Complex N-Linked Glycans on Asn-89 of Kaposi Sarcoma Herpes Virus-encoded Interleukin-6 Mediate Optimal Function by Affecting Cytokine Protein Conformation*" 

Received for publication, July 13, 2009, and in revised form, August 17, 2009. Published, JBC Papers in Press, August 18, 2009, DOI 10.1074/jbc.M109.039115

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Kaposi sarcoma-associated herpesvirus-encoded interleukin-6 (vIL-6) and its human cellular homologue (huIL-6) share similar biological functions. Our previous work showed that N-linked glycosylation was required for optimal function of vIL-6 but not huIL-6 (1). Here we describe heterogeneity in the composition of the glycans of the two N-linked sites of vIL-6. The Asn-89 site of vIL-6, found to be required for optimal cytokine function, is composed of complex glycans. The Asn-78 site is composed of high mannose glycans, which are dispensable for cytokine function. N-Linked glycosylation at the Asn-89 site was required for intracellular production of functional vIL-6, but endoglycosidase-mediated removal of N-linked glycans from secreted vIL-6 did not impair protein function. With the use of a conformation-specific antibody and tryptic digestion assays, we showed that glycosylation at the Asn-89 site of vIL-6 affected protein conformation. Human IL-6, but not vIL-6, requires IL-6Ra for binding to gp130. We tested the hypothesis that the Asn-89 complex glycan of vIL-6 alone was sufficient to confer binding to gp130 independently of IL-6Ra. Two mutants of huIL-6, made to contain additional complex N-linked glycans in the region that interacts with IL-6Ra, did not confer binding to gp130 independently of IL-6Ra. Our findings support the conclusion that complex glycans on Asn-89 of vIL-6 specifically promote a protein conformation that allows the viral cytokine to bind gp130 independently of IL-6Ra.

Kaposi sarcoma-associated herpesvirus (KSHV), herpessivirus 8, is etiologically associated with Kaposi sarcoma, multicentric Castleman disease, and primary effusion lymphomas. Large DNA viruses, such as KSHV, are notorious for their ability to evade the host immune response (2). They have evolved to acquire and modify host-encoded cytokines, chemokines, and their receptors. Thus far, KSHV is the only virus known to encode a homologue of interleukin-6 (IL-6). The K2 open reading frame of KSHV encodes viral IL-6 (vIL-6), a structural and functional homologue of human IL-6 (huIL-6) (3). vIL-6 exhibits 24.8% amino acid identity and 62.2% amino acid similarity with huIL-6 (4).

Hull-6 exhibits a wide spectrum of biological activities, including mediation of fever and acute phase inflammatory response to trauma and pathogens, stimulation of osteoclast formation, and promotion of neuronal regeneration (5). huIL-6 is an important growth factor for multiple myeloma and placymcytoma cells. vIL-6 mimics a number of huIL-6 activities such as stimulating B cell growth and activating the Janus tyrosine kinase (JAK) 1 and signal transducer and activator of transcription (STAT)1/3 pathway (6, 7). vIL-6 is expressed in KHSV-infected B cells and endothelial cells. It induces proliferation, angiogenesis, and hematopoeisis in IL-6-dependent cell lineages (4, 8). vIL-6 can serve as an autocrine growth factor for primary effusion lymphoma cell lines and other IL-6-dependent tumor cell lines (9); however, vIL-6 is less potent than huIL-6, requiring 2 to 3 log higher levels more compared with its human counterpart (1, 6). KHSV-infected cells secrete vIL-6, but they also retain a proportion of the cytokine intracellularly, which can activate the STAT1/3 pathway (10). Both autocrine and paracrine functions of vIL-6 may be involved in tumorigenesis. vIL-6 accelerates hematopoeisis in mice and induces expression of vascular endothelial growth factor, a factor implicated in primary effusion lymphoma, and Kaposi sarcoma pathogenesis (11). Cellular huIL-6 itself is also induced in KHSV-infected cells by the viral immediate-early lytic protein Rta (ORF50) (12), a transcriptional activator that regulates lytic gene expression, and by vGPCR (ORF74) (13), a viral G-protein-coupled receptor with significant homology to the high affinity IL-8 receptor, which functions as a CXC type chemokine receptor.

Despite similarities in their function and domain, huIL-6 and vIL-6 differ in their mode of receptor usage (14, 15). vIL-6 and huIL-6 share with other cytokines, such as ciliary neurotrophic factor, leukemia inhibitory factor, oncostatin M, cardiotropin-1, and IL-11, the use of a gp130 receptor for signal transduction (16). Cytokine binding to gp130 causes JAKs to phosphorylate STATs, which dimerize and translocate to the nucleus, where they bind specific enhancer sequences to stimulate or repress transcription of target genes. Although both cellular IL-6 and vIL-6 bind gp130, the common signal transducing receptor subunit for the IL-6-type family of cytokines,
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huIL-6 requires a nonsignaling coreceptor, IL-6Rα (gp80), for assembly of a 2:2:2 hexameric signaling complex (16). In contrast, vIL-6 directly binds gp130 and does not require IL-6Rα for signal transduction (8, 17). Interaction of huIL-6 with IL-6Rα is mediated by a specific motif on huIL-6 known as site I. Site I on vIL-6 is not occupied when the viral cytokine binds gp130. The interaction of IL-6Rα with site I of huIL-6 is thought to alter the conformation of the cellular cytokine to enable it to bind to gp130 (18).

vIL-6 may act as an anti-apoptotic factor to promote survival of KSHV-infected cells and enhance the proliferation of KSHV-infected cells or potential host target cells (19–21). Interferon-α produced in response to KSHV infection in lymphoma cells specifically down-regulates IL-6Rα, making it difficult for huIL-6 to interact with its gp130 receptor (21). Because vIL-6 binds directly to the signaling gp130 receptor without first binding to IL-6Rα, vIL-6 may exert its proliferative effects on B cells without being affected by interferon-α. Moreover, vIL-6 independence from IL-6Rα allows it to interact with a broader group of target cells.

We previously showed that the differences in biological activity and receptor usage of cellular IL-6 and vIL-6 could be influenced by differences in glycosylation of the two cytokines (1). vIL-6 is glycosylated when expressed in primary effusion lymphoma cells in which the KSHV is lytically activated. vIL-6 contains two N-linked glycosylation sites, Asn-78 and Asn-89, both of which are located in site I. N-Linked glycosylation at Asn-89, but not Asn-78, of vIL-6 was critical for optimal gp130 receptor binding. JAK/STAT signaling, and stimulation of B cell proliferation. Neither O- nor N-linked glycosylation of cellular IL-6 was required for the full functional activity. We considered two hypotheses to account for the essential role of glycosylation of Asn-89 of vIL-6. One hypothesis was that N-linked glycosylation of vIL-6 was required for optimal binding to gp130, either via sugar-sugar or sugar-protein interactions. The second, but not mutually exclusive, hypothesis was that N-linked sugars on Asn-89 were required for proper protein folding. The current study highlights the heterogeneity of N-linked glycans in the vIL-6 protein and demonstrates that the Asn-89 glycans, consisting of complex sugars, play a critical role in the initial folding and conformation of vIL-6. The Asn-89 glycan is dispensable once the protein has properly folded. Site mutants with similar N-linked complex glycans were generated in site I of huIL-6. These homologues of the Asn-89 glycan of vIL-6 did not alter the dependence of huIL-6 on IL-6Rα to interact with gp130. This finding indicated that the N-linked glycan itself did not mediate interaction with the gp130 receptor. Our results show that a virally encoded cytokine, such as vIL-6, distinguishes itself from its cellular homologue through the functional importance of N-linked sugars in modulating protein conformation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—HKB5/B5, a hybrid cell line of Burkitt lymphoma-derived HH514 –16 cells and 293 human embryonic kidney cells (22). HKB5/B5 was used for transient transfections to produce extracellular mammalian-derived cellular IL-6 and vIL-6. Cells were grown in RPMI 1640 medium supplemented with 5% FCS at 37 °C in 5% CO₂. Optimal transfection conditions were achieved using the DMRIE-C (Invitrogen). To produce vIL-6 and huIL-6 lacking N-linked glycans, 0.2 or 1.0 μg/ml tunicamycin (TM) was added to growth media. Culture supernatants and cell extracts were prepared 48 h after transfection. B9.11, an IL-6-dependent mouse plasmacytoma cell line, was used to measure IL-6 activity.

Plasmid Constructions—Plasmids encoding wild-type and glycosylation site mutants of vIL-6 were constructed using pcDNA3.1 vector (Invitrogen) (1) and the SF2 insect cell vector. Glycosylation site mutants of human IL-6 (E109T and F102N) were generated using QuikChange site-directed mutagenesis (Stratagene). The vIL-6 and huIL-6 genes in all clones were sequenced in their entirety.

Western Blot Analysis—Extracts were prepared from transfected HKB5/B5 cells grown in culture for 48 h. For detection of vIL-6 or huIL-6 protein in HKB5/B5 transfectant supernatants, −10 ng of protein was loaded. A 1:200 dilution of vIL-6 rabbit polyclonal antiserum for vIL-6 protein (1) or 1 μg/ml huIL-6 polyclonal antibody (R&D Systems), followed by a 1:200 dilution of a rabbit anti-goat Ig bridge (Sigma-Aldrich) was used for vIL-6 or huIL-6 protein detection, respectively. Immunoreactive bands were detected with 125I-labeled protein A and autoradiography.

ELISA for vIL-6 or huIL-6—To detect vIL-6 by ELISA, wells were coated with 2 μg/ml protein A-agarose-purified Ig fraction of rabbit antiserum to vIL-6 in coating buffer (0.1 M carbonate, pH 9.5) and incubated at 4 °C overnight. The wells were washed three times with PBS/0.05% Tween and blocked with PBS/10% FCS in PBS for 3 h at room temperature. Serially diluted culture supernatants were added. Purified Escherichia coli-derived vIL-6 was used to generate a standard curve. Samples were incubated overnight at 4 °C. After further washes, 100 μl of biotinylated anti-KSHV IL-6 rabbit antiserum at 2 μg/ml was added for 1 h at room temperature. After PBS/Tween washes, streptavidin conjugated to horseradish peroxidase (Zymed Laboratories Inc.) at a dilution of 1:3000 in FCS blocking buffer for 30 min was added. After 10 washes in PBS/Tween, peroxidase activity was detected by adding 50 μl/well tetramethylbenzidine substrate reagent (BD Biosciences). Reaction was stopped using 50 μl of 2 N H₂SO₄. Absorbances were read at 450 nm using a Coulter MR5000 ELISA reader (Dynatech). For the conformation-specific vIL-6 ELISA, 2 μg/ml protein A-agarose-purified Ig fraction of rabbit antiserum to vIL-6 was used as capturing antibody, and 2 μg/ml protein G-agarose purified-monoconal M12 anti-KSHV IL-6 mouse antibody (kind gift of Y. Aoki and G. Tosato, National Institutes of Health) was used as the detecting antibody followed by secondary antibody donkey anti-mouse IgG (Jackson Immuno-Research Laboratories) at a dilution of 1:3000. Signals obtained using the biotinylated anti-KSHV IL-6 rabbit antiserum for detection were normalized and used to standardize signals detected with the M12 anti-vIL-6 antibody. To detect huIL-6, 2 μg/ml polyclonal anti-huIL-6 goat antibody (R&D Systems) was used as the capturing antibody, and 0.5 μg/ml monoclonal anti-huIL-6 mouse antibody was used as the detecting antibody (R&D Systems) followed by the secondary antibody donkey anti-mouse IgG horseradish peroxidase (Jackson Immuno-
Research Laboratories). Recombinant huIL-6 (R&D Systems) was used as standard.

**ELISA for Binding of IL-6 to gp130**—ELISA plates were coated with soluble gp130 (R&D Systems) at 625 ng/ml in carbonate buffer overnight. After washing with PBS/Tween and blocking with PBS/10% FCS for 3 h, wells were loaded with the vIL-6 supernatants and incubated at 4 °C. After washing, vIL-6 that bound to the sgp130 was detected using 100 μl of biotinylated anti-KSHV IL-6 rabbit antisera at 2 μg/ml and the secondary reagent of streptavidin conjugated to horseradish peroxidase. For huIL-6, ELISA plates previously coated with sgp130 were washed and blocked with PBS/10% FCS before the addition of huIL-6 supernatants mixed with 625 ng/ml sIL6-Rα (R&D Systems). After overnight incubation at 4 °C and multiple washings, bound huIL-6 was detected with 2 μg/ml polyclonal anti-huIL-6 goat antibody and donkey anti-goat IgG horseradish peroxidase followed by chromogenic substrates.

**B9.11 Cell Proliferation Assays**—B9.11 cells were washed three times in RPMI 1640 medium without IL-6. Cells were plated at 4 × 10⁴ cells per well in the presence or absence of vIL-6 and huIL-6 preparations. Recombinant forms of both cytokines were expressed in HKB5/B5 cells. After 2 days of culturing B9.11 cells in the presence of serially diluted IL-6 samples, cells were pulsed with 1 μCi per well of (³H) thymidine for 12–16 h. Proliferation of cells was determined by tyridine incorporation using a 96-well cell harvester and a β-scintillation counter. B cell proliferative responses were expressed as mean [³H] thymidine incorporation (cpm) ± S.E. values of triplicate cultures.

**Tryptic Digestion Assays**—One-dimensional peptide separations was performed using porcine-modified trypsin (Promega, Madison, WI) to digest recombinant vIL-6 at 30 °C; the digestion reaction was stopped after addition of HCl (final concentration 0.016 N). Digested products were separated in discontinuous polyacrylamide electrophoresis gel made of 30:0.8 acrylamide:bis (18.2% separating gel, 10% spacing gel, and 5% stacking gel). Separate cathode buffer (0.1M Tricine, 0.1% SDS) and anode buffer (0.2M Tris, pH 8.9) were used. Peptides were transferred to a nitrocellulose filter, which was immunoblotted with rabbit polyclonal anti-KSHV IL-6 antisera and probed with secondary reagent of streptavidin conjugated to horseradish peroxidase. To per-
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Differential Glycosylation Patterns of N-Glycosylation Site Mutants of KSHV IL-6 Accounts for the Heterogeneity—KSHV IL-6 has two N-linked glycosylation consensus sites, represented by Asn-X-Ser/Thr, where X can be any amino acid; these sites are located at Asn-78 and Asn-89. To study the nature of N-linked glycosylation at each site, we generated site-directed mutants, N78K and N89K, and the double mutant N78K/N89K. Single mutant forms of vIL-6, N78K and N89K, both migrated faster than the wild-type protein at ~25 kDa (Fig. 2A, compare lanes 1, 6, and 11). The double mutant N78K/N89K, which lacked both N-linked glycosylation sites, migrated fastest at ~22 kDa (Fig. 2A, lane 16). When both sites were N-glycosylated, PNGase treatment produced partial digestion of wild-type vIL-6 (Fig. 2A, lane 2). However, PNGase treatment of either single mutant yielded a completely digested non-glycosylated form of the protein, which co-migrated with the double mutant N78K/N89K (Fig. 2A, lanes 7, 12, and 16). This result suggests that the enzyme PNGase works more efficiently when there is only one N-linked sugar chain on the vIL-6 protein. Endo F1 digestion of WT vIL-6 protein resulted in two species; one was susceptible to the enzyme and the other was partially resistant (Fig. 1, lane 3 and Fig. 2A, lane 3). The N78K single mutant was predominantly resistant to Endo F1 digestion (Fig. 2A, lane 8). In contrast, the N89K single mutant vIL-6 protein was sensitive to Endo F1 digestion (Fig. 2A, lane 13). This result indicated that high mannose type N-linked sugars are present on site Asn-78 of the vIL-6 protein, whereas most of the sugars in Asn-89 site are not of the high-mannose type (Fig. 2A, lanes 8 and 13). The results with Endo F1 digestion confirmed that there was heterogeneity in the nature of high mannose glycosylation at the two N-linked sites of vIL-6. Endoglycosidase F2 and F3 did not digest either wild type or the single mutants (Fig. 2A, lanes 4–5, 9–10, and 14–15).

Asn to Gln mutants were also generated to produce single (N78Q and N89Q) and double vIL-6 mutants (N78Q/N89Q). This Asn to Gln change maintains the neutral amide content of the amino acid. The molecular weights of the protein products of the Asn to Gln substitution mutants and their sensitivity to Endo F1 digestion were similar to those seen with the Asn to Lys single and double mutant forms of vIL-6 (Fig. 2B). The single mutant form of vIL-6 N89Q was sensitive to Endo F1 digestion; the vIL-6 N78Q counterpart was resistant. These experiments with two different amino acid substitution mutants confirmed that the two N-glycosylation sites have different patterns of glycosylation.

KSHV Interleukin-6 Produced in Insect Cells—We examined the N-linked glycosylation state of the two sites on vIL-6 proteins expressed in insect cells, where high mannose glycosylation predominates (24). Wild-type vIL-6 expressed in insect cells was relatively more sensitive to Endo F1 digestion than was WT vIL-6 produced in mammalian cells (Fig. 2C, lane 4 versus 2). Each of the single mutants was less heavily glycosylated in insect cells than in mammalian cells, because the untreated protein had faster electrophoretic mobility (Fig. 2C, lanes 5 versus 7, 9 versus 11). Both the Asn-89 site and the Asn-78 site were more sensitive to Endo F1 digestion in insect cells. All of these observations support the idea that insect cells produce proteins with simpler sugars containing mannose. However, there remained a significant difference between the two sites in their sensitivity to Endo F1 digestion. The Asn-89 site was partially resistant, and Asn-78 site was completely sensitive to Endo F1 digestion (Fig. 2C, lanes 6, 8, 10, and 12). This result supports the idea that the two N-linked sites of vIL-6 are differentially glycosylated, even in insect cells.

Influence of Glycosylation Inhibitors on the Electrophoretic Mobility of KSHV IL-6 Protein—N-Glycosylation of wild-type vIL-6 and both single mutants was inhibited by growth in the presence of TM (Fig. 2D). TM inhibits the first step of the N-glycosylation pathway, which involves the transfer of GlcNAc-1-P from UDP-GlcNAc to dolichol-P to form dolichyl-P2-GlcNAc. TM treatment of both single point mutants of vIL-6 resulted in the production of high mobility, fully unglycosylated products (Fig. 2D, lanes 5 and 8). Because the abundance of secreted protein was lower after TM treatment, TM may also decrease the synthesis or secretion of the protein into the media. Wild-type vIL-6 became more glycosylated in the presence of castanospermine (CST), an inhibitor of glucosidases I and II in the ER (Fig. 2D, lane 3). There was a differential effect of CST on the two N-glycosylation sites. The Asn-78 site in the N89K mutant protein remained hyperglycosylated, whereas a hyperglycosylated band was not present in the N78K mutant protein after CST treatment. Because the Asn-89 site appeared not to be affected by CST, Asn-89 is not likely to be modified by sugars, which are substrates for glucosidases.

Differential Binding of KSHV IL-6 to ConA Based on the Heterogeneity of Its N-Glycans—The foregoing results indicated that the Asn-78 site was predominantly modified by high mannose sugars, whereas the Asn-89 site was composed of complex sugars. We next sought to determine whether the difference in glycosylation patterns inferred from reactivity to glycosidases affected the binding of vIL-6 glycosylation site mutants to lectins. The lectin ConA is specific for α-mannose units and has been used as an affinity agent for glycosylated proteins. More WT vIL-6 bound to ConA-coated plates than the unglycosy-
lated double mutant N78K/N89K vIL-6 protein (Fig. 3A). The amount of the proteins used in the binding experiments was controlled by vIL-6-specific ELISA. To determine whether the different mannose contents on Asn-78 and Asn-89 of vIL-6 affected lectin binding, we next compared the binding of WT, single, and double site mutants to ConA-agarose beads. There was a gradation of binding to ConA-agarose beads by the different glycosylated forms of vIL-6: WT > N89K > N78K > N78K/N89K (Fig. 3, B and C). However, when the data were corrected for input protein, the N89K mutant vIL-6 bound most avidly to the ConA-agarose (Fig. 3D), and the N78K/N89K vIL-6 was most abundant in the flow through fraction (Fig. 3E). These data are consistent with the results of glycosidase and digestion studies showing that the Asn-78 site in vIL-6 is the major high mannose site.

**Effect of Glycosidase Treatment of WT and N-Glycosylation Site Mutants of vIL-6 on Their Function in a B9.11 Cell Proliferation**—Previously we showed that vIL-6 made in cells treated with TM was not functional in a B9.11 cytokine-dependent cell proliferation assay (1). The effect of TM was to change the secreted vIL-6 from a fully glycosylated to an unglycosylated form (Fig. 2D, lanes 1 and 2). As shown previously, the N89K mutant was impaired in its ability to stimulate proliferation of B9.11 cells (Fig. 4C). This result suggested that glycosylation at the N89K site was important for vIL-6 function. To explore this requirement for glycosylation further, WT vIL-6 and the single mutant proteins were expressed in HKB5/B5 mammalian cells, and the secreted proteins were subsequently treated with either PNGase or Endo F1 glycosidases. Surprisingly, the function of secreted WT vIL-6 protein was not altered by treatment with glycosidases (Fig. 4A). Untreated N78K single mutant vIL-6 protein had comparable functional activity at stimulating B9.11 cells as the WT protein (Fig. 4B). The N78K mutant was still functional after PNGase treatment, although it was decreased ~4-fold in activity. N78K vIL-6, like wild-type, was Endo F1-resistant, and digestion with this enzyme did not alter the functional ability to stimulate B9.11 proliferation. The N89K mutant was inactive in the absence or presence of glycosidase digestions (Fig. 4C). These results showed that high mannose sugars are not absolutely required for functional activity of secreted vIL-6.

**Evidence that N-Glycosylation Influences Conformation of vIL-6**—Two of the previous results suggested that N-linked glycosylation might affect the conformation of the vIL-6 protein. First, the fully glycosylated wild-type vIL-6 was not completely susceptible to PNGase (Fig. 1, lane 2). Second, PNGase-treated WT vIL-6 was fully functional (Fig. 4A), even though vIL-6 produced in the presence of TM was not functional (1). We
hypothesized that the conformation of vIL-6 protein was important for its function. The epitope of the M12-neutralizing monoclonal antibody has been mapped to residues Asp-81 to Cys-93 of vIL-6. This epitope lies within site I of the viral protein (25). The comparable site of human IL-6 interacts with IL-6Rα (15, 26). To explore the possibility that such an antibody could probe for potential glycosylation-dependent conformational differences of site I of vIL-6, M12 antibody was used to detect vIL-6 in an ELISA. The M12 antibody recognized WT vIL-6 8-fold more efficiently than vIL-6 produced in the presence of TM (Fig. 5A). Fig. 5B showed that equivalent amounts of proteins were used for the binding assay. However, when WT vIL-6 was produced in the absence of TM but the secreted form was subsequently treated with PNGase F1, which removes N-glycans from their cores, the M12 antibody could detect the glycanase-treated unglycosylated vIL-6 as efficiently as vIL-6 that had not been exposed to PNGase (Fig. 5C). Immunoblotting confirmed that the PNGase treatment was effective (see Fig. 5D). These results support the functional data of Fig. 4 and suggest that, once the protein is secreted, the conformation is fixed. The N78Q/N89Q mutant vIL-6 protein had impaired ability to be recognized by the M12 antibody (Fig. 5C).

Tryptic Digestions of Glycosylated and Unglycosylated vIL-6—To examine further the effect of glycosylation on the conformation of vIL-6, we compared the sensitivity to trypsin of fully glycosylated and unglycosylated vIL-6 protein. In an initial assay, WT vIL-6 produced in the presence or absence of TM was digested with trypsin for 0, 5, or 10 min. One prominent trypsin-resistant product of WT vIL-6 was not observed in the vIL-6 produced in the presence of TM (Fig. 6A, lanes 2 and 3, compared with lanes 5 and 6). In addition, WT vIL-6 made in the presence of TM was more susceptible to trypsin digestion than vIL-6
produced without TM treatment. No full-length vIL-6 remained after 10 min of trypsin digestion of the TM-treated sample (Fig. 6A, lanes 6).

In a second assay, HKB5/B5 cells were metabolically labeled with Met-35 and Cys-35 and transfected with plasmids for WT or N89Q vIL-6. The secreted radiolabeled vIL-6 was immuno-precipitated with a polyclonal antibody against vIL-6. Trypsin digestion was performed for 10 min to digest the labeled vIL-6 while it was still bound to the antibody beads. The released peptides were then separated in a two-dimensional gel (electrophoresis in one axis, TLC in the y axis). There were several notable differences in the two-dimensional gel profile between WT vIL-6 and N89Q vIL-6. Two prominent spots were observed in the WT vIL-6 sample but not in N89Q vIL-6 (hatched circles, Fig. 6B). Three new spots were found in N89Q vIL-6 but not in the WT protein (solid circles, Fig. 6B). There were at least four intense spots (dotted circles, Fig. 6B) in WT vIL-6 that were markedly reduced in intensity in N89Q vIL-6 protein. These studies using trypptic digestions to compare WT and an Asn-89 glycosylation mutant support the conclusion that glycosylation affects the conformation of secreted vIL-6.

**Soluble IL-6Ra Does Not Enhance Binding of vIL-6 to gp130**

It is known that vIL-6 binds to gp130 independently of soluble IL-6Ra (27). WT and all N-glycosylation mutant forms of vIL-6 failed to bind to soluble IL-6Ra (Fig. 7A). In contrast, huIL-6 bound to soluble IL-6Ra in a dose-dependent manner (Fig. 7B). One hypothesis is that the Asn-89 glycan on vIL-6 induces a conformational change that allows the human cytokine to bind to gp130 in the absence of IL-6Ra. The binding of human IL-6 to IL-6Ra may promote a similar conformational change that allows it to bind to gp130. An ELISA gp130 binding assay was used to explore the possibility that IL-6Ra could rescue the ability of N89K vIL-6 to bind gp130. Binding of WT or N89K vIL-6 to gp130 was assessed in the presence or absence of IL-6Ra (Fig. 7A). Soluble IL-6Ra did not increase the binding of WT vIL-6 to gp130 and was not able to rescue gp130 binding by N89K vIL-6 to wild-type levels (Fig. 7C). Under the same conditions, soluble IL-6Ra enabled human IL-6 to bind to gp130 (Fig. 7, A and B, middle two lanes).

**Comparing the Glycosylation Pattern of Human IL-6 with That of KSHV IL-6**

Our previous studies showed that N-linked glycosylation was not required for the activity of human IL-6 (1). Human IL-6 has two N-linked consensus sites, Asn-73 and Asn-172, but only Asn-73 is used. We explored the possibility that the type of N-linked glycosylation on Asn-73 of human IL-6 differed from that of vIL-6 and could account for a difference in dependence on IL-6Ra. The predominant form of secreted huIL-6 was unglycosylated. Only one-third of secreted human IL-6 was glycosylated (Fig. 8A, lane 1) compared with vIL-6 which, when secreted, was exclusively in the glycosylated form (Fig. 1, lane 1). Human IL-6 was fully PNGase F-sensitive, consistent with the fact that it has a single functional N-glyco-
sialylation at or near site I. Such a mutant may gain the ability to bind gp130 directly, without the requirement for IL-6Rα. Sequence alignment of vIL-6 and huIL-6 revealed two sequences on huIL-6 in the vicinity of Asn-89 of vIL-6 that could readily be converted into N-glycosylation consensus sequences (Fig. 8B). F102N and E109T huIL-6 site-directed mutants were generated for the purpose of installing complete N-glycosylation consensus sequences within site I of huIL-6. The F102N huIL-6 mutant contained an extra N-glycosylation site in addition to the native Asn-73 site. This extra N-glycan could be demonstrated most clearly when F102N was produced in the presence of monensin, which inhibited O-glycosylation (Fig. 8C, lane 6). However, this mutation did not cause the secreted huIL-6 protein to be fully glycosylated. The secreted E109T huIL-6 mutant protein, however, was fully glycosylated, in contrast to WT huIL-6. Moreover, the E109T protein contained an additional N-glycan (Fig. 8D, lane 6). The newly generated N-glycans in the F102N and E109T huIL-6 mutants were not sensitive to Endo F1 digestion (Fig. 8E, lanes 3–4 and 8F, lanes 2 and 4, respectively), indicating that these N-glycans were likely complex. The Asn-73 glycan on huIL-6 was also complex as indicated by its resistance to Endo F1 digestion (Fig. 8A, lane 3). However, despite the creation of additional complex N-glycans in site I in huIL-6 in the form of F102N or E109T, both these mutants failed to bind to gp130 in an IL-6Rα-independent manner (Fig. 9, A and B). Both mutants bound effectively to gp130 when sIL-6Rα was present (Fig. 9, A and B), indicating that the newly installed N-linked sites did not destroy the function of the protein. This experiment indicated that additional complex N-linked glycans, comparable to Asn-89 of vIL-6, were not sufficient to confer upon huIL-6 independence of IL-6Rα in binding to gp130.

**DISCUSSION**

Heterogeneity of N-linked Glycosylation Sites of KSHV IL-6—Our previous work highlighted distinct functional roles of N-linked glycosylation in KSHV-encoded viral IL-6 (vIL-6) and cellular IL-6. We found that optimal function of vIL-6 but not cellular IL-6 required N-glycosylation. Viral IL-6 is glycosylated at two sites, Asn-78 and Asn-89. The N-glycosylation
site Asn-89 was important for the optimal function of the vIL-6 protein, whereas the Asn-78 site glycan was dispensable. This report extends this finding and provides novel information about functional importance of heterogeneity in the nature of the sugars in the two N-glycosylation sites of vIL-6. We observed several key differences in the glycosylation profile between the two N-glycosylation sites of vIL-6. Site Asn-78 is primarily made up of high mannose sugars, whereas site Asn-89 is composed of hybrid or complex sugars, as determined by their Endo F1 glycosidase sensitivities. The Endo F1 sensitivity of the Asn-78 glycan is indicative of its processing in the endoplasmic reticulum. Complex glycosylations are Endo F1-resistant and require further processing in the Golgi apparatus. The heterogeneity of N-linked glycosylation at the two sites was confirmed using two different N-glycosylation substitution mutants in mammalian cells and in an insect cell expression system. The two N-glycosylation sites also showed differential sensitivities to a glycosidase inhibitor, castanospermine. The nature of glycosylation at these two sites of vIL-6 influenced the binding of the vIL-6 to the lectin concanavalin A. The Asn-78 site, with its high mannose content, contributed significantly to the ability of vIL-6 to bind ConA. The critical Asn-89 site of vIL-6, which was required for its optimal function, was composed of more complex glycan sugars and bound less effectively to ConA compared with the Asn-78 N-glycan site. These results
highlighted the differential glycosylation patterns between the two N-glycosylation sites of vIL-6.

We considered two main hypotheses about the functional significance of the Asn-89 glycan sugars. The Asn-89 glycan sugar might directly play a role in the binding of the vIL-6 protein to gp130 receptor through sugar-sugar or sugar-protein interaction. Asn-89 might also affect protein conformation and folding, and thus protein stability and function. We performed a series of experiments to explore the functional role of the Asn-89 glycan on vIL-6 protein.

Effects of Removing N-Linked Glycans Once vIL-6 Protein Was Secreted—We found that wild-type vIL-6 was still optimally functional in a cell proliferation assay if the sugars were removed by endoglycosidases after the cytokine was secreted (Fig. 4). In contrast, vIL-6 produced in the presence of tunicamycin was drastically impaired in its functional ability to bind...
to soluble gp130 and to stimulate cell proliferation. This finding suggested that \(N\)-glycosylation could be important in protein folding during protein synthesis; once the folded protein was secreted, subsequent removal of \(N\)-glycan sugars would not have deleterious impact on the function of protein. This result, suggesting that the Asn-89 glycan on vIL-6 was dispensable once vIL-6 has properly folded, would imply that the sugars do not necessarily perform a direct receptor interaction function, but mainly help with protein folding.

One potential explanation that could not be excluded was that some sugars, which themselves were important in receptor binding, remained because PNGase incompletely digested Asn-89 resulted in enhanced binding of N89K single mutant vIL-6 protein to ConA compared with wild-type protein, likely through increased accessibility to the high mannose sugars at site Asn-78 (Fig. 3D).

A previous study demonstrated that vIL-6 in cell lysates contained a high proportion of high mannose sugars, although complex sugars were also present (10). Our study demonstrates that high mannose and complex glycans are present on two different \(N\)-linked sites. A recent study suggests that vIL-6 associates with gp130 intracellularly (28). This association is sensitive to Endo F1 and co-fractionates with calnexin in the endoplasmic reticulum. This result suggests that the Asn-78 high

![Graph](image_url)
N-Linked Glycans and KSHV IL-6 Conformation

mannose site may be involved in intracellular signaling via gp130. However, our results show clearly that high mannose glycan on the Asn-78 site of secreted vIL-6 is not required for function. These data raise the intriguing possibility that the two N-linked glycans on vIL-6 have different functions. The Asn-78 high mannose glycans may perform an autocrine function as the result of intracellular signaling. The Asn-89 complex glycans may be responsible for the secreted protein assuming a conformation suitable for a paracrine function.

Evidence That N-linked Glycans Affect KSHV vIL-6 Protein Conformation—We obtained two main lines of evidence to suggest that N-glycosylation could be important in protein conformation. The M12-neutralizing monoclonal antibody recognizes a conformation-specific epitope in the sequence of vIL-6 spanning residues Asp-81 to Cys-93 within the site I region of the protein (25). We showed that the M12 antibody detected the glycosylated form of vIL-6 more efficiently than the unglycosylated form in a binding assay (Fig. 5). The recognition of the unglycosylated form of vIL-6, which was produced in the presence of tunicamycin, was reduced by ~8-fold, compared with untreated wild-type vIL-6. Tryptic digestion experiments, followed by separation of the products on one- and two-dimensional gels, showed that glycosylated vIL-6 and unglycosylated vIL-6 elicited different tryptic digestion patterns. It is possible that the N89Q mutation also affects conformation independently of changes in glycosylation. However, other data in Fig. 2 (B and C) clearly show that N89Q changes glycosylation. The M12 antibody may preferentially recognize the glycosylated vIL-6, because the Asn-89 glycan altered the conformation of vIL-6 protein, potentially allowing the M12 epitope to be exposed and easily recognized by the antibody. Site Asn-89 is within the region that is recognized by the M12 antibody. Alternatively, the glycosylated and unglycosylated vIL-6 may have identical conformations, but the Asn-89 glycan increased the binding affinity of the vIL-6 protein to the M12 antibody. However, the data from the tryptic digestion studies suggest that glycosylated vIL-6 and unglycosylated vIL-6 are conformationally different, and favor the explanation that N-glycans on vIL-6 is important for protein conformation.

Although TM treatment of vIL-6 impaired detection by M12 antibody, PNGase F1 treatment of vIL-6 did not impair the ability of M12 antibody to detect vIL-6. This difference in recognition by the M12 antibody between PNGase-treated vIL-6 and TM-treated vIL-6 corroborated our earlier observation that PNGase-treated vIL-6 was not impaired in its function in the B9.11 cell proliferation assay. The double site N78K/N89K mutant vIL-6 also was impaired relative to wild-type protein in binding M12 antibody. This result supports the idea that the N-linked glycan plays a role in initial protein folding but can be dispensable once the properly folded protein is secreted.

Different Patterns of N-Linked Glycosylation of KHSV and Cellular IL-6—The patterns of glycosylation of KHSV and cellular IL-6 differ. All of the secreted form of vIL-6 is glycosylated while only a relatively small fraction of secreted human cellular IL-6 is glycosylated (compare Fig. 1, lane 1, and Fig. 8A, lane 1). Human IL-6 has two N-glycosylation consensus sites, but only one site, at Asn-73, is known to be glycosylated. Glycosylation of Asn-73 is not required for functional activity (1). We found that the functional Asn-73 site in huIL-6 is modified by complex sugars.

Binding of huIL-6 to gp130 requires an initial interaction with IL-6Rα. KHSV IL-6 has the special property of binding to gp130 independently of IL-6Rα. Neither wild-type nor mutant forms of vIL-6 protein bind directly to soluble IL-6Rα (Fig. 7A). The N89K vIL-6, which bound poorly to gp130, cannot be rescued by soluble IL6Rα. These results suggest that, in contrast to huIL-6, soluble IL-6Rα cannot confer conformational change to the mutant vIL-6, or substitute for the Asn-89 glycan, allowing it to bind to gp130 (Fig. 7). It is likely that the Asn-89 glycan plays an important role in conferring upon vIL-6 its IL-6Rα-independent gp130-binding property.

Therefore, attempts were made to glycosylate huIL-6 at site I with the purpose of creating an IL-6Rα-independent version of huIL-6. This involved creating additional N-glycan consensus sequences in site I. Both the E109T and F102N huIL-6 mutant proteins acquired additional functional N-glycosylation sites and were modified by complex sugars. Although the F102N huIL-6 mutant protein contained an additional N-glycosylation, it was secreted largely in an unglycosylated form in a similar fashion to the wild-type huIL-6 protein. The F102N mutation did not confer independence of binding to IL-6Rα. We also engineered a hyperglycosylated form of huIL-6, E109T, with certain properties similar to vIL-6. This huIL-6 mutant was N-glycosylated in site I, was fully glycosylated in its secreted form, and, because it was resistant to digestion with Endo F1, the glycosylation was composed of complex sugars. However, despite these changes and similarities to vIL-6, the E109T mutant was not able to bind gp130 in the absence of IL-6Rα. This result suggested that simply creating an N-linked glycan with the same characteristics as Asn-89 of vIL-6 in site I in huIL-6 was not sufficient to confer the ability of the protein to bind gp130 independently of IL-6Rα. The experiment favors the interpretation that complex sugars in site I by themselves are not sufficient to promote IL-6Rα independent interaction of IL-6 with gp130. The results add to the evidence that direct binding of vIL-6 to gp130 is not mediated by the sugars on the secreted protein.

Previous studies tried to identify structural elements needed for IL-6Rα independence of vIL-6 by constructing vIL-6/huIL-6 chimeric proteins and using a reporter assay as a read-out (27). Replacing huIL-6 residues with three different vIL-6-specific tryptophans, selected based on crystal structure studies, did not confer IL-6Rα independence on huIL-6. Helix B of vIL-6, where site Asn-89 is located and where we installed additional N-glycosylation consensus sequences in huIL-6, was found to be absolutely required for IL-6Rα independence property of vIL-6. Substitution studies in helix B suggested that the overall structure of helix B in vIL-6 was important for sIL-6Rα independence. Replacing helix B of huIL-6 with that of vIL-6 did not confer sIL-6Rα-independent signaling ability. The conformational effect of helix B needs to be in the context of the vIL-6 protein.

Recently, in another study of a series of vIL-6/huIL-6 chimeric proteins, huIL-6 carrying all the gp130 interacting sites from vIL-6 was unable to activate gp130 independently of IL-6Rα (29). However, addition of a non-contiguous stretch of
We found that the vIL-6 mutant, which eliminated the Asn-78 high mannose site, was not impaired in its activity as compared with wild-type vIL-6 in the different assays tested. The chaperones, calnexin and calreticulin, lectins present in the endoplasmic reticulum, both recognize glycoproteins containing monoglycosylated high mannose type oligosaccharides (30, 31). Because the Asn-78 glycan consisted mostly of high mannose, one would expect that this glycan would be important for the interaction with either calnexin or calreticulin. Indeed, vIL-6 has been shown to co-fractionate with calnexin intracellularly (28). N78K vIL-6 mutant protein would be expected to remain sequestered in the endoplasmic reticulum as a misfolded protein. Instead, we found that the Asn-78 site was dispensable, whereas the N89K vIL-6 mutant protein was impaired functionally. Therefore, chaperones such as calreticulin and calnexin, which recognize high mannose, are not likely to be the sole mediators of vIL-6 protein folding. Other chaperones, specific for non-high mannose, or complex N-glycans, may be involved. It is also possible that the Asn-89 glycans themselves served as an intrinsic chaperone that assisted in the proper function of vIL-6 without the need for extrinsic chaperone recruitment.

The data presented here are consistent with a model in which Asn-89 glycan plays a critical role in the initial folding of vIL-6, but is dispensable once the protein has folded. It is possible that the Asn-89 glycan on vIL-6 induces a conformational change in the protein that allows it to bind gp130 independently of IL-6Rα. The authors suggested a potential role of IL6R-α binding to site I of huIL-6 at inducing a conformation change in site III for stable and active protein conformation, which requires certain epitopes, including the small loop between helix B and C, that are not directly in contact with gp130. These studies are consistent with our conclusions that sequences in site I of vIL-6 confer conformational changes that enable vIL-6 to bind gp130.

Model for the Role of Asn-89 Glycan in KSHV IL-6 Function—There are two possible roles for the Asn-89 glycan on vIL-6. One is the involvement of this Asn-89 glycan in critical sugar-sugar or sugar-protein interaction at the gp130 receptor interface. The other possible role involves maintaining the proper conformation of the vIL-6 either intracellularly or extracellularly. These roles may not be mutually exclusive. The gp130 receptor may bind to Asn-89 glycan in a lectin-like manner, or may make sugar-sugar contact with the Asn-89 glycan via one of the 9 N-glycosylations on gp130. The Asn-89 glycan may also hold vIL-6 in a conformational state optimal for protein stability and function. The studies of E109T mutant huIL-6 with additional N-linked sites suggest that sugar-sugar or sugar-protein interactions are not sufficient. Results from the M12 conformation-specific antibody binding assay, the tryptic digestion studies, and bioassays showing that PNGase F1-treated vIL-6 is fully functional, all support the hypothesis that the Asn-89 glycan plays an important role in vIL-6 protein conformation.

Amino acids in site III of vIL-6 and a loop between helix B and C to huIL-6 was able to confer upon the human cytokine gp130 activation independently of IL-6-Rα. The authors suggested a potential role of IL6R-α binding to site I of huIL-6 at inducing a conformation change in site III for stable and active protein conformation, which requires certain epitopes, including the small loop between helix B and C, that are not directly in contact with gp130. These studies are consistent with our conclusions that sequences in site I of vIL-6 confer conformational changes that enable vIL-6 to bind gp130.

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Acknowledgments—We gratefully acknowledge Dr. Jack Elias (Yale University, CT) for providing the B9.11 cells and Dr. Robert Means (Yale University, CT) for helpful discussions.

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