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KSHV-Encoded Viral IL-6 Activates Multiple Human IL-6 Signaling Pathways

Julie Osborne, Patrick S. Moore, and Yuan Chang

ABSTRACT: Kaposi's sarcoma-associated herpesvirus (KSHV or HHV8) encodes a structural and functional homologue of human IL-6 called viral IL-6 (vIL-6). Expression of vIL-6 in KSHV-related lymphoproliferative disorders has been implicated in their pathogenesis. vIL-6 has been shown to mimic a number of IL-6 activities including stimulating the growth of IL-6 dependent cell lines and activating the JAK1 and STAT1/3 pathway in HepG2 cells. However, IL-6 and vIL-6 display differences in receptor usage that may give rise to underlying qualitative and quantitative differences in the signaling pathways utilized. While IL-6 has an absolute requirement for both the IL-6 Rα and the gp130 subunits, vIL-6 appears to require only gp130. In addition to JAK1 and STAT1/3 pathways, IL-6 activates multiple other pathways including the direct activation of STAT 5 by JAK1, the Ras-MAP kinase cascade and a novel H7-sensitive pathway. In this study we examined whether vIL-6 is capable of signaling via distinct IL-6 response elements (IL-6 RE) under the control of these different pathways. We show that vIL-6 activates both STAT1/3- and STAT5-dependent Type II IL-6 REs. In addition, vIL-6 induces transcriptional activation via a Type I IL-6 RE that binds C/EBP, indicative of Ras-MAP kinase pathway induction. Furthermore, vIL-6 is capable of activating the IL-6 response element in the *c-jun* promoter (JRE-IL-6). vIL-6 induced activation of JRE-IL-6 requires both the Ets- and Cre-like sites, suggesting that vIL-6 is capable of stimulating the same novel serine/threonine kinase mediated pathway as IL-6. These results demonstrate that vIL-6 can stimulate all of the known IL-6-induced signaling pathways. Therefore, vIL-6 could potentially contribute to KSHV-related disease progression by continued activation of IL-6-stimulated growth and anti-apoptotic pathways even when cells attempt to protect themselves from IL-6 over-stimulation by downmodulating their IL-6Rα subunits. *Human Immunology 60, 921–927 (1999).* © American Society for Histocompatibility and Immunogenetics, 1999. Published by Elsevier Science Inc.

KEYWORDS: KSHV, vIL-6, Signaling, Growth, Anti-apoptotic

ABBREVIATIONS

KSHV	Kaposi's sarcoma-associated herpesvirus
HHV8	human herpesvirus 8
LIF	leukemia inhibitory factor
OSM	oncostatin M
CNTF	ciliary neurotrophic factor
CT-1	cardiotrophin-1
grb2	growth receptor binding protein 2
MAPK	mitogen-activated protein kinase

INTRODUCTION

The human gammaherpesvirus, KSHV (also called HHV8), is the likely infectious cause of a number of neoplastic and lymphoproliferative diseases including Kaposi's sarcoma, pleural effusion lymphoma and a subset of multicentric Castleman's disease [1–3]. KSHV encodes various cellular homologues, including a protein which displays 62.2% amino acid similarity to human IL-6 called viral IL-6 (vIL-6) [4, 5]. This finding is of particular interest since IL-6 activity has been implicated in the development of the polyclonal lymphoid proliferation associated with Castleman's disease [6]. In addition, IL-6 may play a role in enhancing the growth and tumorigenicity of B cells immortalized by another herpesvirus, EBV [7].

Human IL-6 exerts a wide variety of biological functions including growth and anti-apoptotic effects via it's receptor [reviewed in 8]. The IL-6 receptor consists of a high affinity binding α subunit (IL-6Rα) and a signal transducing β subunit, called gp130, which is shared by receptors for other cytokines, including IL-11, LIF,

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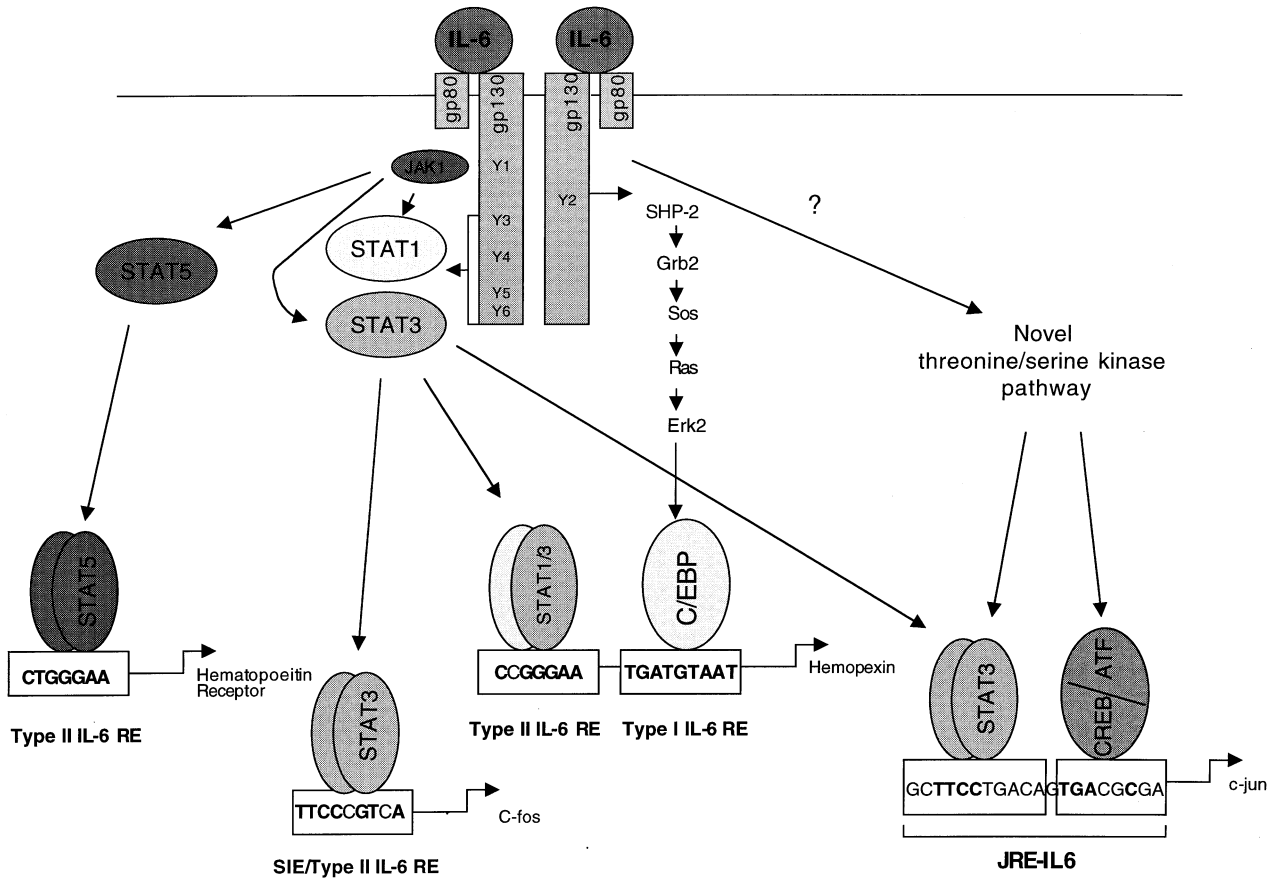

FIGURE 1 IL-6 activates multiple signaling cascades. IL-6 binds to its receptor gp80(IL-6Rα)/gp130(IL-6Rβ) and induces homodimerization of gp130 resulting in activation of multiple signaling pathways. Y1-6 represent the conserved tyrosine residues on gp130 that are critical for IL-6 mediated signaling. The response elements chosen to illustrate the gene activation events are the same gene elements that are present in the CAT constructs used in Figures 2–4. Bases in bold type match the consensus sequences determined for the Type II IL-6 RE [21], SIE [24], Type I IL-6RE [22] and JREIL-6 [16].

OSM, CNTF and CT-1. Following binding of IL-6 to the complex, gp130 homodimerizes leading to the activation of a number of signaling cascades detailed in Fig. 1.

One of the best characterized IL-6 pathways is through Jak/STAT signaling where the initial gp130 dimerization event leads to activation of receptor-associated Jaks, in particular Jak1, 2 and Tyk2, that in turn phosphorylate key tyrosine residues on gp130 [9]. STAT1 and 3 bind these specific phosphorylated tyrosines sites and are themselves phosphorylated by the Jaks resulting in their dimerization and translocation to the nucleus where they bind and activate STAT1 and 3 binding elements in the promoters of specific genes [10]. Recently it has been shown that IL-6 can also activate STAT5 [11]. This STAT is more commonly associated with IL-2 and IL-7 receptor signaling and has been implicated in the regulation of genes important for proliferation and anti-apoptotic functions in hematopoietic cells [12]. However, in contrast to the situation with STAT1 and 3, STAT5 is activated by JAK1 independently of gp130 tyrosine phosphorylation [11] and acts at a regulatory element distinct from that of STAT3 [13]. IL-6 can also activate the growth-promoting Ras-dependent MAPK cascade which is linked to gp130/Jak2 via an adaptor molecule called grb2 [14]. One downstream target of the MAP kinase is NF-IL-6 (C/EBP) which requires threonine phosphorylation to bind to Type I IL-6 response element (IL-6 RE) and cause transcriptional activation [15]. In addition, a novel H7 sensitive pathway is involved in the activation of the IL-6-inducible immediate early gene *junB*. The IL-6 RE in the *junB* promoter (JRE-IL6), is composed of two DNA binding elements; an Ets/low affinity Stat3 binding site and a cAMP responsive element (CRE)-like site [16]. Activation of this promoter requires both sites and the activity of a threonine/serine kinase that is not related to PKA, PKC, Ca^{2+}/CM-dependent kinases or Ras-MAP pathway kinases. However, how this pathway is linked to the gp130 receptor is still unclear.

vIL-6 has been shown to replace IL-6 in maintaining

the survival of IL-6 dependent cell lines [4, 17]. In addition, vIL-6 is able to activate the Jak1/STAT1 and 3 pathway in HepG2 hepatoma cells resulting in the activation of a STAT 1 and 3 inducible IL-6 RE called GAS and hence promoter activation [18]. However, there is one intriguing and possibly fundamental difference between human and vIL-6 in terms of receptor usage. While human IL-6 has an absolute requirement for both the IL-6 Rα and the gp130 subunits of the IL-6 receptor to initiate signaling, vIL-6 appears to require only gp130 [18]. This difference in receptor engagement may give rise to underlying qualitative and quantitative differences in the signaling pathways. Therefore, we examined a number of CAT reporter constructs, containing each of the different known IL-6 response elements, to determine if vIL-6 is capable of activating multiple IL-6 signaling events. In addition, the ability of vIL-6 to induce a differential gene expression profile from that of IL-6 was determined using a human cDNA expression array.

MATERIAL AND METHODS

Cells

The human hepatoma cell line, HepG2 (provided by Dr. Heinz Baumann, Roswell Park Cancer Institute, NY), was grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, glutamine (2 mM), streptomycin (100 μg/ml) and penicillin G (100 U/ml) (all reagents from Gibco, Gaithersburg, MD).

Recombinant vIL-6 Preparation

The vIL-6 coding sequence was amplified from cosmid DNA clone Z6-2 [19] using synthetic oligonucleotide primers vIL6-5H-Bam and vIL-6-3-HindIII, as described previously [17]. The resultant PCR product was digested with BamHI and HindIII and ligated into a GST-tagged prokaryotic expression vector pGEX-KG [20]. This construct, pJO384, directs production of a vIL-6 fusion protein (GSTvIL-6) that has GST at the N terminus and a predicted molecular weight of 54.6 kDa. GSTvIL-6 was expressed in *Escherichia coli* strain BL21 (Novagen, Madison, WI) and extracted under non-denaturing conditions. A single purification procedure using glutathione-agarose beads (Sigma, St. Louis, MO) was sufficient to yield purified fusion protein. To remove the GST portion of the fusion molecule, GSTvIL-6 samples were digested with 1% (w/w) thrombin (Sigma) in 50 mM TrisCl (pH 7.5), 150 mM NaCl and 2.5 mM $CaCl_2$ (TC buffer) at 25°C for 2 h to yield a cleaved product of approximately 23 kDa (TCvIL-6). The vIL-6 protein samples were sterilized by centrifugation using 0.22 μm Spin-X tubes (CoStar, Cambridge, MA). Protein concentrations were determined by Bradford assay (Biorad Laboratories, Richmond, CA) and aliquots were stored at −70°C until use. vIL-6 preparations were also prepared by collecting supernatants from Cos7 cells (American Type Culture Collection, Rockville, MD) transiently transfected with mammalian expression vector pMET7 containing vIL-6 open reading frame in the forward (CosvIL-6) or reverse (Cos6-LIv) orientation, as previously described [4]. In preliminary experiments, the various vIL-6 preparations were determined to be biologically active by B9 bioassay, as previously described [4].

CAT Constructs

Human IL-6 response element constructs pHRRE, pHPX, pSIE and pHPXA were kindly provided by Dr. Heinz Baumann, Roswell Park Cancer Institute. pHRRE contains 8 tandem copies of the modified IL-6RE/APRE of the human hematopoietin receptor gene in the *Bg*/II site of pCAT vector [21]; pHPX contains 5 tandem copies of the Type II IL-6 RE of the rat hemopexin gene in the *Bg*/II site of pCAT vector [22]; pSIE contains 4 tandem copies of the high affinity SIEm67 in the *Bg*/II site of pCAT vector [21]; and pHPXA contains the 5 tandem copies of the A element (Type 1 IL-6 RE) from the human hemopexin gene in the *Bg*/II site of pCAT vector [22]. pJ4, pJ4 M1 and pJ4 M3 were kindly provided by Dr Toshio Hirano, Osaka University, Japan. pJ4 contains the IL-6 RE of the Jun B gene (JRE-IL6) which consists of two distinct DNA elements: a putative EBS and a CRE-like site [16]. pJ4 M1 contains a mutated JEBS and pJ4 M3 contains a mutated Cre-like site. Either mutation abolishes IL-6 activation of the JRE-IL6 indicating that both DNA motifs are necessary for IL-6 responsiveness.

CAT Assay

HepG2 cells were plated at 1×10^6 cells in 10-cm dishes and 24 h later the cells were transfected with DNA using the CellPhect Kit (Amersham Pharmacia Biotech, Piscataway, NJ). Typically 6 μg of the CAT expression construct and 2 μg of an internal transfection marker, pcDNA3.1HisLacZ plasmid (InVitrogen, Carlsbad, CA), were used. Cells were incubated with the DNA precipitate for 16 h and then the cells were subdivided into 6-well plates. Cells were then stimulated for 24 h with serum-free medium containing human IL-6 (100 ng/ml; R&D Systems, Minneapolis, MN), 25% (v/v) CosvIL-6, 25% (v/v) Cos6Liv, GSTvIL-6 (1 μg/ml), GST (1 μg/ml), TCvIL-6 (1 μg/ml) or TC buffer (same volume as TCvIL-6). In experiments where cells were transfected with the pHPXA-CAT construct, subcultures were treated with serum-free medium containing 1 μM dexamethasone (Sigma) as a control or 1 μM dexamethasone plus combinations of human IL-1 (100 ng/ml,

R&D Systems), human IL-6 (100 ng/ml, R&D Systems), GSTvIL-6 (1 μg/ml) or GST (1 μg/ml). IL-6 alone was insufficient to activate this IL-6 RE; significant induction of this construct required the presence of dexamethasone, IL-1 and IL-6 [21]. The CAT activities in the cell extracts after treatment were determined and normalized to the amount of β-galactosidase produced from the pcDNA3.1HisLacZ plasmid and the values expressed relative to the activity in unstimulated cells.

Differential Gene Expression Analysis

Differential gene induction by vIL-6 compared to IL-6 was investigated using Atlas Human cDNA Expression Array (Clontech Laboratories, Inc., Palo Alto, CA). Peripheral blood mononuclear cells (PBMC) were purified from heparinized blood samples from healthy donors by centrifugation on Histopaque 1077 (Sigma) and washed in RPMI 1640 Dutch Modification medium (RPMI, Gibco). PBMC were then resuspended at 5×10^6 cells/ml in RPMI only or RPMI supplemented with 100 ng/ml of IL-6 (R&D Systems) or 1 μg/ml of GSTvIL-6 for 50 min. Total RNA was extracted using Trizol reagent (Gibco) and purified for polyA$^+$ RNA using a PolyATtract mRNA Isolation System IV (Promega, Madison, WI). 1 μg of polyA$^+$ RNA, from each sample, was converted into ^{32}P labeled first-strand cDNA and used to probe the cDNA arrays according to the Atlas kit instructions. Both arrays were probed with ^{32}P labeled first-strand cDNA derived from unstimulated cells, stripped, and then reprobed either with ^{32}P labeled first-strand cDNA derived from IL-6-stimulated cells or from vIL-6-stimulated cells. Autoradiographs were exposed at −70°C using Kodak film and the signal intensities quantified by laser densitometry (Molecular Dynamics, Sunnyvale, CA). Hybridization signals for polyA$^+$ RNA from IL-6 and vIL-6 stimulated cells were expressed relative to the signal on the same blot with polyA$^+$ RNA from the same cells without treatment.

RESULTS

vIL-6 Activates Different STAT Binding Type II IL-6 REs

We first examined whether vIL-6 was able to activate different STAT binding Type II IL-6 REs. HepG2 cells were transfected with CAT reporter gene constructs, pHPX, pHRRE and pSIE, containing the Type II IL-6 RE from three separate genes. pHPX contains the Type II IL-6 RE from the rat hemopexin gene and is activated exclusively by STAT 1 and 3 following IL-6 stimulation [23]. pSIE contains another example of an Type II IL-6 RE, the high affinity mutant form *sis*-inducible element of the *c-fos* gene, which is thought to bind predominantly STAT 3 [24]. In contrast, pHRRE, which contains the Type II IL-6 RE from the rat hematopoietin receptor gene, is activated by STAT5B but not STAT3 [13].

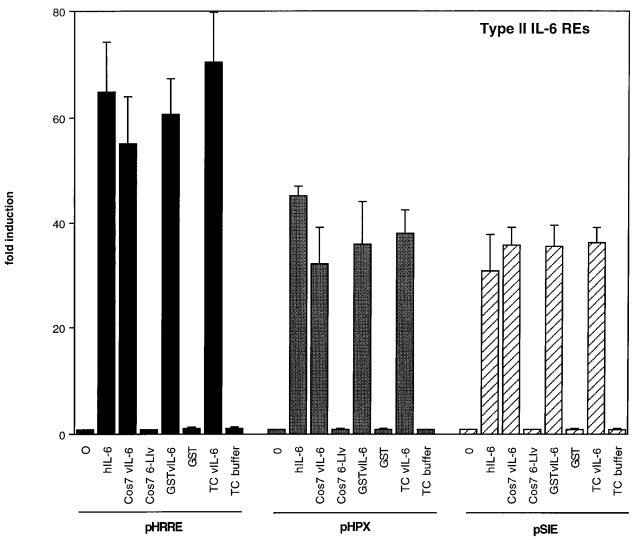

FIGURE 2 vIL-6 activates different Type II IL-6 REs. HepG2 cells, transiently transfected with the IL-6 RE Type II containing CAT reporter constructs pHPX, pSIE and pHRRE, were incubated for 24 h in medium alone or medium plus IL-6 (100 ng/ml), CosvIL-6 (25% v/v), Cos6-LIv (25% v/v), GSTvIL-6 (1 μg/ml), GST (1 μg/ml), TCvIL-6 (1 μg/ml), or TC buffer (same volume as TCvIL-6). The fold induction in CAT activities relative to unstimulated cells represents the mean ± S.D. of three separate experiments.

Our data show that vIL-6 is able to stimulate all three forms of the Type II IL-6 RE (Fig. 2). vIL-6 preparations, from bacteria (GSTvIL-6 and TCvIL-6) or mammalian cells (CosvIL-6), were able to elicit a response similar in magnitude to that of IL-6. Neither GST, Cos6-LIv nor TC buffer were able to activate any of the promoter constructs over the level in unstimulated cells. Therefore, vIL-6 stimulation of gp130 may be able activate STAT 5-mediated gene transcription, in addition to STAT1 and 3. The only apparent difference between vIL-6 and human IL-6 signaling via Type II IL-6 RE was that 10-fold more vIL-6 (1 μg/ml) was required to induce the same level of transcriptional activity as that of IL-6 (100 ng/ml). However, removal of the GST portion of the vIL-6 fusion protein (TCvIL-6) did not result in a higher level of RE activation compared to vIL-6 demonstrating that GST could not be responsible for interfering with vIL-6-mediated signaling.

vIL-6 Activates a C/EBP (NF-IL-6) Responsive Gene Element that is Controlled by the MAP Kinase Cascade

To determine whether vIL-6 was able to activate a Type I IL-6 RE, we tested a CAT construct, pHPXA, containing five copies of the C/EBP transactivating element

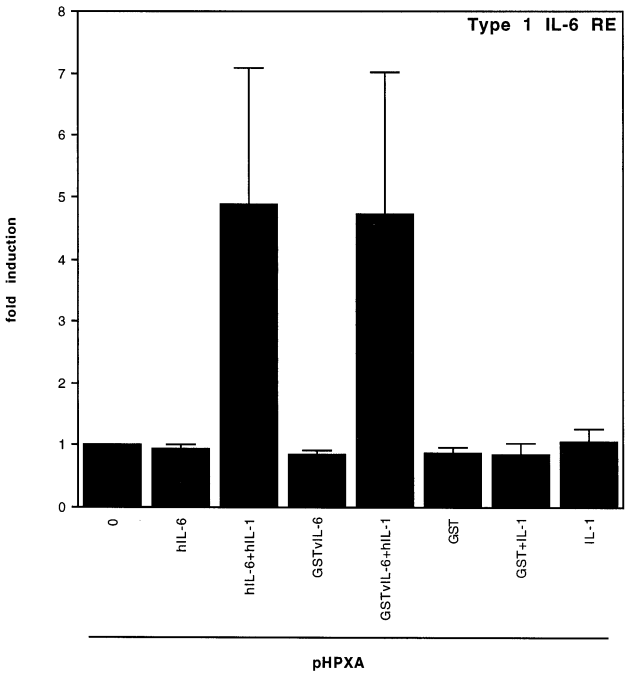

FIGURE 3 vIL-6 activates a C/EBP (NF-IL-6) responsive gene element that is controlled by the MAP kinase cascade. HepG2 cells were transfected with the Type I IL-6 RE-containing pHPXA construct. Transfected cells were then treated with serum-free medium containing 1 μM dexamethasone or 1 μM dexamethasone plus combinations of IL-1 (100 ng/ml), IL-6 (100 ng/ml) or GSTvIL-6 (1 μg/ml) for 24 h. The fold induction in CAT activities relative to cells incubated with dexamethasone alone represents the mean ± S.D. of three separate experiments.

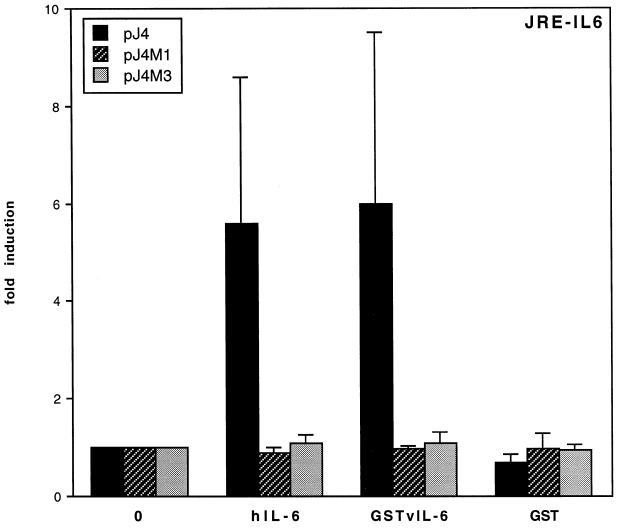

FIGURE 4 vIL-6 activation the JRE-IL-6 is mediated via the Ets and Cre-like binding elements. HepG2 cells were transfected with CAT constructs containing the JRE-IL-6 in the wild-type form (pJ4), with a mutated Ets-binding site (pJ4 M1) or a mutated Cre-like site (pJ4 M3). Transfected cells were then incubated in medium alone or medium plus IL-6 (100 ng/ml), GSTvIL-6 (1 μg/ml) or GST (1 μg/ml) for 5 h. The fold induction in CAT activities relative to unstimulated cells represents the mean ± S.D. of three separate experiments.

from the human hemopexin gene [22]. C/EBP mediated gene transcription is controlled by the MAP kinase cascade [14, 15]. Consistent with previous studies [22], we found that this site was not inducible by IL-6 alone but required treatment with IL-6 plus IL-1 (Fig. 3). Furthermore, GSTvIL-6, but not GST alone, was able to activate this Type I IL-6 RE and similarly required the presence of IL-1. In addition, as was the case in the previous experiment, 10-fold more vIL-6 than IL-6 was required for a comparable level of activation.

vIL-6 Activation the JRE-IL-6 is Mediated via the Ets and Cre-Like Binding Elements

Finally, we examined whether vIL-6 could activate JRE-IL-6. Cells were transfected with pJ4 which contains the wild-type JRE-IL6, pJ4 M1 which contains a mutated Ets-site or pJ4 M3 which contains a mutated Cre-site [16]. Figure 4 shows that treatment with vIL-6 activated the JRE-IL-6 to a level comparable to that of IL-6 stimulation. In addition, activation of JRE-IL-6 by vIL-6, like IL-6, required the presence of the two intact DNA motifs, since mutation in either site abolishes responsiveness. Furthermore, comparable with the result of the two previous experiments, vIL-6 is required in 10-fold excess of IL-6 for equivalent JRE-IL-6 activation.

vIL-6 and IL-6 Stimulate the same Pattern of Gene Expression in PBMC

To investigate whether vIL-6 was capable of stimulating alternative signaling cascades from those of IL-6, we measured the most downstream pathway event: induction of gene transcription, using a human cDNA expression array. The array contains several hundred human cDNAs comprising different classes of genes including intracellular signal transduction modulators and effectors, transcription factors, cytokines, oncogenes and tumor suppressor genes. Two arrays were probed with ^{32}P-labeled cDNA derived from unstimulated cells, stripped, and then reprobed with cDNA derived from the same cells stimulated either with vIL-6 or with IL-6. The hybridization signals obtained for polyA$^+$ RNA from vIL-6 and IL-6 stimulated cells were expressed relative to the signal on the same blot with polyA$^+$ RNA from unstimulated cells. Using this method we detected no significant difference in the pattern of gene expression induced by vIL-6 versus IL-6 (data not shown). Therefore, it appears that vIL-6 does not activate distinct signaling pathways from those of IL-6.

DISCUSSION

The results of this study indicate that there appears to be no downstream differences in the signaling pathways between vIL-6 and IL-6; response elements that are activated by IL-6 are also activated by vIL-6. In addition, data from cDNA array analysis indicates that the overall pattern of gene expression is comparable after vIL-6 versus IL-6 stimulation. Therefore, vIL-6 appears to be capable of mimicking all known IL-6 activated pathways.

Furthermore, the finding that vIL-6 displays a lower specific activity, with respect to IL-6 RE activation than IL-6, is consistent with our preliminary experiments in which vIL-6 exhibited less activity than IL-6 in a B9 proliferation bioassay (data not shown) and with the study by Burger et al. [17] who showed that their bacterially expressed vIL-6 exhibits a lower specific activity than human IL-6 in a human myeloma cell line proliferation assay. This lower specific activity is likely to be a consequence of the apparent inability of vIL-6 to bind the high affinity IL-6 Rα subunit [18]. The difference in receptor engagement between IL-6 and vIL-6 may simply reflect the evolution of an IL-6 like molecule that can deliver an "IL-6 signal" to a cell that expresses only gp130, like endothelial cells, or has downregulated the IL-6Rα subunit. Therefore, by directly mimicking IL-6, vIL-6 could potentially contribute to KSHV-related disease progression by continuing to activate IL-6-stimulated growth and anti-apoptotic pathways in cells that either express gp130 alone or have downmodulated their IL-6Rα subunits in an attempt to protect themselves from IL-6 over-stimulation.

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