Transmembrane glycoproteins with type 1 topology can be retrieved to the endoplasmic reticulum (ER) by a retrieval signal containing a di-lysine (KK) motif near the C terminus. To investigate the structural requirements for ER retrieval, we have constructed mutants of the simian immunodeficiency virus (SIV) envelope (Env) protein with cytoplasmic tails of different lengths and containing a KK motif at the −3 and −4 positions. Such proteins were found to be retained intracellularly when the signal was located 18 amino acids or more away from the membrane-spanning domain. The retrieval signal was found to be functional even when placed at the distal end of the wild-type SIV Env protein with 164 amino acids in the cytoplasmic tail, as shown by the lack of proteolytic processing and lack of cell surface expression of the mutant proteins. However, proteins with a cytoplasmic tail length of 13 amino acids or less having the di-lysine motif at the −3 and −4 positions were not retrieved to the ER since they were found to be processed and transported to the cell surface. The surface-expressed proteins were found to be functional in inducing cell fusion, whereas the proteins retained intracellularly were defective in fusion activity. We also found that the KK motif introduced near an amphipathic helical region in the cytoplasmic tail was not functional. These results demonstrate that the ability of the KK motif to cause protein retrieval and retention in the endoplasmic reticulum depends on the length and structure of the cytoplasmic domain. The ER retrieval of the mutant proteins was found to correlate with increased intracellular binding to β COP proteins.

For type 1 integral membrane proteins, a KKXX or XKKX motif (K residues at the −3 or −3 and −4 positions) functions as an efficient ER retrieval and retention signal (1, 2). The E3/19k protein of adenovirus was found to contain the sequence DEKKMP at the carboxyl terminus, which determined its ER retrieval signal containing a di-lysine (KK) motif near an amphipathic helical region in the cytoplasmic tail and was functional in inducing cell fusion, whereas the proteins retained intracellularly were defective in fusion activity. We also found that the KK motif introduced near an amphipathic helical region in the cytoplasmic tail was not functional. These results demonstrate that the ability of the KK motif to cause protein retrieval and retention in the endoplasmic reticulum depends on the length and structure of the cytoplasmic domain. The ER retrieval of the mutant proteins was found to correlate with increased intracellular binding to β COP proteins.

have demonstrated a requirement for lysine residues at the −3 and −4 positions from the C terminus for ER retrieval (4–8), which appears to be mediated by a 7-subunit receptor called the coatomer complex (9–12).

The cytoplasmic tail length for type 1 transmembrane glycoproteins ranges from a few to several hundred amino acids. It has not been established whether there is a minimum or maximum length of intracytoplasmic amino acids required for the efficient ER retrieval of proteins containing the KK motif. To investigate the possible role of the position of such a signal within the cytoplasmic domain, we have used the envelope glycoprotein of simian immunodeficiency virus (SIV). This envelope glycoprotein was chosen because of the following properties: (a) it is a type 1 transmembrane protein with an unusually long cytoplasmic tail of 164 amino acids; (b) mutants with various truncations in the cytoplasmic tail have been shown to be efficiently transported to the cell surface (13, 14) which enabled us to investigate the effect of placing a KK motif at different positions in the cytoplasmic tail; (c) the cytoplasmic tail includes two amphipathic helical regions which enabled us to determine the effect of placing the KK motif near such a helical region; and (d) the effect of the KK motif on surface expression can be evaluated by functional analysis of membrane fusion activity. The SIV Env protein is synthesized as a precursor (gp160); during its transport to the cell surface, about 10–15% of gp160 is cleaved by a cellular protease resulting in the generation of surface (SU) and transmembrane (TM) subunits (13, 15). In the present study, we have constructed a series of mutant proteins with lysine residues at −3 and −4 positions in the cytoplasmic domain and analyzed their intracellular processing, cellular localization, and ability to induce membrane fusion.

EXPERIMENTAL PROCEDURES

Cells, Virus, and Reagents—HeLa T4 (16) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. HUT 78 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum. The recombinant vaccinia virus vTF7-3, which expresses bacteriophage T7 RNA polymerase in infected cells (17), was obtained from the National Institutes of Health AIDS Research and Reference Reagent Program. The rhesus antiserum to SIV was kindly provided by Dr. P. Marx (Aaron Diamond AIDS Research Center, New York, NY). The cDNA encoding β COP was kindly provided by Dr. T. Kreis (University of Geneva, Geneva, Switzerland), and the antiserum to β COP was purchased from Sigma.

Cloning of SIV Envelope Mutant Genes Carrying ER Retention Signals—Polymerase chain reaction was employed to construct SIV239 env mutants. Briefly, oligonucleotide primers which incorporated coding sequence for lysines at −3 and −4 positions followed by a stop codon were synthesized. The 5′-primer AAT ACG ACT CAC TAT AGG GCG AA was used with a panel of 3′-primers to obtain the desired mutants. Primers 3′-AGA GAA GAA TTC TTA ATA CCC CTT CTT TAA TTA AGC TAG CAT TGG T (SIVenv6RS), 3′-CTG GAA GAA TTC TTA TGG GGA TTT TTT CAC TGG CCT ARA CCC CTG (SIVenv13RS), 3′-TGG GAT GAA TTC CTA CTG GAA TTT TTT GGG TGG GGA AGA GAA

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CAC (SIVenv18RS), 3'-ACC GCC GAA TTC TTA GCT TCT TTT TTT GCC TTC TCT GGT TGG CAG T (SIVenv37RS), 3'-TAG GTA GAA TTC TTA AGT CCT TTT TTT TCG AAT CCT CTG TAG (SIVenv103RS), and 3'-TAT TAT GCA TTA TCA GAA GAT TTT CCT TCG CCC TGT TCT AAT (SIVenv164RS) were used. In addition to having the native coding sequence for lysines and stop codons, the 3'-primers also incorporated an EcoRI restriction site. Polymerase chain reaction was performed using pBSKS+ vector (Stratagene) containing the SIVmac239 env gene as the template. The sense and antisense primers were mixed with the template and appropriate buffer. Vent polymerase (New England Biolabs) was added, and polymerase chain reaction was performed for 30 cycles. After amplification, the products were purified, cut with EcoRI and XbaI, and cloned into the pBSKS+ vector that was cut with appropriate enzymes. The introduced mutations were confirmed by sequencing the plasmid DNA using Sequenase Version 2.0 (U.S. Biochemical Corp.) following the protocol recommended by the manufacturer.

Transfection, Radioimmunoprecipitation, and Protein Analysis—Transfection and protein analyses were done as described previously (5). Briefly, HeLa T4 cells (5 x 10⁵) were infected with vaccinia virus vTF7–3 (multiplicity of infection 10), and DNA (5 μg) and lipofectin (10 μg) were added to the cells. At 7 h post-transfection, the cells were starved in medium lacking methionine and cysteine and then labeled with 100 μCi of [35S]methionine and -cysteine (Amersham Life Sciences) for 30 min. For better detection of the TM proteins, the cells were labeled with 100 μCi of [3H]leucine after preincubation in medium lacking leucine. At the end of the labeling, the label was removed, and Dulbecco’s modified Eagle’s medium with fetal calf serum was added and chased for different times. Cells were lysed in radiolabeled preincipitation buffer and clarified, and proteins were immunoprecipitated with SIV-specific antiserum from an infected rhesus monkey. The immunoprecipitated proteins were extensively washed and analyzed by SDS-PAGE and autoradiography. For separation of the precursor and gp120, and for detection of the TM proteins, aliquots were analyzed on both 7.0 and 10.0% SDS-PAGE. The media collected at different chase times were immunoprecipitated and analyzed similarly.

Immunofluorescence—At 7 h post-transfection, the expression and cell localization of proteins were analyzed after fixing cells for 10 min with paraformaldehyde (3.6%), permeabilizing for 5 min with Nonidet P-40, incubating with primary antibodies specific to the Env protein of SIV, and then using secondary mouse anti-monkey antibodies conjugated with fluorescein isothiocyanate. The coverslips were washed with phosphate-buffered saline, mounted on glass slides, and viewed in a Nikon fluorescence microscope.

Cell Fusion Assay—HeLa T4 cells were infected with vTF7–3 and transfected with plasmids as described above. At 8 h post-transfection, the cells were detached from the dishes using versene and cocultured with an equal number of CD4+ HUT 78 cells. The cells were photographed using a modulation contrast imaging system (18) attached to a Nikon Diaphot microscope.

Assay for Intracellular Interaction of β COP and SIV Env Proteins—HeLa T4 cells transfected with plasmid DNA were labeled with [35S]methionine and -cysteine for 3 h, washed with phosphate-buffered saline, permeabilized with saponin (1%), and incubated with β COP antibody (Sigma) for 30 min at 4 °C. The supernatant was removed, and it was determined that the SIV Env proteins did not leak out due to permeabilization. The cells were then washed in phosphate-buffered saline to remove the β COP antibody and resuspended in CHAPS buffer (20). The lysate of the transfected cells was divided into two equal portions; one portion was reacted with SIV antiserum to analyze the cell-associated Env proteins, and protein A was added to the other portion to detect the β COP-Env protein complex. The proteins were analyzed using SDS-PAGE and a PhosphorImager (Molecular Dynamics).

RESULTS

SIVmac239 Env Protein Mutants with KK Residues at −3 and −4 Positions Are Efficiently Retrieved to the ER—We have used the SIVmac239 Env protein to investigate the function of the KKKX retrieval signal in proteins with cytoplasmic tails of various lengths. In Fig. 1, we report the positions introduced in the cytoplasmic tail, which all contained two lysine residues as the −3 and −4 amino acids from the C-terminal end. Using the recombinant vaccinia virus-based transient T7 expression system (19), cells transfected with plasmid DNA expressing wt or mutant SIV Env proteins were labeled with [35S]methionine and -cysteine for 30 min and chased for up to 6 h. With the wt SIVmac239 Env protein, the precursor protein of 160 kDa was synthesized during the 30-min pulse (Fig. 2, lane 1). During 3- and 6-h chases, a fraction of the precursor protein was cleaved into the SU component gp120 and the TM component gp41 (Figs. 2A, lanes 2 and 3, and 2B, lane 1). These results are consistent with earlier observations (13, 21, 22).

To investigate the structural features necessary for a functional ER retrieval signal, we placed KK residues at the −3 and −4 positions of SIV Env proteins having cytoplasmic tail lengths of 18, 37, or 164 amino acids (Fig. 1). Immunoprecipitation of the protein encoded by construct SIVenv18RS showed the presence of a precursor protein (Fig. 2A, lane 4) which was not proteolytically cleaved during the chase periods, as evidenced by the lack of SU or TM subunits (Figs. 2A, lanes 5 and 6, and 2B, lane 2). With the mutant protein encoded by construct SIVenv37RS, which has 37 amino acids in the cytoplasmic tail and lysines at −3 and −4 positions, similar results were obtained (Figs. 2A, lanes 7–9 and 2B, lane 3). To exclude the possibility that the lack of cleavage of proteins encoded by the constructs SIVenv18RS and SIVenv37RS was due to the deletions in the cytoplasmic tail, we also analyzed truncated proteins with cytoplasmic tails of 18 and 37 amino acids but which lacked the di-lysine motif. These proteins were efficiently cleaved and transported to the cell surface, as evidenced by cell surface immunofluorescence staining and secretion of gp120 into the media (data not shown). This demonstrates that the lack of proteolytic processing was a consequence of di-lysine-mediated ER retrieval and retention.

To determine whether the signal is functional when placed in a protein with 164 amino acids in the cytoplasmic tail, we mutated the coding sequence of full length SIV Env to introduce di-lysines at −3 and −4 positions. When this construct (SIVenv164RS) was analyzed for protein expression, a precursor protein similar in size to wt SIV Env was synthesized during the 30-min pulse. With chases up to 6 h, this protein did not undergo proteolytic processing (Figs. 2A, lanes 10–12 and 2B, lane 4), indicating its retrieval and retention in the ER.

To further investigate the cellular localization of these proteins, their site of expression was analyzed by indirect immunofluorescence. In permeabilized cells, the wild-type SIV Env protein exhibited a reticular staining pattern that extended throughout the cells (Fig. 3, panel A). As expected, the cells also showed surface expression of the envelope protein (panel E). The intracellular staining pattern of the mutant proteins resembled that of the wild-type protein (Fig. 3, panels B–D). However, in contrast to the wild-type Env protein, no cell surface staining was detected for any of the three mutants (panels F–H), confirming that those proteins are efficiently retrieved and retained in the ER. Thus a di-lysine motif func-
tions as a retrieval signal in proteins with a cytoplasmic tail 164 amino acids in length.

**SIV Envelope Proteins with a Di-lysine Motif near the Membrane Spanning Domain Are Transported to the Cell Surface**—To determine if ER retrieval signals placed closer to the membrane spanning domain are functional, we analyzed the mutant proteins encoded by constructs SIVenv6RS and 13RS and having 6 or 13 amino acids in the cytoplasmic tail and lysines at \( \text{positions} \) from the C terminus. The proteins encoded by both constructs were found as a precursor during the 30-min pulse, and chases for 3 and 6 h resulted in cleavage into SU and TM components (Fig. 4). These data indicate that the mutant proteins are not retained in the ER but undergo proteolytic processing in a post-ER-Golgi compartment (15, 23).

For both the mutant proteins, the intracellular immunofluorescence pattern resembled that of the wild-type protein (Fig. 5, A and B). Although the mutants contained the di-lysine motif, cell surface expression was observed on unpermeabilized cells (panels D and E). Thus, these results support the conclusion that a cytoplasmic domain of a minimum length is needed for efficient retrieval and retention in the ER by a di-lysine containing signal.

**A Di-lysine Motif near an Amphipathic Helical Region of the Cytoplasmic Tail Of SIV Env Does Not Function as a Retrieval Signal**—The cytoplasmic domain of HIV is postulated to form amphipathic helical structures between amino acids 770–794 and 824–856 (24), which may associate with the inner surface of the plasma membrane (25). To determine whether a retrieval signal positioned near such an amphipathic region could be functional, we analyzed the construct SIVenv103RS, which encoded a protein truncated after 103 amino acids in the cytoplasmic domain. During a 30-min pulse, a precursor protein of approximately 155 kDa was synthesized in transfected cells (Fig. 6A). After a 3- or 6-h chase, the precursor was cleaved into SU and a TM subunit of approximately 36 Kda, unlabeled methionine and cysteine for 30 min and chased for 3 or 6 h in the presence of unlabeled methionine and cysteine. Samples were immunoprecipitated, analyzed using SDS-PAGE, and visualized using autoradiography. Lane 1 shows the wt SIV Env proteins labeled during the 30-min pulse; lane 2, 3-h chase; lane 3, 6-h chase; lane 4, proteins expressed by plasmid SIVenv164RS; lanes 5 and 6, proteins during the 3- and 6-h chase; lane 7, proteins expressed by plasmid SIVenv37RS; lanes 8 and 9, proteins during the 3- and 6-h chase; lane 10, proteins expressed by plasmid SIVenv164RS, and lanes 11 and 12, proteins during the 3- and 6-h chase. Pre denotes the precursor protein; gp120 and TM denote the surface and transmembrane subunits. B, the lack of proteolytic processing of the mutant proteins was further confirmed by labeling the transfected cells with [\(^{3}H\)]leucine and analyzing the TM proteins (asterisks) by immunoprecipitation and 10% SDS-PAGE. Lane 1, wt TM subunit; lanes 2–4, TM in cells expressing plasmids SIVenv18RS, SIVenv37RS, and SIVenv164RS, respectively; lanes 5–7, TM proteins in cells expressing plasmids SIVenv6RS, SIVenv13RS, and SIVenv103RS, respectively. The numbers on the right side denote the sizes of molecular weight markers.

**Intracellular expression of SIV Env and retrieval signal mutants.** HeLa T4 cells were infected with vTF7–3 and transfected with plasmid DNA encoding the wt envelope, SIVenv18RS, SIVenv37RS, or SIVenv164RS. A, 7 h post-transfection, the cells were labeled with \([^{35}S]\)methionine and -cysteine for 30 min and chased for 3 or 6 h and immunolabeled as described under “Experimental Procedures.” Panels A–D show the intracellular fluorescent staining patterns, and panels E–H show surface fluorescence. Panels A and E, wild-type SIV Env; panels B and F, cells transfected with plasmid SIVenv18RS; panels C and G, cells transfected with plasmid SIVenv37RS; panels D and H, cells transfected with plasmid SIVenv164RS.
indicating that the retrieval signal was not functional (Figs. 6A, lanes 1–3 and 2B, lane 7).

The lack of recognition of the retrieval signal was confirmed by detection of gp120 in the extracellular medium from cells transfected with plasmid SIVenv103RS (Fig. 6B, lanes 1 and 2).

The protein exhibited a reticular immunofluorescence pattern within the cell (Fig. 6C, panel a) and was readily detected on the cell surface (panel b). These results indicate that the di-lysine motif did not function as an effective ER retrieval signal when positioned near an amphipathic helical region.

Membrane Fusion Properties of SIV env Mutants—In cells transfected with expression vectors or SIV-infected cells, membrane fusion occurs when the cell surface-expressed Env protein interacts with the CD4 receptor on neighboring cells (13, 27). Hence, we determined if the mutants with the di-lysine motif in different length cytoplasmic tails were functional in inducing the formation of syncytia. Coculture of HUT 78 cells with HeLa T4 cells expressing the wt SIV Env protein revealed syncytium formation (Fig. 7A). In contrast, the mutant proteins encoded by plasmids SIVenv18RS, SIVenv37RS, and SIVenv164RS failed to induce membrane fusion (panels B–D).

These results are consistent with the observed defect in proteolytic processing and cell surface transport. In contrast, the mutant proteins encoded by plasmids SIVenv18RS, SIVenv37RS, and SIVenv164RS failed to induce membrane fusion (panels B–D). These results are consistent with the observed defect in proteolytic processing and cell surface transport.

Intracellular Retrieval and Retention of Membrane Proteins

Fig. 4. Expression of SIV Env proteins having a retrieval signal close to the transmembrane domain. Upper and lower panels represent the proteins resolved using 7 and 10% SDS-PAGE, respectively. Lane 1 shows the proteins labeled during the 30-min pulse, and lanes 2 and 3 show the proteins during a 3- and 6-h chase, respectively, from cells transfected with plasmid SIVenv6RS. Lane 4 shows the proteins labeled during the 30-min pulse, and lanes 5 and 6 show the proteins during a 3- and 6-h chase, respectively, from cells transfected with plasmid SIVenv13RS. Pre denotes the precursor protein; gp120 and TM denote the surface and transmembrane components. The numbers on the right side denote the sizes of molecular weight markers.

Fig. 5. Cellular localization of SIV Env proteins having a retrieval signal proximal to the membrane spanning domain. HeLa T4 cells were transfected, fixed, and stained as in Fig. 3. Panels A–C show the intracellular fluorescent staining patterns, and panels D–F show surface fluorescent. A and D, cells transfected with plasmid SIVenv6RS; B and E, cells transfected with plasmid SIVenv13RS; and C and F, untransfected HeLa T4 cells.

Fig. 6. A retrieval signal near the amphipathic helical region of SIV Env is not functional. HeLa T4 cells were infected with vTF7–3 and transfected with plasmid SIVenv103RS. Labeling and immunoprecipitation were done as described for Fig. 2, and the proteins were resolved on SDS-PAGE and visualized by autoradiography. A, intracellular expression of protein encoded by plasmid SIVenv163RS. Upper and lower panels represent the proteins resolved using 7 and 10% SDS-PAGE, respectively, and the numbers on the right side indicate the sizes of molecular weight markers. Lane 1, 30-min pulse; lane 2, 3-h chase; lane 3, 6-h chase. B, media were collected at the end of 3- and 6-h chase periods and immunoprecipitated and analyzed by SDS-PAGE. Lane 1, 3-h chase; lane 2, 6-h chase. Pre denotes the precursor protein; gp120 and TM denote the surface and transmembrane components. C, immunofluorescence of cells transfected with plasmid SIVenv103RS. Cells were processed as in Fig. 3. Panel a, intracellular fluorescence; panel b, cell surface fluorescence.
Thus, the results with the syncytium assay are consistent with the other results showing cell surface expression versus ER retrieval of the Env mutants.

Interaction of β COP with SIV Env Proteins—To investigate the mechanism involved in the observed differences in retrieval of SIV mutant proteins, we analyzed the interaction of the Env proteins with β COP proteins. β COP is a 110-kDa protein that is associated with non-clathrin-coated vesicles and the Golgi complex (28) and has been implicated in the transfer of proteins from the ER to the Golgi complex (29). We developed an assay using saponin permeabilization which enabled us to determine binding of the retrieved or retained proteins to β COP. Quantitation of the amount of β COP-associated Env proteins showed that proteins encoded by plasmids 164RS, 37RS, and 18RS bound to β COP at least 3- to 5-fold higher levels than the wt Env protein (Figs. 8A, lanes 2–4, and 8B). In contrast, the KK motif-containing proteins that were not retained showed lower binding to β COP (Figs. 8A, lanes 1 and 5–7, and 8B). The amount of radiolabeled β COP detected in Env-expressing cells was less than that of the coprecipitated Env proteins, presumably because most of the β COP is unlabeled due to the 3-h labeling time used. To confirm the identity of β COP, we transfected cells with a plasmid DNA expressing β COP, and the lysate was immunoprecipitated using β COP antibodies (Fig. 8A, lane 8). The intracellular expression levels of the Env proteins were similar with all the constructs (Fig. 8C). These results demonstrate that an increased binding to β COP is observed in the proteins with a functional ER retention motif.

DISCUSSION

Most previous studies of ER retrieval and retention have analyzed proteins with cytoplasmic tails of more than 15 amino acids although there are examples of retained proteins having only 10 amino acids in the cytoplasmic tail (10, 30, 31). Chimeric proteins used to analyze the di-lysine motif had at least 15 amino acids in the cytoplasmic tail, and attempts to place the lysines closer to the membrane were not reported. The present results provide evidence that potential retrieval signals in the SIV Env proteins with a tail length of 6 or 13 amino acids are not functional, whereas such a signal is functional in proteins with longer cytoplasmic domains. Structural modeling (24) and topogenic analyses of the HIV-1 envelope protein (25)
indicated that the cytoplasmic domain has the propensity to form two amphipathic helical structures. Peptides which correspond to the amphipathic region were found to interact with membranes (32, 33), indicating that such amphipathic regions may promote membrane association. The mutant protein encoded by plasmid SIVenv103RS contains a cysteine residue that is the site for palmitoylation (34), and this modification was postulated to enable the amphipathic regions to tightly associate with cellular membranes. Our observation that a retrieval signal placed near the predicted amphipathic helical region of the SIV Env protein is not recognized by the cellular retention machinery may be explained by the close association of this region with cellular membranes.

The finding that di-lysines introduced at different positions in the SIV envelope protein result in proteins that have different cellular localization phenotypes raises several possibilities. When lysines are positioned very near to the membrane spanning domain, they may not be recognized because of the lack of some structural features. Alternatively, when the signal is located near the membrane, a cellular protein may sterically mask the signal and thereby result in lack of recognition. A masking of a retention signal has been reported to occur during the assembly of the masking of a retention signal has been reported to occur during the assembly of the region with cellular membranes. The mutants in which the KK motif was functional failed to undergo proteolytic processing, were not detected on the cell surface, and were defective in inducing cell fusion. Thus, our studies provide evidence that there is a requirement for lysines to be placed at a minimum distance from the membrane spanning domain.

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