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A Furin-Defective Cell Line Is Able To Process Correctly the gp160 of Human Immunodeficiency Virus Type 1

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Furin, a subtilisin-like mammalian endoprotease, is thought to be responsible for the processing of many proprotein precursors of cellular and viral origin, including gp160 of human immunodeficiency virus type 1, which share the consensus processing site motif, Arg-X-Lys/Arg-Arg, for protease recognition (for reviews, see P. J. Barr, Cell 66:1–3, 1991, and Y. Nagai, Trends Microbiol. 1:81–87, 1993). To confirm and extend the concept that gp160 is processed by furin, we used here a cell line, LoVo, which was recently demonstrated to be furin defective. Unexpectedly, LoVo cells were found to process gp160 as efficiently as normal cell lines do, hence being able to fuse with CD4-expressing HeLa cells and to produce fully infectious virions. On the other hand, the same cell line was almost totally incapable of processing Newcastle disease virus fusion glycoprotein with a similar oligobasic cleavage recognition motif, providing a strong case for furin-mediated processing. Our present study thus raises a further need to search for and identify the proteinases involved in human immunodeficiency virus type 1 gp160 processing rather than supporting the notion that furin is responsible.

The formation of biologically active envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1) involves posttranslational cleavage of the precursor gp160 into gp120 and gp41 (20). Many other membrane proteins of viral and cellular origin require similar proteolytic processing for their maturation. The Arg-X-Lys/Arg-Arg motif is highly conserved at the cleavage site and is thought to be critical for protease recognition. Furin, a Golgi-localized subtilisin-like endoprotease, displays substrate specificity for this consensus motif (3, 5, 9, 13, 21, 25, 31, 36, 38) and is thought to be responsible for processing the precursors with this motif, including HIV-1 gp160 (11, 24).

LoVo is a cell line established from a human colon carcinoma. Its defectiveness in proprotein processing has been exemplified by its inability to cleave the hepatocyte growth factor/scatter factor (HGF/SF) proreceptor (18, 23), insulin proreceptor (23), and a mutant prorenin (32), all of which possess the Arg-X-Lys/Arg-Arg motif for protease recognition. This impaired processing was caused by a particular mutation in furin (32).

The normal human furin gene encodes a polypeptide of 794 amino acids which consists of the unique structural and functional domains, including the signal peptide, propeptide, subtilisin-like catalytic domain, homo B domain, cysteine-rich domain, and transmembrane domain, in this order from the amino terminus (Fig. 1). Three independent furin cDNA clones (32) and two additional ones (unpublished data) isolated from LoVo cells unexceptionally revealed a one-nucleotide deletion at positions 1,283 to 1,286 (numbered according to reference 35), where four successive T residues are present in the wild-type cDNA; only three T residues were found in the LoVo cDNAs. This deletion of one T residue was deduced to cause a frameshift at amino acid position 429, followed by an aberrant termination in the homo B domain of the furin polypeptide (Fig. 1). The requirement of an entire homo B domain for processing activity has already been demonstrated by carboxy-terminal truncation analysis (12). Evidence suggesting that LoVo furin was indeed inactive came from the finding that processing of the HGF/SF proreceptor and the mutant prorenin in LoVo cells was fully restored by transfection with a recombinant DNA expressing intact human furin but not at all with a similar DNA construct of LoVo furin (18, 32).

The concept that furin is responsible for processing HIV-1 gp160 and other viral and cellular proproteins is primarily based upon the observation that furin coexpressed by an expression vector, mostly by vaccinia virus vectors, can promote processing of the respective precursors in normal cells possessing a basal level of processing activity. Normal cells usually express furin at low levels but still appear to be able to process the substrates efficiently. Coexpression systems may often result in furin expression in great excess beyond the basal level and may therefore deviate from a physiological status, perhaps giving rise to a nonspecific result. The use of LoVo cells defective in furin is obviously a novel, different approach and is expected to complement the previous results obtained by those coexpression studies. Here, we examined gp160 of HIV-1 and the F_0 precursor for fusion (F) glycoprotein of a virulent Miyadera strain of Newcastle disease virus (NDV), which share a similar oligobasic cleavage recognition motif.

LoVo cells are unable to process NDV F_0. The 68-kDa F_0 precursor of virulent NDV strains is efficiently processed into 12-kDa F_2 and 56-kDa F_1 in a wide variety of cells and tissues by cleavage at the Arg-Gln-Lys/Arg-Arg site (26, 27, 34). Such efficient processing is exemplified by a pulse-chase labeling study of infected BHK-21 cells, a baby hamster kidney cell line (Fig. 2A). After 30 min of pulse-labeling, portions of the glycoprotein molecules were detected in the cleaved form F_1 (the smaller cleavage product F_2 is not shown in this figure). During the subsequent 1-h chase, F_0 was almost completely processed. By contrast, LoVo cells allowed F_0 to remain almost totally uncleaved even after a 4-h chase (Fig. 2A). There is a

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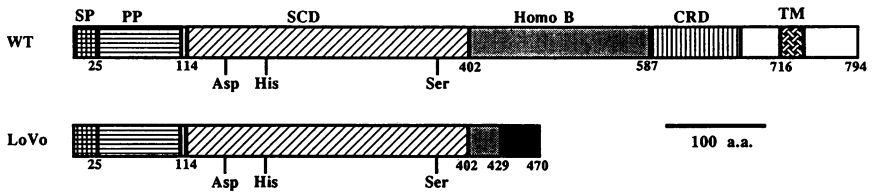

FIG. 1. Structural comparison of wild-type (WT) human furin and the defective furin from LoVo cells, which were deduced from the respective cDNA sequences (32, 35). SP, signal peptide; PP, propeptide; SCD, subtilisin-like catalytic domain; Homo B, homo B domain; CRD, cysteine-rich domain; TM, transmembrane domain; a.a., amino acids.

faint band already migrating to the position of F_1 after the pulse. However, it did not significantly increase in amount during the chase period and was also present in uninfected control cells. This band thus appears to represent at least in part a nonspecific protein, and specific cleavage, if it takes place, could be marginal in LoVo cells.

Uncleaved F_0 is transported to the cell surface and incorporated into the progeny (26). Thus, the medium of infected cells contains largely noninfectious virions with uncleaved F_0, and its treatment with trypsin or furin results in F_0 cleavage and a marked increase in infectivity (9, 26). This was also the case with the present LoVo system (Fig. 2B). With the nearly 50%

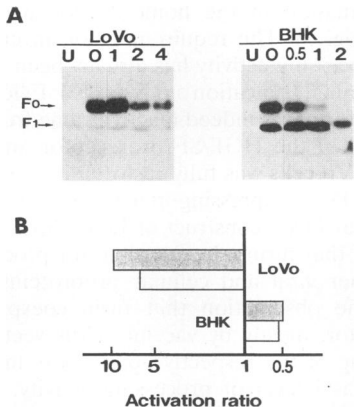

FIG. 2. Proteolytic processing of NDV F_0 glycoprotein in LoVo and BHK-21 cells (A) and the effect of trypsin (■) and furin (□) on the infectivity of NDV grown in these cells (B). (A) Cells were infected with the Miyadera strain of NDV at an input multiplicity of 10 PFU per cell and pulse-labeled with 500 kBq/ml of EXPRE^{35}S^{35}S-protein-labeling mix (>37 TBq/mmol; Du Pont-New England Nuclear, Wilmington, Del.) for 30 min at 4 h after infection (0) or then chased for the hours indicated at the top of each gel lane. Cell lysates were immunoprecipitated with an anti-F monoclonal antibody (33) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (9, 19). Lane U, uninfected cells labeled for 30 min as described above. (B) LoVo and BHK-21 cells were inoculated with NDV as described above and after a 1-h incubation at 37°C, treated with anti-NDV serum (1:50 dilution) for 45 min to minimize the residual infectivity. Twenty-four hours after infection, the culture medium was harvested, treated with trypsin or soluble furin as described previously (9), and assayed for infectivity by plaquing on BHK-21 cell monolayers. The results were presented as ratios of the infectivity of the treated sample to that of the untreated control. In these and the following experiments, LoVo cells were grown in F12 medium buffered with 0.012 M *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and supplemented with 10% heat-inactivated fetal calf serum (FCS), and BHK-21 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% FCS and 10% tryptose phosphate broth.

reduction of infectivity due to nonspecific digestion with either enzyme for BHK-21-grown viruses which are fully active, the 11- and 7-fold increases of LoVo viruses by the enzyme treatments were quite remarkable. This level of activation was comparable to or even higher than that observed for BHK-21-grown avirulent NDV strains by a similar treatment with trypsin, whose F_0 possesses a single arginine at the cleavage site and is therefore uncleavable in these cells (26). Also remarkable was the fact that infected LoVo cells manifested no cell fusion but did so when F_0 cleavage was promoted by trypsin added to the culture medium (data not shown). These results clearly show that the LoVo cell line defective in furin lacks the processing activity for NDV F_0, hence providing for the first time a strong genetic support for the involvement of furin in viral glycoprotein processing.

LoVo cells are able to process HIV-1 gp160. Three different HIV-1 *env* genes, which were derived from the recombinant virus NL432 and the natural isolates SF13 and SF162, respectively, were expressed in LoVo and BHK-21 cells by recombinant vaccinia viruses, and the processing pattern of the gene products was analyzed by pulse-chase labeling. NL432 is T-cell line-tropic, SF162 is macrophagetropic, and SF13 is dualtropic (28, 29). These three glycoprotein substrates are identical in their putative cleavage site sequence (Arg-Glu-Lys-Arg). No virus strain-specific differences in processing kinetics were found at all, and the representative results obtained with NL432 are shown in Fig. 3.

By 30-min pulse-labeling, only the precursor gp160 was detected in both LoVo and BHK-21 cells. During the subsequent 1-h chase, only a small amount of cleavage product gp120 developed in these cells. After a 2-h chase, gp120 became clearly detectable not only in BHK-21 cells but also in LoVo cells. The amount of gp120 reached its maximum at 3 h and remained nearly constant during the subsequent 1-h chase in both LoVo and BHK-21 cells. In good agreement with this developmental kinetics of gp120, the other cleavage product, gp41, was detected after a 3- to 4-h chase in both LoVo and BHK-21 cells (Fig. 3). Furthermore, the two cell types were similar in that they shed gp120 into the media, which could be detected after a 3-h chase and then increased (Fig. 3). These results clearly indicate that LoVo cells defective in furin and incapable of NDV F_0 processing are essentially identical in the processing pattern of gp160 to BHK-21 cells which are fully capable of NDV F_0 processing.

Compared with F_0 in BHK-21 (Fig. 2) and many other cell lines (26), gp160 was processed much more slowly in BHK-21 and LoVo cells (Fig. 3), although the Golgi apparatus and/or the trans-Golgi network are thought to be the processing site for both of them (7, 27, 30). Thus, the apparent slower processing of gp160 could be a result of its slower intracellular transport. Another striking feature of gp160 processing was that only a small portion of the synthesized molecules were

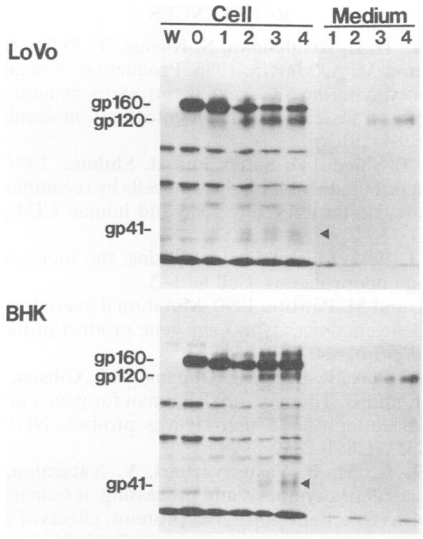

FIG. 3. Proteolytic processing of HIV gp160 in LoVo and BHK cells. The cells were infected with vaccinia virus expressing gp160 of HIV-1 NL432 at a multiplicity of 10 PFU per cell and at 10 h after infection labeled for 30 min (0) with EXPRE^{35}S^{35}S-protein-labeling mix as in Fig. 2 or then chased for the hours shown at the top of each gel lane. Cell lysates were immunoprecipitated with anti–HIV-1 serum from an infected individual and analyzed by SDS-PAGE as in Fig. 2. Lane W, cells infected with the wild-type vaccinia virus strain WR and pulse-labeled for 30 min. The arrowheads indicate gp41. The recombinant vaccinia virus was constructed as described previously (2).

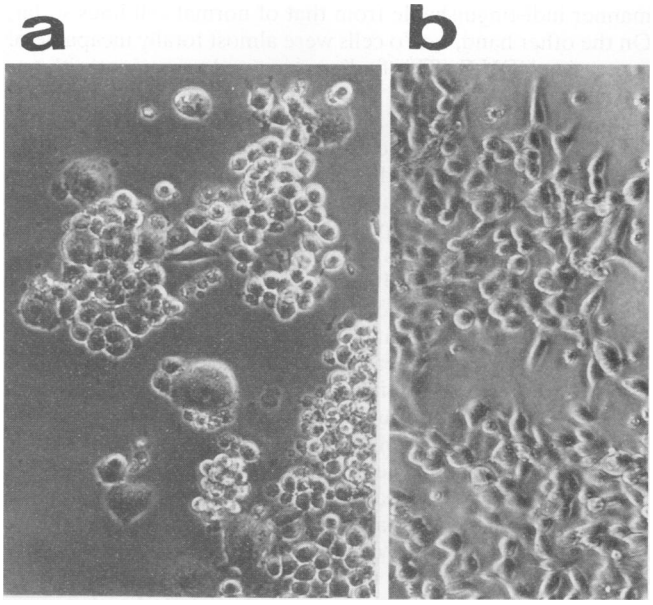

FIG. 4. Development of syncytia after cocultivation of LoVo cells expressing NL432 gp160 and HeLa cells expressing human CD4 (a). At 10 h after infection, LoVo cells with the vaccinia virus carrying the gp160 gene as in Fig. 3 were overlaid with CD4-expressing HeLa cells, grown in MEM supplemented with 10% FCS, and incubated for 5 h. The ratio of CD4-expressing HeLa cells to gp160-expressing LoVo cells was approximately 2.0. Control LoVo cells infected with the wild-type vaccinia virus were similarly cocultured with CD4-expressing HeLa cells (b). Magnification, ×100.

actually cleaved. This has also been a feature of many previous experiments utilizing a vaccinia virus-mediated expression system as well as native HIV-1 (6, 7, 11, 30). It appears that a considerable portion of gp160 is retained in the rough endoplasmic reticulum or delivered to the lysosome via the Golgi apparatus (37).

LoVo cells produce the biologically active fusion glycoprotein and infectious progeny. The following two observations were made in the experiments to assess the capability of LoVo cells to produce the biologically active fusion glycoprotein of HIV-1.

Firstly, remarkable syncytia were formed when LoVo cells expressing gp160 were cocultured for several hours with CD4-expressing HeLa cells (Fig. 4). Although less prominent, syncytium formation was also observed in a similar experiment using BHK-21 cells, instead of LoVo cells (data not shown). Secondly, transfection of LoVo cells with an HIV-1 DNA clone, NL432 (1), resulted in the production of infectious virions. In this experiment, BHK-21 cells were found to be a poor producer of infectious virions. We therefore used as a control a human rhabdomyosarcoma cell line, RD4, which we have routinely used as one of the best cell lines for recovering infectious HIV-1 following transfection with HIV-1 molecular clones. Two days after transfection with NL432 DNA, the culture media of LoVo and RD4 cells were harvested and quantitated for p24 antigen with an HIV AG-1 monoclonal assay kit (Abbott, Wiesbaden-Delkenheim, Germany). The titers were 1.5 and 1.9 ng/ml, respectively, as shown for experiment 1 in Table 1. The infectivity per 1.5 ng of p24 antigen per ml was then determined by both scoring virus-induced cell lysis and examining p24 production in MT4 cells, a human T-cell line. The titers of LoVo and RD4 culture media were $10^{1.7}$ and $10^{2.0}$, respectively, in both of the assays and thus nearly the same (Table 1). In experiment 2, both LoVo and RD4 cells produced larger amounts of p24 antigen than in experiment 1, but the infectivity per 3.0 ng of antigen per ml to MT4 cells was again very similar. These results indicate that LoVo cells are able to produce biologically active HIV-1 glycoproteins and virions with infectivity at levels as high as those of the control cells and, therefore, appear to process gp160 correctly.

Taken together, our data indicate that LoVo cells defective in furin are able to process and activate HIV-1 gp160 in a

TABLE 1. Production of infectious virus after transfection with HIV-1 DNA[a]

Cell line	Expt	p24 (ng/ml)	$TCID_{50}$	
			CPE	p24
LoVo	1	1.5	$10^{1.7}$	$10^{1.7}$
	2	12.5	$10^{1.7}$	$10^{1.7}$
RD4	1	1.9	$10^{2.0}$	$10^{2.0}$
	2	24.5	$10^{1.8}$	$10^{1.8}$

[a] Two days after transfection of LoVo and RD4 cells with the NL432 molecular clone, the culture medium was harvested and assayed for p24 antigen as described in the text. The medium was adjusted to contain 1.5 ng (experiment 1) or 3.0 ng (experiment 2) of p24 antigen per ml, serially diluted, and inoculated to a 94-well plate of MT4 cells. Ten wells were used for each dilution, and the culture medium was changed with fresh medium every three days. Fourteen days after infection, the cytopathic effect (CPE) characterized by cell killing was scored, and the 50% tissue culture infectious dose ($TCID_{50}$) was determined. $TCID_{50}$ was also determined by examining p24 antigen production in each well. RD4 cells were grown in Dulbecco's MEM with 10% FCS, and MT4 cells were grown in RPMI medium with 10% FCS.

manner indistinguishable from that of normal cell lines so far. On the other hand, LoVo cells were almost totally incapable of processing NDV F_0. This finding is in good agreement with our previous one suggesting the superiority of furin to other mammalian subtilisins in cleavage activation of NDV F_0 (9). Thus, it now appears to be established that furin is a bona fide processing protease for this glycoprotein. A mutant cell line (RPE.40) of Chinese hamster ovary cells was reported to exhibit a similar phenotype, the defect in NDV F_0 processing (14), and this defect was able to be complemented by furin cDNA transfection (22). It will be interesting to learn whether the RPE.40 line is also defective in furin.

The strikingly different cleavability of HIV-1 gp160 and NDV F_0 with a similar cleavage site in LoVo cells suggests that not only the cleavage site motif but also other structural features, including the three-dimensional structure, would be important for determining enzyme specificity in processing. The putative secondary cleavage site (Lys-Ala-Lys-Arg-Arg) in HIV-1 gp160, which is located four residues upstream from the primary cleavage site, may also be important for its unique processing pattern in LoVo cells because no such homologs are present in NDV F_0 and the other substrates uncleavable in LoVo cells. Evidence available from site-directed mutagenesis studies strongly suggests that cleavage occurs predominantly at the primary site (4, 10, 20). Approximately 10 to 20% of the gp160 molecules are likely processed at the secondary site, but the corresponding gp41 molecules do not exist as oligomers, a structure possibly important for the intracellular transport and biological properties of the glycoprotein (7, 8). Besides, the addition of polar residues to the gp41 amino terminus as a result of cleavage at the secondary site may disturb gp41 fusogenic activity (17, 20). The secondary site, however, appears to be essential for or to contribute greatly to the proper processing at the primary site, as mutations at the secondary site, if drastic enough (substitution of nonbasic residues for all of the four basic residues), remarkably reduced the cleavage of gp160 (4). Thus, it will be interesting to assess the role of the secondary site in efficient gp160 processing in LoVo cells.

In summary, the most straightforward explanation for our present results is either that furin may be involved in gp160 processing but can be replaced with a different protease or that the processing is mediated by some enzyme(s) other than furin. As such a protease, PACE 4, another mammalian subtilisin, is of concern since it displays, like furin, widespread tissue distribution and is supposed to participate in proprotein processing (16). The 26-kDa protease is also of considerable interest since it has been isolated from a human T-cell line and correctly processes gp160, at least in vitro (15). However, it remains to be elucidated whether the cryptic processing enzyme for gp160 in LoVo cells nonpermissive for HIV-1 is indeed identical to the natural processing protease in cells capable of viral passage. Also, in view of the fact that LoVo furin is defective in presumably regulatory domains such as the homo B and the cysteine-rich domains but does retain the intact catalytic domain (Fig. 1), the possibility that gp160 cleavage does not require such regulatory domains at all and therefore occurs in LoVo cells, whereas these domains are absolutely necessary for NDV F_0 processing, has not been ruled out.

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