Determinants of Secretion and Intracellular Localization of Human Herpesvirus 8 Interleukin-6[∇]

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Human herpesvirus 8 (HHV-8) interleukin-6 (vIL-6) is distinct from human and other cellular IL-6 proteins in that it does not require the nonsignaling α -receptor subunit for the formation of gp130-based signal transducing complexes and also is largely retained intracellularly rather than being secreted. We and others have reported that vIL-6 is retained and is active in the endoplasmic reticulum (ER) compartment, and data from our laboratory have demonstrated that intracellular vIL-6 is functional in the autocrine promotion of proliferation and survival of HHV-8 latently infected primary effusion lymphoma cells. It has also been reported that vIL-6 secretion in gp130-deficient cells can be enhanced by introduced gp130, thereby implicating the signal transducer in vIL-6 trafficking to the cell surface. We examine here the requirements for intracellular retention and localization of vIL-6. Using vIL-6-hIL-6 chimeric and point-mutated vIL-6 proteins, we identified regions and residues of vIL-6 influencing vIL-6 secretion. However, there was no correlation between vIL-6 secretion and gp130 interaction. We found that vIL-6, but not hIL-6, could associate stably with ER-resident chaperone protein calnexin. Glycosylation-dependent interaction of vIL-6 with calnexin correlated with proper protein folding, but there was no direct relationship between vIL-6-calnexin interaction and intracellular retention. While calnexin depletion had little influence on absolute amounts of secreted vIL-6, it led to markedly reduced levels of intracellular cytokine. This was reversed by gp130 transduction, which had no detectable effect on vIL-6 secretion, but redistributed vIL-6 into ER-distinct locations in calnexin-depleted cells, specifically. Our data reveal that calnexin plays a role in ER localization of vIL-6 and that gp130 promotes ER exit, but not secretion, of the viral cytokine.

The viral homologue of interleukin-6, vIL-6, specified by human herpesvirus 8 (HHV-8) shows only 25% amino acid identity to human IL-6 (hIL-6) but is highly related structurally (2, 5). Despite the high degree of conservation of three-dimensional structure and equivalence of receptor interaction interfaces (1, 6), the viral cytokine can associate functionally with the gp130 signal transducer in the absence of the gp80 α -subunit, absolutely required for cellular IL-6 signaling through gp130. The nonsignaling gp80 subunit can be incorporated into vIL-6-induced signaling complexes and indeed seems to have a stabilizing effect that enhances signal transduction (1, 3, 11). Another major difference between vIL-6 and cellular IL-6 proteins, including hIL-6, is that the viral cytokine is very inefficiently secreted, retained largely within the endoplasmic reticulum (ER) compartment, where it is able to transduce signal via gp80-deficient vIL-6₂/gp130₂ tetrameric complexes, exclusively (4, 15). Thus, the unique ability of vIL-6 to signal intracellularly may be explained by its gp80 independence; hIL-6 cannot signal in the ER even when targeted to this compartment (4). The biological significance of intracellular, strictly autocrine signaling by vIL-6 was demonstrated recently in primary effusion lymphoma (PEL) cells, which are latently infected with HHV-8; these cells grew with markedly reduced

kinetics and displayed higher rates of apoptosis upon shRNA-mediated vIL-6 depletion relative to cocultured untransduced cells (4). Thus, vIL-6 appears not only to be expressed in latently infected PEL cultures but also to be biologically active in this setting via intracrine signaling.

Despite these findings and other mechanistic studies of vIL-6, the means by which the viral cytokine is retained in the ER and secreted so inefficiently is unknown. The elegant work of Meads and Medveczky (15) demonstrated the slow secretion kinetics of vIL-6 relative to hIL-6 and implicated gp130 as a necessary cofactor for vIL-6 secretion. Thus, vIL-6 expressed in gp130-negative Ba/F3 cells was able to be secreted only if gp130 was supplied via expression vector transduction. However, most cell types express gp130; thus, while the signal transducer may be involved in vIL-6 trafficking, the underlying explanation for the very slow rate of vIL-6 secretion must involve other factors.

We report here investigations of the structural requirements for vIL-6 intracellular retention, the influence of gp130 on this process, and the possible involvement of ER-resident chaperon proteins for retention of vIL-6 in the ER. Our data identify effects of structural alterations and point mutations of vIL-6 on secretion efficiency, the lack of gp130 involvement in these observed effects, mechanistically relevant interactions of calnexin with the viral cytokine, and the influence of gp130 on vIL-6 subcellular localization and stability in the context of calnexin depletion. The results presented thus further advance our understanding of vIL-6–cellular protein interactions that impact upon its intracellular function.

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MATERIALS AND METHODS

Cell culture, transfections, and lentiviral transduction. HEK293T cells were grown in Dulbecco modified Eagle medium with 10% fetal calf serum. For transfection, cultures were passaged 12 to 24 h prior to transfection to produce 70% confluent monolayers, to which were added calcium phosphate-DNA coprecipitates (generated by mixing DNA, CaCl_2 , and HEPES-buffered saline). Cells were harvested 48 h posttransfection for determinations of vIL-6 expression, STAT3 activation, and induced receptor complexing. For vIL-6 secretion, vIL-6-CBD was captured with chitin beads (catalog no. S6651; New England Biolabs, Beverly, MA) from media harvested 48 h posttransfection. For infection of HEK293T cells with retroviral vectors, 70% confluent monolayers were inoculated in the presence of 5 μg of Polybrene/ml and incubated at 37°C for 6 h, after which the inoculum was replaced with fresh medium. Transduced cells were selected in puromycin (1 $\mu\text{g}/\text{ml}$) added 2 days postinfection.

Plasmids and retroviral vectors. pSG5-based eukaryotic expression vectors for untagged and KDEL motif-tagged vIL-6-CBD fusion proteins, CBD fusions of vIL-6 domain substitution and C-helix point variants, vIL-6-RFP and GFP-KDEL have been described previously (3, 4). The gp130 expression vector, pEFBOS-gp130, was obtained from M. Narazaki and K. Kishimoto. Viral vectors utilized in the present study comprised pSuper.Retro.puro from Oligoengine (Seattle, WA) containing the H1 promoter for expression of calnexin or calreticulin mRNA-complementary shRNA sequences GACGATACCGATGAT GAAA and GGTTCATGTCATCTCAAC, respectively; annealed, complementary oligonucleotides corresponding to each were cloned between the BglIII and HindIII sites of pSuper.Retro.puro. Retroviral vectors pSuper-CNX and pSuper-CRT (5 μg) were transfected into 70% confluent monolayers of Phoenix cells (Orbigen, San Diego, CA) in 10-cm culture dishes. Two days posttransfection, viruses were harvested from culture media.

Subcellular fractionation. Phosphate-buffered saline (PBS)-washed cells were suspended in ice-cold homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM HEPES [pH 7.4]) and disrupted by repeated passage through a 27-gauge syringe needle. Homogenates were centrifuged at $1,000 \times g$ for 10 min at 4°C to remove nuclei, and supernatants subjected to density gradient ultracentrifugation for membrane fractionation. Iodixanol discontinuous gradients were prepared by using OptiPrep (Accurate Chemical and Scientific, Westbury, NY) layered successively at 30, 25, 20, 17.5, 15, 12.5, 10, 7.5, and 5%. Postnuclear supernatants were applied and centrifugation carried out at 25,000 rpm in an SW41 Ti rotor for 3 h at 4°C. The gradient was removed in 10 to 12 equal fractions from the top of the gradient. For analysis of vIL-6 membrane association, crude microsomal preparations were made by Dounce homogenization of PBS- and 0.25 M sucrose-washed cells in 2 ml of buffer containing 0.25 M sucrose–10 mM Tris-HCl (pH 7.4), low-speed centrifugation to remove cell debris and nuclei, and final centrifugation at 28,000 rpm in a Beckman SW41 Ti rotor for 1 h to pellet total microsomal membranes; the pellets were then resuspended in sucrose buffer for subsequent analysis.

Analysis of microsomal membranes for vIL-6 association. Crude microsomal membrane preparations were left untreated or treated with either 0.1 M sodium carbonate (pH 11.5) or 0.5 M potassium acetate in a final volume of 7 ml and incubated on ice for 30 min. The mixtures were then subjected to centrifugation at 29,000 rpm in a Beckman SW41 Ti rotor for 1.5 h to pellet membranes. 10% trichloroacetic acid was applied to supernatants to precipitate proteins; pellets obtained by centrifugation were dissolved in 100 μl of sodium dodecyl sulfate (SDS) sample buffer. Membrane pellets were resuspended directly in 100 μl of SDS sample buffer. The samples were analyzed by SDS–12% polyacrylamide gel electrophoresis (PAGE) and immunoblotting using antiserum or monoclonal antibodies specific for vIL-6, calnexin, and calreticulin.

Antibodies, Western blotting, and coprecipitations. Antiserum to vIL-6, generated in this laboratory, has been described previously (24). Commercially obtained antibodies used for immunoblotting were as follows: CBD (New England Biolabs, Beverly, MA), catalog no. E8034S; gp130 (BD Biosciences, Rockville, MD), catalog no. 555755; phospho-STAT3 (Cell Signaling Technology, Danvers, MA), catalog no. 9131; STAT3 (Santa Cruz Biotech, Santa Cruz, CA), catalog no. sc-482; Na^+/K^+ -ATPase (Abcam, Cambridge, MA), catalog no. ab7671-50; EEA1 (BD Biosciences, Rockville, Md.), catalog no. 610457; Calnexin, BD Biosciences (Rockville, MD), catalog no. 610523; Calreticulin, BD Biosciences (Rockville, MD), catalog no. 612136; ERGIC-53/p58, Sigma-Aldrich (St. Louis, MO), catalog no. E 1032; Bip/GRP78, BD Biosciences (Rockville, MD), catalog no. 610978; β -actin, Sigma-Aldrich (St. Louis, MO), catalog no. A2228; and hIL-6 (R&D Systems, Minneapolis, MN), catalog no. AB-206-NA. For Western blotting, Tricine-SDS-PAGE and electrophoretic transfer to nitrocellulose membranes was carried out by using standard procedures. Membranes were blocked in PBS-T buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 ,

1.4 mM KH_2PO_4 , 0.1% Tween 20) containing 5% nonfat milk prior to the addition of primary antibody (0.1 to 1.0 $\mu\text{g}/\text{ml}$) and incubation at 4°C overnight. PBS-T-washed membranes were incubated with horseradish peroxidase-conjugated anti-rabbit (vIL-6) or anti-mouse secondary antibodies in PBS-T–5% nonfat milk, washed with PBS-T, and membrane-bound antibody visualized by using chemiluminescence assay. For coprecipitation-based vIL-6–receptor complexing assays, gp130-Fc was precipitated with protein A-agarose after overnight incubation with cell lysates or ER membrane fractions, sedimented material washed with PBS, and pellets heated in SDS-PAGE load buffer prior to gel loading. For interaction of vIL-6 with calnexin or calreticulin, cells were lysed in lysis buffer (10 mM HEPES [pH 7.5], 50 mM NaCl, 5 mM CaCl_2 , 0.2% NP-40), vIL-6-CBD was precipitated with chitin beads, and pellets were heated in SDS-PAGE load buffer prior to gel loading.

Immunofluorescence microscopy. For studies of subcellular localization of vIL-6, 293T cells were seeded onto poly-L-lysine-coated chamber slides and transfected with expression plasmids for vIL-6-RFP and/or ER-directed KDEL-tagged green fluorescent protein (GFP). At 24 h after transfection, cells were fixed with 4% paraformaldehyde in PBS and permeabilized 0.25% Triton X-100. Nuclei were counterstained with DAPI (4',6'-diamidino-2-phenylindole) and viewed by confocal microscopy.

Trypsin digestion. Chitin captured vIL-6-CBD and vIL- ΔN -CBD were resuspended in 100 mM Tris (pH 8) were digested by trypsin (0.1 $\mu\text{g}/\text{ml}$) at 37°C for 0, 10, 20, 30, 40, 50, and 60 min. The reactions were stopped by adding SDS-PAGE loading buffer and boiling for 10 min.

Pulse-chase analysis of vIL-6–calnexin interaction. HEK293T cells were seeded in 10-cm culture dishes and transfected with pSG5-vIL-6 and pSG5-calnexin-CBD. After 24 h, the cells were rinsed twice with PBS and incubated in methionine-cysteine-free medium for 30 min at 37°C, and pulse-labeled with 200 $\mu\text{Ci}/\text{ml}$ of [^3S]methionine-cysteine (Perkin-Elmer, Boston, MA; catalog no. NEG07200) for 20 min. The cells were rinsed twice with warm PBS and chased with 5 ml of warm culture medium containing 2 mM methionine and 2 mM cysteine. Cells were collected at various times during the chase. Calnexin-CBD and associated proteins were precipitated from aliquots of cell lysates with chitin beads, proteins bound to calnexin dissociated by heating to 95°C in 1% SDS buffer, and samples diluted in Tris-buffered saline containing 1% Triton X-100 prior to immunoprecipitation of vIL-6 with rabbit antiserum (24), and size fractionation and detection by SDS-PAGE and autoradiography of dried gels. vIL-6 was also immunoprecipitated directly from samples of cell lysates and culture media, subjected to SDS-PAGE, and visualized by autoradiography.

RESULTS

vIL-6 domains and sequences influencing vIL-6 secretion.

To investigate the structural requirements for vIL-6 intracellular retention, we utilized vIL-6 proteins in which each domain (N terminus, helices A-D, A-B loop) were individually replaced with equivalent regions of hIL-6 (Fig. 1A). These domain-substitution variants of vIL-6 were shown previously to retain functional integrity and therefore are not significantly altered with respect to structure or expression (3). Coding sequences for the various vIL-6 proteins were cloned into a chitin-binding domain (CBD)-encoding expression vector to allow their expression in transfected cells as CBD fusion proteins and subsequent precipitation (from culture media) with chitin beads (3, 4). HEK293T cell cultures were transfected with each of these vectors, and the relative amounts of encoded protein in media and cytoplasmic extracts were determined by Western blotting. The results of these experiments identified markedly increased secretion of the C-helix substitution variant and some increase in secretion upon substitution of the distal half of vIL-6 helix-D with that of hIL-6 (Fig. 1B). However, these sequences were unable to confer retention to hIL-6 when introduced into the human cytokine (Fig. 1C), indicating that the contribution of these regions to vIL-6 intracellular retention is context dependent.

Since it has been reported that the signal sequence of vIL-6 is uncleaved in the mature protein (15), we tested the possi-

FIG. 1. Secretion of domain-substitution variants of vIL-6 and hIL-6. (A) Diagrammatic representation of vIL-6 domain substitution variants, involving exchange of N-terminal, α -helical, and A-B loop regions of vIL-6 with equivalent, colinear sequences of hIL-6 (3). (B) Secretion versus intracellular retention properties of the vIL-6 domain-substitution variants. Proteins were expressed as CBD fusion proteins in transfected HEK293T cells, and chitin bead-precipitated material from cell lysates and culture media analyzed by SDS-PAGE and immunoblotting to detect intracellular and secreted vIL-6 proteins, reacting with anti-CBD antibody (α -CBD). Lower and upper bands in lysates represent unglycosylated and N-glycosylated vIL-6 species, respectively. Antibody to β -actin was used to control for equal protein amounts extracted from the various transfected cultures. P, precipitation; cntl, control (pSG5, empty vector transfection). (C) Testing of secretion versus retention properties of hIL-6 domain substitution variants containing vIL-6 C-helix or distal D-helix and C-tail sequences, using procedures analogous to those outlined for panel B.

bility that vIL-6 may actually be membrane inserted and that this may play a role in ER retention. Microsomal membrane preparations (see Materials and Methods) were treated either with potassium acetate (500 mM) or sodium carbonate (100 mM) to release peripherally associated proteins or liberate soluble ER luminal proteins, respectively; membrane pellets and corresponding supernatants were analyzed by immunoblotting for vIL-6 content. Calreticulin (receptor-interacting soluble ER luminal protein) and calnexin (ER integral membrane protein) were also detected, to provide controls. The results failed to provide evidence of membrane insertion of vIL-6 but rather indicated that vIL-6 was present in the ER

FIG. 2. Distinguishing between membrane-free and membrane-inserted vIL-6 in microsomes. (A) Microsomal membrane preparations (see Materials and Methods) from vIL-6 vector-transfected HEK293T cells were either untreated ("input") or treated with potassium acetate (to release peripheral proteins) or sodium carbonate (to release ER luminal proteins). After SDS-PAGE, membrane-transferred proteins were probed with vIL-6 antiserum or antibodies to calreticulin (ER luminal protein) or calnexin (ER membrane-inserted protein) to provide appropriate controls. vIL-6 was found to mirror the extraction profile of calreticulin, not calnexin, demonstrating that it is soluble, not membrane inserted. Pell., pellet (membrane); Sup., supernatant (cytosol). (B) Immunoblots to verify appropriate preparation of ER membrane fractions and separation from cytosolic components. Antibodies specific for lactate dehydrogenase (LDH), a cytosolic marker, and calnexin and calreticulin (ER markers) were used for quality control.

lumen as a soluble protein, released by carbonate treatment and cofractionating with calreticulin, free of (pelleted) membranes (Fig. 2).

C-helix residues and vIL-6 secretion. The influence of the helix-C region on secretion of vIL-6 led us to explore the effects of amino acid substitutions within these sequences on intracellular retention of the viral cytokine. The experiments were conducted as before, with expression of the various mutated proteins (Fig. 3A) as CBD fusions, to allow rapid and effective precipitation of vIL-6 from culture media prior to Western analysis. The results of these experiments identified several point mutations as enhancing the secretion of vIL-6, most notably mutations of residues V₁₁₉/I₁₂₀, D₁₂₃/V₁₂₄, W₁₃₅/D₁₃₆, and N₁₄₁/K₁₄₂ (variants C2, C4, C8, and C11, respectively) (Fig. 3B). Since residues within this region are known to be involved in interactions with gp130 (3, 6, 14), our data suggested the possibility that altered interactions with gp130 may underlie the observed effects of the introduced mutations. This idea was reinforced by the reported promotion of vIL-6 secretion in gp130-negative Ba/F3 cells when gp130 was introduced via expression vector transduction (15).

FIG. 3. Secretion properties of C-helix point variants of vIL-6. (A) Diagrammatic representation of C-helix point variants (3). Bold lettering represents vIL-6 amino acid residues of the C-helical region (top line); gray lettering corresponds to colinear hIL-6 amino acids; the bottom line corresponds to the complete sequence of the C-helical region of hIL-6, substituted for vIL-6 C-helix in variant cIL-6.21. (B) Lysate versus media Western blots for analysis of the secretion properties of each of the vIL-6 variants, essentially as outlined for Fig. 1B. α-CBD, anti-CBD; v, vIL-6 (wild-type); h, hIL-6.

To determine possible correlations between vIL-6–gp130 functional interactions and secretion, we screened our vIL-6 proteins for their abilities to signal through endogenously expressed gp130 in HEK293T cells. Signal transduction induced by vIL-6 was measured by Western analysis to detect tyrosine-phosphorylated (activated) STAT3 in cell extracts derived from the various transfected cultures or from the empty vector (pSG5.CBD)-transduced control. The data (Fig. 4A) revealed that several of the vIL-6 C-helix variants were impaired in signal transduction, indicative of weakened “site II” interactions with gp130 domains 2 and 3 (5), but that this did not correlate, positively or negatively, with the effects of the respective mutations on vIL-6 secretion. Therefore, these data indicated that vIL-6 association with gp130 is not a primary determinant of the retention/secretion properties of vIL-6.

This was further investigated, independently of functional analyses requiring vIL-6-induced dimerization of gp130, by assaying for physical association of the C-helix variants with gp130. Our experiments were designed to test the interactions of wild-type and C-helix variant vIL-6 proteins with cotransduced gp130-Fc and gp130-CBD fusion proteins using gp130-Fc/protein A-agarose coprecipitation assays. The results revealed general concordance between vIL-6-induced gp130 dimerization (coprecipitated gp130-CBD) and the previously obtained signal transduction data, and the dimerization-independent gp130-Fc interaction results (vIL-6 coprecipitation) mirrored these (Fig. 4B). Variants C2, C3, C5, and C6 showed diminished precipitation by gp130-Fc, a finding consistent with their reduced signaling abilities, and variants C2, C4/C11, and C8, all secreted much more efficiently than wild-type vIL-6,

FIG. 4. Signaling and gp130-binding properties of vIL-6 C-helix point variants. (A) Functional interaction of vIL-6 proteins with gp130, as determined by Western blotting for phosphorylated (active) STAT3 (pSTAT3) in cell extracts from vIL-6 vector-transfected HEK293T cells. (B) gp130 binding and dimerization interactions by C-helix point variants. HEK293T cells were cotransfected with expression vectors for gp130-Fc, gp130-CBD and each of the vIL-6 proteins in turn. Protein A-agarose was used to precipitate gp130-Fc from cell lysates, and coprecipitated vIL-6 and gp130-CBD* (indicative of gp130 functional dimerization) identified by Western analysis of SDS-PAGE size fractionated and membrane-transferred proteins. P, precipitation; IB, immunoblot; v, vIL-6; h, hIL-6; cntl, control (pSG5, empty vector).

displayed no, strong and intermediate interactions, respectively, with gp130. Thus, the physical vIL-6–gp130 interaction data revealed no detectable correlation, positive or negative, between the ability of vIL-6 variants to be secreted and their interactions with gp130 (Table 1).

vIL-6 interaction with ER-resident chaperone. We hypothesized that the observed intracellular localization and ER retention of vIL-6 (4) may be due to interaction of vIL-6 with ER-resident proteins. Obvious candidates were chaperone proteins calnexin and calreticulin (26), which were therefore tested for their interactions with vIL-6 by coprecipitation assays. We found that calnexin, but not calreticulin, could associate with vIL-6 (Fig. 5A). This was not the case for hIL-6,

TABLE 1. Characteristics of vIL-6 C-helix variants

vIL-6 type	Secretion ^a	Signaling ^b	gp130 ^c	
			Binding	Dimerization
Wild type	–	+++	+++	+
C1 (GK ₁₁₇ ES)	–	++	++	+
C2 (VI ₁₂₀ EE)	+++	–	–	–
C3 (NV ₁₂₂ QA)	–	++	++	+
C4 (DV ₁₂₄ RA)	+++	+++	+++	+
C5 (ME ₁₂₆ VQ)	+	+	+	–
C6 (LL ₁₂₈ MS)	+	+	+	–
C7 (TLG ₁₃₃ VLI)	+	+++	+++	+
C8 (VD ₁₃₅ QF)	+++	++	++	–/+
C9 (IQE ₁₃₈ LQK)	++	+++	+++	+
C10 (EL ₁₄₀ KA)	+	+++	+++	+
C11 (NK ₁₄₂ KN)	+++	+++	+++	+

^a From immunoblot detection of vIL-6 in transfected cell lysates and culture media (Fig. 3).
^b From immunoblot detection of pSTAT3 in transfected cell lysates (Fig. 4A).
^c From gp130-based coprecipitation assays (Fig. 4B).

FIG. 5. vIL-6-calnexin interactions and vIL-6 conformation. (A) Coprecipitation assays to assess IL-6-chaperone protein interactions, using vIL-6-CBD (v) and hIL-6-KDEL-CBD (hK) and immunoblot detection of chitin-bead coprecipitated calnexin and calreticulin. cntl, control (pSG5); P, precipitation; IB, immunoblot. (B) Calnexin binding by monoglycosylated and glycosylation-deficient vIL-6 variants vIL-6(N₇₈A) (v1N) and vIL-6ΔN (vΔN), as determined by coprecipitation assay and immunoblotting. m, monomer; d, dimer. (C) Detection of multimeric, unresolved disulfide-linked forms of vIL-6 and vIL-6ΔN by immunoblotting of SDS-PAGE fractionated proteins either treated (R) or not treated (NR) with reducing agent (β-mercaptoethanol) prior to gel loading. (D) Protease sensitivity of vIL-6ΔN relative to vIL-6, as determined by trypsin digestion of chitin bead-precipitated vIL-6-CBD proteins from transfected HEK293T cell extracts. Monomeric (m) and dimeric (d) vIL-6 forms are indicated, along with glycosylated (Gly) and unglycosylated (UnGly) wild-type vIL-6 and trypsin digestion products of vIL-6ΔN (*).

retained in the ER via KDEL motif-tagging (4), which also did not associate detectably with calreticulin.

As important determinants of protein binding by calnexin are glycan moieties (23, 25), we tested glycosylation site-altered vIL-6 variants, mutated at one (N₈₉A) or both (N₇₈A+N₈₉A) N-linked glycosylation sites, for their abilities to interact with calnexin. While the N₈₉A vIL-6 variant was able to bind calnexin equivalently to wild-type vIL-6, the double-mutated vIL-6 was refractory to interaction with the ER chaperone (Fig. 5B). Interestingly, this vIL-6 variant was found to be conformationally altered relative to wild-type vIL-6, as revealed by its increased susceptibility to protease digestion and also by the formation of increased amounts of β-mercaptoethanol-sensitive high-molecular-weight species, indicative of disulfide-linked homologous (vIL-6 antiserum-reactive) multim-

ers (Fig. 5C and D). These results are consistent with a model in which appropriate folding of vIL-6 involves its interaction with calnexin (26). While it is conceivable that alteration in the primary structure of vIL-6(N₇₈A+N₈₉A) is directly responsible for conformational alterations and its lack of interaction with calnexin, this seems unlikely for two reasons: first, numerous other (including more substantial) alterations in vIL-6 structure have been introduced while retaining its signaling activity (3, 14), indicative of conservation of overall structure, and second, calnexin can interact with misfolded proteins. However, we cannot on the basis of these data exclude the possibility that interactions, perhaps transient, with other ER chaperones are involved in vIL-6 folding.

vIL-6-calnexin interactions and vIL-6 secretion. By analogy with our gp130-vIL-6 interaction studies, we wanted to see whether there was a correlation between vIL-6 intracellular retention and interactions with calnexin. Calnexin-vIL-6 coprecipitation analysis of our domain substitution variants of vIL-6 (Fig. 1A) failed to reveal evidence of altered vIL-6-calnexin interactions in relation to increased secretion, notably of the C-helix substitution variant (Fig. 6A). As an alternative means of assessing the function of calnexin with regard to vIL-6 secretion, we generated HEK293T cells in which calnexin was depleted, via retrovirus-mediated shRNA transduction and drug selection of retrovirus vector-positive cells. Similar cultures were generated for calreticulin depletion to provide a negative control. Immunoblot analysis of calnexin and calreticulin expression in these shRNA-transduced cultures and untransduced HEK293T cells was undertaken to check for specific depletion of the targeted mRNA-encoded proteins (Fig. 6B). These cells were then used to examine vIL-6 intracellular retention versus secretion upon calnexin, or calreticulin, depletion. The data from these experiments showed that calnexin depletion, specifically, led to an increased ratio of secreted to retained protein, but intracellular vIL-6 levels were markedly reduced (Fig. 6C). This was specific to vIL-6, since no corresponding depletion of ER-retained hIL-6-KDEL occurred in a similar experiment (Fig. 6D). Interestingly, transfection of gp130 expression vector into calnexin-depleted cells led to increased vIL-6 expression, indicating a direct influence of the signal transducer on stability of the viral cytokine, countering the effect of calnexin depletion (Fig. 6E).

In further investigations of the possible role of calnexin for vIL-6 intracellular retention, the secretion properties of vIL-6 were investigated in the absence or presence of α-glucosidase I and II inhibitor castanospermine (CAS) (7, 9). Deglycosylation of Glc₃Man₉GlcNAc₂ to Glc₁Man₉GlcNAc₂ after transfer to nascent proteins is necessary for recognition by calnexin (and calreticulin). We included in our analyses vIL-6ΔN to examine its secretion properties relative to wild-type vIL-6 and also to provide a control for possible indirect (nonspecific) effects of CAS on vIL-6 secretion. Inclusion of vIL-6.C2 provided a positive control for detection of secreted vIL-6 in culture media. The results of immunoblotting experiments to detect vIL-6 in lysates and culture media of transfected cells failed to reveal significant influence of CAS on vIL-6 secretion (Fig. 6F). These data suggest that calnexin is not necessary for intracellular retention of vIL-6, although failure to achieve native conformation may restrict ER egress.

Finally, pulse-chase analysis was used to examine the kinetics of

FIG. 6. Influences of calnexin and gp130 on secretion and expression vIL-6. (A) Interactions of calnexin with vIL-6 domain substitution variants (see Fig. 1A) by coprecipitation. SP, signal peptide; A-D1/D2, helices A-C and proximal (D1) and distal (D2) regions of helix D; AB, A-B loop; cntl, control (pSG5). (B) Western analysis of ER chaperone protein expression relative to β-actin (load control) in HEK293T cell lines stably transduced with shRNAs directed to calnexin (Cnx) or calreticulin (Crt) mRNAs to ensure appropriate and specific depletion. BiP/GRP78, ER chaperone protein (control). (C) Retention versus secretion of vIL-6 in chaperone-depleted cell lines (Cnx⁻, Crt⁻) relative to parental HEK293T ("293") cells, revealing increased secretion/retention ratios specifically in calnexin-depleted cells but also markedly reduced intracellular vIL-6. exp., exposure. (D) Control and confirmatory experiment, verifying specificity of vIL-6 (not hIL-6-KDEL) depletion in calnexin-knockdown cells. (E) Western analysis of transfected cell lysates and media to determine effects of gp130 on vIL-6 secretion in HEK293T and calnexin-depleted cells. The data show no apparent influence on gp130 on vIL-6 secretion in either cell type, but pronounced increases in vIL-6 expression in response to gp130 in calnexin-depleted cells. vIL-6ΔC (vIL-6.21), used as control for detection of secreted protein. Empty expression vector pEFBOS (-) was transfected in place of pEFBOS-gp130 (+) to provide comparisons for effects of gp130 overexpression. (F) Effects of CAS, an inhibitor of α-glucosidases I and II (essential for generation of monoglucosylated glycans and calnexin recognition) on vIL-6 secretion. Inclusion of vIL-6ΔN (ΔN) and vIL-6.C2 (C2) in addition to wild-type vIL-6 (v) served as controls for vIL-6 glycosylation-specific effects of CAS on secretion of the viral cytokine and assessment of its relative secretion, respectively. (G) Radiolabel pulse-chase analysis to compare kinetics of vIL-6-calnexin association (calnexin-CBD-precipitable vIL-6) with vIL-6 intracellular retention (vIL-6 in total cell lysates). Cells were cotransfected with vIL-6 and calnexin-CBD expression vectors, after 24 h pulsed for 20 min with [³⁵S]methionine-cysteine, and then monolayers were washed and chased with fresh medium before harvesting of cells and media at the indicated times. Chitin-bead precipitated material (bound to calnexin-CBD) from cell lysates was dissociated in 1% SDS prior dilution and immunoprecipitation (IP) with vIL-6 antiserum (see Materials and Methods). vIL-6 in these immunoprecipitates and obtained by direct immunoprecipitation from cell lysates, in addition to culture media, was identified by autoradiography of PAGE-fractionated proteins. Calnexin-CBD and β-actin were identified by immunoblotting (IB) to provide expression and load controls, respectively.

vIL-6-calnexin association and determine whether duration of interaction and intracellular retention were comparable. Calnexin-CBD was expressed with vIL-6 in appropriately transfected HEK293T cells, allowing subsequent precipitation from cell lysates with chitin beads and then secondary immunoprecipitation of vIL-6 after dissociation of calnexin-protein complexes (see Materials and Methods). The data obtained from this experiment revealed that vIL-6-calnexin association was detectably reduced by 20 min postchase and greatly diminished at 8 h, clearly distinct from the kinetics of intracellular retention (Fig. 6G). Again, these data indicate that calnexin:vIL-6 interaction is insufficient, by itself, to account for intracellular retention of the viral cytokine.

Subcellular localization of vIL-6 in response to calnexin depletion and gp130. In view of the potential influence of calnexin-vIL-6 interactions for ER retention and the reported positive effect of gp130 on vIL-6 secretion in Ba/F3 cells (15), we next examined by confocal fluorescence microscopy the distribution of

vIL-6 (RFP-tagged) relative to GFP-KDEL (ER marker) in response to calnexin depletion and gp130 expression. Calnexin depletion had no detectable effect on the distribution of vIL-6 relative to ER, with strong colocalization of RFP and GFP signals (where both were detected) in regular and calnexin-deficient HEK293T cells (Fig. 7). However, in calnexin-depleted cells, specifically, gp130 vector transfection led to clear segregation of RFP and GFP signals. These results demonstrate that the signal transducer influences vIL-6 intracellular distribution, apparently effecting ER egress under conditions of diminished calnexin-vIL-6 interaction. These data indicate that calnexin has a role in maintaining normal localization of vIL-6 within the ER and that in calnexin-depleted (or calnexin-saturated) cells gp130 can promote vIL-6 relocation to extra-ER compartments.

gp130 promotes distribution of vIL-6 to ER-distinct membrane fractions. To further examine the effects of gp130 on vIL-6 intracellular localization, we used iodixanol density gra-

FIG. 7. Confocal fluorescence microscopy analysis of vIL-6 distribution in response to gp130. HEK293T (293T) and calnexin-depleted (Cnx⁻) cells were cotransfected with expression plasmids for vIL-6-RFP and ER-targeted GFP-KDEL, with or without cotransfected gp130 expression vector (gp). Colocalization of RFP and GFP signals was evident in all but calnexin depleted cells overexpressing gp130. High magnification, single-layer (slice) images of cells with high-intensity RFP and GFP staining are shown, providing representative examples of the staining patterns observed.

dient membrane fractionation to compare the distributions of vIL-6 in parental, calnexin-depleted and calreticulin-depleted HEK293T cells. Fractions were analyzed by Western analysis for the identification of vIL-6 and protein markers of various membrane types. The results revealed a clearly distinct vIL-6 distribution profile in calnexin-depleted cells as a function of gp130 overexpression, with gp130 effecting a shift of vIL-6 into lighter membrane fractions, partially overlapping but distinct from the ER-localized calnexin (Fig. 8). This distribution was also distinct from that of ERGIC-53, a marker of the ER-Golgi intermediate compartment. The gp130-induced redistribution of vIL-6 was not observed in the case of HEK293T or calreticulin-depleted cells. Importantly, all vIL-6 was able to be digested with endonuclease H, diagnostic of the presence of immature high-mannose glycans, suggesting that gp130 promotes relocation of vIL-6 to pre- or early-Golgi complex compartments.

DISCUSSION

We and others previously reported the slow secretion rates of vIL-6, relative to hIL-6, and the predominant ER localiza-

FIG. 8. Iodixanol gradient membrane fractionation for identification of vIL-6 subcellular distribution in HEK293T (293T) and calnexin (Cnx⁻)- and calreticulin (Crt⁻)-depleted cells in the absence or presence of transfected gp130. Chitin bead-precipitated vIL-6-CBD was subjected to endonuclease H (Endo H) digestion (+) or left untreated (-) prior to gel loading and immunoblotting. Upper and lower bands in vIL-6 immunoblots represent glycosylated and unglycosylated/glycan-digested protein, respectively. Fractions derived from HEK293T cells were probed to detect protein markers of early endosomes (EEA1), plasma membrane (Na/K-ATPase), ERGIC (ERGIC53), and ER (calnexin).

tion of the viral cytokine and activity in this compartment (4, 15), but the molecular basis for intracellular retention and ER localization was not investigated. The experimental analyses presented here have addressed this issue directly, identifying the C-helix domain and its particular structure as contributors to intracellular retention of vIL-6, showing that ER chaperone protein calnexin both binds to vIL-6 and influences its stability and localization, and demonstrating that vIL-6 signal transducer gp130 plays a role in promoting ER egress of vIL-6 but apparently not in trafficking of the cytokine to the cell surface for secretion. While our studies expand our understanding of the factors influencing the intracellular retention, stability, and distribution of vIL-6, they also implicate the involvement of other, currently unknown factors in intracellular retention; furthermore, the amino acid and/or tertiary structural determinants of ER retention have yet to be identified.

Three clearly defined ER retention motifs are currently known: the KDEL motif present in many ER-luminal proteins, and the COP-I-interacting dilysine (KKXX) motif present at the cytoplasmic C termini of some type I ER-retained transmembrane proteins and related diarginine (XXRR) found in some type II ER membrane proteins (8, 16, 19, 20, 22). vIL-6 lacks these motifs; indeed, the addition of KDEL-containing sequences to vIL-6 enhances its intracellular retention (4). Here, we have formally excluded the possibility that vIL-6, the signal sequence of which in uncleaved (15), may be present in ER as a membrane-inserted (type II) protein. vIL-6 could be extracted from membrane preparations along with calreticulin,

a soluble (receptor-associated) ER luminal protein, rather than cofractionating with ER membrane-inserted calnexin in carbonate-treated microsomal precipitates. Thus, the presence of vIL-6 in the ER is most likely to involve a receptor-mediated ER retention and/or ERGIC-to-ER retrieval (21). Using domain substitution variants and then point-mutated vIL-6 proteins, we have identified the requirement of C-helix residues for full intracellular retention of the protein; we also found in these investigations that substitution of the distal region of vIL-6 helix-D with the equivalent region of hIL-6 led to increased secretion of the viral cytokine. While these particular domains were shown previously to play a role in vIL-6 interactions with gp130 (3) and the signal transducer was reported to enable secretion of vIL-6 in gp130-transduced Ba/F3 cells (lacking endogenous gp130) (15), we found here that gp130 interactions and secretion properties of vIL-6 were not correlative. For example, variants vIL-6.C2 and vIL-6.C5 both showed diminished binding to gp130 but displayed distinct secretion abilities, while vIL-6.C4 and vIL-6.C11 were secreted as efficiently as vIL-6.C2 but bound gp130 similarly to wild-type vIL-6. Furthermore, overexpression of gp130 was not able to increase the secretion of vIL-6. On the basis of these data, we do not believe that gp130 is a primary determinant of vIL-6 secretion. The apparent discrepancy between our results and those reported previously for gp130 enhancement of vIL-6 secretion in Ba/F3 cells (15) may reflect relative abilities to detect small positive effects of gp130 on vIL-6 secretion against a background of no secretion in cells normally lacking the signal transducer versus similar effects in cells expressing endogenous gp130 in which some vIL-6 secretion occurs (i.e., in HEK293T cells). Whether gp130 might be required for vIL-6 secretion in cell types other than Ba/F3 cells is unknown, but our findings from analyses of vIL-vIL-6.C2, which does not interact with gp130 intracellularly, suggest that gp130 is not universally required.

With regard to structural determinants of vIL-6 retention in the ER, our data do not favor a model in which a primary amino acid sequence motif analogous to KDEL, C-terminal dilysine, or N-terminal diarginine is operative. First, both C-helix and (to a lesser extent) distal D-helix substitutions led to increased vIL-6 secretion; second, several disparate mutations within the C-helical region of vIL-6 promoted vIL-6 secretion; third, neither the vIL-6 C-helix nor the distal D-helix region of vIL-6 could confer intracellular retention to hIL-6. Rather, we think it more likely that the overall three-dimensional structure of vIL-6 is the major determinant or a necessary factor for ER retention.

Similar to our analysis of vIL-6 secretion in relation to gp130 interaction of the viral cytokine, we detected no correlative differences in binding to calnexin of the more highly secreted vIL-6 Δ C (cIL-6.21) and vIL-6 Δ D2 (cIL-6.23) relative to other domain substitution variants or wild-type vIL-6. Also, calnexin depletion did not lead to increases in absolute levels of secreted vIL-6 (which were comparable with those observed in transfected HEK293T cultures), although the ratio of secreted to intracellular vIL-6 was increased, in the background of vIL-6 destabilization. Using pulse-chase analysis we were able to dissociate the kinetics of vIL-6–calnexin association and intracellular retention, the latter being appreciably slower than the former and suggestive that calnexin interaction is not sufficient

explanation for the slow secretion rate of vIL-6. Notwithstanding, the relatively long-lived association of vIL-6 with calnexin (still detectable after 8 h) seems distinct from the fairly transient interactions normally seen between ER chaperones and nascent proteins during protein folding (26). Calnexin depletion-mediated destabilization of vIL-6 may reflect the promotion of ER-associated degradation (ERAD) of the viral cytokine, which would not be unexpected if its normal folding requires calnexin. Cellular proteins that are terminally misfolded are ultimately degraded via ERAD (13, 26). The stability of the glycosylation-deficient vIL-6 Δ N indicates glycosylation dependence of instability induced by calnexin depletion, consistent with the involvement of both calnexin and ERAD-associated mannose-recognizing lectin EDEM in ERAD-related ER-to-cytosol translocation (10, 12, 13, 17, 18). Regardless of the mechanism of vIL-6 destabilization, it is clear that overexpression of gp130 in the context of calnexin depletion led both to stabilization of vIL-6 and to redistribution to non-ER locations, as identified in both confocal fluorescence and membrane fractionation experiments. It is likely that the two phenomena are linked, probably involving the removal of vIL-6 from ERAD mechanisms. In this respect, it may be significant that gp130 overexpression led to a greater proportion of unglycosylated vIL-6, which would, like vIL-6 Δ N, presumably not be available for ER-to-cytosol translocation. Importantly, gp130 did not increase vIL-6 secretion, despite apparent relocalization of vIL-6 out of the ER, nor did it effect detectable relocalization of vIL-6 in regular (non-calnexin-depleted) HEK293T cells. The implications of these results are twofold: first, there is likely to be an ER-independent intracellular-retention mechanism for vIL-6, preventing vIL-6 from entering the secretion pathway even after “escape” from ER; second, calnexin is likely to play a role in ER retention of vIL-6, because gp130 can promote ER egress of vIL-6 only after calnexin depletion. Thus, despite the ability of secreted domain substitution variants to bind calnexin in our coprecipitation assays and the dissociation of vIL-6–calnexin interaction and intracellular retention by pulse-chase analysis, our results may in fact indicate that calnexin acts as an ER receptor for vIL-6 and that it is involved in normal ER retention and localization of the viral cytokine.

Combined, our data are consistent with model in which calnexin plays a role in ER retention, while other proteins, within and/or outside the ER, are coparticipants of intracellular retention. We speculate that gp130 can effectively compete with calnexin when the chaperone is depleted, allowing ER exit of vIL-6, but that secretion is prevented by other, as-yet-unknown mechanisms. While verification of this model, requiring identification of novel intracellular receptors of vIL-6 and their functional testing, falls outside the scope of the present investigation, we predict that secreted variants of vIL-6 identified here will show weakened interactions with such binding partners. Indeed, these vIL-6 variants should prove useful for the identification of such receptors.

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