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A Kaposi's Sarcoma-associated Herpesvirus-encoded Cytokine Homolog (vIL-6) Activates Signaling through the Shared gp130 Receptor Subunit*

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The present studies analyzed the biologic activity of a gene product (vIL-6) encoded by the recently discovered Kaposi's sarcoma-associated herpesvirus (KSHV) bearing 24.8% amino acid identity with human interleukin-6 (huIL-6). Based on this similarity, we hypothesized that this viral homolog might trigger the JAK/STAT pathway, which typically is engaged by IL-6 and other cytokines. Activation of receptor-associated Janus tyrosine kinases (JAKs) results in the subsequent phosphorylation of signal transducers and activators of transcription (STATs) leading to nuclear entry and transcriptional regulation of target genes. Treatment of HepG2 cells with culture medium containing recombinant KSHV-encoded vIL-6 led to rapid induction of JAK1 phosphorylation and a nuclear DNA-binding activity found to contain STAT1 and STAT3. An antibody to the IL-6 receptor (IL-6R) α subunit effectively neutralized the response to huIL-6 but failed to block STAT activation by vIL-6. In contrast, an antibody reactive with the gp130 subunit of IL-6R abrogated signaling of both responses. Moreover, a transfected cell line expressing human gp130 without IL-6Rα exhibited a robust response to vIL-6 but not to huIL-6. These results demonstrate that KSHV encodes a cytokine that activates specific JAK/STAT signaling via interactions with the gp130 signal transducing subunit independently of the IL-6Rα chain. This activity may have an impact on gp130-mediated signaling in response to native cytokines and thereby influence disease pathogenesis upon KSHV infection.

A recently discovered virus belonging to the subfamily of gamma herpesviruses, Kaposi's sarcoma-associated herpesvirus (KSHV),[1] is a likely causative factor for the development of Kaposi's sarcoma (KS) (1) as well as other neoplasias associated with KS, including body cavity based lymphoma (BCBL) (also known as pleural effusion lymphomas, or PEL) (2–4) and multicentric Castelman's disease (5). Multiple KSHV genome-containing cell lines have been cultured out of PELs, including BCBL-1, BC-1, and BCP-1 (6–8). Sequencing of the viral genome has revealed co-linearity and a notable degree of sequence homology with herpesvirus saimiri (9, 10). Like herpesvirus saimiri, KSHV contains multiple open reading frames encoding cellular homologs, including a G-protein-coupled receptor, cyclin D1 (11, 12), and bcl-2 (10, 13, 14) genes. The KSHV open reading frame K2 gene (15) encodes a protein (vIL-6) with 24.8% amino acid sequence identity with that of human IL-6 (huIL-6) that can promote survival of an IL-6-dependent cell line (15, 16). vIL-6 is expressed in KSHV-infected hematopoietic cells including PEL and derived cell lines (15) and some forms of multicentric Castelman's disease[2] but is rarely found in KS lesions (15).

Human IL-6 may contribute to a variety of B cell neoplastic disorders (17–20). Normally, IL-6 functions in the regulation of the immune system and other tissues. It is known to stimulate B cells (18, 21), and it has the ability to induce growth arrest and differentiation of M1 myeloid precursor cells into macrophages (22–24). Other studies demonstrate an anti-apoptotic function induced by IL-6 in some B cell neoplasms and mediated through apoptotic antagonists such as Bcl-xL (25, 26). IL-6 also plays a pivotal role in hepatic tissue, where it causes the expression of multiple acute phase response (APR) proteins (27).

The receptor for IL-6 contains two heterologous subunits, IL-6Rα and gp130. Both receptors belong to the cytokine receptor superfamily containing described canonical motifs (28). To initiate signaling by the IL-6 receptor complex, the ligand must first make a low affinity contact with the IL-6Rα subunit which then recruits the signal transducing gp130 subunit. Importantly, receptor function depends on the dimerization of gp130 subunits (29). IL-6 thus belongs to a small family of cytokines that function via the homodimerization of gp130 or the heterodimerization of gp130 with LIFRβ, another signal transducing subunit.

A prominent signaling event induced by this dimerization of signal transducing subunits is activation of the JAK-STAT system, a recently described pathway employed by numerous cytokine receptor systems (30) and known to be activated specifically by the IL-6 family of cytokines (31, 32). Ligand-induced receptor assembly causes the activation of associated Janus protein tyrosine kinases (JAKs) resulting in phosphorylation of receptor tyrosines and signal transducers and activators of transcription (STAT) factors that interact physically with specific receptor phosphotyrosines. Finally, phosphorylation of the STAT factors directs their dimerization and relocalization to

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[1] The abbreviations used are: KSHV, Kaposi's sarcoma-associated herpesvirus; KS, Kaposi's sarcoma; huIL-6, human interleukin-6; IL-6, interleukin-6; R, receptor; JAK, Janus tyrosine kinases; STAT, signal transducers and activators of transcription; gp, glycoprotein; EMSA, electrophoretic mobility shift assay; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; APR, acute phase response.

[2] Corbellino *et al.*, manuscript in preparation.

the nucleus where they influence the transcription of target genes (30, 33) by binding to specific promoter elements. Previous work in the IL-6 system provided evidence that the gp130 subunit can associate variously with the JAK kinases, JAK1, -2, and Tyk2, and that JAK activation leads to STAT1 and STAT3 DNA binding and gene transactivation in a cell context-dependent manner (34–36). Earlier reports suggest that some APR genes contain STAT3 binding motifs (37–39). In addition, STAT3 may be the essential factor in mediating many of the principal functions of IL-6, such as growth arrest and differentiation of M1 cells into macrophages (22–24) and survival of IL-6-responsive cells (40).

In these studies we endeavored to determine whether the JAK-STAT signaling program was induced by the KSHV cytokine homolog vIL-6, whether this response mimicked that of huIL-6, and whether the cell surface receptor engaged by the viral cytokine is related to the classical IL-6 receptor complex.

EXPERIMENTAL PROCEDURES

Cell Culture, vIL-6 Preparation, and Cytokine Stimulation—HepG2 cells were cultured in minimal essential medium (Life Technologies, Inc.), 10% fetal bovine serum (Gemini, Calabasis, CA), 1% 100 × non-essential amino acids and 1% 100 × sodium pyruvate. BCBL-1 cells (a gift of Dr. Don Ganem) were grown in RPMI 1640, 10% fetal bovine serum, and 0.05 M 2-mercaptoethanol.

vIL-6 and the reverse vIL-6 (6-LIv) control were prepared as described previously. Open reading frame K2 was cloned into the pMET7 expression vector in forward and reverse orientations and transfected into COS7 cells. COS-derived supernatants were then concentrated 10-fold with a Centriplus 10 filter. HepG2 cells were treated for 10–15 min with 0.5–1.0 ml COS media containing vIL-6 or reverse vIL-6, 100 ng/ml human IL-6 (R & D Systems, Minneapolis, MN), or 1 nM human LIF (R & D Systems) prior to preparation of nuclear extracts. Soluble IL-6Rα fragments (sIL-6Rα, R & D Systems) were used where indicated at 50 ng/ml.

BCBL-1 cells were stimulated with 20 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma), and then cell culture supernatant was harvested. HepG2 cells were then incubated with 1 ml of supernatant for 10–15 min before nuclear extraction.

BAF-130 cells, representing Ba/F3 cells expressing transfected human gp130 but not IL-6Rα (41), were kindly provided by Dr. M. Narazaki (Osaka University Medical School). They were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 10% WEHI 3B-conditioned medium as a source of IL-3, and geneticin 0.3 mg/ml (Life Technologies, Inc.).

Electrophoretic Mobility Shift Assay (EMSA)—10–40 × 10^6 HepG2 cells or 40–50 × 10^6 BAF-130 cells were incubated with COS7 supernatants and then washed in calcium- and magnesium-free phosphate-buffered saline. Nuclear extracts were prepared as described previously (42, 43).

The STAT-response element from the $F_c\gamma$RI gene promoter was used for EMSA assays. The probe was prepared by end-labeling oligonucleotides with [γ-^{32}P]dATP (Amersham Corp.) and polynucleotide kinase (Boehringer Mannheim). Binding reactions were performed using 10^5 cpm, 3 μg of poly[d(I-C)], binding buffer, and 10 μg of nuclear extract (52). Probe specificity competition was done with 50 ng of $F_c\gamma$RI and IRF-1 oligonucleotides. Sequences of oligonucleotides are as follows: $F_c\gamma$RI, GTATTTCCCAGAAAAAGGAC; IRF-1, GCCTGATTTCCCCGAAATGACGG. Preincubations of nuclear extracts with antibodies were performed for 45 min on ice in the absence of poly[d(I-C)] and binding buffer as described previously (42). The STAT1 antibody was from Transduction Laboratories (Lexington, KY), and STAT2 and -3 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), JAK1, 4G10, and anti-SHC were from Upstate Biotechnology (Lake Placid, NY), and MOPC21 (monoclonal IgG_1) and MOPC195 (IgG_{2b} control) were from Cappel (Durham, NC).

Receptor neutralization studies were performed using monoclonal antibodies reactive with human IL-6Rα, gp130, and a polyclonal antibody against IL-6Rα (R & D Systems, Minneapolis, MN) or with MOPC21 (monoclonal IgG_1) and anti-SHC (polyclonal IgG) antibodies as controls. 10–40 × 10^6 HepG2 cells were incubated for 1 h with various concentrations of antibody and then subjected to cytokine stimulation for 15 min, and nuclear extracts were subsequently prepared as described above.

Immunoprecipitations—Cell lysates were prepared from 30–40 × 10^6 cells using a buffer containing 1% Nonidet P-40, 20 mM Tris·HCl, pH 8.0, 150 mM NaCl, 50 mM NaF, 100 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, leupeptin (10 mg/ml), aprotinin (10 mg/ml), pepstatin A (1 mg/ml) as described previously (63) and pre-cleared once overnight and twice for 30 min with 5 μg of rabbit preimmune serum (Cappel, Durham, NC) and 100 μl of protein A-agarose (Boehringer Mannheim). JAKs were immunoprecipitated with 5 μg of rabbit polyclonal antibody against JAK1, -2, and -3 (Upstate Biotechnology, Lake Placid, NY) and Tyk2 (a gift of Dr. Oscar Colaminici) for 1 h at 4 °C and then incubated with 30 μl of protein A-agarose for 1 h. Immunoprecipitates were washed 3 times in lysis buffer and boiled in SDS sample buffer for 5 min. Immunoblots were performed with 4G10 and visualized by enhanced chemiluminescence (Amersham Corp.). To assess protein loading, blots were stripped (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris·HCl, pH 6.7) for 30 min at 55 °C and reblotted with appropriate JAK antibodies.

Luciferase Assay—HepG2 cells were plated at 1.8 × 10^6 cells/well density in 6-well plates 16 h before transfection. Transfections were performed with LipofectAMINE reagent (Life Technologies, Inc.) as per manufacturer's instructions using 1 μg per well of reporter plasmid DNA containing 4 IRF-1 promoter STAT-response elements upstream of a minimal TK promoter and firefly luciferase gene (a gift of Ligand Corp.) (44). 48 h after transfection cells were incubated in media containing 100 ng/ml IL-6, 1 ml of vIL-6, or 1 ml of control reverse vIL-6 (6-LIv) media for 6 h. Cells were washed in phosphate-buffered saline and lysed in 200 μl of Reporter Lysis Buffer (Promega, Madison, WI). Lysates were exposed to luciferase substrates (Analytical Luminescence Laboratory, Ann Arbor, MI) and counted for luminescence units on a Monolight 2000 (Analytical Luminescence Laboratories). Lysates were assayed for protein concentration using the Bio-Rad Protein Assay (Bio-Rad) to control for protein quantity.

RESULTS

The Viral Cytokine Activates Signal Transduction through the JAK-STAT Pathway—Concentrated COS7 cell supernatants expressing recombinant vIL-6 or the reverse 6-LIv as a negative control were used to stimulate HepG2 cells, a human hepatoma line that expresses IL-6 receptors (45). Serial immunoprecipitations of JAKs were carried out in HepG2 cells after cytokine stimulation, followed by immunoblotting with anti-phosphotyrosine antibodies to measure the tyrosine phosphorylation of these kinases as a marker of their activation (Fig. 1*A*). Phosphorylation of JAK1 occurred in response to both vIL-6 and huIL-6 but not the 6-LIv control supernatant. Stripping and re-blotting with anti-JAK1 antibodies demonstrated equivalent levels of JAK1 protein expression in each sample. However, the phosphorylation status of other JAK kinases revealed no reproducible activation of either JAK2 or Tyk2 in samples treated with either huIL-6 or vIL-6 (data not shown).

Subsequently, the induction of STAT factors by the expressed viral cytokine was assessed. Treatment of HepG2 cells with the recombinant supernatants resulted in the appearance of three discrete nucleoprotein complexes in electrophoretic mobility shift assays (EMSA) employing the STAT-binding probe Fc_γRI (Fig. 1*B*). This tripartite DNA-binding activity mirrors that found upon stimulation with huIL-6. To demonstrate that these DNA-binding activities were binding the oligonucleotide probe specifically, cold competition was performed with a related gamma activation sequence element probe, from the IRF-1 promoter (Fig. 2*A*). Preincubation of extracts with excesses of either $F_c\gamma$RI or IRF-1 oligonucleotide sequences caused complete elimination of the 3 bands. Furthermore, incubation of these extracts with anti-phosphotyrosine antibodies caused a diminution of DNA-binding activity, providing evidence that this activity requires tyrosine phosphorylation sites, characteristic of STAT factors (Fig. 2*B*). Finally, antibody supershift experiments with antibodies raised against STAT proteins were performed to elucidate precisely the components involved in these complexes. An antibody against the N terminus of STAT1 caused a band supershift concomitant with the loss of the lower two bands in the tripartite band pattern (Fig.

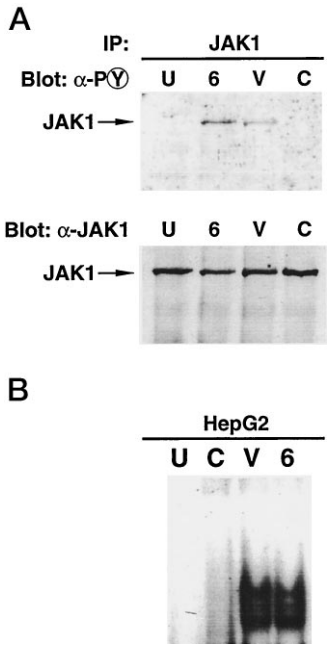

FIG. 1. **KSHV vIL-6 stimulates the activation of the JAK-STAT pathway.** *A*, HepG2 cells were unstimulated (*U*) or incubated for 10 min with 100 ng/ml human IL-6 (*6*), COS culture media containing the viral cytokine (*V*), or the recombinant control COS culture media (*C*). JAK1 immunoprecipitation was performed, followed by immunoblotting with either an antibody reactive with phosphotyrosine residues (4G10) or JAK1. *B*, an EMSA utilizing the $Fc_\gamma RI$ probe is shown displaying nuclear extracts obtained from HepG2 cells that were untreated (*U*) or stimulated for 15 min with control COS culture media (*C*), COS culture media containing the viral cytokine (*V*) or 100 ng/ml human IL-6 (*6*).

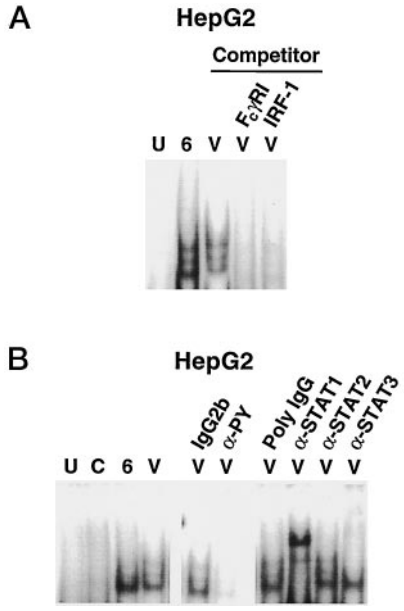

FIG. 2. **The DNA binding complexes induced by vIL-6 contain STAT1 and STAT3.** *A*, an EMSA shows nuclear extracts from HepG2 cells as prepared in Fig. 1. Cold competition of ^{32}P-labeled probe from the vIL-6-stimulated extracts was performed using 50 ng of $F_c\gamma RI$ and IRF-1 promoter region unlabeled probes (42). *B*, nuclear extracts were incubated with antibody against phosphotyrosine, an IgG_{2b} control antibody (MOPC195), a monoclonal antibody against STAT1, rabbit polyclonal sera against STAT2 and STAT3, and polyclonal serum against the SHC protein was used as an antibody control. A monoclonal IgG_1 antibody (MOPC21) was used as a control for the STAT1 antibody and did not cause a supershift or dimunition of the DNA binding complex (data not shown). The abbreviations used are: *U*, untreated; *C*, stimulated for 15 min with control COS culture media; *V*, COS culture media containing the viral cytokine; *6*, 100 ng/ml human IL-6.

2*B*). Additionally, the anti-STAT3 antibody completely abolished the upper two bands but did not yield a supershifted complex. This indicates that the lower and upper bands consist of STAT1 and STAT3 homodimers, respectively, whereas the intermediate band contained STAT1/STAT3 heterodimers. A similar profile of STAT activation has been described in response to huIL-6 (36) and other gp130-interacting cytokines (46–48).

vIL-6 Induces STAT-dependent Transcription—To examine vIL-6 induced gene expression, a reporter plasmid containing the firefly luciferase gene downstream of the STAT-responsive IRF-1 element linked to a minimal promoter was transiently expressed in HepG2 cells. A 30-fold increase in luciferase activity in response to huIL-6 relative to the unstimulated condition was observed in these cells (Fig. 3). Others have reported similar levels of transcriptional transactivation in response to IL-6 (33). Likewise, exposure of reporter-expressing cells to vIL-6 caused a comparable increase in transcriptional activity. The lower level of transcriptional activity triggered by vIL-6 relative to that of huIL-6 may be due either to lower specific activity of vIL-6 compared with huIL-6 or to differences in relative concentration. These data demonstrate that STAT factors activated following stimulation with the viral cytokine are capable of transactivating gene expression and therefore may play a role in modulating gene expression in cells expressing the appropriate cell surface receptors.

The KSHV Cytokine Interacts with the gp130 Subunit but Not the IL-6Rα Subunit—The IL-6Rα subunit contains an extracellular domain that binds the ligand at low affinity but is not alone sufficient to spark an intracellular signal. Signal initiation depends on the ligation of IL-6/IL-6Rα with gp130 subunits. In light of the similarities found between huIL-6 and vIL-6 in terms of JAK/STAT activation, we asked whether these two molecules also mimic each other in their interactions with receptor subunits. To this end, HepG2 cells were treated with increasing concentrations of a monoclonal antibody against IL-6Rα and then stimulated with huIL-6. A loss of signaling capacity was observed at high antibody concentrations using STAT DNA binding activity in EMSA as an indicator (Fig. 4*A*). Unexpectedly, when the same procedure was carried out using vIL-6 instead of huIL-6, no loss of STAT activation was detected. Furthermore, when cells preincubated with antibody were exposed to a 1:3 dilution of vIL-6 supernatant, no loss of STAT-activated DNA binding activity was observed. Similar studies employing a polyclonal antibody to neutralize IL-6Rα also demonstrated no inhibition of vIL-6 activity, while substantially diminishing that of huIL-6 (data not shown). A similar antibody competition approach was then employed to elucidate the role of the gp130 subunit in signaling by the KSHV cytokine. In contrast to the anti-IL6-Rα antibody, an anti-gp130 monoclonal antibody at high concentrations inhibited both the huIL-6 and vIL-6 signals (Fig. 4*B*). These findings suggest that the viral cytokine, unlike huIL-6 itself, utilizes gp130 without necessarily interacting with IL-6Rα; IL-6Rα apparently does not facilitate the association of vIL-6 with gp130 as it does with IL-6. Therefore, by these criteria, it appears unlikely that the viral cytokine must rely on recruitment of IL-6Rα to elicit its intracellular signal.

As an alternate test of the hypothesis that vIL-6 differs from huIL-6 by selectively engaging gp130, signaling studies were performed using a transfected IL-3-dependent murine cell line expressing exclusively human gp130 but not IL-6Rα. These BAF-130 cells have been shown previously to respond to huIL-6

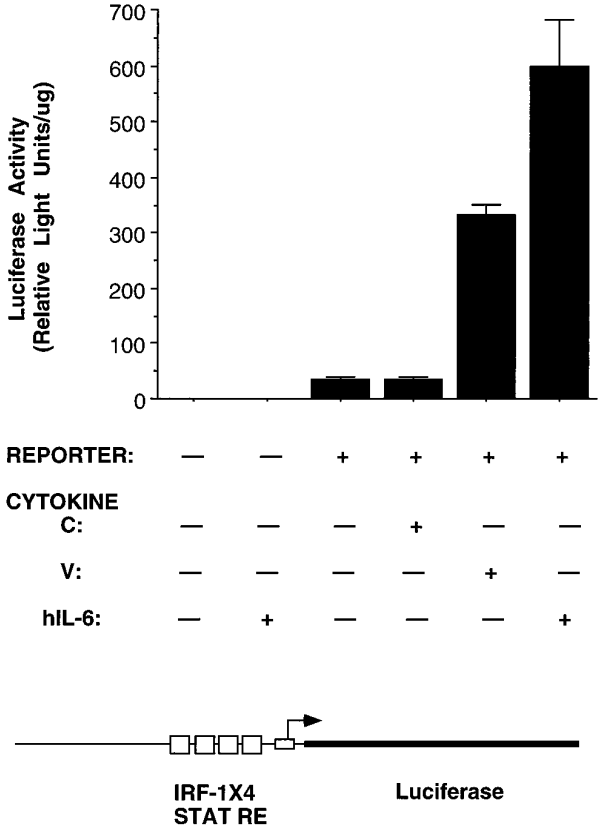

FIG. 3. **vIL-6 activates STAT-dependent transcription.** HepG2 cells were transfected with a reporter construct containing an IRF-1 promoter STAT-response element and cultured for 48 h. Cells were either stimulated with COS supernatant from reverse construct transfectants (*C*), COS media containing vIL-6 (*V*), or 100 ng/ml human IL-6 (*6*) for 6 h each, and the luciferase activity was assessed.

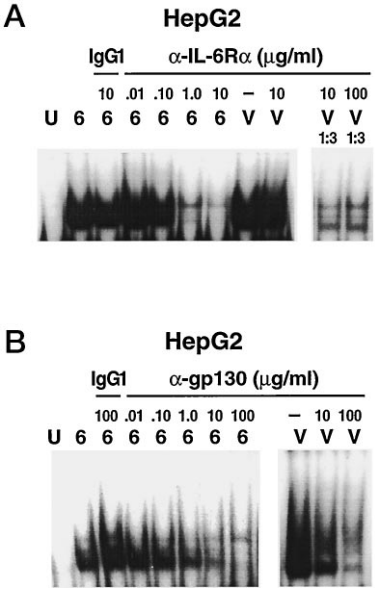

FIG. 4. **gp130 is a necessary component of the receptor complex engaged by the KSHV cytokine.** *A*, HepG2 cells were incubated with 10 μg of MOPC21, a control monoclonal IgG_1 antibody, or with increasing concentrations (0.01–10 mg/ml) of a monoclonal antibody against the human IL-6Rα for 1 h and then treated with 100 ng/ml human IL-6. Similarly, HepG2s were treated with 10 and 100 mg/ml anti-IL6Rα and then incubated for 15 min with the COS media containing the viral cytokine and, on a subsequent occasion, a 1:3 dilution of the viral cytokine. *B*, HepG2 cells were cultured for 1 h with several increasing doses (0.01–100 μg/ml) of an antibody reactive to human gp130 and then exposed to human IL-6. Likewise, cells treated with 10 and 100 mg/ml anti-gp130 antibody were stimulated with the viral cytokine containing media. See Fig. 2 legend for abbreviations.

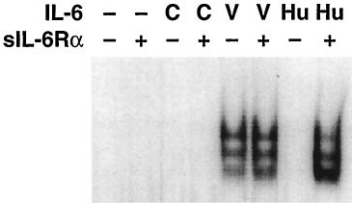

FIG. 5. **vIL-6 functionally engages human gp130 independently of IL-6Rα.** BAG-130 cells expressing human gp130 but not IL-6Rα were exposed to huIL-6 (*Hu*, 20 ng/ml), vIL-6 *V*), control supernatant (*C*), or no IL-6 (−) for 15 min. Stimulations were performed in either the presence (+) or absence (−) of sIL-6Rα. Nuclear extracts were subsequently prepared from these cultures and analyzed by EMSA with the Fcγ RI probe as described under "Experimental Procedures."

only upon the addition of soluble fragments of the IL-6Rα chain (sIL-6Rα) to the culture (41). Likewise, we observed that the induction in these cells of STAT DNA binding by huIL-6 was undetectable by EMSA with the STAT-binding probe in the absence of sIL-6Rα but was readily evident as a three-component nucleoprotein complex when sIL-6Rα was added (Fig. 5). Strikingly, supernatants containing vIL-6 (but not the 6LI-v negative control) induced the indistinguishable tripartite nucleoprotein species in BAF-130 cells regardless of the presence or absence of sIL-6Ra (Fig. 5). These findings confirm that vIL-6 engages the IL-6R in a markedly different manner from that of huIL-6 and that this interaction is functionally independent of the IL-6Rα subunit.

Induction of a KSHV-containing Cell Line with Phorbol Ester Causes Secretion of an IL-6-like Activity—BC-1 and BCP-1, cell lines that are coinfected with KSHV and Epstein-Barr virus or KSHV alone, respectively, have been demonstrated to produce transcripts encoding vIL-6 in response to TPA (15). BCBL-1 is a body cavity-based lymphoma (BCBL) cell line that is KSHV-positive and Epstein-Barr virus-negative, similar to BCP-1. To assess further the potential biologic activity of vIL-6 within the viral life cycle, we subjected BCBL-1 cells to TPA treatment (20 ng/ml for 48 h) and harvested the culture medium; this treatment of BCBL-1 cells has been shown to induce both the expression of KSHV genes (including that encoding vIL-6) and the lytic replication of KSHV, which is otherwise latent and transcriptionally suppressed in these cells (7, 49). HepG2 cells subsequently were exposed to the TPA-induced BCBL-1 supernatant for 15 min (Fig. 6). EMSA of nuclear extracts from these cells using a probe containing a STAT-binding motif demonstrated induction of a DNA binding complex similar to that seen after stimulation by huIL-6, with a pattern that suggested activation of multiple STAT factors. In contrast, exposure of the HepG2 cells to TPA itself or uninduced BCBL-1 supernatant did not result in a similar banding pattern. Further studies have indicated that BCP-1, when induced by TPA, also secretes a similar biologic activity (data not shown). Therefore, induction of KSHV-infected cells by phorbol ester results in the secretion of a bioactivity with signaling properties that resemble those of vIL-6.

DISCUSSION

The goal of the present studies was to explore the signaling capabilities of a putative cytokine encoded by KSHV. Previous reports have shown that this protein, deemed vIL-6, supports

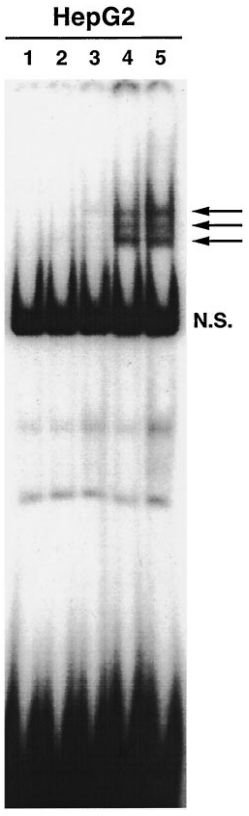

FIG. 6. **The phorbol ester TPA induces the secretion of an IL-6-like activity from BCBL-1 cells.** HepG2 cells were untreated (*lane 1*) or treated for 15 min with 20 ng/ml TPA (*lane 2*), normal BCBL-1 culture media (*lane 3*), culture media from BCBL-1 cells that were induced for 48 h with 20 ng/ml TPA (*lane 4*), or, as a positive control, 100 ng/ml human IL-6. Nuclear extracts were prepared as described under "Experimental Procedures," and DNA binding activity was assessed using a ^{32}P-labeled STAT-specific probe derived from the promoter region of the F_cgRI gene (42). *Arrows* indicate the nucleoprotein complexes, and *N.S.* designates a nonspecific band that occurs in each sample.

the survival of B9 plasmacytoma cells and can substitute for huIL-6 in this capacity (15, 16, 50). In view of this IL-6-like activity, we sought to elucidate in more detail the functional relationship between huIL-6 and vIL-6 by assessing the JAK-STAT intracellular signaling pathway using HepG2 cells. Indeed, vIL-6 was found to trigger the phosphorylation of JAK1, a result in agreement with the literature surrounding IL-6. Several groups have shown that IL-6 is responsible for the activation of various JAKs in a pattern that is cell context-dependent (34, 35, 51), and in HepG2 cells both IL-6 and vIL-6 selectively activated JAK1.

JAK1 has been suggested to be the primary kinase mediating STAT activation after IL-6 stimulation, and the present findings suggest that JAK1 is also integral to mediating downstream STAT activation events by KSHV vIL-6. Both cytokines induced a tripartite pattern of DNA binding complexes as detected by EMSA; these nucleoproteins were found to represent STAT1 and STAT3 in homo- and heterodimeric formations. The same pattern of induction has been observed previously in the IL-6 system as detected by both EMSA analysis and phosphotyrosine immunoblot analysis (35, 51, 53, 54). There is reported evidence that the pattern of STAT activation, like that of the JAKs, differs in a cell context-dependent manner and that in many situations STAT1 is induced by huIL-6 to a lesser extent than is STAT3 (36). In fact, EMSA experiments utilizing extracts from the TF-1 erythroleukemia line revealed that stimulation with huIL-6, LIF, or vIL-6 induced exclusively homodimers of STAT3 (data not shown). Despite such variation among different cell types, huIL-6 and vIL-6 exert indistinguishable effects upon a given cell type.

Target gene transactivation is the key biologic effect of STAT induction by cytokines. Reporter systems directly assaying transcriptional activity have shown this function for many of the known STAT factors (33). Using a reporter construct containing a STAT-response element from the promoter region of the IRF-1 gene, we detected a level of STAT-dependent transcriptional activity triggered by vIL-6 that was comparable to that induced by huIL-6. This activity directly implies downstream effector function which would result from viral cytokine stimulation of target cells. Specifically, STAT3 has been documented as an integral factor in the APR necessary for the transcriptional activation of APR genes in hepatic cells (37–39). Additionally, STAT3 function has been associated with differentiation of myeloid precursor cells into macrophages (23) and with cell survival (40). The function of STAT1 in IL-6 signaling may be partially clarified by studies of STAT1 gene-disrupted mice. These mice, while incompetent in their ability to respond to interferon stimulation, were able to respond to a host of other cytokines that usually trigger STAT1 activation, including IL-6 (55, 56). *In vitro* studies have also shown that STAT1 and STAT3 have largely overlapping response element specificity (33, 57). Therefore, it seems likely that STAT3 determines the specificity of the STAT transcriptional effects in response to both IL-6 and vIL-6. Further work is needed to define more precisely the pertinent gene targets.

In addition to measuring signal transduction events after cytokine stimulation we examined the interactions of vIL-6 with human cell surface receptors. Binding assays performed with radiolabeled huIL-6 and HepG2 cells demonstrated that the KSHV vIL-6 was able modestly (15–25%) to inhibit radiolabeled ligand binding (data not shown), indicating that vIL-6 employs at least some of the human IL-6 receptor components to initiate its signal. Subsequent dissection of the huIL-6R with neutralizing antibodies found that gp130, but not IL-6Rα, is a critical component of the receptor complex engaged by the viral cytokine. In fact, the vIL-6 molecule apparently contains a conserved Trp residue homologous to part of the site 3 gp130-binding site of IL-6 (58, 59). These findings do not exclude the possibility that IL-6Rα may contact the viral cytokine under normal circumstances, but such an interaction may not be essential in the initiation of the signaling program. This hypothesis is strongly supported by the marked functional responses we observed in BAF-130 cells treated with vIL-6, but not with huIL-6, in the absence of IL-6Rα. These findings differ from a recent report providing evidence with neutralizing antibodies that IL-6Rα may play a role in the vIL-6 signal in murine cells (16). This distinction raises the possibility that the viral cytokine may differ in the way it interacts with the human and murine receptors and that vIL-6, as the product of a virus that is tropic for human cells, has a higher affinity for human gp130 allowing the two to interact without huIL-6Rα. Alternatively, vIL-6 may react with human and murine IL-6Rα chains with similar affinity, but at a contact site that differs from huIL-6 and that is not blocked by antibodies that neutralize the huIL-6/huIL-6Rα interaction. In any event, it is clear from our experiments that huIL-6Rα chains are dispensable for the functional interaction of vIL-6 with human gp130 leading to effective signal transduction, a feature that substantially distinguishes the viral cytokine from native human IL-6.

The promiscuity of the gp130 subunit within multiple receptor complexes makes conceivable the notion that the viral cytokine could interact with a number of other receptor subunits, although vIL-6 is more similar in its primary sequence to IL-6

than it is to IL-11, OSM, CNTF, LIF, or CT-1. LIFRβ, CNTFRα, OSMRα, and IL-11Rα are all known to be involved in receptor complexes containing gp130 (21). Interestingly, at least one report has indicated that an antibody to the gp130 subunit is sufficient to cause dimerization and subsequent signal transduction (60), leaving such homodimerization as a possible mechanism of vIL-6 action. These possibilities are beyond the scope of the present studies but are grounds for further examination. In any event, the engagement of gp130 by vIL-6 is the likely molecular basis of intracellular signaling initiation by this viral cytokine.

KSHV is preferentially found in peripheral B lymphocytes (61, 62), a feature that is consistent with its classification as a gamma herpesvirus (9). Furthermore, transcription from the KSHV genome in latently infected cell lines is tightly regulated (49). The observation that the KSHV-containing BC-1 and BCP-1 cell lines express vIL-6 transcripts when induced by phorbol ester suggests that this protein may play a role in the maintenance of replicating virus, although vIL-6 is also expressed in latently infected cells (15). Indeed, we observed that phorbol ester induced secretion of protein with IL-6-like signaling activity from KSHV-infected cells, as demonstrated by the profile of STAT activation. The recent findings that STAT3 plays a role in anti-apoptosis of IL-6-stimulated cells (40) and that IL-6 prevents B cell apoptosis (25, 26) argue that vIL-6 may function in protection from apoptotic responses of these cells to viral infection. Additionally, this cytokine may act synergistically with other KSHV products, such as bcl-2 and cyclinD1 (11, 12) homologs, to prolong the survival of infected cells.

In situ immunostaining to detect the viral cytokine demonstrated cells in KS lesions generally do not express vIL-6, in marked contrast to the more prevalent expression in cells derived from a PEL (15). In light of the evidence, it may be more likely that vIL-6 exerts the bulk of its effects on hematopoietic cells rather than on the endothelial cells involved in KS lesion formation. It remains to be seen, however, whether hematopoietic cells are the only cell lineage affected by vIL-6 expression and precisely which *in vivo* events invoke this expression. Nonetheless, the stimulation of a signaling program resembling that of the gp130-utilizing family of cytokines may play a pivotal role in the life cycle of KSHV and the pathogenesis of associated diseases and may therefore provide a useful therapeutic target.

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A Kaposi's Sarcoma-associated Herpesvirus-encoded Cytokine Homolog (vIL-6) Activates Signaling through the Shared gp130 Receptor Subunit

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