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Endocytosis Plays a Critical Role in Proteolytic Processing of the Hendra Virus Fusion Protein

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The Hendra virus fusion (F) protein is synthesized as a precursor protein, F_0, which is proteolytically processed to the mature form, F_1+F_2. Unlike the case for the majority of paramyxovirus F proteins, the processing event is furin independent, does not require the addition of exogenous proteases, is not affected by reductions in intracellular Ca^{2+}, and is strongly affected by conditions that raise the intracellular pH (C. T. Pager, M. A. Wurth, and R. E. Dutch, J. Virol. 78:9154–9163, 2004). The Hendra virus F protein cytoplasmic tail contains a consensus motif for endocytosis, YXXΦ. To analyze the potential role of endocytosis in the processing and membrane fusion promotion of the Hendra virus F protein, mutation of tyrosine 525 to alanine (Hendra virus F Y525A) or phenylalanine (Hendra virus F Y525F) was performed. The rate of endocytosis of Hendra virus F Y525A was significantly reduced compared to that of the wild-type (wt) F protein, confirming the functional importance of the endocytosis motif. An intermediate level of endocytosis was observed for Hendra virus F Y525F. Surprisingly, dramatic reductions in the rate of proteolytic processing were observed for Hendra virus F Y525A, although initial transport to the cell surface was not affected. The levels of surface expression for both Hendra virus F Y525A and Hendra virus F Y525F were higher than that of the wt protein, and these mutants displayed enhanced syncytium formation. These results suggest that endocytosis is critically important for Hendra virus F protein cleavage, representing a new paradigm for proteolytic processing of paramyxovirus F proteins.

Paramyxoviruses, a family of enveloped negative-strand RNA viruses, include important human pathogens such as human respiratory syncytial virus (RSV), measles virus, and the recently emerged Hendra and Nipah viruses (17). The entry of paramyxoviruses is promoted by the two major surface glycoproteins, namely, an attachment protein (HN, H, or G) required for primary receptor binding and a fusion (F) protein, which promotes both virus-cell and cell-cell membrane fusion. Paramyxovirus F proteins, with the exception of the F protein from SER virus (29), promote fusion at neutral pH, and viral entry is thought to occur at the plasma membrane of the target cell (17).

Proteolytic processing of the inactive precursor forms of paramyxovirus F proteins as well as of many viral fusion proteins is required to form fusogenically active molecules (reviewed in reference 8). The majority of paramyxovirus F proteins, including those from simian virus 5 (11), measles virus (4), and RSV (2, 12, 25), are proteolytically processed by furin, a Ca^{2+}-dependent serine protease primarily localized to the *trans*-Golgi network. Furin-mediated F protein cleavage is thought to occur within the exocytic pathway. Proteolytic cleavage mediated by furin has also been demonstrated for a number of other viral proteins (1, 14, 16, 30, 33–35). In contrast, the Sendai virus F protein has a single basic residue at its cleavage site and is not cleaved intracellularly. However, the F_0 precursor that is expressed at the cell surface and incorporated into released virions can be cleavage activated by exogenous proteases (15, 28).

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The Hendra virus F protein is a type I integral membrane protein of 546 amino acid residues and is also made as a precursor form, F_0, that is subsequently cleaved into a disulfide-linked heterodimer, F_1+F_2 (21, 26). The Hendra virus F protein contains several common elements seen in other paramyxovirus fusion proteins, including a hydrophobic fusion peptide at the N terminus of the F_1 subunit, heptad repeats abutting the fusion peptide and the putative transmembrane domain (13), and multiple N-linked carbohydrates (6). However, proteolytic processing of the Hendra virus F protein differs significantly from that seen for the majority of paramyxovirus F proteins. The processing of F_0 to F_1+F_2 occurs in a furin-independent manner (21, 26), does not require the addition of exogenous proteases, displays no sensitivity to the removal of intracellular Ca^{2+}, and is strongly inhibited by increases in intracellular pH (26). The closely related Nipah virus F protein has also been shown to be cleaved in a furin-independent manner and to not require a basic residue at the site of processing (22).

Paramyxovirus F proteins display great diversity in both the length and composition of their cytoplasmic tails. The Hendra virus F protein cytoplasmic tail is 22 amino acids long and contains three tyrosine residues. Of these, tyrosine 525 is part of a YXXΦ motif that has been implicated in the promotion of endocytosis. To identify the role of this motif in the Hendra virus F protein, we prepared Hendra virus F proteins with the mutations Y525A and Y525F (Hendra virus F Y525A and Hendra virus F Y525F, respectively) and analyzed them for changes in the endocytosis rate, proteolytic processing, and promotion of membrane fusion. We observed rapid endocytosis for the wild-type (wt) Hendra virus F protein and a much slower rate of internalization for the Y525A mutant, which no

longer has a consensus endocytosis motif. An intermediate rate of endocytosis was observed for the Y525F mutant, consistent with reports that phenylalanine can function in place of tyrosine in this endocytosis motif (10). While both Hendra virus F mutants were rapidly transported to the cell surface in a manner similar to that of the wt protein, we observed dramatic reductions in the rate of proteolytic processing for Hendra virus F Y525A. These results indicate that endocytosis mediated by the YXXΦ motif is critical for proteolytic processing of the Hendra virus F protein.

MATERIALS AND METHODS

Cell lines. Vero cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Invitrogen, Carlsbad, California) supplemented with fetal bovine serum (FBS [10%]), penicillin (1%), and streptomycin (1%) and were incubated at 37°C.

Plasmids and mutagenesis. The Hendra virus F and G genes, kindly provided by Lin-Fa Wang, Australian Animal Health Laboratory, were subcloned into the pCAGGS mammalian expression vector (24, 26). Tyrosine 525 in the C-terminal domain of Hendra virus F was mutated to alanine (Y525A) and phenylalanine (Y525F) by site-directed mutagenesis using a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers were designed according to the QuikChange II protocol and obtained from Invitrogen by custom primer design.

Antibodies. Polyclonal antibodies to residues 526 to 539 of the cytoplasmic tail of Hendra virus F protein were produced by Genemed Custom Peptide Antibody Service, San Francisco, California (26).

Expression of the F protein. For expression using the pCAGGS expression system (24), subconfluent monolayers of Vero cells were transiently transfected with empty vector, pCAGGS-Hendra F, or pCAGGS-Y525A or -Y525F using Lipofectamine Plus (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol. After 3 to 5 h at 37°C, the cells were washed twice in phosphate-buffered saline plus calcium and magnesium chloride (PBS^+) and incubated overnight at 37°C in DMEM (10% FBS, 1% penicillin, 1% streptomycin).

Pulse-chase experiments and immunoprecipitation. Following transfection, cells were washed twice with PBS^+ and starved for 45 min in cysteine-methionine-deficient DMEM. Cells were then labeled for 30 min to 2 h using cysteine-methionine-deficient DMEM to which Tran[^{35}S] was added (100 μCi/ml; MP Biomedicals, Irvine, CA). Following labeling, cells were washed twice with PBS^+ and then chased (DMEM, 10% FBS, 1% penicillin-streptomycin) for various lengths of time. After the chase, cells were again washed twice with PBS^+ and then lysed with RIPA buffer (100 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% deoxycholic acid) containing 1:100 aprotinin (Calbiochem, San Diego, California), 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, Missouri), and 25 mM iodoacetamide (Sigma). Following centrifugation at 136,500 × *g* for 10 min, the lysates were immunoprecipitated as previously described (27) with 10 μl Hendra virus F protein-specific antiserum (26). Samples were analyzed in a 15% polyacrylamide gel under reducing conditions and visualized using the Typhoon imaging system (Amersham, Piscataway, NJ).

Biotinylation of cell surface proteins. Surface biotinylation was performed following transient transfection of Vero cells with wt or mutant Hendra virus F and radiolabeling. Cells were placed on ice and washed three times with ice-cold PBS deficient in calcium and magnesium chloride (PBS^-) at pH 8. Cells were then biotinylated using 0.8 ml EZ-Link sulfo–*N*-hydroxysuccinimide–biotin (sulfo-NHS-biotin, 0.5 mg/ml; Pierce, Rockford, Illinois) in PBS^- (pH 8) at 4°C. The cells were rocked gently on ice for 10 min and then incubated at 15°C for 20 min. Cells were then washed three times with PBS^- (pH 8) at 4°C and lysed in RIPA buffer as described for pulse-chase experiments. Lysates were immunoprecipitated, 40 μl of 10% SDS was added to the protein A-Sepharose beads, and the samples were boiled for 10 min. The supernatant was removed and saved. Sixty microliters of 10% SDS was added to the protein A-Sepharose beads, samples were boiled for another 10 min, and the supernatant was removed and added to the previous supernatant. Fifteen microliters of the combined supernatant was saved for analysis of the total F protein in the lysed cells. To the remaining 85 μl, 500 μl biotinylation dilution buffer (20 mM Tris [pH 8], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.2% bovine serum albumin) and 40 μl immobilized streptavidin beads (Pierce) were added, and the samples were rocked at 4°C for 1 h. The samples were washed with RIPA buffer as described previously. Both total and surface portions were analyzed in a 15% polyacrylamide gel under reducing conditions and visualized using the Typhoon imaging system (Amersham).

Endocytosis assay. The endocytosis assay was performed as described previously (19). wt and mutant F proteins were expressed in Vero cells using the pCAGGS system, and cells were radiolabeled as described above. To inhibit endocytosis, cells were put on ice in a 4°C cold room and then washed twice with PBS^+ at 4°C and twice with PBS^+ freshly adjusted to pH 8 at 4°C. Cells were then biotinylated with EZ-Link sulfo-NHS-SS-biotin (Pierce) as described previously (19). All plates, except for that for the 0-min time point, were then flooded with prewarmed (37°C) DMEM (supplemented with 10% FBS, 1% penicillin, 1% streptomycin) and returned to 37°C for 15 or 30 min to allow endocytosis to proceed. At the end of each time point, plates were returned to the cold room to inhibit further endocytosis and were washed twice with PBS^+ (pH 8) at 4°C. One of the duplicate plates was treated with 2-mercaptoethanesulfonic acid sodium salt (MESNa; 100 mM in MESNa buffer [50 mM Tris, pH 8.6, 100 mM NaCl, 1 mM EDTA, 0.2% bovine serum albumin]; Sigma), a membrane-impermeable reducing agent, three times (for 15 min each) with rocking. The other duplicate plate was treated with MESNa buffer (excluding MESNa) in the same manner. Following treatment with MESNa or buffer, all plates were washed twice with PBS^+ (pH 8) at 4°C and then incubated in iodoacetamide (5 mg/ml) in PBS^+ (pH 8) for 5 min with gentle rocking. The cells were again washed twice with PBS^+ (pH 8) at 4°C and then lysed in RIPA buffer. Lysates were immunoprecipitated, and streptavidin pull-down assays were performed as described above. Samples were analyzed in a 15% polyacrylamide gel under reducing conditions and visualized using the Typhoon imaging system (Amersham).

Analysis of surface expression following overnight labeling. Following transient transfection of Vero cells with wt or mutant Hendra virus F, cells were radiolabeled for 20 h using 2 ml overnight labeling medium (90% cysteine-methionine-deficient DMEM, 10% normal DMEM supplemented with 10% FBS, 1% penicillin, 1% streptomycin) to which Tran[^{35}S] was added (40 μCi/ml). Biotinylation, immunoprecipitation, and streptavidin pull-down assays were performed as described above, and samples were analyzed in a 15% polyacrylamide gel under reducing conditions and visualized using the Typhoon imaging system (Amersham).

Syncytium assay. Subconfluent monolayers of Vero cells in six-well plates were transiently cotransfected with pCAGGS-Hendra G (1.3 μg) and pCAGGS vector, pCAGGS-wt Hendra F, or pCAGGS-mutant Hendra F (1.6 μg) using Lipofectamine Plus (Life Technologies) according to the manufacturer's protocol. At 24 h posttransfection, the cells were washed twice with PBS^+, and 2 ml DMEM (supplemented with 10% FBS, 1% penicillin-streptomycin) was added. Pictures were taken at 43 h posttransfection using a Nikon Diaphot inverted phase-contrast microscope and a Kodak DCS digital camera.

Luciferase reporter gene assay. Subconfluent monolayers of Vero cells in six-well plates were transiently cotransfected with empty vector, wt F, or mutant F plasmid DNA (1.4 μg), G plasmid DNA (1.1 μg), and the T7 control plasmid (0.8 μg) containing the luciferase gene under control of the T7 promoter, using Lipofectamine Plus (Life Technologies) according to the manufacturer's protocol. At 18 h posttransfection, BSR cells, which constitutively express T7 polymerase (kindly provided by Klaus Conzelmann), were overlaid on the transfected Vero cells at a 1:1 ratio for 3 h. Luciferase activity was analyzed using a luciferase assay system (Promega) according to the manufacturer's protocol, and light emission was read using an Lmax luminometer (Molecular Devices, Sunnyvale, CA).

RESULTS

Expression of Hendra virus F endocytosis mutants. To determine the role of the putative endocytosis motif, site-directed mutagenesis was used to create Hendra virus F Y525A, which eliminates the consensus endocytosis motif, and Hendra virus F Y525F, which could retain some of the endocytic properties of the motif (10) while deleting potential phosphorylation of the hydroxyl on the tyrosine side chain (Fig. 1). To determine whether these mutations affected either protein expression or proteolytic cleavage, the wt Hendra virus F protein or the F protein mutants transiently expressed in Vero cells via the pCAGGS expression system (24) were analyzed by pulse-chase analysis followed by immunoprecipitation (26). The polypeptides were separated by SDS-polyacrylamide gel electrophore-

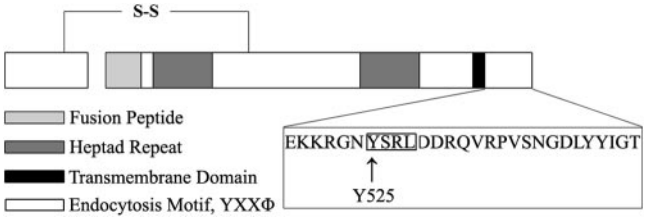

FIG. 1. Schematic representation of Hendra virus F protein. The magnified region shows the sequence of the cytoplasmic tail, identifying the relevant tyrosine residue. Mutants Y525A and Y525F were obtained by site-directed mutagenesis.

sis (SDS-PAGE) and visualized using the Typhoon imaging system (Fig. 2). The wt Hendra virus F protein and mutant Hendra virus F Y525F undergo efficient proteolytic processing, with the large majority of the protein being cleaved within 3 h, which is consistent with previous reports demonstrating rapid proteolytic cleavage in the absence of exogenous proteases (26). Surprisingly, while mutant Hendra virus F Y525A was expressed at similar levels to those of the wt protein, proteolytic processing was much less efficient, with only 22% of the protein undergoing processing by 3 h and 38% being processed by 6 h. A similar dramatic reduction in the rate of proteolytic cleavage of Hendra virus F Y525A was observed when the proteins were expressed in BHK cells (data not shown), indicating that the slower processing observed is not specific to one cell type. These results show that the mutation of tyrosine 525 to alanine dramatically affects proteolytic cleavage of the Hendra virus F protein.

Examination of the cell surface population of wt and mutant Hendra virus F proteins. A reduction in proteolytic processing could occur if proper transport through the secretory pathway is inhibited. To determine whether the mutants Y525A and Y525F efficiently transit through the secretory pathway to the cell surface, Vero cells transiently expressing wt Hendra virus F or the mutant Hendra virus F Y525A or Hendra virus F Y525F were metabolically labeled for 2 h. Cell surface proteins were then biotinylated at various times postlabeling, after

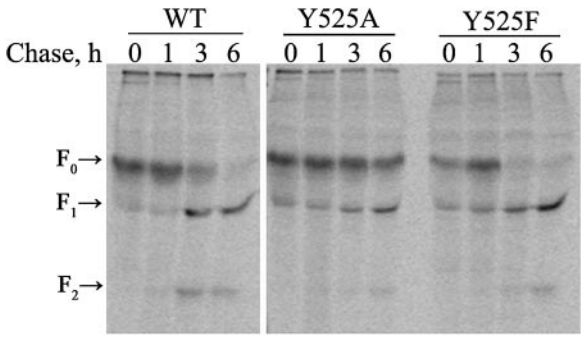

FIG. 2. Expression of wt and mutant Hendra virus F proteins. Vero cells were transfected with pCAGGS-wt Hendra F, pCAGGS-Hendra F Y525A, or pCAGGS-Hendra F Y525F. Cells were metabolically labeled for 45 min with Tran[^{35}S], chased for the indicated times, and then lysed. Lysates were immunoprecipitated with a polyclonal antibody specific to Hendra virus F, resolved via 15% polyacrylamide gel electrophoresis under reducing conditions, and visualized using storage phosphorimage autoradiography.

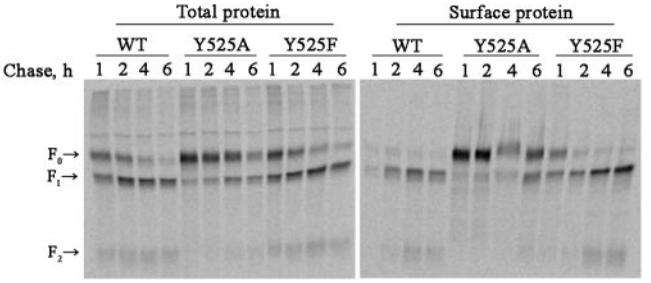

FIG. 3. Biotinylation of cell surface proteins. Vero cells were transfected with pCAGGS-wt Hendra F, pCAGGS-Hendra F Y525A, or pCAGGS-Hendra F Y525F. Cells were metabolically labeled for 2 h with Tran[^{35}S] and chased for the indicated times prior to the biotinylation of surface proteins. Cells were put on ice, biotinylated, and then immediately lysed, and the lysates were immunoprecipitated. Fifteen percent of the immunoprecipitated lysate was reserved as a representative of the total protein. The remaining 85% of the lysate was subjected to a streptavidin pull-down assay to isolate biotinylated proteins. Both total protein and biotinylated surface proteins were resolved via 15% polyacrylamide gel electrophoresis under reducing conditions and visualized using storage phosphorimage autoradiography.

which the Hendra virus F wt and mutant proteins were purified by immunoprecipitation. The biotinylated F proteins were then identified by streptavidin pull-down assays. Total protein levels (biotinylated plus nonbiotinylated proteins) were similar for the wt and two mutant proteins (Fig. 3). For the wt protein, only a small portion of the protein at 1 h postlabeling is present on the cell surface (biotinylated), with the cell surface population and the percentage of cleaved protein at the cell surface gradually increasing over time (Fig. 3). A similar pattern is seen with the mutant Hendra virus F Y525F, although the surface expression of this mutant is higher than that seen for the wt Hendra virus F protein. The mutant Hendra virus F Y525A rapidly reaches the cell surface, as evidenced by the high level of surface protein seen at 1 h postlabeling. However, little cleaved protein was present at the surface at 1 and 2 hours postlabeling, and by 6 h postlabeling, only 38% of the cell surface population of Hendra virus F Y525A had undergone proteolytic processing, compared to 96% and 98% for the wt and Y525F proteins, respectively. These results indicate that the slow proteolytic processing of the mutant Hendra virus F Y525A is not due to a defect in initial transport through the secretory pathway to the cell surface. Consistent with this, the wt Hendra virus F protein and mutant Y525A showed similar kinetics of conversion of N-linked carbohydrate chains from the high-mannose to the complex form in the medial Golgi, as judged by the acquisition of resistance to endo-β-*N*-acetylglycosaminidase digestion (data not shown).

Endocytosis assay of wt and mutant Hendra virus F proteins. Both the wt and mutant Hendra virus F proteins showed an increase in proteolytically processed protein on the cell surface over time (Fig. 3). This would be predicted if proteolytic cleavage occurs during endocytic recycling of the protein. However, this result would also be seen if proteolytic processing occurred during transport through the exocytic pathway but transport of the cleaved product to the cell surface was slower than transport of the uncleaved protein. To differentiate between these two possibilities and to verify changes in endocy-

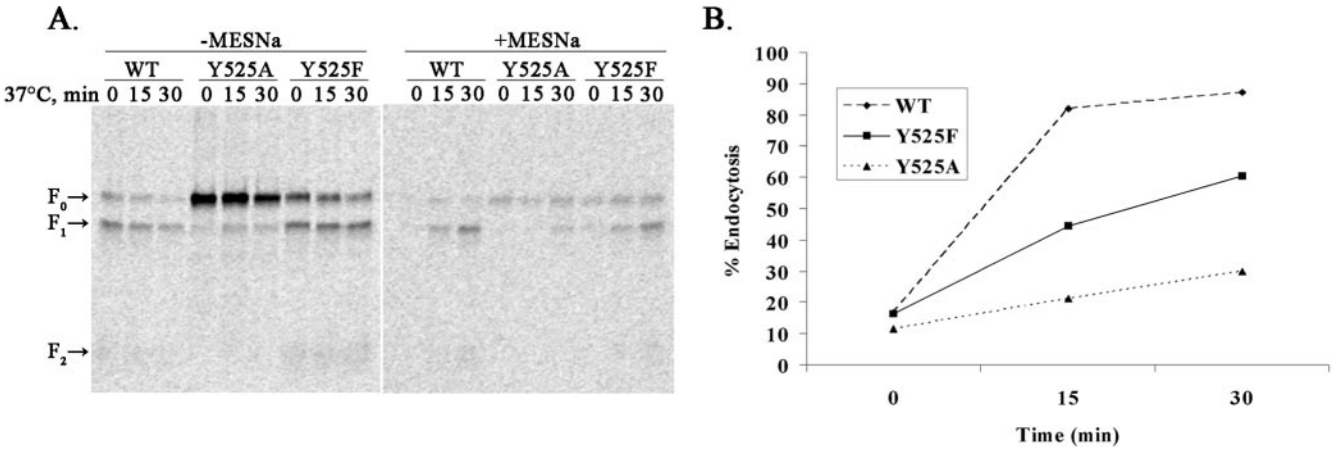

FIG. 4. Endocytosis assay. (A) Vero cells were transfected in duplicate with pCAGGS expressing wild-type or mutant Hendra virus F. Cells were metabolically labeled for 2 h with Tran[^{35}S] and chased for 1 h. Plates were moved to 4°C to inhibit endocytosis, biotinylated with a cleavable form of biotin, flooded with prewarmed DMEM, and returned to 37°C for various lengths of time to permit endocytosis. Following the warm-up, cells were returned to 4°C on ice, treated or not treated with MESNa, a membrane-impermeant reducing agent, to cleave any accessible biotin, and then lysed. Lysates were then immunoprecipitated, biotinylated proteins were pulled down with streptavidin beads, and samples were resolved via 15% polyacrylamide gel electrophoresis under reducing conditions and visualized using storage phosphorimage autoradiography. (B) Percent endocytosis of Hendra virus F and mutant F proteins averaged over three experiments.

tosis rates in the mutant proteins, we performed an endocytosis assay (19). Vero cells expressing the wt or mutant Hendra virus F proteins were metabolically labeled for 2 h, chased for 1 h, and then biotinylated with the reducible agent sulfo-NHS-SS-biotin. Cells were subsequently treated with the membrane-impermeant agent MESNa (to remove biotin present on cell surface proteins) immediately or were incubated at 37°C for 15 or 30 min to allow endocytosis prior to MESNa treatment. The wt Hendra virus F protein was rapidly endocytosed, with >60% of the biotinylated protein resistant to MESNa reduction after 15 min (Fig. 4A and B). The rate of endocytosis was much slower for the mutant Hendra virus F Y525A protein (Fig. 4B), consistent with slow bulk flow internalization rather than signal-directed endocytosis. These results confirm the functional importance of the endocytosis motif on the Hendra virus F protein cytoplasmic tail. An intermediate rate of endocytosis was observed for Hendra virus F Y525F. For all three proteins, the percentage of protein that had undergone proteolytic cleavage increased following endocytosis (Fig. 4A). These results show that endocytosis plays a critical role in the proteolytic processing of the Hendra virus F protein.

Membrane fusion promotion by wt and mutant Hendra virus F proteins. Our previous experiments indicated that the cell surface populations of the mutants Hendra virus F Y525A and Hendra virus F Y525F are significantly modified compared to that of the wt Hendra virus F protein, with increases in total cell surface expression, and in the case of the Y525A mutant, an increased amount of uncleaved protein on the cell surface. Since both changes can significantly affect the promotion of membrane fusion, we first determined the steady-state surface levels of the wt and mutant F proteins and then used two assays to examine the promotion of membrane fusion.

To examine surface populations, a 20-h metabolic label was performed on Vero cells transiently expressing the wt or mutant Hendra virus F proteins, followed by biotinylation. Both mutants had greatly increased cell surface expression of the proteolytically cleaved form, F_1 (Fig. 5). In addition, a significant amount of the uncleaved precursor protein, F_0, was observed for Hendra virus F Y525A.

Membrane fusion promotion of the wt and mutant Hendra virus F proteins was first assayed by determinations of syncytium formation. The wild-type or mutant Hendra virus F proteins were coexpressed with the Hendra virus G attachment protein in Vero cells (Fig. 6A). The formation of multinucleated giant cells (syncytia) was observed at 43 h posttransfection. Both mutant proteins promoted the formation of syncytia, indicating that both mutants are fusogenically active. Moreover, the syncytia formed were larger for Hendra virus F Y525A than for the wt protein and were largest for the mutant Hendra virus F Y525F protein. The enhanced fusogenic activity observed could be due to the higher surface density observed with these mutants, as increases in surface density have

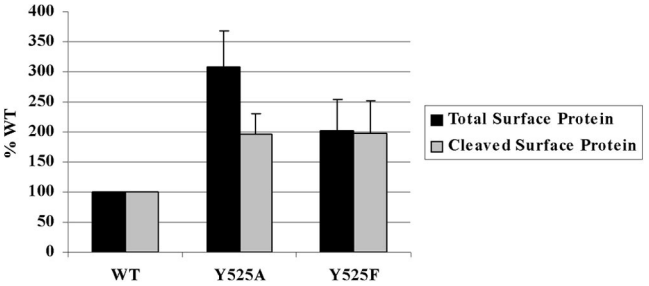

FIG. 5. Surface expression of wt and mutant Hendra virus F. Vero cells transfected with pCAGGS-wt or mutant Hendra virus F were metabolically labeled overnight with Tran[^{35}S], biotinylated, and lysed. Lysates were immunoprecipitated, and surface proteins were separated using streptavidin beads. Biotinylated surface proteins were resolved via 15% polyacrylamide gel electrophoresis under reducing conditions and quantitated using storage phosphorimage autoradiography. The results shown are the averages of three separate experiments.

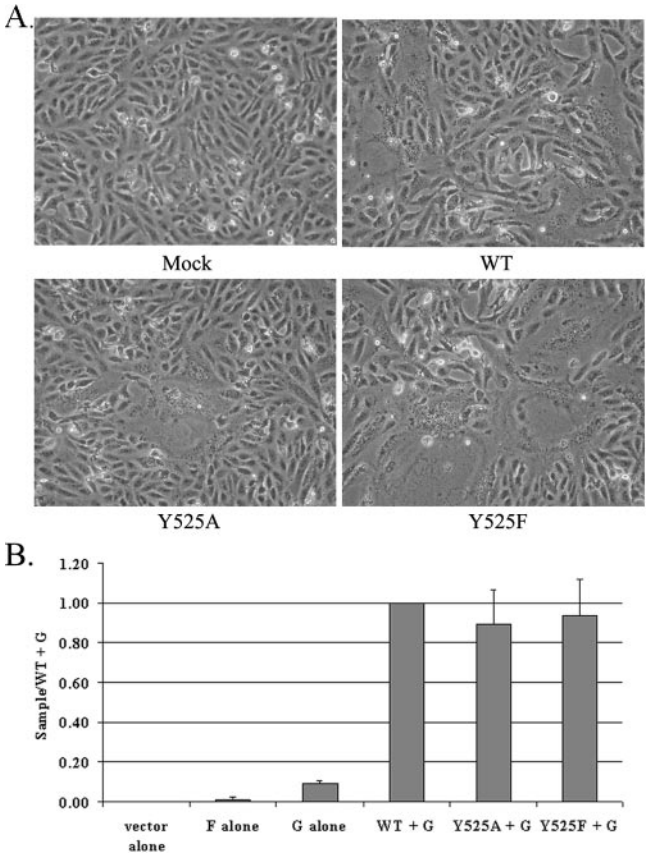

FIG. 6. Fusion assays. (A) Syncytium assay. Vero cells were cotransfected with empty pCAGGS vector, pCAGGS-wild type F, pCAGGS-Hendra FY525A, or pCAGGS-Hendra F Y525F and pCAGGS-Hendra G. Pictures were taken at 43 h posttransfection using a Nikon Diaphot inverted phase-contrast microscope and a Kodak DCS digital camera. (B) Reporter gene assay. pCAGGS expressing Hendra virus F wt or the two mutants, along with pCAGGS-Hendra G and a plasmid containing the luciferase gene under control of the T7 promoter, was transfected into Vero cells. BSR cells, which stably express the T7 polymerase, were overlaid onto the F- and G-expressing cells, and the mixed cell populations were incubated at 37°C for 3 h. Cells were lysed and analyzed for luciferase activity on a luminometer. The results are averages of duplicates or triplicates, with the vector alone set as background, and are representative of three separate experiments.

been found for other paramyxovirus F proteins to lead to increased fusion (9). Interestingly, the presence of uncleaved cell surface protein for Hendra virus F Y525A did not result in a significant reduction in membrane fusion.

Membrane fusion was also assayed using a quantitative luciferase reporter gene assay (Fig. 6B). Wild-type or mutant Hendra virus F proteins were coexpressed with the Hendra virus G attachment protein in Vero cells containing a luciferase plasmid under control of the T7 promoter. The F- and G-expressing cells were overlaid with BSR cells (5), which possess a stably transfected T7 polymerase, and the combined cell populations were incubated at 37°C for 3 h and then lysed and analyzed for luciferase activity. Fusion required the presence of both Hendra virus F and G proteins, as reported previously (Fig. 6B) (5). However, comparable levels of fusion were observed when the wt Hendra virus F protein or the mutants were coexpressed with the Hendra virus G protein. This suggests that syncytium formation is more strongly affected by surface expression levels than a short-term fusion assay and confirms that the uncleaved protein present on the cell surface with Hendra virus F Y525A does not significantly inhibit fusion promotion.

DISCUSSION

Our results demonstrate that endocytosis plays an important role in the proteolytic processing of the Hendra virus F protein. A Hendra virus F protein containing a point mutation within the endocytosis motif, Hendra virus F Y525A, showed a greatly reduced endocytosis rate compared to the wt protein (Fig. 4B), consistent with a previous report that this motif promotes endocytosis of the related Nipah virus F protein (32). Hendra virus F Y525A underwent much slower proteolytic processing than the wt Hendra virus F protein (Fig. 2), but this reduction in processing was not due to an effect on exocytic transport, as Hendra virus F Y525A rapidly reached the cell surface (Fig. 3). An examination of protein that had been on the cell surface (as judged by biotinylation) but subsequently had undergone endocytosis (and thus was resistant to reduction with MESNa) demonstrated an increased percentage of cleaved protein after endocytosis for both wt Hendra virus F protein and the mutants Hendra virus F Y525A and Hendra virus F Y525F (Fig. 4A).

The requirement for endocytosis for proteolytic processing of the Hendra virus F precursor protein is novel for type I viral fusion proteins. Previously characterized type I fusion proteins that are cleaved intracellularly undergo proteolytic processing within the exocytic transport pathway. Furin, a proprotein convertase localized primarily to the *trans*-Golgi network, is involved in the cleavage of many F proteins from paramyxoviruses, including simian virus 5 (11), measles virus (4), and RSV (25), with the RSV F protein recently demonstrated to undergo two furin-promoted proteolytic cleavage events (2, 12). Proteolytic cleavage mediated by furin has also been demonstrated for a number of other viral proteins, including the influenza virus HA protein of H5 and H7 avian strains (16, 34), the human immunodeficiency virus (HIV) and simian immunodeficiency virus gp160 proteins (1, 14), the human cytomegalovirus glycoprotein B (30), the Ebola virus glycoprotein (Gp) (33), and the baculovirus envelope fusion protein (35). The recently discovered SKI-1/S1P protease, which resides primarily in the endoplasmic reticulum, promotes cleavage of the Lassa virus precursor protein and the Crimean-Congo hemorrhagic fever virus and lymphocytic choriomeningitis virus glycoproteins (3, 18, 31).

The importance of endocytosis for proteolytic processing of the Hendra virus F protein suggests that an enzyme within the endocytic pathway is involved in this critical processing event. Our previous characterization of Hendra virus F proteolytic cleavage demonstrated that the protease involved did not require Ca^{2+} (26). However, the addition of basic amines such as chloroquine or the inhibition of vacuolar ATPases by the addition of bafilomycin or concanamycin rapidly inhibited Hendra virus F protein proteolytic processing (26), indicating a requirement for a low pH. Recent studies from our laboratory indicate that a reduction of the activity of the endosomal/

lysosomal protease cathepsin L with either chemical inhibitors or small interfering RNA knockdowns prevents proteolytic processing of the Hendra virus F protein (26a). Taken together, these data suggest a very different model for the primary proteolytic cleavage of the Hendra virus F protein compared to the processing of other viral fusion proteins. The protein is synthesized as the large precursor, F_0, and is transported via the exocytic pathway to the cell surface in this form. Endocytosis promoted by the motif within the cytoplasmic tail brings the protein in contact with cathepsin L, and proteolytic cleavage occurs. The mature cleaved form, F_1+F_2, could then be recycled to the cell surface.

Interestingly, our experiments with the endocytosis mutant Hendra virus F Y525A demonstrate that it efficiently promotes membrane fusion, as judged by two different assays (Fig. 6), even though 33% of the protein on the cell surface was not proteolytically cleaved (Fig. 5). These results differ somewhat from studies of the Nipah virus F protein, where mutation of the endocytosis motif was found to decrease syncytium formation (32). However, no examination of the cleavage state of the proteins was performed in that study. Proteolytic processing of the precursor forms of type I fusion proteins is thought to be required to form fusogenically active molecules (reviewed in reference 8), with mutations that affect cleavage abrogating fusion promotion. For the Newcastle disease virus F protein, strains with a furin consensus cleavage site are virulent and systemically disseminate through the host, while strains with F_0 molecules having single basic residues are avirulent and tend to be restricted to the respiratory tract, where the necessary secreted protease can be found (23). Newcastle disease virus F protein cleavage site mutants inhibit fusion promotion by the wt protein when expressed in the same cell, suggesting that the uncleaved protein serves as a dominant-negative protein, likely by the formation of mixed trimers with the wt protein (20). For the henipaviruses, F protein cleavage is clearly also important for function. A Nipah virus F protein mutant which cannot be proteolytically processed because it lacks the residues at the cleavage site is properly folded and transported but cannot promote membrane fusion (22). Recent studies from our laboratory have found that the inhibition of Hendra virus F protein cleavage by the reduction of cathepsin L activity dramatically reduces fusion activity (26a). It is possible that the negative effects of uncleaved protein are partly balanced by the increased surface expression seen for both Hendra virus F Y525A and Hendra virus F Y525F (Fig. 5), as higher levels of surface expression have been shown to result in increased membrane fusion promotion (9). Alternatively, the uncleaved protein may exist either in separate trimers from the cleaved protein or in different membrane microdomains, especially since the cleaved protein has clearly undergone at least one round of endocytic recycling.

While our results clearly show that the Hendra virus F protein can undergo proteolytic cleavage during endocytic recycling, the fact that processing occurs within the endosomal pathway raises the intriguing possibility that cleavage could also occur during virion entry. Isolated Hendra virus has up to 55% uncleaved F protein in its envelope, depending on the cell types from which the virions originate (21). Proteolytic processing of uncleaved protein on the surface of the virion could therefore occur if the virus were endocytosed. Recently, endocytosis and a secondary proteolysis event by cathepsin B have been implicated in Ebola virus infection (7). Future experiments to examine the role of endocytosis in the entry of Hendra virus will determine whether proteolytic activation of this virus within the endosome can occur.

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