The Vesicular Stomatitis Virus Matrix Protein Inhibits Glycoprotein 130-Dependent STAT Activation

Lara Terstegen, Petros Gatsios, Stephan Ludwig, Stephan Pleschka, Willi Jahnen-Dechent, Peter C. Heinrich and Lutz Graeve

*J Immunol* 2001; 167:5209-5216; doi: 10.4049/jimmunol.167.9.5209

http://www.jimmunol.org/content/167/9/5209

**References** This article cites 49 articles, 33 of which you can access for free at: http://www.jimmunol.org/content/167/9/5209.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
The Vesicular Stomatitis Virus Matrix Protein Inhibits Glycoprotein 130-Dependent STAT Activation

Lara Terstegen,* Petros Gatsios, Stephan Ludwig, Stephan Pleschka, Willi Jahnken-Dechent, Peter C. Heinrich, and Lutz Graeve

Infection of cells by vesicular stomatitis virus (VSV) results in the inhibition of host transcription. We show in this study that infection of HeLa cells with VSV leads to a strongly diminished activation of STAT3 and STAT1 by the inflammatory cytokine IL-6. This effect was mimicked by forced expression of a single viral protein, the matrix (M)-protein of VSV, which blocked STAT activation via chimeric receptors containing the cytoplasmic domain of the IL-6 signal transducer gp130. Western blot analysis revealed that VSV M-protein did not inhibit the nuclear translocation of activated STAT3 but did inhibit its tyrosine phosphorylation. Inhibition of STAT activation was not dependent on tyrosine 759 of the IL-6 signal transducer gp130, suggesting that the inhibitory action of VSV M-protein is not mediated by the induction of the suppressor of cytokine signaling 3. VSV M-protein inhibited gene transcription from cotransfected α₂-macroglobulin or antichymotrypsin promoter/luciferase reporter constructs which contain STAT3-binding sites. However, transcription from a STAT5-dependent construct was not negatively affected. In conclusion, our data suggest that infection by VSV and specifically overexpression of the viral M-protein interferes with an important signaling pathway necessary for triggering antiviral and inflammatory responses. *The Journal of Immunology, 2001, 167: S209–S216.

The Janus kinase (Jak)/STAT pathway is a major signaling pathway for numerous cytokines, growth factors, and differentiation factors. It transduces the signal from surface receptors to respective target genes in the nucleus (1, 2, 3). Four JakS (Jak1–3 and Tyk2) and seven STAT factors (STAT1–4, STAT5a, STAT5b, and STAT6) are currently known in the mammalian system (2). JakS are noncovalently associated with receptor intracellular parts and are usually activated by ligand-induced receptor homo- or heterodimerization resulting in Jak autophosphorylation. Subsequently, JakS phosphorylate tyrosine residues within the cytoplasmatic part of the receptor chains, thereby creating docking sites for SH2-domain-containing STATs (4). Upon tyrosine phosphorylation STATS dimerize and translocate to the nucleus where they act as transcription factors. The members of the IL-6-type cytokine family activate the tyrosine kinases Jak1, Jak2, and Tyk2, resulting in the recruitment and activation of STAT3 and STAT1 via the common signal transducer gp130.

In addition to STATS, other signaling molecules are also recruited to tyrosine-phosphorylated cytokine receptors. These either serve as adapter molecules to other signaling pathways, such as the mitogen-activated protein (MAP) kinase cascade (5), or exhibit a negative regulatory effect on signaling as recently described for the phosphotyrosine-phosphatase SHP-2 (6, 7) and the suppressors of cytokine signaling (SOCS) (8, 9, 10).

We recently focused our attention on mechanisms that modulate the IL-6-induced activation of the Jak/STAT pathway by cross-talk with other signaling cascades. We demonstrated in different cell types that preactivation of the MAP kinase pathway either by PMA or by fibroblast growth factor leads to an inhibition of IL-6-induced activation of the Jak/STAT pathway (11). This inhibition was paralleled by the induction of SOCS-3, a negative regulator of Jak/STAT activation. SOCS-3 can bind to a phosphorylated tyrosine module of gp130 (12, 13). This tyrosine residue 759 is crucial for the inhibitory action of activated MAP kinases (11), suggesting that PMA exhibits its modular role via induction of SOCS-3. A similar negative cross-talk is found in monocytes. LPS and TNF-α both induce SOCS-3 mRNA expression via activation of the p38 MAP kinase, resulting in the inhibition of a subsequent STAT activation by IL-6 or IFN-γ (14, 15).

Infection of cells with the cytopathic rhabdovirus vesicular stomatitis virus (VSV) results in a rapid inhibition of host RNA and protein synthesis (16). The inhibition of host transcription occurs at the level of initiation of host RNA polymerases (17). Black and Lyles (18) demonstrated that the VSV matrix (M)-protein is a potent inhibitor of the transcription of cotransfected reporter gene constructs. Furthermore, VSV M-protein inhibits transcription from chromosomally integrated HIV type 1 provirus, suggesting that the negative effect of the M-protein is not restricted to transcription from plasmids (19). VSV M-protein inhibits not only viral but also cellular promoters such as the human IFN-β promoter (20), indicating that VSV directly interferes with the antiviral response of the cell. VSV M-protein interferes with host gene transcription at the level of RNA polymerases. Thus, it has been
shown that M-protein inhibits host transcription via RNA polymerases I and II and also partially through RNA polymerase III (21). Alternatively, M-protein can interfere with host gene expression at the level of nuclear transport. It was recently demonstrated that the M-protein of VSV and of other vesiculoviruses inhibits Ran-dependent nucleocytoplasmic transport (22–25).

In this paper we analyzed whether infection of cells by the pathogenic virus VSV can also affect the Jak/STAT pathway. We found that VSV infection of HeLa cells results in a rapid and sustained inhibition of IL-6-induced STAT activation. This inhibition was also observed when the VSV M-protein was overexpressed. VSV M-protein specifically inhibited STAT3-dependent gene transcription. This inhibition was not due to a block in nuclear translocation of activated STAT3 but occurred upstream of STAT3 tyrosine phosphorylation.

Materials and Methods

Cell culture, viruses, and infection

Cells were grown in DMEM containing 10% FCS, streptokinase (100 mg/ml), and penicillin (60 mg/ml) at 5% CO₂ in a water-saturated atmosphere. Plaque-titered VSV (Indiana serotype) propagated on HeLa cells was used for infection of HeLa cells. HeLa cells grown 90% subconfluent in 10-cm dishes were washed with PBS and infected with VSV at a multiplicity of infection (moi) of 13 in PBS/BSA (PBS containing 0.2% BSA, 1 mM MgCl₂, 0.9 mM CaCl₂, 100 U of penicillin/ml, 0.1 mg of streptomycin/ml) for 30 min at room temperature. The inoculum was aspirated and cells were incubated with DMEM/BSA (medium containing 0.2% BSA and antibiotics).

DNA constructs and transfection procedures

cDNAs for STAT3 tagged with a hemagglutinin (HA) epitope, Eg. and EgY759F (EgYFYYYY) were generated as described (6, 26, 27). pGL3αs-M-215Luc contains the promoter region –215 to +8 of the rat α₂-macroglobulin (α₂-M) gene upstream of the luciferase-encoding sequence of plasmid pGL3-Basic (Promega, Mannheim, Germany) (6). pACT-357Luc contains a 246-bp upstream promoter fragment of the human α1-antichymotrypsin gene (28). SIE-Luc contains three copies of STAT3 consensus binding site linked to the thymidine kinase (tk)-promoter and a luciferase reporter gene (29). 3xk-BtkLuc contains three copies of the NF-κB motif (30). To generate tk-GL3-casein, the tk-promoter was introduced into pGL3-Basic via BamHI and BglII restriction sites. An oligonucleotide encoding for three STAT3 binding sites was introduced into tk-pGL3 via Xhol restriction sites: 5′-CTAGATTTAATCTTAAATTCGTG-3′. The VSV M-protein cDNA (kindly provided by J. Kruppa, Universitätskrankenhaus Eppendorf, Hamburg, Germany) and anti-M-protein polyclonal Ab (kindly provided by J. Kruppa, Universität, H9252/University of Wisconsin, Madison, WI) were inserted in pcDNA3.1 (Invitrogen, Breda, The Netherlands) according to the manufacturer’s instructions.

EMSA

HeLa cells were stimulated with erythropoietin (Epo; 7 U/ml) or IL-6 (200 U/ml) and sgp80 (1 μg/ml) for 15 min. Nuclear extracts were prepared as described (31). EMSAs were performed using a double-stranded [32P]-labeled mutated m679IE-oligonucleotide from the c-fos promoter (m679IE: 5′-GATCGAATCTTGAATATTTAAATCATTGATCTTAAATTCGTG-3′). The protein-DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% acrylamide and 0.25% Tris-boric acid (0.5 mM EDTA) at 20 V/cm for 4 h. Gels were fixed in 10% methanol, 10% acetic acid, and 80% water for 1 h, dried, and autoradiographed.

Immunoprecipitation

Cells were stimulated as described above, washed twice with PBS, and solubilized in 1 ml of lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM Na₃VO₄, 0.25 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 15% glycerol) for 30 min at 4°C. Insoluble material was removed by centrifugation, and cell lysates were incubated with specific Abs at 4°C for a minimum of 2 h. The immune complexes were bound to protein A-Sepharose (5 mg/ml in lysis buffer) for 1 h at 4°C. When Abs were used, rabbit anti-mouse IgG was bound to the protein A-Sepharose beads. After centrifugation, the Sepharose beads were washed three times with wash buffer (0.05% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM NaF, 1 mM EDTA, 1 mM Na₃VO₄, and 15% glycerol). The samples were boiled in gel electrophoresis sample buffer and the precipitated proteins were separated on 10% SDS-polyacrylamide gels. The following Abs were used: anti-gp130 mouse polyclonal Ab (Upstate Biotechnologie, Lake Placid, NY), anti-M-protein polyclonal Ab (kindly provided by J. Kruppa, Universität, H9252/University of Wisconsin, Madison, WI), and HA.11 mouse mAb (Berkeley Antibody, Richmond, CA).

Immunoblotting and immunodetection

The electrophoretically separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by the semidy Western blotting method. Nonspecific binding was blocked with 10% BSA in TBS-N (20 mM Tris-HCl, 150 mM NaCl, 1 mM NaF, 1 mM Na₃VO₄, 0.25 mM PMSF, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 15% glycerol) for 15 min. Nuclear extracts were separated by SDS-PAGE and blotted onto PVDF membranes. Phosphorylated and supernatants were incubated with specific antisera to either c-Jun N-terminal kinase (JNK1), p38, or extracellular signal-related kinase (ERK2) (Santa Cruz Biotechnologie, Heidelberg, Germany) and protein A-agarose for 2 h at 4°C to precipitate the endogenous kinases. Immune complexes were used for in vitro kinase assays as previously described (35). Briefly, immunoprecipitated kinases were washed twice, both in Triton X-100 (10 mM MgCl₂, 25 mM β-glycerophosphate, 25 mM HEPES (pH 7.5), 5 mM benzamidin, 0.5 mM DTT, and 1 mM Na₃VO₄). The assays were performed in kinase buffer supplemented with 5 μCl of [32P]-ATP, 0.1 mM ATP, and 1 μg of GST-c-Jun (1–155), ATF-2 (New England Biolabs), or myelin basic protein (Sigma Biochemicals, München, Germany) as substrates. JNK, p38, or ERK, respectively, at 30°C for 15 min. Proteins were separated by SDS-PAGE and blotted onto PVDF membranes. Phosphorylated substrates were detected by a Fuji BAS 2000 Bio Imaging Analyzer (Fujiﬁlm, Tokyo, Japan) and by autoradiography. Equal loading of the immunoprecipitated kinases was confirmed by Western blotting in a standard ECL reaction.

Luciferase assay

HeLa cells were grown on 60-mm dishes to 50% confluence and transfected in DMEM using SuperFect (Qiagen) with 3.5 μg of reporter construct plasmid DNA, 1.5 μg of internal control plasmid DNA pCH110 (Amersham Pharmacia Biotech), and 5 μg of the VSV M-protein expression plasmid. Cells were incubated with the precipitate for 3 h, cultured in fresh medium for at least 24 h, and stimulated with IL-6 (200 U/ml) and soluble IL-6R (0.5 μg/ml), IL-1β (100 ng/ml), or prolactin (5 μg/ml) over night. Cell lysates were precipitated and luciferase activities were measured according to the manufacturer’s instructions (Promega). Luciferase activities were normalized to β-galactosidase activities. For statistical evaluation, Student’s t test was performed. Value of p < 0.05 was considered to be significant. Values are expressed as means ± SD (n = 3); *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Results

Infection of HeLa cells with VSV inhibits IL-6-induced STAT activation

To determine whether viral infection interferes with IL-6-induced STAT activation, HeLa cells were infected with VSV, Indiana serotype (moi = 13), and cultured at 37°C for 1–4 h. To activate the Jak/STAT pathway, infected cells were stimulated at different
times with 200 U/ml IL-6 and 0.5 μg/ml soluble IL-6R for 15 min at 37°C. Although HeLa cells do express both subunits of the IL-6R complex, the agonistically acting soluble IL-6R was added to increase the STAT activation (36). After 15 min of cytokine stimulation cells were harvested and nuclear extracts were prepared. EMSAs were performed with a STAT1/3-specific probe (32, 33). Fig. 1 shows that stimulation of mock-infected cells results in a prominent broad band in the gel-shift. This band contains a mixture of STAT1, and STAT1/STAT1 (from the three different activated STAT dimers, STAT3/STAT3, STAT3/sPAT1, and STAT1/STAT1 (from top to bottom), as determined by supershift experiments with specific Abs (data not shown). Infection of HeLa cells with VSV resulted in a dramatic decrease in IL-6-induced DNA binding of nuclear STATs which was already apparent at 1 h postinfection (Fig. 1).

The VSV M-protein inhibits STAT3 activation

It was recently demonstrated that the VSV M-protein inhibits host RNA polymerases I, II, and III transcription from the human IFN-β promoter (20, 21). In addition, inhibition of the Ran GTPase-dependent nuclear transport by the VSV M-protein was reported (22–24). We therefore questioned whether expression of the VSV M-protein interferes with translocation of activated STATs into the nucleus. HeLa cells were transiently transfected with expression vectors coding for the VSV M-protein and an HA-tagged STAT3. In this instance, STAT3 activation was achieved by cotransfecting an Epo receptor/gp130 chimeric receptor construct (Eg) and stimulation with Epo. The Eg chimera consists of the extracellular domain of the Epo receptor and the transmembrane and cytoplasmic parts of gp130 (Fig. 2A, right panel) and allows us to study the activation of STAT factors in transfected cells independently of the endogenous gp130 (27). Coexpression with the VSV M-protein did not significantly affect the protein levels of either the Eg chimera (Fig. 2A, left panel) or the STAT3-HA (Fig. 2B). Cells transfected with the Eg expression vector alone displayed the typical STAT activation pattern in the gel-shift assay after stimulation with 7 U/ml Epo (Fig. 2C, lanes 1 and 2). Coexpression of M-protein in unstimulated cells did not show any effect on STAT activation (Fig. 2C, lane 3). However, coexpression of M-protein almost completely abrogated the STAT activation after Epo stimulation (Fig. 2C, lane 4). This became even more apparent when STAT3-HA was coexpressed. In this case, Epo stimulation resulted in a very prominent STAT3 activation which was blocked completely upon coexpression of the M-protein (Fig. 2C, lanes 5–9). Thus, the inhibition of STAT activation seen in VSV-infected cells can be achieved by expression of a single viral protein, the VSV M-protein.

VSV M-protein inhibits tyrosine phosphorylation of STAT3

Because it was found that the VSV M-protein inhibits nuclear transport (22–24), one possible explanation for the above results is that the M-protein interferes with the transport of activated STATs from the cytoplasm to the nucleus. We therefore analyzed the distribution and phosphorylation status of STAT3-HA in cytosolic extracts vs nuclear extracts before and after stimulation and in the absence or presence of the M-protein. HeLa cells were transfected with Eg and STAT3-HA as well as M-protein or empty vector and stimulated for 15 min with Epo. Nuclear and cytosolic extracts were prepared and immunoprecipitated with an anti-HA Ab. After SDS-PAGE, Western blots were performed using specific Abs directed against STAT3 protein as well as against the tyrosine-phosphorylated form of STAT3. Lanes 1 and 5 of Fig. 3 show that in unstimulated cells in the absence of the M-protein immunoprecipitated STAT3-HA was not tyrosine-phosphorylated and was found both in the cytosol and the nucleus. After Epo stimulation for 15 min STAT3 became tyrosine-phosphorylated and phosphorylated STAT3 was also detected in both compartments (Fig. 3, lanes 2 and 6). The increase in the STAT3 protein signal in the nuclear compartment indicates that a net transport of STAT3 into the nucleus has occurred (Fig. 3, lanes 1 and 2). Surprisingly, an increase in cytosolic STAT3 was also observed. This is probably due to an unmasking of STAT3 upon activation that increases the immunoprecipitable fraction of STAT3 (37). Coexpression of the M-protein resulted in a slight reduction of the STAT3 protein level in all compartments. Most importantly, however, it completely blocked the appearance of tyrosine-phosphorylated STAT3 in either compartment. Thus, the VSV M-protein does not block translocation of phosphorylated STAT3 into the nucleus but inhibits phosphorylation itself.

Inhibition of STAT activation by VSV M-protein does not depend on tyrosine 759 of gp130

We have recently demonstrated that PMA preincubation results in an inhibition of IL-6-induced STAT activation in different cell types (11). This inhibition was mediated via the activation of MAP kinases and was paralleled by an induction of SOCS-3 expression. It was also found that the PMA-mediated attenuation of STAT

FIGURE 1. Infection with VSV inhibits IL-6-induced STAT activation. HeLa cells were infected with VSV at an moi of 13. At different times after infection cells were stimulated with IL-6 (200 U/ml) and sgp80 (0.5 μg/ml) for 15 min. Cells were harvested and nuclear extracts were prepared as described in Materials and Methods. Nuclear extracts (10 μg) were mixed with a 32P-labeled oligonucleotide (mutated SIE probe of the c-fos promoter) and EMSAs were performed. The positions of the activated STAT homo- and heterodimers are indicated by arrows.

Inhibition of STAT activation by VSV M-protein does not depend on tyrosine 759 of gp130

We have recently demonstrated that PMA preincubation results in an inhibition of IL-6-induced STAT activation in different cell types (11). This inhibition was mediated via the activation of MAP kinases and was paralleled by an induction of SOCS-3 expression. It was also found that the PMA-mediated attenuation of STAT...
activation required tyrosine 759 of the cytoplasmic domain of gp130, which upon phosphorylation might recruit SOCS-3 to the activated receptor complex (12, 13). Using in vitro kinase assays we analyzed whether VSV infection activates MAP kinases. We found that only 1 h postinfection JNK1, p38, and ERK2 are activated in VSV-infected HeLa cells (Fig. 4A). Next we studied whether the inhibition of STAT activation by VSV M-protein is dependent on tyrosine 759 of gp130 by using a mutant Eg chimera in which the tyrosine 759 is replaced by phenylalanine. In this case, activation of endogenous STATs via the Eg chimera after Epo stimulation was detected by an EMSA. Fig. 4B shows that the inhibitory effect of VSV M-protein on STAT activation was not dependent on tyrosine 759. One should note that in this experimental setting the inhibition of STAT activation is not as strong as in STAT3 overexpressing cells. We currently have no explanation for this phenomenon. From these experiments we conclude that although MAP kinases are rapidly activated upon VSV infection, the observed inhibition appears not to involve SOCS-3, through which MAP kinases can negatively modulate the Jak/STAT pathway.

**Inhibition of STAT-dependent gene transcription by VSV M-protein**

Recent studies have suggested that VSV M-protein directly inhibits its RNA polymerases (21). Our results suggest a more specific inhibition of transactivation through the M-protein. To address this question reporter gene assays with STAT-specific promoter-reporter gene constructs were performed. Initial studies were performed with a fragment from the α2-M promoter, which contains two STAT3 binding sites (38). HeLa cells were transfected with an α2-M promoter-luciferase gene construct, a β-galactosidase plasmid as an internal control, and a VSV M-protein expression vector or an empty vector, cultured for 24 h, and stimulated overnight with IL-6 and soluble IL-6R. As shown in Fig. 5A, IL-6 stimulation led to a 15 to 20-fold induction of the reporter gene. Coexpression of VSV M-protein reduced this induction to about 5-fold. This inhibitory effect was dose-dependent (Fig. 5B). For comparison, the M-protein of influenza A virus when coexpressed did not reduce the activity of the α2-M promoter (Fig. 5A). The observed inhibitory effect of the VSV M-protein was not due to an overall inhibition of the transcription machinery, because a reporter construct containing an NF-κB-responsive promoter showed an increased IL-1β response in the presence of VSV M-protein (Fig. 5C). As recently reported, the influenza A M-protein strongly upregulated the NF-κB-dependent gene transcription (Fig. 5C) (35).
When additional STAT-dependent promoter-reporter gene constructs were analyzed, we observed a similar inhibitory effect of VSV M-protein on the IL-6 induction of the STAT3-responsive antichymotrypsin promoter (Fig. 5D, left columns). More specifically, an artificial construct containing three optimized binding sites for STAT3 (SIE-Luc) was equally inhibited (Fig. 5D, middle columns). In contrast, the prolactin-induced activation of a promoter construct containing three STAT5 binding sites of the casein promoter was not sensitive to the inhibitory action of the M-protein (Fig. 5D, right columns). Other promoters, such as the tissue inhibitor of metalloproteinases-3 promoter or the SV40 promoter, were also insensitive to the action of VSV M-protein (data not shown).

Discussion
In this study we report on a novel way in which VSV interferes with host transcription, namely by blocking the Jak/STAT pathway, an important signal transduction pathway used by many cytokines and growth factors to induce novel genes. Viral inhibition of Jak/STAT signaling has been extensively studied in the IFN system (reviewed by Cebulla and Sedmak, Ref. 39). EBV, human CMV, and adeno-, Ebola, hepatitis B and C, papilloma, and mumps viruses were all reported to inhibit STAT-dependent gene transcription (39, 40). The mechanisms by which this inhibition occurred were dependent on the virus studied. Adenovirus infection, for example, led to a decreased expression of p48 and, in some cell lines, STAT1, two components of the IFN-stimulated gene factor 3 (ISGF3) complex formed after IFN-αβ stimulation (41, 42). The viral protein responsible for this effect was identified as E1A. This protein was also reported to inhibit the IL-6-dependent activation of the junB promoter (43). The human papillomavirus E7 protein also disrupts ISGF3 formation possibly by direct binding to p48 (44). In contrast, the E6 oncoprotein of human papillomavirus-18 directly interacts with Tyk2 and thereby blocks STAT activation after IFN-α (45). Human cytomegalo virus (HCMV) infection results in reduced Jak1 protein levels, probably through increased degradation via the proteasome (46). Also, p48 protein levels were down-regulated in HCMV-infected cells (47). Inducible expression of the entire hepatitis virus C genome also inhibited the Jak/STAT pathway. In this case, both IFN-α signaling via ISGF3 and LIF-induced STAT3 activation were blocked (40). TNF-α-induced NF-κB activation was not affected. The block in STAT activation was not at the level of STAT tyrosine phosphorylation or nuclear translocation but probably at the level of STAT-DNA binding (40).

In our studies the VSV M-protein upon overexpression was capable of inhibiting Jak/STAT activation by IL-6 in a similar fashion as the viral infection, suggesting that the M-protein is the main inhibiting principle. It is theoretically possible that other viral proteins or virus-induced processes also contribute to the activation block. However, when Ferran et al. (20) tested all five VSV proteins in cotransfection studies, they found that only the M-protein is capable of inhibiting transcription from the human IFN-β promoter after stimulation of cells with double-stranded RNA. At which step of the Jak/STAT signal transduction cascade does the interference by VSV M-protein occur? In the experiments shown in Fig. 2, VSV M-protein expression had no significant effect on the receptor (Eg) or STAT protein levels. In contrast, M-protein clearly inhibits the appearance of activated tyrosine-phosphorylated STATs in the nucleus (Fig. 3). Thus, it could inhibit the translocation step or the activation itself. That VSV M-protein can affect the nuclear transport of proteins has recently been demonstrated by Her et al. (22) in the oocyte system. They injected radioactively labeled *Xenopus laevis* nuclear proteins into the...
cytoplasm of oocytes and their translocation into nuclei was measured. VSV M-protein expressed from coinjected mRNA effectively inhibited nuclear uptake of these labeled proteins. In addition, import as well as export of messenger ribonucleoprotein particles was impaired in the presence of the M-protein. Recently, the localization of VSV M-protein at the nuclear rim of the nuclear pore complex and an interaction with Nup98 was reported (23, 24). Furthermore, evidence was provided that VSV M-protein has to be within the nucleus to inhibit nucleocytoplasmic transport (23).

If M-protein inhibits the nuclear translocation of activated STAT proteins, one would expect an accumulation of STATs in the cytoplasm of oocytes and their translocation into nuclei was measured. VSV M-protein expressed from coinjected mRNA effectively inhibited nuclear uptake of these labeled proteins. In addition, import as well as export of messenger ribonucleoprotein particles was impaired in the presence of the M-protein. Recently, the localization of VSV M-protein at the nuclear rim of the nuclear pore complex and an interaction with Nup98 was reported (23, 24). Furthermore, evidence was provided that VSV M-protein has to be within the nucleus to inhibit nucleocytoplasmic transport (23).

If M-protein inhibits the nuclear translocation of activated STAT proteins, one would expect an accumulation of STAT dimers in the cytoplasm. As shown in Fig. 3, this clearly does not occur. Thus, VSV M-protein obviously directly interferes at the level of STAT tyrosine phosphorylation. This could be due to the inhibition of the respective Jak tyrosine kinase. We tried to analyze the phosphotyrosine level of Jak1 or Eg in our cotransfection system because Jak1 was shown to be necessary for the IL-6-dependent receptor phosphorylation and STAT activation (48). However, we could not detect tyrosine-phosphorylated Jak1 or Eg in Epo-stimulated cells, probably because of the low transfection efficiency. Jak overexpression would not solve this question, because this leads to constitutive (stimulation-independent) Jak and STAT activation (data not shown). Alternatively, M-protein could have an effect on the expression of crucial Jaks as was shown for HCMV-infected cells for Jak1 (46). VSV M-protein could also block STAT dimerization by directly binding to STATs in a way that interferes with tyrosine phosphorylation, but no coimmunoprecipitation of STAT1 or STAT3 with M-protein (or vice versa) was observed (data not shown). It is also possible that the M-protein activates a tyrosine phosphatase that rapidly dephosphorylates activated STATs. However, the tyrosine phosphatase SHP-2 which was shown to exhibit a negative regulatory role in IL-6 signaling is not a likely candidate, because its function is dependent on tyrosine 759 of the gp130 cytoplasmic tail and, as shown in Fig. 4B, expression of a mutant receptor lacking this tyrosine had no effect on the inhibition by M-protein. This finding also makes an involvement of SOCS-3 in this inhibition very unlikely because the inhibitory effect of SOCS-3 (at least at physiologic levels) was found to depend on Tyr759. Of course, we cannot exclude that other phosphatases or SOCS-like factors mediate the inhibitory effect of VSV M-protein. Future studies have to identify the exact molecular mechanism by which M-protein interferes with the IL-6-induced STAT activation.

Whatever the mechanism of interference with STAT3 activation is, it very clearly results in a shut-off of IL-6-dependent gene transcription (Fig. 5). Transcription via both the α2-M promoter and the antichymotrypsin promoter, as well as an artificial STAT3-responsive promoter, were severely compromised. It has been shown in the past that VSV M-protein blocks transcription of several viral and host genes (18, 20) and it was recently demonstrated that VSV M-protein interferes with the activity of transcription factor IID, also in this case by an unknown mechanism (49). However, in our transfection system not all promoter-reporter constructs tested were down-regulated. Thus, we found no significant inhibition of a STAT5-dependent reporter construct, and a promoter-reporter gene containing three NF-κB binding sites was even stimulated by M-protein coexpression (Fig. 5). Other promoters, like the tissue inhibitor of metalloproteinases-3 promoter or the SV40 promoter, were
also not affected. The latter result is in contrast to Ferrant et al. (20), who reported an inhibitory effect of M-protein on the SV40 promoter. These obviously conflicting results might be explained by the different cells used or different expression levels obtained for the M-protein. Our results indicate that the observed inhibition of the STAT3-dependent gene transcription by VSV M-protein is a STAT3-specific effect.

The Jak/STAT pathway is a major signaling pathway used by many cytokines and growth factors during development and defense against microbial invaders. Specifically, IFNs are important mediators of antiviral and antibacterial responses. Also, IL-6-type cytokines are involved in acute and chronic inflammatory processes of viral or microbial origin. Thus, by specifically inhibiting the Jak/STAT pathway, certain viruses have developed means to counteract this defensive mechanism. Understanding how they achieve this will probably enable us to develop new therapies that may help to control these pathogens more efficiently.

Acknowledgments

We thank Douglas S. Lyles for graciously supplying the mAb 23H12 directed against the VSV M-protein, Joachim Kruppa for providing VSV Indiana serotype and the rabbit antiserum against total VSV, and John K. Rose for the VSV M-cDNA. We also thank Hugues Gascan for the SIE-Luc construct, Fred Schaper for providing the Rc/CMV-EgY759F cDNA, Petra May for the tk-pGL3-casein cDNA, and Wiltrud Frisch for her technical assistance.

References

49. Yuan, H., S. Puckett, and D. S. Lyles. 2001. Inhibition of host transcription by vesicular stomatitis virus involves a novel mechanism that is independent of phosphorylation of TATA-binding protein (TBP) or association of TBP with TBP-associated factor subunits. J. Virol. 75:4453.