol isomerases with a CXXC motif are involved in disulfide bond reduction in viral fusion glycoproteins and in virus entry.

Transfection. The transfections of COS-7 cells were accomplished using Lipofectamine or Lipofectin (Invitrogen) as recommended by the manufacturer. For each transfection, a mixture of DNA (0.5 μg/35-mm plate) and 7 μl of Lipofectamine or Lipofectin in OptiMEM medium (Gibco/Invitrogen) was incubated at room temperature and added to cells previously washed with OptiMEM. The cells were incubated for 5 h, and OptiMEM was replaced with 2 ml of supplemented DMEM and incubated for 40 to 48 h.

Surface biotinylation. COS-7 monolayers were grown in 35-mm plates and transfected with cDNAs encoding the HHN and F proteins and/or cDNAs encoding PDI or ERdj5. When added, PDI inhibitors were added at 24 h posttransfection. After 48 h, the monolayers were washed three times with PBS-CM (phosphate-buffered saline with 0.1 mM CaCl₂ and 1 mM MgCl₂). PBS-CM containing 0.5 mg/ml sulfo-NHS-SI-sulfosuccinimidyl-2-(biotinamido)-ethyl-1,3-dithiopropionate-biotin (Pierce) was added, and cells were incubated for 40 min at 4°C. Unbound biotin was absorbed with 2 ml DMEM containing fetal calf serum (10%), and cells were washed three times with PBS and lysed with RSB lysis buffer (0.01 M Tris-HCl [pH 7.4], 0.01 M NaCl, 1.5 mM MgCl₂) containing 1% Triton X-100, 0.5% sodium deoxycholate, 2.5 mg of N-ethyl maleimide per ml, and 0.2 mg of DNase per ml. Lysates were incubated for 1 h at room temperature or overnight at 4°C with neutravidin-agarose (Pierce) containing 0.3% sodium dodecyl sulfate (SDS), and they were washed with PBS containing 0.5% Tween-20 and 5 mg/ml bovine serum albumin (BSA) and then with PBS containing 0.5% Tween-20 and 1 mg/ml BSA. Precipitates were washed three times with PBS containing 0.5% Tween-20 and 0.4% SDS, resuspended in gel sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol) with 0.7 M β-mercaptoethanol, and resolved by polyacrylamide gel electrophoresis (PAGE). Proteins were detected by Western blot analysis.

**PAGE** and Western blot analysis. Proteins in cell extracts or immunoprecipitates, diluted in gel sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol) with 0.7 M β-mercaptoethanol, were resolved by 10% polyacrylamide gels. After electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol) and transferred to Immobilon-P (Millipore Corp.) membranes. The membranes were blocked overnight at 4°C in PBS containing 0.5% Tween-20 and 5% nonfat milk, washed with PBS containing 0.1% SDS, incubated with primary antibody, washed with PBS containing 0.1% SDS, and then incubated with HRP-conjugated secondary antibody (anti-mouse IgG coupled to horseradish peroxidase) (Amersham Biosciences) diluted 1:1,000 in PBS, washed with PBS containing 0.5% Tween-20 and 0.2% SDS, and then washed extensively in PBS. Development of the blue color was quantitated by measuring the absorbance at 650 nm (12, 21).

**RESULTS**

**Construction of MPB.** MPB [3-[3-(maleimidobutyrylamidino) biocytin] (Molecular Probes) was used to biotinylate free thiols in cell surface proteins. At 48 h after transfection, cells, grown in 35-mm plates, were washed with PBS-CM (PBS with 0.1 mM CaCl₂ and 1 mM MgCl₂) and incubated with MPB (0.5 mM in PBS) at 25°C for 30 min. Cells then were washed once with DMEM and twice with PBS and lysed using RSB lysis buffer as described above. Proteins in extracts were precipitated with neutravidin-agarose containing 0.3% SDS after having been washed once with PBS containing 0.5% Tween-20 and 5% BSA, and then washed with PBS containing 0.5% Tween-20 and 1 mg/ml BSA. Precipitates were washed three times with PBS containing 0.5% Tween-20 and 0.4% SDS and resolved by SDS-PAGE and analyzed by Western blotting using anti-F (anti-HR2) antibody.

**PAGE and Western blot analysis.** Proteins in cell extracts or immunoprecipitates, diluted in gel sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, and 10% glycerol) with 0.7 M β-mercaptoethanol, were resolved by 10% polyacrylamide gels. After electrophoresis, gels were equilibrated in transfer buffer (25 mM Tris, pH 8.2, 192 mM glycine, 15% methanol) and transferred to Immobilon-P (Millipore Corp.) membranes. The membranes were blocked overnight at 4°C in PBS containing 0.5% Tween-20 and 10% nonfat milk, washed with PBS-Tween 20, and incubated for 1 h at room temperature with primary antibody diluted to 1:1,000 in PBS-Tween 20. Membranes were washed, incubated for 1 h at room temperature with secondary antibody (goat anti-mouse IgG, goat anti-rabbit IgG, or anti-mouse IgG coupled to horseradish peroxidase) (Amersham Biosciences) diluted to 1:4,000 in PBS-Tween 20, and then washed extensively in PBS-Tween 20. Bound antibody was detected using the ECL Western blotting detection reagent (Amersham Biosciences). The quantification of the signal was accomplished using a Fluor-S imager (Bio-Rad).

**Cell viability.** COS-7 cell monolayers plated on a 12-well plate were transfected with empty vector or with ERdj5 or PDI cDNA and with or without HHN and F cDNAs. At 48 h posttransfection, cells were incubated with 1 ml of MTT [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) isothiazolium, monosulfate (Promega)] (0.5 mg/ml in PBS) for 3 h at 37°C. After removing MTT, cells were treated with 1 ml acidified isopropanol (0.04 M HCl in absolute isopropanol) to solubilize blue formazan crystals, and the development of blue color was quantitated by measuring the absorbance at 570 nm and subtracting the background measurement at 650 nm (12, 21).

**Syncytium formation.** COS-7 cells, grown in 35-mm plates, were transfected with HHN and F cDNAs with or without ERdj5 or PDI cDNA (0.5 μg/plate) using Lipofectamine as described above. The average number of nuclei per syncytium was counted in 20 fusion areas for each plate at 24, 48, and 72 h. Values obtained after transfection with empty vector were subtracted.

**Content mixing.** Content mixing was measured by using a modification of a previously described protocol (19). Briefly, cells plated on 12-well plates were transfected with pCAGGS-HHN (0.04 μg/well), pCAGGS-F (0.04 μg/well) cDNAs, and a plasmid carrying a tetracycline-responsive transcriptional activator, TTA (Clontech) (0.4 μg/well). A separate population of cells was transfected with a plasmid encoding the β-galactosidase protein under the control of the β-galactosidase promoter. Transfections were performed using Lipofectamine (Gibco) as recommended by the manufacturer. After transfection, a mixture of DNA (0.5 μg/35-mm plate) and 7 μl of Lipofectamine or Lipofectin (0.04 μg/plate) using Lipofectamine as described above. The average number of nuclei per syncytium was counted in 20 fusion areas for each plate at 24, 48, and 72 h. Values obtained after transfection with empty vector were subtracted.
FIG. 1. Overexpression of ERdj5 and PDI and its effect on HN and F protein expression and cell viability. (A) Proteins in extracts of COS-7 cells, transfected with empty vector or cDNA encoding ERdj5 (top) or PDI (bottom), were resolved by SDS-PAGE and analyzed by Western blotting. The blots, with actin as a loading control, are shown below each panel. Numbers at bottom of each panel represent the expression of the respective proteins in transfected cells as a percentage of expression in untransfected cells. The quantification was performed in films with protein bands in the linear range. (B) Cells, untransfected (UT) or at 48 h after transfection with ERdj5 or PDI cDNA and with or without F and HN protein cDNA, were incubated with MTT for 3 h at 37°C. The blue metabolic product of MTT, measured as the optical density at 570 nm, is represented as a percentage of the value obtained for untransfected cells. The values represented are averages from three different experiments, and error bars represent the ranges. (C) Cells were transfected with empty vector (lanes 2 and 6) or were cotransfected with HN and F protein cDNAs (lanes 3 and 7) or HN and F protein with ERdj5 cDNA (lanes 4 and 8) or with PDI cDNA (lanes 5 and 9). Surface HN protein (top) or F protein (bottom), biotinylated using sulfo-NHS-SS-biotin, was precipitated with neutravidin-agarose (lanes 6 to 9), and total F or HN protein in the extracts (lanes 2 to 5) was resolved by SDS-PAGE and analyzed by Western blotting using anti-F protein antibody (anti-HR2) or anti-HN protein antibody (anti-AS). The amount of the total extract loaded represents one-third of the amount of extract used to precipitate biotinylated surface proteins. Lane 1 shows the infected cell extract that was used as a marker. (D) Surface expression of PDI. Cells were transfected with increasing amounts of cDNAs encoding PDI and, 48 h post transfection, cells were incubated with sulfo-NHS-SS-biotin. Biotinylated cell surface proteins in aliquots of the resulting cell extracts were precipitated with neutravidin (lanes 2, 4, 6, 8, and 10). Lanes 1, 3, 5, 7, and 9 show protein precipitated with equivalent amounts of aprotinin coupled to protein G as a negative control. PDI in the precipitates was detected with anti-PDI protein antibody in a Western blot. Top panel, lanes 1 and 2, no PDI cDNA; lanes 3 and 4, 0.25 μg PDI cDNA; lanes 5 and 6, 0.5 μg PDI cDNA; lanes 7 and 8, 0.75 μg PDI cDNA; and lanes 9 and 10, 1 μg PDI cDNA. Bottom panel, the detection of actin in each extract.

RESULTS

Overexpression of ERdj5 and PDI in COS-7 cells and effects on cell viability and NDV glycoprotein expression. To explore the effects of the overexpression of PDI-like isomerases on membrane fusion, we first examined the expression of two different members of the PDI isomerase family, PDI and ERdj5, after the transfection of cells with cDNAs encoding these isomerases. As shown in Fig. 1A, COS-7 cells transfected with ERdj5 or PDI cDNA expressed the respective proteins at 1.5- and 1.8-fold the levels of untransfected cells.

To determine if the increased expression of these isomerases affected cell viability, MTT, a compound that is metabolized to form blue formazan crystals in live cells (21), was used. COS-7 cells transfected with cDNA encoding ERdj5 or PDI and with or without HN and F protein cDNA were incubated with MTT, and the development of blue color in these cells was compared to that of untransfected cells or cells transfected with HN and
F cDNAs only. As shown in Fig. 1B, the overexpression of ERdj5 or PDI did not decrease cell viability. In addition, as MTT is a measure of metabolic activity (21), the results suggest that the overexpression of isomerases did not increase cell division.

To determine the effect of the overexpression of these isomerases on the surface expression and the total expression of HN or F protein, surfaces of COS-7 cells expressing HN and F proteins with or without ERdj5 or PDI were biotinylated using sulfo-NHS-SS-biotin. Lysates of these cells were precipitated with neutravidin-agarose to detect the biotinylated surface proteins. The total HN and F proteins in the resulting cell lysates were determined by Western blot analysis. The results in Fig. 1C show that neither the total expression of HN or F protein (lanes 3 to 5) nor the surface expression of HN or F protein (lanes 7 to 9) was affected by the overexpression of ERdj5 or PDI.

To verify that, as previously reported, PDI can be detected at cell surfaces and that increased expression resulted in the increased surface expression of this host protein, surfaces of cells transfected with increasing amounts of cDNAs encoding PDI were biotinylated with sulfo-NHS-SS-biotin. Proteins in lysates of these cells were precipitated with neutravidin-agarose to precipitate, specifically, cell surface molecules. Figure 1D, top, shows that PDI was biotinylated and that increased expression results in increased amounts of the protein on cell surfaces. The surface expression of ERdj5 was not detected in a similar assay. The amount of total PDI detected on cell surfaces was approximately 0.5 to 1.5% of total cell-associated PDI. This level of ERdj5 was below the limits of detection of this protein using available antibodies, which may account for the failure to detect the protein at cell surfaces.

ERdj5 or PDI overexpression in cells expressing HN and F proteins enhances cell-cell fusion.

To determine the effect of the overexpression of these thiol isomerases on cell-cell fusion, the sizes of syncytia in cells cotransfected with cDNAs encoding HN and F proteins with or without ERdj5 or PDI were compared to those in cells transfected only with cDNAs encoding HN and F proteins. Figure 2A shows representative cell monolayers visualized by immunofluorescence using anti-NDV antibody. Syncytia in monolayers overexpressing ERdj5 or PDI isomerases were noticeably larger than syncytia in monolayers expressing HN and F proteins only. This result was quantified by determining the average number of nuclei per syncytium in cells

FIG. 2. Effect of ERdj5 and PDI overexpression on cell-cell fusion. (A) Syncytia were visualized by the immunofluorescence of cells using anti-NDV antibody. Cells transfected with empty vector or cotransfected with HN and F protein cDNAs with or without ERdj5 or PDI cDNA were processed for immunofluorescence at 48 h posttransfection. (B) Average sizes of syncytia after transfection with HN and F protein cDNAs (HN+F), HN, F, and ERdj5 cDNAs (HN+F+E), or HN, F, and PDI cDNAs (HN+F+P) were determined by counting the number of nuclei per syncytium in 20 different fusion areas at 24, 48, and 72 h posttransfection. The results shown are the averages of three independent experiments, with the error bars indicating standard deviations. (C) Effects of the overexpression of ERdj5 and PDI on content mixing were assayed using a β-galactosidase reporter assay. Effector cells cotransfected with HN and F protein cDNAs as well as with pTA were mixed with target cells (transfected with pB1-G with or without ERdj5 or PDI). Content mixing between effector and target cells was quantified by measuring β-galactosidase in cell extracts and is shown as a percentage of the activity detected in cells transfected with HN and F cDNAs (HN+F). The results shown are the averages of three independent experiments, and the error bars indicate the range.
Effector cells were transfected with cDNAs encoding the isomerases, the enhancement of fusion was further stimulated by approximately 20% (not shown). That enhanced fusion was observed when target cells overexpress only PDI or ERdj5 is consistent with the conclusion that it is cell surface thiol isomerases that are involved in the enhanced fusion and not intracellular effects of the isomerases on the F or HN protein.

To determine if this increase in cell-cell fusion in cells overexpressing ERdj5 or PDI protein was due directly to the increased production of free thiols, we characterized the effect of an inhibitor of free thiols on cytoplasmic protein mixing in cells overexpressing ERdj5 or PDI proteins. DTNB, a nonspecific inhibitor of free thiols (6), efficiently inhibited the content mixing in cells overexpressing ERdj5 or PDI protein as well as in cells expressing NDV glycoproteins alone (Fig. 3A). This result showed that the increased fusion also was dependent on thiol isomerase activity.

To determine if the increased fusion was due directly to the overexpression of the isomerases, we determined the effect of antibodies specific to the PDI protein (Fig. 3B) or ERdj5 protein (Fig. 3C) on the enhanced cell-cell fusion. In the presence of antibody to PDI or ERdj5, content mixing in cells overexpressing PDI or ERdj5, respectively, was significantly reduced to levels below those seen in cells not overexpressing the isomerases. This result suggests that increased fusion is due directly to the increased levels of surface PDI or ERdj5. The results also are consistent with the proposal that other thiol isomerases also can function in fusion, since neither antibody could completely inhibit fusion, as shown here and previously (12).

**Effect of overexpression of ERdj5 and PDI on detection of free thiols in F protein expressed with or without HN protein.**

To determine directly if the overexpression of ERdj5 or PDI protein leads to an alteration in the detection of free thiols in F protein, surfaces of cells expressing F protein with either of the isomerases were labeled with a thiol-reactive biotin, MPB. MPB links to the free thiols in proteins on cell surfaces and thus biotinylates those proteins (11). We have previously reported that the surface-expressed F protein, but not the HN protein, is labeled with this reagent (12). The cell surface proteins labeled with MPB were precipitated with neutravidin-agarose, and the amount of protein precipitated was normalized to the amount of total F protein in the extracts. Figure 4A shows that the detection of free thiols in both F₀ and F₁ proteins, expressed in the absence of HN protein, was increased in cells cotransfected with cDNA encoding PDI or ERdj5. The labeling of F₁ was increased by slightly more than twofold in cells overexpressing the isomerases (Fig. 4A).

We also determined the effects of the overexpression of the isomerases on the detection of free thiols in F protein coexpressed with HN protein. We previously reported that levels of MPB binding to F protein were only slightly affected by coexpression with HN protein (12). Interestingly, the overexpression of ERdj5 or PDI protein resulted in the significantly decreased detection of free thiols in F protein in the presence of HN protein (Fig. 4B). This result is consistent with the proposal that, due to the increased fusion in cells overexpressing the isomerases, more of the steady-state cell surface F protein is in a postfusion conformation, and that this conformation is not a substrate for thiol isomerases.

FIG. 3. Effect of inhibitors of PDI isomerases on cell-cell fusion in cells overexpressing ERdj5 or PDI. The effects of DTNB (A), anti-PDI antibody (B), or anti-ERdj5 antibody (C) were assayed using a β-galactosidase reporter assay, as described in Materials and Methods. Effector cells cotransfected with HN and F protein cDNAs as well as pTAX were mixed with target cells (transfected with pBI-G with or without ERdj5 or PDI) in the presence of DTNB (A), anti-PDI antibody (B), or anti-ERdj5 antibody (C). β-Galactosidase activity in cell extracts represents content mixing and is shown as a percentage of the activity detected in cells transfected only with HN and F cDNAs (HN+F). The results represent the average of three independent experiments with the error bars representing the range.

expressing HN and F proteins with or without isomerases. As shown in Fig. 2B, the average number of nuclei per syncytium, determined at different time points after transfection, was twofold higher in cells overexpressing ERdj5 or PDI than in cells expressing HN and F proteins only.

Cell-cell fusion also was measured by a cytoplasmic protein mixing assay as described in Materials and Methods. Results in Fig. 2C and 3A show that the overexpression of ERdj5 or PDI increased content mixing by three- to fourfold. This increase was observed when only target cells were transfected with cDNA encoding PDI or ERdj5 (Fig. 2C). Identical results were obtained when only effector cells were transfected with cDNA encoding either isomerase (not shown). When both target and effector cells were transfected with cDNAs encoding the
No free thiols were detected in the HN protein (12), even when PDI or ERdj5 was over expressed (not shown).

**Overexpression of ERdj5 or PDI in cells expressing HN and F proteins favors the postfusion conformation of F protein.** Previously we showed that the binding of two different conformation-sensitive anti-F protein antibodies, anti-Fu1a and anti-HR1 antibodies, to cell surface F protein expressed with HN protein was altered in the presence of the inhibitors of free thiols (12). Anti-HR1 antibody was raised against an HR1 peptide and, thus, binds the HR1 domain. Anti-Fu1a likely binds the fusion peptide, since a point mutation in the fusion peptide inhibits the binding of the antibody to the mature protein (29). Anti-Fu1a binding was increased and the binding of anti-HR1 was decreased by the inhibition of isomerases and fusion (12). This result suggested that these antibodies differentially bind to pre- and postfusion conformations of F protein. We further analyzed the binding of these antibodies to cell surface F protein expressed with HN protein in the presence or absence of exogenous neuraminidase (Fig. 5) in order to define further the form of F protein recognized by these antibodies. In cells incubated overnight with exogenous neuraminidase, the majority of cell surface F protein is likely to be in a prefusion conformation, since the absence of cell receptors for HN protein will inhibit HN protein binding and F protein activation. In cells grown in the presence of exogenous neuraminidase, the binding of anti-Fu1a antibody (Fig. 5A and B)

**FIG. 4.** Effect of ERdj5 and PDI overexpression on the production of free thiols in F protein when expressed without (A) or with (B) HN protein. Cells transfected with empty vector, F protein cDNA, or HN and F protein cDNAs with or without ERdj5 or PDI cDNA were labeled with MPB as described in Materials and Methods. Proteins in resulting cell extracts were precipitated with neutravidin-agarose (lanes 1 to 4) and resolved by SDS-PAGE. Total F protein in cell extracts (lanes 5 to 8) was resolved as a control for the expression of the proteins. The quantification of F1 labeled by MPB is shown at the bottom of each panel and was performed in films with protein bands in the linear range.

**FIG. 5.** Binding of conformation-sensitive antibodies to F protein on cell surfaces in the presence of MPB or neuraminidase (NAase). (A, C, and E) Cells transfected with pSVL vector (top panels) or with pSVL-HN and pSVL-F (bottom panels) were processed for surface immunofluorescence using anti-Fu1a antibody, anti-HR1 antibody, and anti-NDV antibody, respectively (indicated on the left). Column 1 shows cells without any treatment, and columns 2 and 3 show cells treated with MPB and neuraminidase, respectively, as described in Materials and Methods. (B, D, and F) Quantification of the fluorescence intensities of the cells shown in panels A, C, and E, respectively. The results shown are averages of the mean fluorescence intensity of 10 different cells and are normalized for background fluorescence by subtracting the mean fluorescence of cells transfected with empty vector. Error bars represent the range of fluorescence intensity of 10 different cells within one experiment, and the experiment shown is representative of three similar experiments.
was increased five- to sixfold compared to that of untreated cells (first lane), and the binding of anti-HR1 antibody (Fig. 5C and D) was inhibited. The binding of anti-NDV antibody, a control antibody (Fig. 5E and F), was not affected. These results suggest that anti-Fu1a antibody recognizes primarily F protein in prefusion conformation, and anti-HR1 antibody binds better to F protein in an intermediate conformation or, perhaps, a postfusion state. Similar results were seen when fusion was inhibited by MBP linkage (12) (Fig. 5, middle lane). These results are consistent with our previous results that the binding of anti-Fu1a antibody is increased, and the binding of anti-HR1 antibody is inhibited, when cells are treated with inhibitors of free thiols, DTNB and bacitracin, conditions that also inhibit fusion (12). Taken together, these results argue that anti-Fu1a preferentially detects prefusion F protein, while anti-HR1 detects F protein after its activation.

We next determined the effects of the overexpression of ERdj5 and PDI proteins on the binding of these conformation-sensitive antibodies (Fig. 6A to D). As shown in Fig. 6A and B, the binding of anti-Fu1a antibody was decreased by the overexpression of isomerases, while the binding of anti-HR1 antibody (Fig. 6C and D) was increased by the overexpression of isomerases. As shown above, the overexpression of these isomerases enhanced fusion and, thus, increased the amount of the postfusion form of F protein. The binding of anti-NDV antibody (Fig. 6E and F) was not affected. These results suggested that the majority of F protein in these cells is in a postfusion conformation when fusion is stimulated by the increased expression of isomerases.

DISCUSSION

In this study, we evaluated the effect of the overexpression of the thiol isomerases PDI and ERdj5 on the activity and conformation of the NDV F protein. The overexpression of either isomerase resulted in significantly increased cell-cell membrane fusion mediated by NDV glycoproteins. The overexpression of either isomerase also resulted in the increased detection of free thiols in F protein expressed in the absence of HN protein and a decreased detection of free thiols in F protein expressed with HN protein. The overexpression of these isomerases also altered the binding of two different conformation-sensitive antibodies in a manner consistent with an enhanced population of surface-expressed F protein in a postfusion conformation in the presence of HN protein.

While increased fusion upon the overexpression of the thiol isomerases could be an indirect effect of this overexpression, results presented here are most consistent with the proposal that the increased fusion activity of the F protein is a direct result of increased cell surface thiol isomerase activity. First, the overexpression of PDI or ERdj5 increased the levels of each of these isomerases in total cell extracts. Furthermore, the
levels of surface PDI were significantly increased. Second, the overexpression of either isomerase increased the detection of free cysteines in the population of cell surface F protein nearly twofold over the levels we previously detected in the F protein (12), suggesting a direct effect on F protein. Third, we showed that increased fusion was observed only when target cells were overexpressing either thiol isomerase. This result argues against an indirect effect of the thiol isomerases on the folding of intracellular F protein. The result also is consistent with the increased levels of PDI or ERdj5 on target cell surfaces. While the increased expression of ERdj5 in target cells could indirectly affect the expression of another protein on target cell surfaces, the finding that antibody specific to ERdj5 inhibited the increased fusion is more consistent with the direct effects of ERdj5 on cell surfaces. Fourth, we have shown previously that the inhibition of thiol isomerases with membrane-impermeable, specific inhibitors such as DTNB or bacitracin blocked the appearance of free cysteines in the F protein and blocked fusion (12). Here, we showed that DTNB also blocked fusion in cells overexpressing PDI or ERdj5. This observation is consistent with the conclusion that the increased expression of thiol isomerase activity at cell surfaces directly affected fusion. Fifth, the increased fusion, resulting from the overexpression of either isomerase, was significantly decreased by antibodies specific for the isomerase overexpressed. That both antibodies significantly decreased the enhanced fusion activity suggested that the enhanced fusion is due directly to the increased expression of the specific thiol isomerases and that both isomerases are at cell surfaces. That neither antibody completely inhibited fusion likely is due to the redundancy in cells of proteins with thiol isomerase activity. In the presence of specific thiol isomerase antibodies, other isomerases are still functional.

An alternative possibility to account for the results presented here is that the increased expression of thiol isomerases increased the efficiency of the proper folding of the F protein, increased concentrations of fusion-competent F protein, and, therefore, increased fusion. However, our results showed that there are not increased levels of F protein on cell surfaces in the presence of excess thiol isomerases. Furthermore, our results showed that fusion is increased significantly when isomerases are expressed on target cells only. This increase was identical to increases observed when the isomerases were transfected into effector cells only. This result argues that the increased thiol isomerase activity affects F protein at cell surfaces. It is unlikely that an improperly folded F protein is delivered to cell surfaces for repair by cell surface isomerases, since improperly folded proteins are not likely transported to cell surfaces but are subject to ER quality control mechanisms that degrade abnormally folded proteins (22).

The combined results presented here and previously (12) suggest that, prior to fusion activation, cell surface F protein exists in several forms that are in equilibrium, and the concentration of each form reflects the concentration of surface isomerases (illustrated in the model shown in Fig. 7). These results, as well as previous results that showed that the binding of thiol-reactive MPB blocked fusion (12), suggest that it is the form containing free thiols that proceeds through the conformational changes, which results in cell-cell fusion (Fig. 7).

When HN protein was coexpressed with F protein and the thiol isomerases, we found that the MPB labeling of F protein was reduced to about 50% of the level of MPB labeling of F protein when expressed with HN protein in the absence of the enhanced expression of an isomerase. One possibility for the decreased MPB labeling of F protein seen in this case is that, in the presence of HN and the isomerases, cell-cell fusion is enhanced and more steady-state F protein is in the postfusion conformation. Postfusion F protein is thought to be a very stable structure and, therefore, may not be a substrate for thiol isomerases and may not be labeled by MPB.

We utilized conformation-sensitive antibodies to explore this hypothesis. We found that the binding of anti-Fu1a antibody was significantly enhanced under conditions that inhibited fusion, while anti-HR1 antibody bound preferentially to F protein when fusion was not inhibited. Anti-Fu1a antibody is thought to bind near the fusion peptide, since the mutation of amino acid 123, located seven amino acids from the amino-terminal end of F1, eliminated the binding of this antibody to F protein (29) without having an effect on the intracellular processing of the protein. Thus, this mutation does not likely have global effects on the conformation of the protein, which could account for the failure to bind anti-Fu1a. Anti-HR1 antibody, raised against an HR1 peptide, binds the HR1 domain in F protein.

According to current models proposed for conformational changes in F protein during membrane fusion (26, 37), the first conformational change in F protein is the dissociation of HR2 domains in the trimer. Subsequently, the HR1 domain unfolds and then refolds into an extended trimeric helix, resulting in the insertion of the fusion peptide, which is at the tip of the helix, into target cell membranes. F protein in this state is referred to as prehairpin intermediate. After the insertion of fusion peptide into the target membrane, the next proposed major conformational change involves the refolding of F protein into a stable trimer of hairpins formed by the complexing of the HR1 and HR2 domains into a six-helix bundle (6HB). The refolding of F protein into this postfusion form leads to the close approach and fusion of the target and effector membranes.

![FIG. 7. Model for the role of free thiols in NDV F protein. (A) F protein is present in a prefusion, metastable conformation with intact disulfide bonds. Disulfide bonds are cleaved by PDI-like isomerase, resulting in free thiols in F protein (B) before the major conformational changes in F protein required for fusion (C). MPB binds to the free thiols in F protein, and the binding of MPB prevents further conformational changes and the fusion of membranes. Anti-Fu1a antibody binds to F protein in the prefusion state, while anti-HR1 antibody binds preferentially to the activated or postfusion form of F protein. The enhanced expression of PDI or ERdj5 favors the conformation shown in panel C at steady state. S-S, disulfide bond; SH, free or reduced cysteine.]
Since anti-Fu1a binds preferentially to F protein when fusion activation is inhibited, it is likely that the anti-Fu1a antibody binds preferentially to a prefusion conformation of F protein before any major conformational change. In the proposed prefusion conformation of the paramyxovirus 5 F protein, the amino acid sequence of the NDV F protein amino acid 123 in the fusion peptide is exposed on the surface of the trimer. This structure was formed with an uncleaved F protein in the absence of the HN protein. However, if this structure represents the conformation of the prefusion, cleaved NDV F protein in the presence of HN protein, the region around amino acid 123 should be accessible to anti-Fu1a in the prefusion F protein. Upon insertion into a membrane, the fusion sequence, buried in a target membrane, would be masked from antibody binding. In contrast, it is likely that anti-HR1 antibody will bind preferentially to the HR1 trimer in the prehairpin conformation or subsequent intermediate forms of the F protein. The results of antibody binding in cells treated with neuraminidase, which prevents receptor binding and, consequently, fusion, were consistent with the predicted binding for these antibodies. Our previous findings that inhibitors of thiol isomerases blocked fusion and increased the binding of anti-Fu1a and decreased anti-HR1 binding (12) also are consistent with this conclusion. We have shown previously that MBP binding to free thiols also blocks fusion (12). Here, we showed that, after MBP binding, the anti-Fu1a antibody binding increases and anti-HR1 antibody binding decreases. These combined results suggest that the binding of MBP leads to the presence of more F protein in the prefusion state. In addition, MBP also may bind to and inactivate cell surface thiol isomerases, and this inactivation may result in a decrease in disulfide bond reduction in F protein, the inhibition of fusion, and the presence of more F protein in the prefusion form. In either case, this result further confirmed that when free thiols are blocked, F protein is in the prefusion conformation.

In contrast, when isomerases were overexpressed, fusion was significantly enhanced, and the binding of anti-Fu1a antibody decreased and that of the anti-HR1 antibody increased. These results suggested that the overexpression of the isomerases significantly changed the conformation of the F protein, and it is likely that the majority of F protein, under these conditions, is in a postfusion conformation.

The overall implication of these results is that, in the absence of free thiols, F protein is in a prefusion conformation, as illustrated in Fig. 7. The overexpression of isomerases increases the concentration of an early conformational intermediate with free thiols. In the absence of HN protein expression, no further changes take place. However, when disulfide bonds are reduced, F protein, in the presence of HN protein, may move into conformations that reflect the postfusion or intermediate forms. The reduction of disulfide bonds in F protein may facilitate the conformational changes in F protein required for membrane fusion (Fig. 7).

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REFERENCES


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