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Identification of Two Sequences in the Cytoplasmic Tail of the Human Immunodeficiency Virus Type 1 Envelope Glycoprotein That Inhibit Cell Surface Expression

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During synthesis and export of protein, the majority of the human immunodeficiency virus type 1 (HIV-1) Env glycoprotein gp160 is retained in the endoplasmic reticulum (ER) and subsequently ubiquitinated and degraded by proteasomes. Only a small fraction of gp160 appears to be correctly folded and processed and is transported to the cell surface, which makes it difficult to identify negative sequence elements regulating steady-state surface expression of Env at the post-ER level. Moreover, poorly localized mRNA retention sequences inhibiting the nucleocytoplasmic transport of viral transcripts interfere with the identification of these sequence elements. Using two heterologous systems with CD4 or immunoglobulin extracellular/transmembrane domains in combination with the gp160 cytoplasmic domain, we were able to identify two membrane-distal, neighboring motifs, *is1* (amino acids 750 to 763) and *is2* (amino acids 764 to 785), which inhibited surface expression and induced Golgi localization of the chimeric proteins. To prove that these two elements act similarly in the homologous context of the Env glycoprotein, we generated a synthetic gp160 gene with synonymous codons, the transcripts of which are not retained within the nucleus. In accordance with the results in heterologous systems, an internal deletion of both elements considerably increased surface expression of gp160.

The human immunodeficiency virus type 1 (HIV-1) glycoprotein gp160 is processed into the transmembrane subunit (TM) gp41 and the nonconvalently linked gp120 glycoprotein, which binds to the CD4 receptor and chemokine coreceptor molecules. Cleavage mediated by a cellular furin protease during the protein transport through the *cis* or medial Golgi appears to be mandatory for membrane fusion (18, 29, 42, 57, 66). Nascent gp160 molecules are bound to GRP78-BiP, calnexin, and calreticulin chaperones and are highly glycosylated, sulfated, and palmitoylated (5, 18, 24, 36, 49, 71). Correct folding, as well as glycosylation and oligomerization, was found to be necessary for efficient protein transport (6, 14, 17, 18, 24, 30, 35, 48, 50). Previous studies demonstrated that the majority of the Env glycoprotein is intracellularly retained and remains endoglycosidase H (Endo H) sensitive (31, 32, 53, 69). Only a minor fraction leaves the endoplasmic reticulum (ER) and is transported to the cell surface. Recently we showed that the ER-retained Env glycoprotein is ubiquitinated and degraded by the proteasome (9; A. Bültmann and J. Haas, unpublished data). Glycoprotein surface expression, however, not only depends on ER-mediated quality control and the retention of misfolded or disassembled Env in the ER but also involves subsequent steps, including Golgi export and internalization of surface-expressed Env (58). A tyrosine-based, membrane-proximal YXXΦ motif (amino acids [aa] 713 to 716) in the cytoplasmic gp41 domain was previously reported to be responsible for endocytosis, but additional, more distal elements were expected (4, 58).

There are several lines of evidence suggesting that the cytoplasmic domain of the TM glycoprotein modulates surface expression. Deletion of the carboxy terminus, which has been observed after long-term culture of chronically HIV-1-infected cells, increases both protein transport and processing of gp160 but leads to a decreased incorporation of glycoproteins into virions, probably because of a direct interaction between Env and viral matrix proteins (13, 15, 18, 22, 25, 28, 39, 72, 73). Moreover, deletion of the cytoplasmic domain appears to reduce infectivity of virions due to postentry events (26, 72). Similar to the case with HIV-1, protein synthesis and processing of the TM protein in simian immunodeficiency virus (SIV) is modulated by the cytoplasmic domain. Most SIVs isolated by culturing in human cells possess a premature stop codon truncating the cytoplasmic tail of gp41 (10, 67; V. M. Hirsch, P. Edmondson, C. M. Murphey, B. Arbeille, P. R. Johnson, and J. I. Mullins, Letter, Nature **341:**573–574, 1989). This truncation of the cytoplasmic domain of gp160 increases surface expression, fusogenicity, and, in contrast to the case with HIV-1, infectivity (10, 54, 63, 64, 74). Regarding HIV-2, which is highly related to SIV, similar observations have been made (43).

In HIV-1 infection, unspliced and singly spliced viral mRNA is not transported into the cytoplasm unless the posttranscriptional regulator Rev is present (21, 23, 38). This nuclear retention of HIV-1 mRNA is caused by multiple poorly localized negative sequence elements present throughout the viral genome (2, 7, 12, 21, 45, 56, 60, 61). In the *env* gene, inhibitory sequence elements were found in the region containing the

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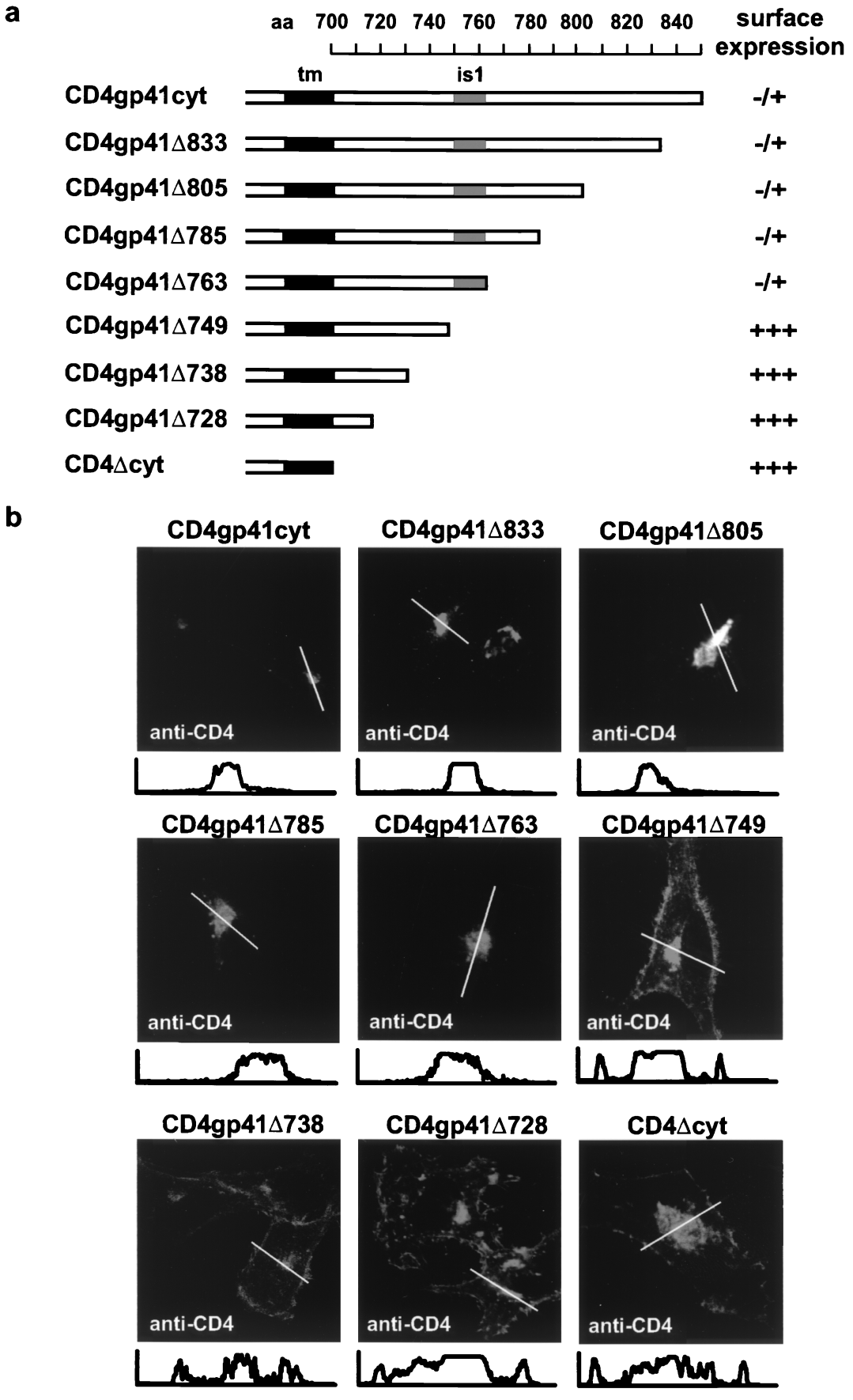

a
aa 700 720 740 760 780 800 820 840
surface expression
tm
is1
CD4gp41cyt -/+
CD4gp41Δ833 -/+
CD4gp41Δ805 -/+
CD4gp41Δ785 -/+
CD4gp41Δ763 -/+
CD4gp41Δ749 +++
CD4gp41Δ738 +++
CD4gp41Δ728 +++
CD4Δcyt +++
b
CD4gp41cyt
CD4gp41Δ833
CD4gp41Δ805
CD4gp41Δ785
CD4gp41Δ763
CD4gp41Δ749
CD4gp41Δ738
CD4gp41Δ728
CD4Δcyt
anti-CD4

Rev-responsive element, but also in other regions, including both gp120 and gp41 (7, 21, 45, 56). Thus, sequences suppressing the nucleocytoplasmic mRNA transport colocalize with peptide motifs acting at the protein level and make it difficult to dissect both phenomenons.

To identify sequence elements influencing the steady-state surface expression of Env, we used two different approaches. First, we investigated the gp41 cytoplasmic domain in two heterologous systems using chimeras with CD4 and immunoglobulin extracellular/transmembrane domains, thus rendering the protein independent of the complex folding and processing which causes ER retention and degradation of the majority of the Env glycoprotein. Second, we investigated the sequence elements identified with the first approach using a synthetic *env* sequence with synonymous codons, which proved to be refractory to mRNA retention. We identified two membrane-distal sequence elements which suppress Env surface expression and cause Golgi localization. Deletion of the two elements led to a significant increase in surface expression of the chimeric reporter constructs and of the homologous Env glycoprotein.

MATERIAL AND METHODS

Cell culture and transfection. 293T, an adenovirus transformed human kidney cell line, and HeLa cervix carcinoma cells were maintained in Dulbecco modified Eagle medium supplemented with 10% (vol/vol) heat-inactivated fetal calf serum and 100 IU of penicillin/ml, 100 μg of streptomycin/ml, and 2 mM L-glutamine. For RNA analysis, 293T cells were transfected by calcium phosphate coprecipitation according to standard protocols. For intracellular localization experiments, HeLa cells were transfected by microinjection.

Plasmid constructs. To generate an *Mlu*I restriction site 3′ of the transmembrane region, human CD4 was amplified by PCR using a cDNA clone as a template and subsequently cloned into *Hin*dIII and *Not*I restriction sites of vector pCR3. Similarly, the extracellular domain of human immunoglobulin G1 (IgG1) hooked up to the CD7 transmembrane region followed by an *Mlu*I restriction site was cloned into the *Hin*dIII and *Not*I restriction sites of plasmid pRK (33). The gp41 cytoplasmic domain and mutants thereof were generated by PCR amplification using the synthetic gp160 sequence as a template to avoid nuclear mRNA retention. After the PCR, they were cloned into the *Mlu*I and *Not*I sites of both the CD4 and the surface immunoglobulin constructs. The syngp160 mutant constructs were generated by PCR using an *Esp*I site in the transmembrane region and a *Not*I site in the 5′ untranslated region. For generation of stably transfected cell lines, the syngp160 constructs were subcloned in the plasmid pBRep. The HIV-1 *gag* construct contained a Rev-independently expressed, synthetic, codon-optimized *gag* sequence (MN isolate) cloned in pND14. All constructs were confirmed by sequencing. The following oligonucleotides were used for PCR amplification: CD4 for, CGCGGGAAGCTTGCCG CCACCATGAACCGGGGAGTCCC; CD4 rev, CGCGGGGCGGCCGCTTA AATGGGGCTACATGTCTTC; CD4Δcyt MluI rev, CGCGGGGCGGCCGCT TATTACCTTCGGTGCCGGCAACGCGTACAGAAGAAGATGCC; CD4gp41 MluI for, CGCGGGACGCGTGTGCGCCAGGGCTAC; cdm7 rev, CCACAG AAGTAAGGTTCC; gp41Δ833 rev, CGCGGGGCGGCCGCTTATTAGAGC ACCTCGATCACGC; gp41Δ805 rev, CGCGGGGCGGCCGCTTATTACTGG CTCCAATACTGGAG; gp41Δ785 rev, CGCGGGGCGGCCGCTTATTATAG GAGTTCCACGATGCG; gp41Δ763 rev, CGCGGGGCGGCCGCTTATTAGC TGCGGAGGTCGAC; gp41Δ749 rev, CGCGGGGCGGCCGCTTATTACCT GCCGCTG; gp41Δ738 rev, CGCGGGGCGGCCGCTTATTAGCCCTCCTCC TCGATGC; gp41Δ728 rev, CGCGGGGCGGCCGCTTATTAGGGCCCGCG CGGCAC; CD4gp41:749 for, CGCGGGACGCGTCTCGTGCACGGCTTCCT GG; gp41Δ768 rev, CGCGGGGCGGCCGCTTATTAGCTGAACAGGAACA GGC; CD4gp41:750–763 for, CGCGTGTGCACGGCTTCCTGGCGATCATC TGGGTCGACCTCCGCAGCTAATAAGC; CD4gp41:750–763 rev, GGCCGC TTATTAGCTGCGGAGGTCGACCCAGATGATCGCCAGGAAGCCGTGC ACA; CD4gp41:763–785 for, CGCGGGACGCGTCTGTTCCTGTTCAGCTA CCACCACCGCGACCTGCTGCTGATCGCC; CD4gp41:763–785 rev, CGCG GGGCGGCCGCTTATTATAGGAGTTCCACGATGCGGGCGGCGATCA GCAGCAGGTCGCG; gp41Δis1 for, GAACAGGAACAGGAGCCTGCCGC TGGTGTCG; gp41Δis1 rev, GCGGCAGGCTCCTGTTCCTGTTCAGCTAC; gp41Δis1+2 for, GCGGCAGGGGCCGCCGCGGCTGGG; gp41Δis1+2 rev, CCCAGCCGCGGCGGCCCCTGCCGCTGGTGTCGC; gp41Δis12 for, GACC TCCGCAGCGGCCGCCGCGGCTGG; gp41Δis2 rev, CCCAGCCGCGGCG GCCGCTGCGGAGGTCGA; gp41L776/7A for, CTGGCCGCCATCGCCGCC CGCATCGTG; gp41L776/7A rev, CCACGATGCGGGCGGCGATGGCGGC CAGGTCGCGGTGGTGGTAGC; gp41L784/5A for, GAAGCCGCCGGCCG CCGCGGCTGG; gp41L784/5A rev, CCCAGCCGCGGCGGCCGGCGGCTT CCACGATGCGGGCGGCG; gp41L776/7/784/5A for, GCCGCCATCGCCGC CCGCATCGTGGAAGCCGCCGGCCGCCGCGGCTGGG; gp41L776/7/784/5A rev, GGCGGCTTCCACGATGCGGGCGGCGATGGCGGCCAGGTCGCG GTGGTGGTAGC; syngp160Δcyt for, TGAGCATCGTGAACCGCTAGC; syngp160Δcyt rev, GGCCGCTAGCGGTTCACGATGC; gp41 EspI for, CGCG GGGCTGAGCATCGTGAACCGCGTGCGCCAGGGCTA; pCR3 rev, ATTT AGGTGACACTATAG; β-actin for, CGCGGGGAATTCAGCTGTGCTACG TCGC; β-actin rev, CGCGGGGGATCCTCGTGGATGCCACAGG; syngp160 for, GTGCTGAAGTACTGGTGG; wtgp160 for, ATTGGTGGAATCTCCT AC; pcdm7 rev, CCACAGAAGTAAGGTTCC.

Generation of a synthetic gp160 sequence. The synthetic gp160 sequence was generated by 15 oligonucleotides used as templates for PCR as described previously (27). PCR primers of adjacent fragments were overlapping with primers and included unique restrictions sites at the 5′ end. PCR products were either used for overlapping PCR with adjacent fragments or directly cloned after phenol extraction, precipitation, and restriction. Subfragments of gp120 and gp41 were cloned into modified pCdm7 and pUC plasmids containing *Nhe*I/*Pst*I/*Mlu*I/*Eco*RI/*Bam*HI/*Not*I and *Bam*HI/*Dra*III/*Kpn*I/*Ear*I/*Esp*I/*Sal*I/*Bst*XI/*Not*I polylinkers, respectively. Subsequently, both fragments were added together using a short oligonucleotide adapter. Finally, the synthetic gp160 sequence was subcloned into a pCDM7-derived plasmid under the control of a human cytomegalovirus immediate-early promotor and a CD5 signal peptide (62). The correct gp160 sequence was confirmed by double-stranded DNA sequencing.

RNA isolation and cDNA synthesis. Transfected 293T cells were harvested, washed with cold phosphate-buffered saline (PBS), and lysed with 2 ml of lysis buffer (10 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 10 mM NaCl, 0.5% NP-40) for 5 min on ice. Subsequently, nuclei and cytoplasms were separated by centrifugation at 3,000 × *g* for 10 min at 4°C, 8 ml of 4 M guanidine thiocyanate solution (4 M guanidine thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% lauryl sarcosyl, 0.7% β-mercaptoethanol) was added to the nuclear fraction, and 6 ml of 6 M guanidine thiocyanate solution (6 M guanidine thiocyanate, 37.5 mM sodium citrate [pH 7.0], 0.75% lauryl sarcosyl, 0.7% β-mercaptoethanol) was added to the cytoplasmic fraction. After shearing of the genomic DNA, 5.7 M CsCl was overlayed with the guanidine thiocyanate solution in ultracentrifugation tubes. Centrifugation was performed in an SW40 rotor at 35,000 rpm for 18 h at 20°C. The RNA pellet was dissolved in diethyl pyrocarbonate (DEPC)-treated H_2O, phenol-chloroform extracted once, and ethanol precipitated.

Prior to cDNA synthesis, the RNA was treated with RQ1 RNase-free DNase (Promega, Madison, Wis.) for 30 min at 37°C. After inactivation of the DNase by incubation at 65°C for 10 min, 2 μg of RNA was heat denaturated, and 2 μl 10-fold reverse transcriptase (RT) buffer, 1 μl of deoxynucleoside triphosphate (25 mM each), 1 μl of RNasin (Roche, Mannheim, Germany), 1 μl of oligo(dT) primer (250 ng/μl), 1 μl of Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Beverly, Mass.), and H_2O was added to a final volume of 20 μl. After incubation at 37°C for 90 min, the reaction was stopped by heat inactivation at 67°C for 15 min.

Southern blot analysis. After electrophoresis, the agarose gel was incubated in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 30 min, rinsed with H_2O, and submerged in neutralization solution (0.5 M Tris-HCl [pH 7.5], 3 M NaCl)

FIG. 1. The peptide motif *is1* in the cytoplasmic gp41 domain is responsible for suppressed surface expression and Golgi localization. (a) Schematic diagram of the chimeric constructs with the CD4 extracellular/transmembrane and gp41 cytoplasmic domains used to identify the retention elements. (b) HeLa cells were microinjected with the constructs indicated in panel a, stained with the CD4-specific monoclonal antibody Q4120 and a FITC-conjugated secondary antibody, and subsequently analyzed by confocal laser scanning microscopy. The cross-section levels are indicated by white bars. The cells shown are representative of all cells microinjected with the respective constructs. Similar results were obtained in at least three independent experiments.

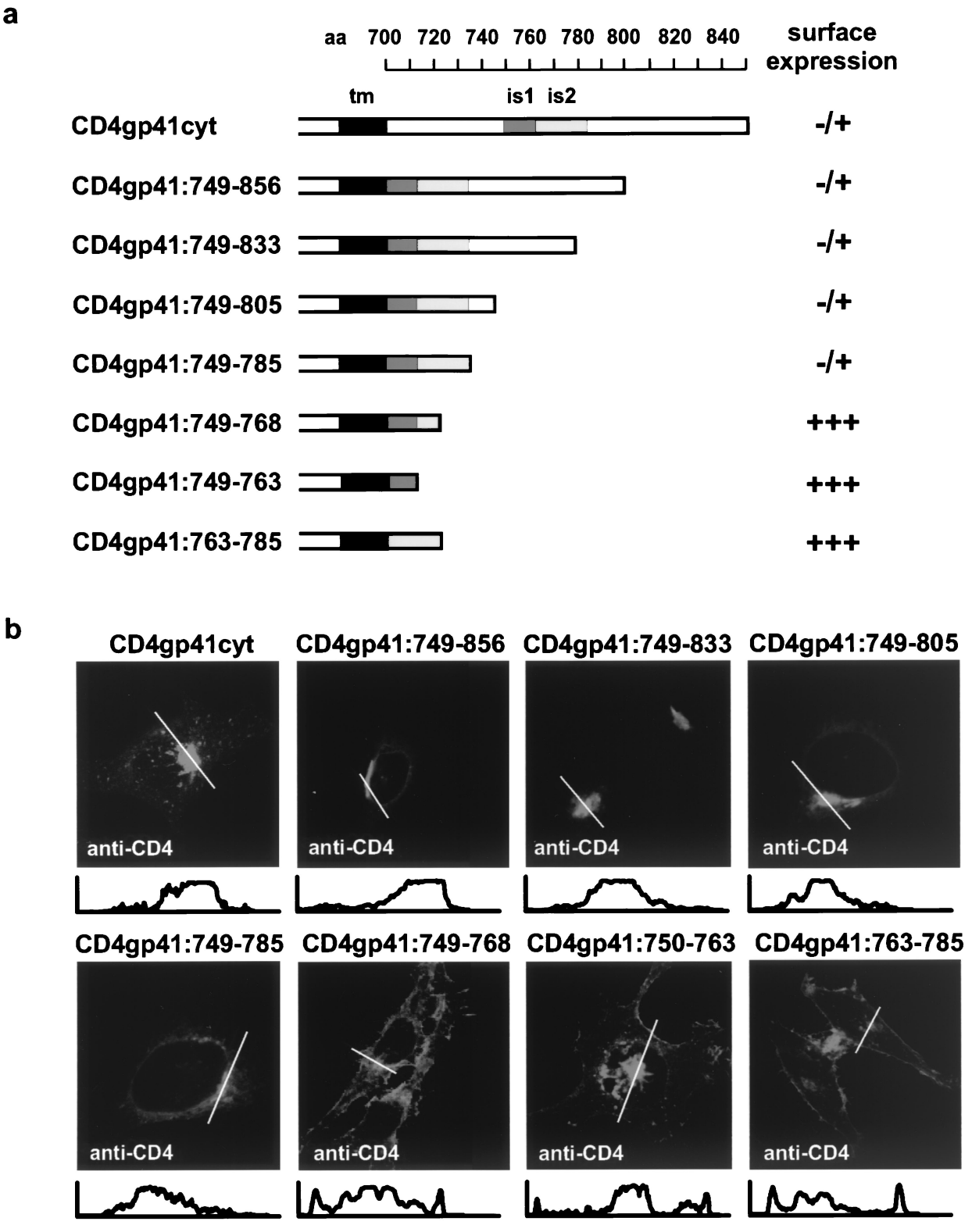


FIG. 2. A second peptide motif, *is2*, in the cytoplasmic gp41 domain is necessary for suppressed surface expression and Golgi localization. (a) Schematic diagram of the chimeric constructs with the CD4 extracellular/transmembrane and gp41 cytoplasmic domains used to identify the retention elements. (b) HeLa cells were microinjected with the constructs indicated in panel a, stained with the CD4-specific monoclonal antibody Q4120 and a FITC-conjugated secondary antibody, and subsequently analyzed by confocal laser scanning microscopy. The cross-section levels are indicated by white bars. The cells shown are representative of all cells microinjected with the respective constructs. Similar results were obtained in at least three independent experiments.

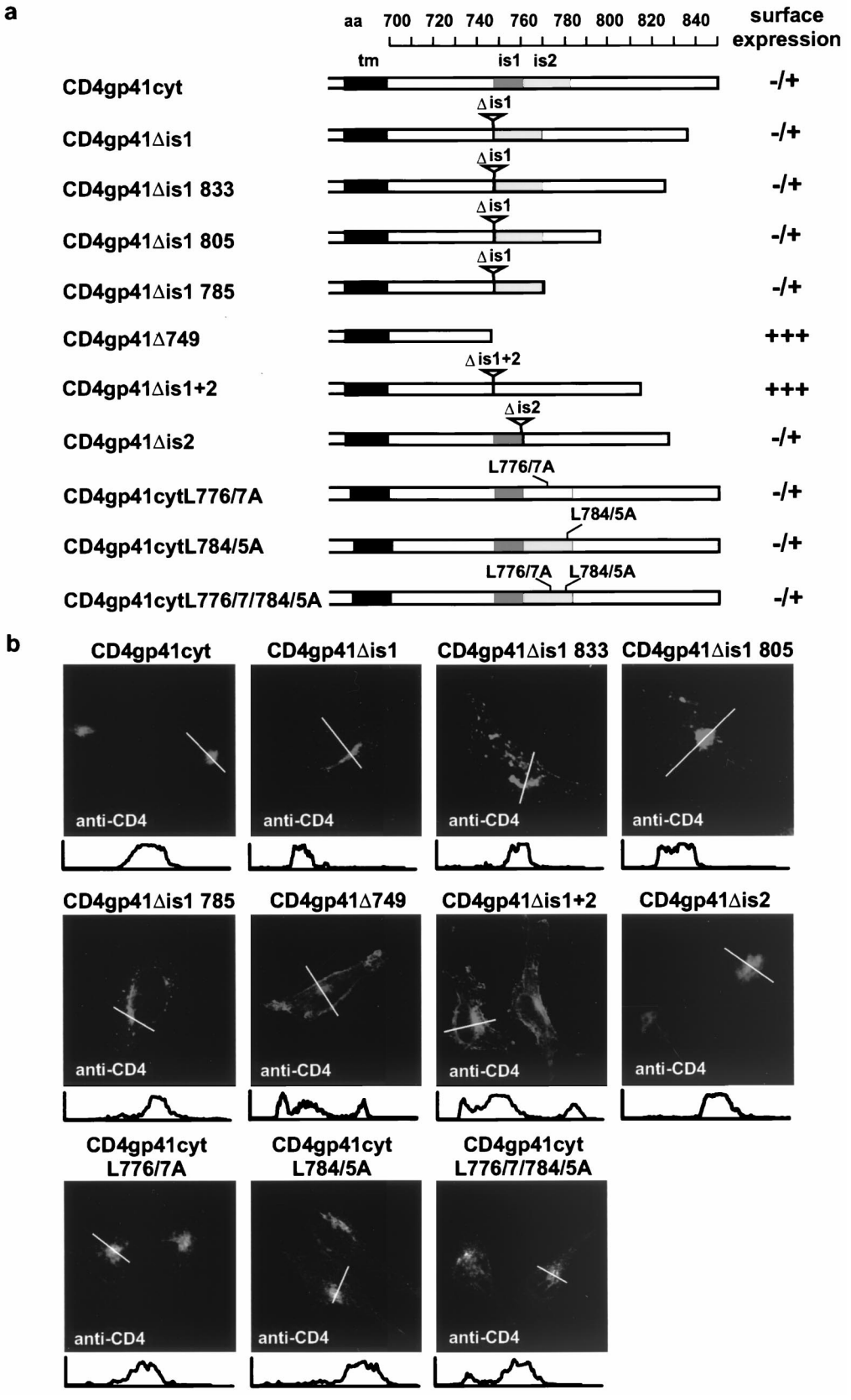
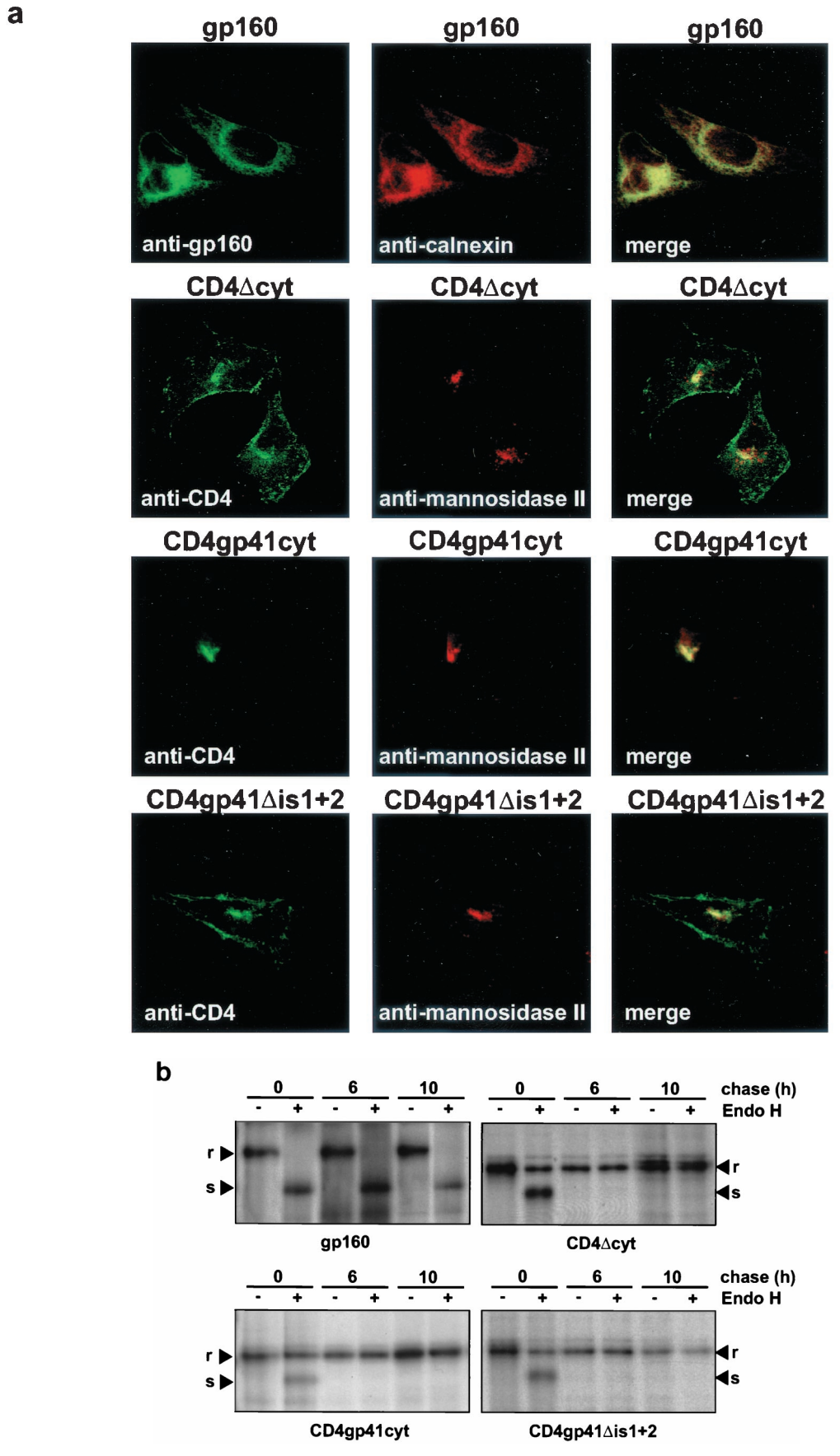


FIG. 3. Either *is1* or *is2* is sufficient for Golgi localization in the sequence context of the gp41 cytoplasmic domain. (a) Schematic diagram of the chimeric constructs with the CD4 extracellular/transmembrane and gp41 cytoplasmic domains used to identify the retention elements. (b) HeLa cells were microinjected with the constructs indicated in panel a, stained with the CD4-specific monoclonal antibody Q4120 and a FITC-conjugated secondary antibody, and subsequently analyzed by confocal laser scanning microscopy. The cross-section levels are indicated by white bars. The cells shown are representative of all cells microinjected with the respective constructs. Similar results were obtained in at least three independent experiments.


a
gp160
gp160
gp160
anti-gp160
anti-calnexin
merge
CD4Δcyt
CD4Δcyt
CD4Δcyt
anti-CD4
anti-mannosidase II
merge
CD4gp41cyt
CD4gp41cyt
CD4gp41cyt
anti-CD4
anti-mannosidase II
merge
CD4gp41Δis1+2
CD4gp41Δis1+2
CD4gp41Δis1+2
anti-CD4
anti-mannosidase II
merge
b
0 6 10 0 6 10 chase (h)
- + - + - + - + - + - + Endo H
r
s
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s
gp160
CD4Δcyt
0 6 10 0 6 10 chase (h)
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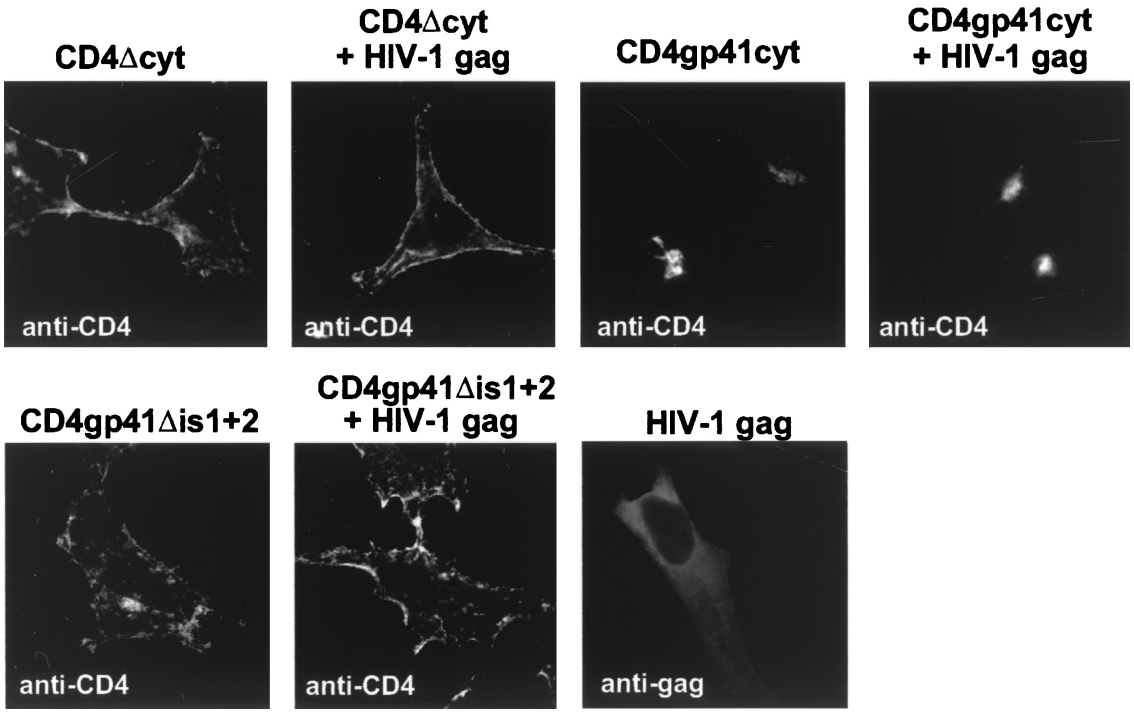

FIG. 5. The *is1*- and *is2*-induced Golgi localization is not influenced by HIV-1 Gag. HeLa cells were microinjected with either CD4Δcyt, CD4gp41cyt, or CD4gp41Δis1+2 alone or in combination with HIV-1 *gag* at a DNA ratio of 1:1. Subsequently, cells were stained with the CD4-specific monoclonal antibody Q4120 and a FITC-conjugated secondary antibody and analyzed by confocal laser scanning microscopy. To control expression of the HIV-1 *gag* plasmid, HeLa cells microinjected with HIV-1 *gag* were stained with monoclonal antibody EH12E1 against Gag.

for a further 30 min. DNA was blotted overnight by capillary transfer onto a polyvinylidene difluoride nylon membrane using 20× SSC buffer (3 M NaCl, 300 mM sodium citrate [pH 7.0]). The DNA was subsequently fixed to the membrane by UV cross-linking. Prehybridization was performed in hybridization buffer (5× SSC, 1% blocking reagent, 0.1% *N*-lauryl sarcosine, 0.02% sodium dodecyl sulfate [SDS]) at 68°C for 1 h, followed by hybridization with the digoxigenin-labeled probe in hybridization buffer at 68°C overnight. After two washes with wash solution I (2× SSC, 0.1% SDS) at room temperature for 5 min and wash solution II (0.1× SSC, 0.1% SDS) at 68°C for 15 min, the membrane was equilibrated in Tris-buffered saline (TBS) buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl). Subsequently the membrane was blocked with 5% milk powder–TBS for 1 h, incubated with a peroxidase-conjugated anti-digoxigenin antibody (Roche, Mannheim, Germany) for 1 h, and washed with TBST (10 mM Tris-HCl [pH 8], 150 mM NaCl, 0.05% Tween 20). For chemiluminescence substrate detection, the ECL Western blot detection reagents (Amersham Pharmacia Biotech, Uppsala, Sweden) were used.

Microinjection and immunofluorescence. HeLa cells grown on glass coverslips were microinjected with 0.05 μg of plasmid DNA/μl with an injection time of 0.5 s and a pressure of 60 hPa using the Eppendorf 5242/5170 microinjector system (Eppendorf, Hamburg, Germany). After further growth overnight, cells were washed with PBS and fixed with 3% paraformaldehyde–PBS for 20 min followed by incubation with 50 mM NH_4Cl–20 mM glycine–PBS for 10 min. Cells were permeabilized with 0.2% Triton X-100–PBS for 10 min. Subsequently, cells were blocked with 0.2% gelatin–PBS for 10 min and incubated with the primary antibody for 30 min in the same buffer. HIV-1 Env was detected using the human polyclonal antiserum 95-1, human CD4 by mouse antibody Q4120 (Sigma, St. Louis, Mo.). After being washed three times with PBS, cells were incubated with the secondary, fluorescein isothiocyanate (FITC)-conjugated antibodies (Dianova, Hamburg, Germany) for a further 30 min. After three final washes, the coverslips were mounted on glass slides and viewed with a Leica TCS NT confocal laser scanning microscope (Leica, Bensheim, Germany).

Flow cytometry. For immunofluorescence analysis, transfected cells were detached from culture dishes by treatment with 1 mM EDTA–PBS. Cells were washed with FACS buffer (PBS–0.5% fetal calf serum–0.03% NaN_3) and incubated for 30 min at 4°C with the human antiserum 95-1 at a dilution of 1:50. Subsequently, cells were washed again and incubated for 30 min at 4°C with a secondary anti-human IgG FITC-conjugated antibody at a dilution of 1:100. Finally, cells were washed, resuspended in FACS buffer, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, Calif.).

RESULTS

Identification of two sequence elements in the gp41 cytoplasmic domain responsible for suppressed surface expression. The analysis of post-ER events regulating Env surface expression is difficult, since the majority of the glycoprotein is retained in the ER due to incorrect processing and interferes with subsequent events (see Fig. 4). We thus decided to generate chimeric constructs using a CD4 extracellular/transmembrane domain and the gp41 cytoplasmic domain (56). Surface expression was tested by immunofluorescence analysis of mi-

FIG. 4. *is1* and *is2* are responsible for Golgi localization. (a) The cytoplasmic gp41 domain induces Golgi localization. HeLa cells microinjected with either gp160, CD4Δcyt, CD4gp41cyt, or CD4gp41Δis1+2 were costained with antibodies against gp160 or CD4 (green fluorescence) and either the ER marker calnexin or the Golgi marker mannosidase II (red fluorescence). (b) Chimeric CD4 fusion constructs exit the ER. 293 cells transfected with either gp160, CD4Δcyt, CD4gp41cyt, or CD4gp41Δis1+2 were pulsed with [^{35}S]Cys-[^{35}S]Met for 1 h and subsequently chased as indicated for 0, 6, or 10 h. Cell lysates were precipitated, digested with endoglycosidase H (Endo H), and subjected to electrophoresis on a 10% polyacrylamide gel.

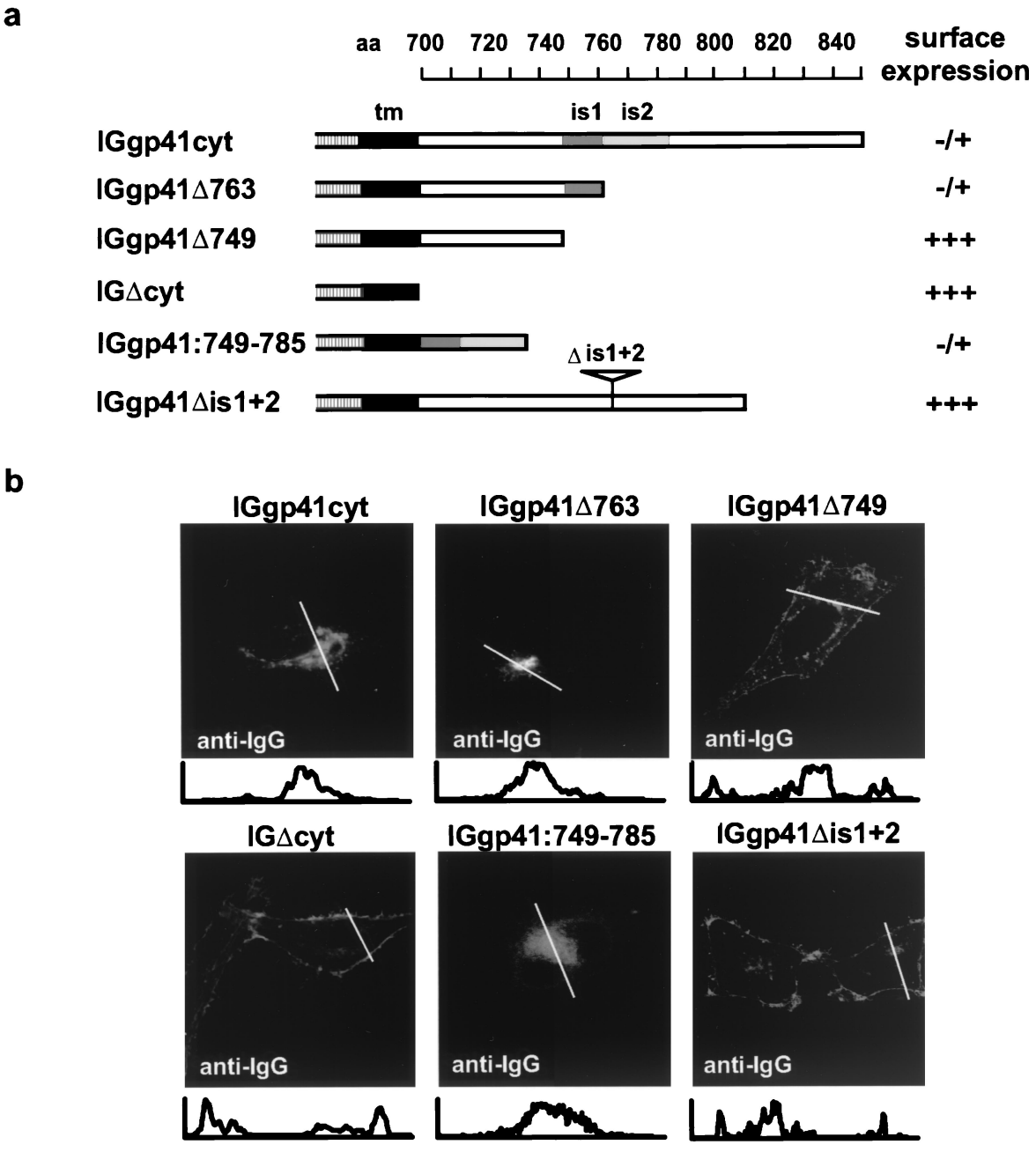

FIG. 6. Either *is1* or *is2* is sufficient for Golgi localization in a second heterologous system using an immunoglobulin extracellular domain. (a) Schematic diagram of the chimeric constructs with the immunoglobulin (IG) extracellular domain, CD7 transmembrane stock, and gp41 cytoplasmic domain used to identify the retention elements. (b) HeLa cells were microinjected with the constructs indicated in panel a, stained with a FITC-conjugated antibody against human immunoglobulin G, and subsequently analyzed by confocal laser scanning microscopy. The cross-section levels are indicated by white bars. The cells shown are representative of all cells microinjected with the respective constructs. Similar results were obtained in at least three independent experiments.

croinjected HeLa cells using confocal laser scanning microscopy (Fig. 1). This approach was chosen since microinjection of single cells with defined amounts of plasmid DNA proved to be more reliable than analysis of bulk-transfected cultures. As anticipated, addition of the gp41 cytoplasmic domain drastically reduced surface expression of the chimeric molecule, which was even better illustrated if the immunofluorescence staining of microinjected cells was analyzed by cross-section (Fig. 1b). Intriguingly, the constructs with carboxy-terminal deletions between aa 856 (CD4gp41cyt) and 763 (CD4gp41Δ763) were localized in the Golgi compartment, but the shorter deletion mutants, CD4gp41Δ749, CD4gp41Δ738, CD4gp41Δ728, and CD4Δcyt, all showed significant surface expression (Fig. 1). Thus, the sequence motif between aa 749 and 763 appears to be responsible for predominant Golgi localization. To analyze whether this element, termed *is1* (inhib-

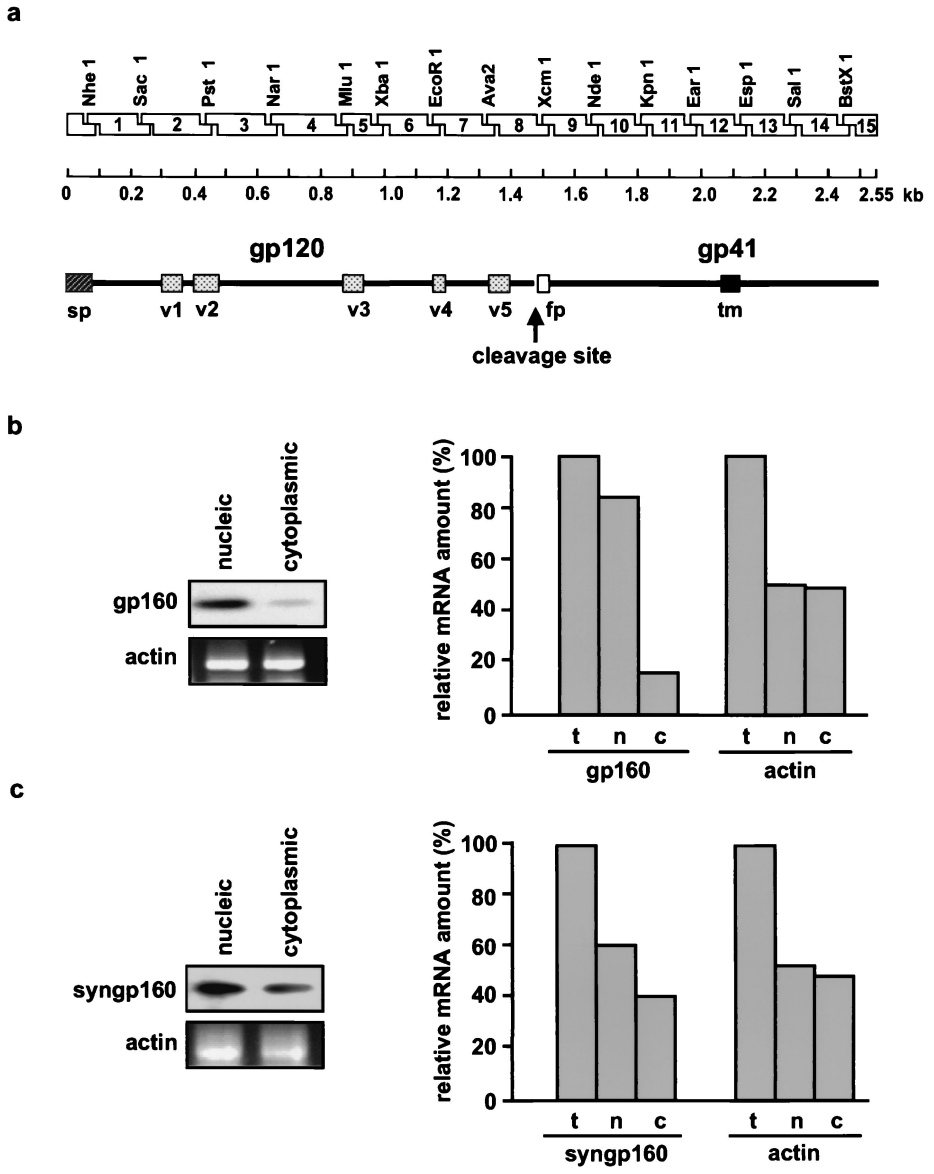

FIG. 7. The nucleocytoplasmic mRNA export of a synthetic gp160 sequence is not inhibited. (a) Generation of a synthetic Env sequence (scheme). The synthetic gp160 sequence (syngp160) was generated by 15 long oligonucleotides which were PCR-amplified using primers with the indicated restriction sites. Amplification products were sequentially cloned into plasmids containing adapted multicloning sites and subcloned into a pCDM7-derived expression plasmid. sp, signal peptide; v1 to v5, variable regions; fp, fusion peptide; tm, transmembrane region; ↑, cleavage site. (b) RT-PCR analysis of nuclear and cytoplasmic RNA isolated from 293T cells transiently transfected with wild-type gp160. RNA was isolated by CsC1 gradient isolation to avoid contamination with plasmid DNA and, after DNase treatment, transcribed into cDNA using an oligo(dT) primer. Subsequently, the cDNA was used as a PCR template with either gp160 or actin primers. PCR products were separated on an agarose gel, blotted onto a polyvinylidene difluoride membrane, and subsequently hybridized with a gp160-specific probe. Quantitative analysis was performed using Image Master ID Elite software (Amersham Pharmacia Biotech). (c) RT-PCR analysis of nuclear and cytoplasmic RNA isolated from 293T cells transiently transfected with syngp160.

itory sequence), is sufficient to prevent surface expression, we cloned it directly 3′ to the transmembrane region (Fig. 2). The chimeric molecule CD4gp41:749–763 was expressed at the cell surface, indicating that *is1* is not sufficient for Golgi localization if not expressed in the correct sequence context. However, if the complete region between aa 749 and the carboxy-terminal end was expressed 3′ to the transmembrane region (CD4gp41:749–856), surface expression was inhibited. Carboxy-terminal deletions of this construct identified a second element, *is2*, which, in combination with *is1*, appeared to be sufficient for Golgi localization without further sequence context and the correct distance from the plasma membrane (Fig. 2). Subsequently, *is1* and *is2*, separately or together, were deleted in the context of the complete gp41 cytoplasmic domain (Fig. 3). Either of the two elements alone was able to inhibit surface expression, and the chimeric protein was detected on the plasma membrane only if both elements *is1* and *is2* together were deleted. To test whether the two dileucine motifs

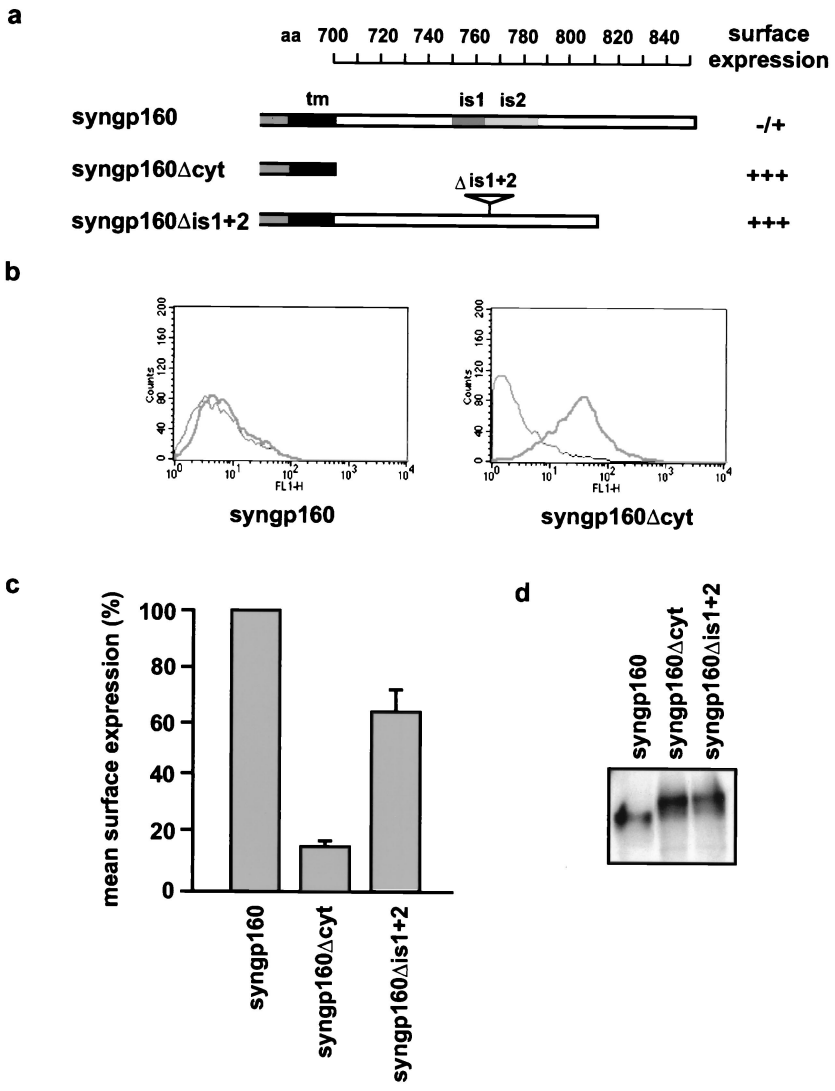

FIG. 8. Deletion of *is1* and *is2* restores surface expression of Env. (a) Schematic diagram of the syngp160 constructs used to evaluate surface expression. (b) The cytoplasmic domain of gp41 is responsible for suppressed surface expression. HeLa cells were stably transfected with syngp160 and syngp160Δcyt using a bovine papillomavirus-based, replicating vector. Nonpermeabilized bulk cultures were stained with the Env-specific human antiserum 95-1 and a FITC-conjugated secondary antibody and subsequently analyzed by flow cytometry. As a negative control (dashed line), cells were treated only with the secondary antibody. (c) Quantitative analysis of Env surface expression. 293T cells transiently transfected with either syngp160, syngp160Δcyt, or syngp160Δis1+2 were stained with the Env-specific human antiserum 95-1 and a FITC-conjugated secondary antibody without prior permeabilization and subsequently analyzed by flow cytometry. Surface expression (mean ± standard error of the mean of seven independent experiments) was calculated by subtracting the mean of the negative control from the mean of the cells stained with both the primary and the secondary antibody. (d) Western blot analysis of 293T cells transiently transfected with either syngp160, syngp160Δcyt, or syngp160Δis1+2. Cells from one experiment were split into two groups and used for either immunofluorescence or Western blotting. The Western blot was reacted with the Env-specific human antiserum 95-1.

present in *is2* are responsible for suppressed surface expression and Golgi retention or retrieval, we mutated either of the two separately or both of them together (Fig. 3). However, there was no surface expression with either of these chimeric constructs, similar to the construct in which the *is2* region has been deleted.

To test that the identified sequence motifs *is1* and *is2* in fact cause Golgi localization, we performed colocalization experiments with ER and Golgi marker proteins. Whereas HIV-1 gp160 colocalized predominantly with the ER marker calnexin, the chimeric CD4gp41cyt fusion protein was detected mainly in the Golgi and was thus found to costain with mannosidase II (Fig. 4a). In contrast to CD4gp41cyt, a considerable portion of CD4Δcyt and CD4gp41Δis1+2 was detected at the cell surface, and only a minor amount of the protein colocalized with mannosidase II. To confirm biochemically that the chimeric CD4 fusion proteins had left the ER and were not subjected to ER retention and degradation due to incorrect folding, we performed Endo H digests of lysates from cells transfected with the key mutants in pulse-chase experiments. In accordance

with the colocalization experiments, we found that CD4Δcyt, CD4gp41cyt, and CD4gp41Δis1+2 were completely Endo H resistant after a 6-h chase, indicating that all these fusion proteins had reached the medial Golgi and that the cytoplasmic gp41 domain thus did not influence the ER exit (Fig. 4b). In contrast, the gp160 glycoprotein was still almost completely Endo H sensitive after both 6 and 10 h of chase, indicating that it was retained within the ER.

Subsequently, we tested whether the gp41 cytoplasmic domain-induced Golgi localization would be influenced by HIV-1 Gag. A plasmid carrying *gag* was coinjected into HeLa cells at a DNA ratio of 1:1 (Fig. 5). Since the DNA was coinjected rather than cotransfected, all cells expressing the chimeric CD4 proteins also expressed HIV-1 Gag. Correct expression of the HIV-1 *gag* construct used was tested by staining with an anti-Gag monoclonal antibody. HIV-1 Gag had no effect on the localization of either of the chimeric CD4 proteins, and CD4gp41cyt was found in the Golgi independent of HIV-1 Gag expression.

To control that this effect was not artifically caused by the CD4 extracellular/transmembrane region, we generated a second set of chimeric constructs using surface-anchored IgG1 in combination with the gp41 cytoplasmic domain (Fig. 6). Similarly to the CD4 chimeras, surface expression was considerably reduced if the gp41 cytoplasmic domain was added 3′ to the transmembrane region and could be restored by deletion of both *is1* and *is2*. Moreover, surface expression was suppressed if both elements were added directly 3′ to the immunoglobulin transmembrane region. In conclusion, in both heterologous systems the elements *is1* and *is2* behaved similarly and induced localization to the Golgi. Either of the two elements is sufficient to inhibit surface expression if deleted separately, and only deletion of both of them restores surface expression.

***is1* and *is2* inhibit surface expression of gp160.** In contrast to most cellular transcripts, the majority of HIV-1 Env mRNA is retained in the nucleus (Fig. 7b). RT-PCR analysis of nuclear and cytosolic mRNA isolated from 293T cells expressing HIV-1 gp160 indicated that only 18% of the viral mRNA can be found in the cytosol, whereas more than 80% was retained in the nucleus. In contrast to the case with gp160, approximately 50% of the cellular actin mRNA was detected in the cytosol. Thus, we confirmed previous data indicating that gp160 contains inhibitory sequence motifs retaining the viral mRNA in the nucleus (2, 7, 12, 21, 45, 56, 60, 61). To identify peptide motif(s) responsible for inhibition of protein export, we thus had to choose strategies which eliminated the interfering mRNA retention.

To test whether the identified sequence elements are also responsible for inhibited surface expression in the homologous context, we decided to generate a synthetic gp160 gene in which the primary sequence codons have been replaced by synonymous codons. In the HIV-1 *env* gene, codons with either adenine or thymine at the third codon position are preferentially used (11; P. Grantham and P. Perrin, Letter, Nature **319:**727–728, 1986). We substituted codons with guanine or cytosine for most codons with adenine or thymine at the third position. The synthetic gp160 sequence (syngp160) was generated by long 150- to 200-mer oligonucleotides, which were amplified by PCR and sequentially cloned into two plasmids containing suitable polylinkers. Finally, the two sequences coding for gp120 and gp41 were subcloned into a pCDM7-based expression plasmid containing the immediate-early promoter of human cytomegalovirus and tested by Western blot analysis of cell lysates from transiently transfected cells for the correct length (Fig. 7a). RT-PCR analysis of nuclear and cytoplasmic mRNA indicated that in contrast to the wild-type *env* sequence, the syngp160 mRNA is not retained in the nucleus (Fig. 7c). More than 40% of the syngp160 mRNA was found in the cytoplasmic fraction, similarly to actin mRNA (48% in the cytoplasmic fraction). Since we were unable to detect intron-containing transcripts in the cytoplasmic fractions by RT-PCR using primers annealing in the intron (the vector used contains a small intron in the 3′ untranslated region), we conclude that the cytosolic fractions were properly isolated and not contaminated by nuclear RNA (data not shown).

Deletion of the gp41 cytoplasmic domain considerably increased surface expression in 293T cells stably or transiently transfected (Fig. 8). Quantitative flow-cytometric analysis of 293T cells transiently transfected with either syngp160 or syngp160Δcyt indicated that surface expression was reduced to less than 20% by the cytoplasmic domain (Fig. 8c). In contrast, the total cellular glycoprotein amount was similar in the Western blot analysis, indicating that reduced surface expression was not due to decreased production rates (Fig. 8d). Similarly to the results in the two heterologous systems, elimination of both *is1* and *is2* (syngp160Δis1+2) resulted in an almost fourfold increase of surface expression compared to syngp160 containing the complete cytoplasmic domain, suggesting that the two elements *is1* and *is2* act similarly in the context of homologous Env.

DISCUSSION

In this study, we were able to identify sequence elements in the cytoplasmic tail of the HIV-1 Env glycoprotein which inhibit cell surface expression. To investigate post-ER events without interference of ER-retained, misfolded glycoprotein, we used two heterologous systems. We found that two elements, *is1* and *is2*, inhibit surface expression and induce localization to the Golgi. Generally, Golgi localization can be caused by either retrieval or retention signals. Retention signals of resident Golgi transmembrane proteins are located mainly in the transmembrane domain (44, 46). Two models have been proposed for transmembrane Golgi retention motifs: the first is based on retention through oligomerization to large aggregates, and the second postulates retention through a different length of membrane-spanning domains regarding the differences in membrane thickness along the exocytic pathway. Retrieval signals have been found in several trans-Golgi proteins that recycle between the plasma membrane and the Golgi complex and are composed of either tyrosine-based or dileucine motifs (8, 51, 59).

The gp41 cytoplasmic domain is a strong inducer of endocytosis, and *is1* and *is2* appear to act predominantly as retrieval motifs (data not shown). In time course analyses, approximately 40% of a chimeric CD4 molecule with the cytoplasmic gp41 domain was endocytosed after 15 min, and 60% was endocytosed after 60 min (data not shown). In contrast, only approximately 5% of the CD4 control molecule without the cytoplasmic gp41 domain was internalized after 15 min, and

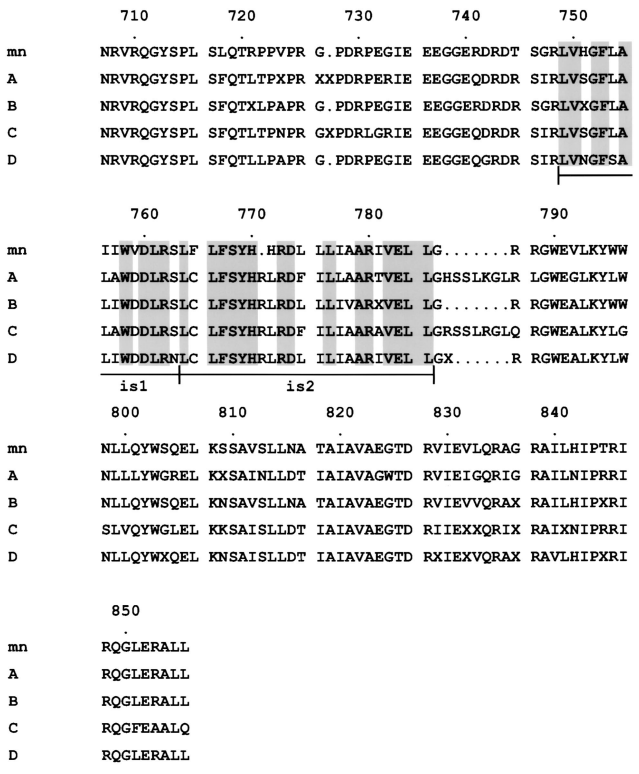

FIG. 9. *is1* and *is2* are conserved between different HIV-1 isolates. The gp41 cytoplasmic domain (aa 707 to 856) of the HIV-1 MN isolate and the consensus sequences of subtype A, B, C, and D isolates (according to the Los Alamos National Library Database) were compared using standard software of the University of Wisconsin Genetics Computer Group (CLUSTAL program). The Golgi retention or retrieval motifs *is1* and *is2* are marked below the sequence, and identical amino acids are indicated by grey boxes.

10% after 60 min. Similar results were presented in a recent study with recombinant vaccinia virus expressing gp160 with or without the cytoplasmic domain (58). In this study, it was found that >50% of the gp160 was internalized after 60 min, but only approximately 15% was internalized if the cytoplasmic domain was absent. A membrane-proximal, tyrosine-based YXXΦ motif (aa 713 to 716) was recently found to mediate endocytosis of gp160 (4, 58). This motif appears to be bound by clathrin-associated m1 and m2 subunits of AP adaptor complexes, and its function is suppressed in the presence of the HIV-1 Gag precursor polyprotein (4, 19). However, both groups report that additional determinants distal to the YXXΦ motif may be involved (4, 58). Intriguingly, we observed only little or no effect of the gp160 amino acids 707 to 749 including the YXXΦ motif (Fig. 1) (CD4Δ749, CD4Δ738, and CD4Δ728) on steady-state surface expression (in comparison to results with CD4Δcyt) in this study. We conclude that in terms of steady-state surface expression, the two membrane-distal elements *is1* and *is2* determined in this study are even more important than the proximal tyrosine-based YXXΦ motif.

The gp41 cytoplasmic domain (148 aa) contains several dileucine motifs known to mediate Golgi retrieval in a variety of other molecules (1). The dileucine motifs present in *is2* appear to be rather conserved between different isolates (Fig. 9). The aa 784/5 dileucine motif was found to be completely conserved between all consensus subtype A to D sequences, and in the aa 776/7 dileucine motif the first leucine was found to be replaced by an isoleucine in the subtype A, C, and D consensus sequences. Previous reports have shown that isoleucine-leucine motifs act similarly as a dileucine motif in Golgi retrieval (47). In this study, the two dileucine motifs present in *is2* were not sufficient to inhibit surface expression and needed additional sequence context to inhibit surface expression.

Intriguingly, the inhibitory element *is1* does not contain any known retrieval or retention signal. Beyond its rather hydrophobic structure and three leucine residues in an equal distance of seven amino acids, there are no other apparent features of this motif. The inhibitory element *is2* is nearly identical to the previously identified lentivirus lytic peptide LLP2 (aa 768 to 788), which was described (together with the lentivirus lytic peptide LLP1 (aa 828 to 855) as an amphipathic structure that associates with membranes and was reported to alter membrane permeability by channel formation (20, 40, 41, 65, 68). Furthermore, LLPs possess calmodulin-binding capacity, modulate intracellular signaling, and contribute to the cytopathogenic effect of Env (3, 34, 41).

In HIV-1 and other retroviruses, glycoprotein surface expression is strictly regulated. Willey and colleagues reported that in HIV-1-infected peripheral blood mononuclear cells, less than 15% of the total gp160 amount is cleaved to gp120 within 24 h, indicating that the majority of the protein is not transported to the plasma membrane (69). In this study, we show that the cytoplasmic domain is responsible for restricted expression, which is in accordance with previous reports indicating its involvement in intracellular trafficking, surface expression, and incorporation into virus particles (52, 55, 70). There are several lines of evidence suggesting that restricted surface expression of glycoproteins is crucial for the virus in vivo. Whereas in vitro culture of SIV and HIV-2 selects for TM truncations, revertants which express full-length Env again can be detected after administration of SIV mutants with truncated cytoplasmic TM domains into rhesus macaques (37). Second, the virus has obviously developed several independent strategies on different levels to suppress glycoprotein expression: inhibition of mRNA transport and glycoprotein Golgi retention or retrieval, both of which appear to be highly coordinated (19, 21, 23, 38). Stressing their importance, both strategies appear to be conserved in all HIV-1 viruses independently of subtype or isolate, despite the high mutation rate. In accordance with this observation, the main features of the two elements, *is1* and *is2*, presented in this report appear to be conserved between different isolates (Fig. 9). Currently, we have no hints for an alternative function of Golgi retention or retrieval of the Env glycoprotein. The elimination of the YXXΦ motif, for example, had no effect on major histocompatibility complex class II-restricted presentation of Env-derived peptides (58).

The transcripts of the synthetic sequence with synonymous codons generated in this study were not retained within the nucleus, indicating that the approach to eliminating inherent, poorly localized negative elements by synonymous codons might represent a novel strategy to increase viral glycoprotein expression (Fig. 7). Due to a direct interaction between Env and viral matrix proteins, the carboxy-terminal end of the gp41

cytoplasmic domain appears to influence the incorporation of glycoproteins into virions (13, 15, 16, 22, 25, 28, 39, 72, 73). Thus, the gp160 mutant with the short internal deletion of *is1* and *is2*, syngp160Δis1–2, which is considerably more highly expressed at the cell surface in comparison to wild-type Env, should still be incorporated into virus particles. In consequence, this construct might prove to be useful for further studies on HIV-1 Env-pseudotyped viruses and for gene therapeutical applications, since elements necessary for virion incorporation are still present.

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