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Association of Mumps Virus V Protein with RACK1 Results in Dissociation of STAT-1 from the Alpha Interferon Receptor Complex

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It has been reported that mumps virus protein V or the C-terminal Cys-rich region of protein V (Vsp) is associated with blocking of the interferon (IFN) signal transduction pathway through a decrease in STAT-1 production. The intracellular target of the V protein was investigated by using a two-hybrid screening system with Vsp as bait. Full-length V protein and Vsp were able to bind to RACK1, and the interaction did not require two WD domains, WD1 and WD2, in RACK1. A significant interaction between V protein and RACK1 was also demonstrated in cells persistently infected with mumps virus (FLMT cells), and the formation of the complex was not affected by treatment with IFN. On the other hand, in uninfected cells, STAT-1 was associated with the long form of the β subunit of the alpha IFN receptor, and this association was mediated by the function of RACK1 as an adaptor protein. Immunoprecipitation and glutathione *S*-transferase pull-down experiments revealed that the association of RACK1 or mumps virus V protein with the IFN receptor was undetectable in mumps virus-infected cells. Furthermore, RACK1 interacted with mumps virus V protein with a higher affinity than STAT-1 did. Therefore, it is suggested that mumps virus V protein has the ability to interact strongly with RACK1 and consequently to bring about the disruption of the complex formed from STAT-1, RACK1, and the IFN receptor.

Many functions of interferon (IFN) are induced by activation of the JAK (Janus protein kinase)/STAT (proteins in the family of signal tranducers and activators of transcription) signaling pathway. In the alpha IFN (IFN-α) signaling pathway, STAT-2 and STAT-1α are phosphorylated by IFN-α to form the IFN-stimulated gene factor 3 complex through activation of the tyrosine kinases Jak-1 and Tyk-2. The IFN-stimulated gene factor 3 complex formed from the STAT-1α, STAT-2, and IFN regulatory factor-9 (IRF-9) components strongly binds to the consensus sequence of the IFN-α-stimulated response element in the promoter region of the IFN-stimulated gene. Gamma interferon (IFN-γ) mediates the phosphorylation of STAT-1α to form a homodimer, the IFN-γ-activated factor complex, through the activation of Jak-1 and Jak-2 caused by tyrosine phosphorylation. The IFN-γ-activated factor complex binds to the IFN-γ-activated sequence element in the regulatory region of IFN-γ-inducible genes. Tyrosine kinase Jak-1 and STAT-1α are the essential components of the IFN-α and IFN-γ signaling pathway.

The antiviral activity of IFN, whose function is largely due to the intracellular induction of 2′,5′-oligoadenylate synthetase, double-stranded RNA-activated protein kinase, and Mx protein through the JAK/STAT pathway, is the most important defense mechanism against viral infection. However, it has been reported that some viruses have the ability to break down IFN functions through suppression of the IFN signal transduction pathway and to inhibit antiviral protein or enzyme, 2′,5′-oligoadenylate synthetase, or double-stranded RNA-activated protein kinase activity (4, 9, 10, 11).

Evidence showing a decrease in the basal expression of STAT-1α and the dysfunction of the response to IFN-α and IFN-γ in cells persistently infected with mumps virus (MuV) has been reported (7, 12, 21). Furthermore, Didcock et al. reported that the expression of simian virus 5 structural protein V induces the proteosome-mediated degradation of STAT-1α (3). The expression of MuV structural protein V (MuV-V) or the C-terminal Cys-rich region of protein V (MuV-Vsp) inhibited the establishment of the antiviral state induced by IFN-α and IFN-γ (13). This inhibition was caused by a decrease in STAT-1α production and a subsequent suppression of the IFN signal transduction pathway. Investigation of the effect of proteosome inhibitor MG132 on the levels of STAT-1 showed that it was able to protect STAT-1α from degradation, even though the levels after recovery were lower than the basal levels in control cells. These results indicate that STAT-1α disappeared as a result of the function of MuV-V. It is therefore very important to determine which intracellular molecules or proteins are involved in these events through their association or interaction with MuV-V. In this article, we report the interaction of MuV-V with RACK1.

MATERIALS AND METHODS

Cells. Human amnion cells (FL), human lymphoblastoid cells of B-cell origin (Akata cells), and their counterparts persistently infected with MuV (FLMT cells and Akata-MP cells, respectively) were cultured and maintained as described previously (7, 21).

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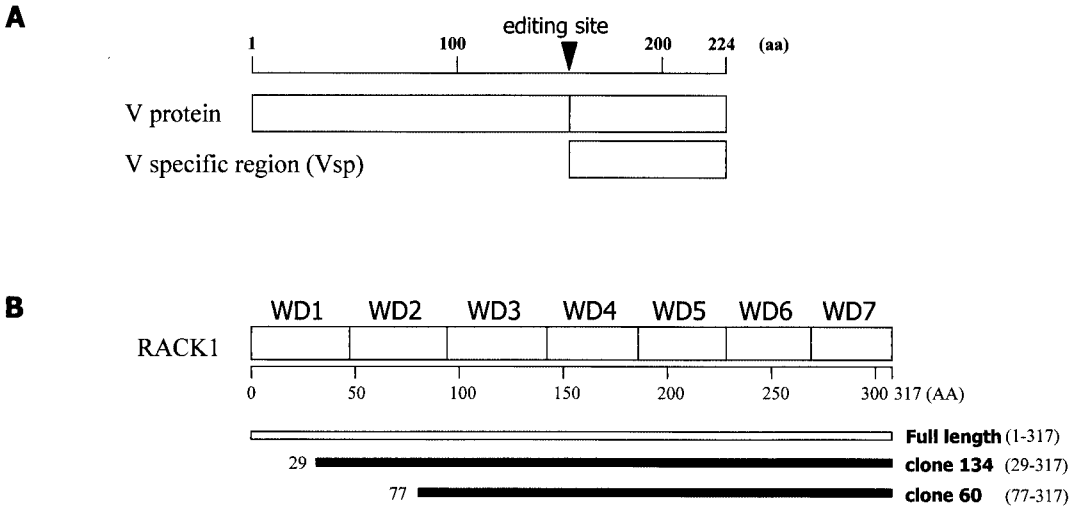

FIG. 1. Diagrams of MuV-V (A) and RACK1 (B). (A) The position of the RNA editing site is indicated. Vsp, which does not contain P protein, was used in a two-hybrid screening as bait. (B) The positions of the seven WD repeats are indicated. Full-length RACK1 (white box) and two overlapping RACK1 clones (black boxes), clone 60 and clone 134, are indicated. AA, amino acids.

Antibodies. Rabbit polyclonal antibodies against STAT-1 p84/p91 (E-23) and STAT-2 p133 (C-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Mouse antibody against RACK1 (immunoglobulin M; clone 20) was obtained from Transduction Laboratories (San Diego, Calif.). Rabbit antibody against MuV-V was provided by A. Kato and K. Takeuchi (National Institute of Infectious Diseases, Tokyo, Japan).

Yeast two-hybrid screening. To construct the pAS2-1 bait plasmid, cDNA corresponding to the C-terminal region (amino acids [aa] 154 to 224) of MuV-V (Fig. 1A) was generated by PCR and cloned into Gal4 DNA binding domain plasmid pAS2-1 (Clontech, Palo Alto, Calif.). Yeast host strain AH109 was cotransformed with both the pAS2-1 bait plasmid and the pACT2 library plasmid (K562 cDNA library construct in pACT2; Clontech). To screen the K562 library, positive clones were initially selected on the basis of growth efficiency in the absence of histidine and adenine (without 3-aminotriazole). After transfer of the clones to patches, positive clones were identified by MEL1 reporter activity with 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-Gal) spread plates. The inserts from positive library plasmids were isolated and then sequenced to confirm their original genes.

For additional two-hybrid experiments, the full open reading frame (ORF) of RACK1 (Fig. 1B) and the full ORF of MuV-V (Fig. 1A) were cloned into pACT2 and pAS2-1, respectively. The ORFs of two mutant V proteins (Vc189a and Vc207a; single amino acid substitution of cysteine with alanine at positions 189 and 207, respectively) (Table 1) were also cloned into the pAS2-1 bait plasmid. The RACK1 clones were tested against the V and Vm proteins and two other, unrelated bait plasmids (pLAM5′-1, which encodes a DNA binding domain fusion with human laminin C, and pVA3-1, which encodes a DNA binding domain fusion with murine p53) as negative controls. The V proteins were also tested against pTD1-1 (which encodes an activator domain fusion with simian virus 40 large T antigen).

TABLE 1. Interaction of MuV-V with RACK1 in a yeast two-hybrid system[a]

Bait	β-Galactosidase activity with the following activator domain (amino acids):			
	RACK1 (1–317)	Clone 134 (29–317)	Clone 60 (77–317)	pTD1-1
Full-length V	+	+	+	−
Vsp	+	+	+	−
Vc189a	+	+	+	−
Vc207a	+	+	+	−
pLAM5′-1	−	−	−	ND
pAV3-1	−	−	−	ND

[a] MuV-V, MuV-Vsp, and two mutants (Vc189a and Vc207a) were fused to the activator domain and used as bait. The presence (+) or absence (−) of β-galactosidase activity is indicated. ND, not determined.

Immunoprecipitation experiments. FL and FLMT cells were cultured for various times with or without 1,000 IU of IFN-α (Serotec, Oxford, United Kingdom)/ml, and cytoplasmic protein extracts were prepared for analysis of protein-protein interactions among RACK1, V protein, and STAT-1 with appropriate antibodies as described previously (22).

Expression of GST fusion or His_6-tagged proteins. For the construction of a glutathione *S*-transferase (GST) fusion protein expression vector, DNA fragments corresponding to MuV-V, MuV-Vsp, RACK1, or the cytoplasmic region of the long form of the β subunit of the IFN-α receptor (IFN-αRβL) chain (IFNαRβLc) were prepared by PCR and then cloned into an appropriate pGEX6p vector (Amersham Pharmacia Biotech, Piscataway, N.J.). The expression vectors for His_6-tagged proteins were constructed by using the MuV-V, MuV-Vsp, or RACK1 inserts described above and pQE31 or pQE32 (Qiagen, Santa Clarita, Calif.). *Escherichia coli* strain DH5α transformed with each expression vector was grown in 100 ml of Luria-Bertani medium to an optical density at 600 nm of 0.5 and then treated with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h. The bacterial pellets were harvested by centrifugation, washed, resuspended in radioimmunoprecipitation assay buffer (50 mM HEPES [pH 7.5], 4 mM EDTA, 150 mM NaCl, 1% Nonidet P-40), and sonicated for 10 min. After the removal of cell debris, GST fusion proteins and His_6-tagged proteins were purified with a GSTrap FF column (Amersham Pharmacia Biotech) and a HisTrap kit (Amersham Pharmacia Biotech), respectively. The purified recombinant proteins were incubated with protein A-Sepharose beads to remove nonspecifically binding proteins. The supernatants separated from the beads were subjected to immunoprecipitation analysis.

GST pull-down experiments. Cell extracts from Akata or Akata-MP cells were incubated with preequilibrated glutathione-agarose beads for 1 h at 4 C. After removal of the beads, the supernatants were incubated with GST-fused IFN-αRβLc immobilized on glutathione-agarose beads for 3 h at 4°C, and then the beads were collected and washed. The complex trapped on the beads was eluted in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer (21a) and analyzed by immunoblotting with anti-RACK1 antibody or anti-STAT-2 antibody.

RESULTS

Two-hybrid screening and interaction between MuV-V and RACK1 in the yeast system. To investigate the cell-specific target protein for MuV-V, a human erythroleukemia cell line

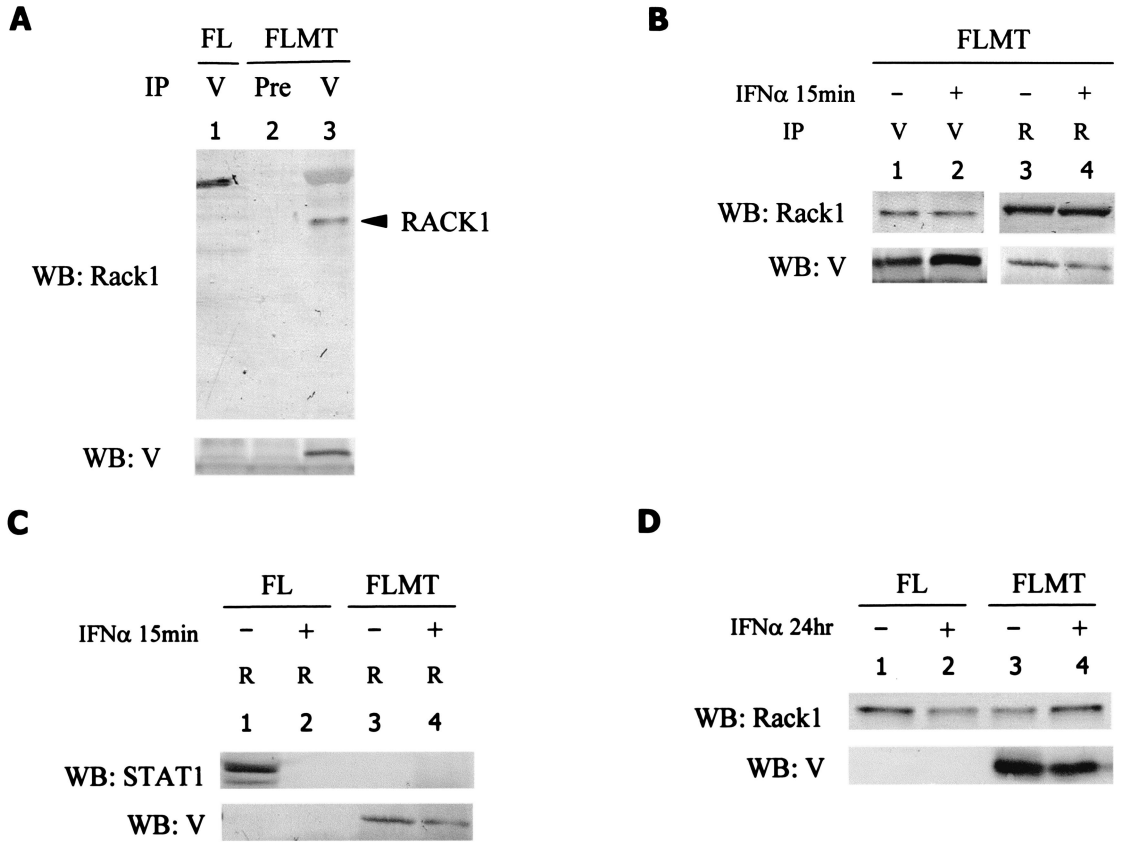

FIG. 2. Association between RACK1 and MuV-V in MuV-infected cells. (A) Whole-cell lysates of FL cells (lane 1) and FLMT cells (lanes 2 and 3) were immunoprecipitated (IP) with anti-V protein antibody (lanes 1 and 3) or preimmune serum (Pre) (lane 2) and detected by Western blot (WB) analysis with anti-RACK1 antibody (upper panel) or anti-V protein antibody (lower panel). (B) FLMT cells were treated (+) or not treated (−) with IFN for 15 min, and then cell lysates were immunoprecipitated with anti-V protein antibody (V) (lanes 1 and 2) or anti-RACK1 antibody (R) (lanes 3 and 4) and subjected to immunoblotting with anti-RACK1 antibody (upper panel) or anti-V protein antibody (lower panel). (C) FL cells (lanes 1 and 2) and FLMT cells (lanes 3 and 4) were treated (+) or not treated (−) with IFN for 15 min, and then cell lysates were immunoprecipitated with anti-RACK1 antibody and analyzed by Western blotting with anti-STAT-1 antibody (upper panel) or anti-V protein antibody (lower panel). (D) FL cells (lanes 1 and 2) and FLMT cells (lanes 3 and 4) were treated (+) or not treated (−) with IFN for 24 h, and then the levels of production of RACK1 (upper panel) and MuV-V (lower panel) were investigated by immunoblotting.

(K562) cDNA library was screened with the cDNA of a fusion of MuV-Vsp with the Gal4 DNA binding domain as bait (Fig. 1A). Three different clones were isolated from a number of positive yeast clones because of a strong binding signal and expected functions. One clone (clone 177) encoded the C-terminal region of Nmi. Sequence analysis followed by database research for the other two clones revealed that the cDNA fragments of the clones encoded the C-terminal 289 aa (clone 134) and 241 aa (clone 60) of the previously described receptor for activated C kinase (RACK1) (Fig. 1B).

It has been reported that RACK1 is a 36-kDa protein that has seven repeating units of Trp-Asp (WD) motifs and that mediates the interaction between the human IFN-α receptor and STAT-1 (2, 14, 17, 20). Therefore, it has been suggested that RACK1 plays an important role in early signaling through the JAK/STAT pathway.

In order to clarify the role of MuV-V in the decrease in STAT-1 production and the dysfunction in the IFN signal transduction pathway, the specificity of the interaction between MuV-V and RACK1 was examined by using pairs of different plasmids (Table 1). Transformed AH109 strains were cultured and the stringency of the identified interactions was analyzed as described in Materials and Methods. All four clones derived from MuV-V specifically bound to full-length RACK1 and to the RACK1 clones (clone 60 and clone 134) isolated from the two-hybrid screening (Table 1).

Immunoprecipitation analysis of the interaction between MuV-V and RACK1 in MuV-infected cells. To confirm the interaction between MuV-V and RACK1 observed in the yeast system, immunoprecipitation analysis was carried out with cell lysates from MuV-infected cells (FLMT cells) and anti-RACK1 or anti-MuV-V antibody. As shown in Fig. 2A, the anti-MuV-V antibody coimmunoprecipitated RACK1 in FLMT cells. This interaction was specific because there was no detection by preimmune serum (Fig. 2A) and it was not affected by stimulation with IFN (Fig. 2B). The anti-RACK1 antibody also coimmunoprecipitated MuV-V from lysates of FLMT cells (Fig. 2B). However, STAT-1– RACK1 or STAT-1– MuV-V interactions were impossible to study in FLMT cells in this assay due to the low levels of STAT-1 in the

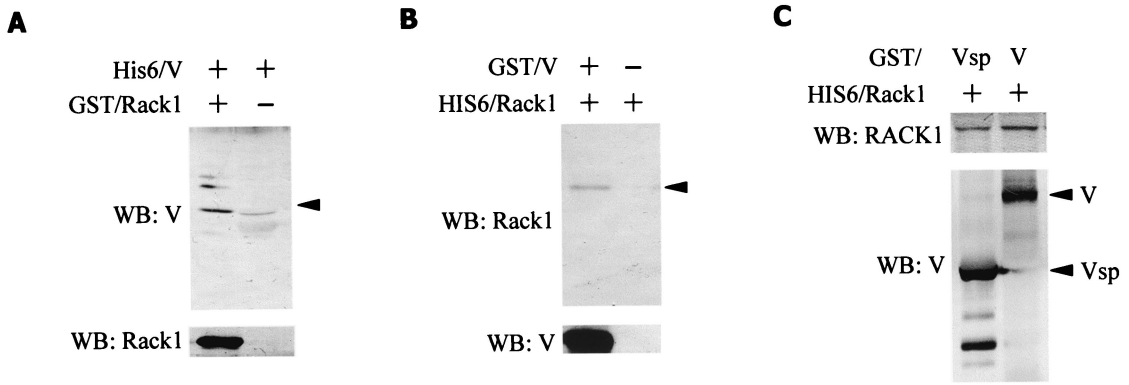

FIG. 3. Interaction of MuV-V with RACK1 in vitro. GST-fused RACK1 (A), GST-fused MuV-V (B and C), and GST-fused MuV-Vsp (C) were incubated with His_6-tagged MuV-V (A) and His_6-tagged RACK1 (B and C). Each mixture was pulled down by using glutatione-agarose beads, and MuV-V, MuV-Vsp, and RACK1 were detected by immunoblotting (Western blotting [WB]).

cells (13, 21). On the other hand, the interaction between RACK1 and STAT-1 was demonstrated in virus-uninfected FL cells (Fig. 2C). Treatment of FL cells with IFN for 15 min resulted in a great reduction in the association because of the phosphorylation of STAT-1 (Fig. 2C). The results obtained here with FL cells are consistent with those reported by Usacheva et al. (20). Incubation of FL and FLMT cells with 1,000 IU of IFN/ml for 24 h had no effect on the constitutive level of production of RACK1 (Fig. 2D).

The interaction between MuV-V and RACK1 was also recognized in an in vitro binding assay system with recombinant protein expressed in *E. coli*. GST fusions with RACK1 (full length), MuV-V, and MuV-Vsp were immobilized on glutathione-agarose beads and incubated with His_6-tagged MuV-V (Fig. 3A) or His_6-tagged RACK1 (Fig. 3B and C). Proteins binding to RACK1, MuV-V, or MuV-Vsp were pulled down with glutathione-agarose beads. Figure 3 shows that GST-RACK1 and GST MuV-V (or GST MuV-Vsp) were capable of binding to His_6-tagged MuV-V (or His_6-tagged MuV-Vsp) and His_6-tagged RACK1, respectively.

The association between IFNαRβL and RACK1 is affected by MuV infection. We next constructed a GST fusion with the IFN receptor to analyze the role of MuV-V in the association between the IFN receptor and RACK1. cDNA fragments of the cytoplasmic region of IFNαRβLc were generated by PCR and then cloned into a pGEX6p vector. GST-IFNαRβLc was purified and incubated with lysates from Akata cells (non-MuV-V-producing cells) or Akata-MP cells (no detection of STAT-1). After incubation of the mixtures, proteins binding to IFNαRβLc were pulled down with glutathione-agarose beads and analyzed by Western blot analysis. As shown in Fig. 4, IFNαRβLc interacted with RACK1 (lane 2), STAT-1 (lane 5), and STAT-2 (lane 8) in lysates from uninfected control Akata cells. On the other hand, the cytoplasmic domain of IFNαRβLc associated only with STAT-2 (Fig. 4, lane 9) and not with RACK1 (lane 3), STAT-1 (lane 6), or MuV-V (lane 11) in Akata-MP cell lysates. Therefore, it is reasonable to speculate that MuV-V promotes disruption of the IFN receptor RACK1 interaction by binding to RACK1 with a high affinity, resulting in the subsequent release of STAT-1 from the IFN receptor.

Influence of MuV-V on the association between RACK1 and STAT-1 in the in vitro system. RACK1 is thought to act as an adaptor between STAT-1 and the IFN receptor (2, 20). The interaction is critical for the IFN signal transduction pathway. A drastic decrease in or disappearance of STAT-1 production has been reported in MuV-infected cells (7, 21). Therefore, a stable IFN receptor complex containing RACK1 and STAT-1

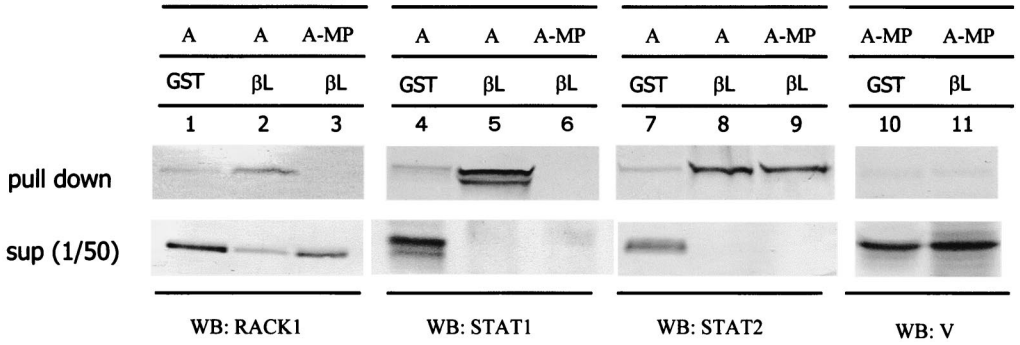

FIG. 4. Effect of MuV infection on the association between IFN-αRβL and RACK1. A GST fusion protein containing the entire cytoplasmic domain of IFN-αRβLc (βL) was used to pull down RACK1 (lanes 2 and 3), STAT-1 (lanes 5 and 6), STAT-2 (lanes 8 and 9), and MuV-V (lane 11) from lysates obtained from Akata cells (A) (lanes 1, 2, 4, 5, 7, and 8) or Akata-MP cells (A-MP) (lanes 3, 6, 9, 10, and 11). GST alone was used as a negative control. WB, Western blotting. Sup(1/50), 1/50 volume of supernatant after pull-down experiment.

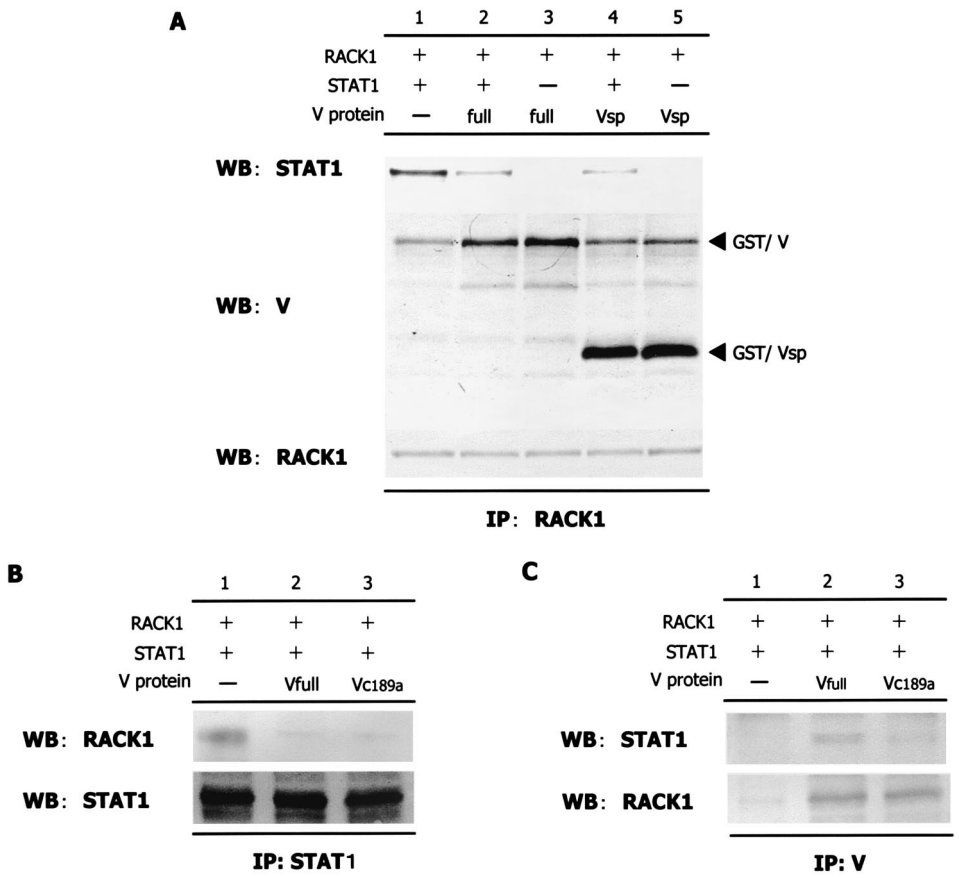

FIG. 5. Influence of MuV-V on the association between RACK1 and STAT-1 in vitro. (A) His_6-tagged STAT-1 (lanes 1, 2, and 4), GST-fused MuV-V (lanes 2 and 3), GST-fused MuV-Vsp (lanes 4 and 5), and GST-fused RACK1 (lanes 1 to 5) were mixed as shown and coimmunoprecipitated (IP) with anti-RACK1 antibody. Precipitates were subjected to immunoblotting (Western blotting [WB]) with anti-STAT-1, anti-MuV-V, and anti-RACK1 antibodies. (B and C) His_6-tagged STAT-1, GST-fused RACK1, GST-fused MuV-V, and GST-fused Vc189a were mixed as shown and coimmunoprecipitated with anti-STAT-1 antibody (B) or anti-MuV-V antibody (C). RACK1 and STAT-1 in the precipitates were investigated by Western blotting analysis with anti-RACK1 antibody or anti-STAT-1 antibody.

may be modified in the presence of MuV-V. In other words, MuV-V may affect the interaction between RACK1 and STAT-1 and consequently provide modulation of complex formation with RACK1, STAT-1, and IFNαRβL. It has been postulated that MuV-V interacts with RACK1 with a high affinity and causes resultant disruption of the complex formed with the IFN receptor, RACK1, and STAT-1. This possibility was supported by the results of pull-down experiments with GST-IFNαRβLc (Fig. 4).

We therefore investigated fluctuations in the levels of association between RACK1 and STAT-1 in the presence of MuV-V. The anti-RACK1 antibody coimmunoprecipitated STAT-1 (Fig. 5A, upper panel, lanes 1, 2, and 4), Muv-V (lane 2), and MuV-Vsp (lane 4) from the protein mixture. A larger amount of STAT-1 was coimmunoprecipitated by the anti-RACK1 antibody in the absence of MuV-V (Fig. 5A, lane 1) than in the presence of MuV-V (lane 2). Almost identical results were obtained in the experiment with MuV-Vsp (Fig. 5A, lane 4). Furthermore, the presence of IFNαRβLc hardly affected the interaction of MuV-V with RACK1 and STAT-1 indicated in Fig. 5A (data not shown). This result is consistent with the data shown in Fig. 4. Therefore, it was suggested that the interaction between RACK1 and STAT-1 was diminished in the presence of MuV-V or MuV-Vsp. This suggestion was clearly confirmed by immunoprecipitation analysis with the anti-STAT-1 antibody. As shown in Fig. 5B, MuV-V and mutant MuV-V (Vc189a) significantly inhibited the association of STAT-1 with RACK1 (Fig. 5B, compare lanes 1 and 2 or 3). Furthermore, in the presence of RACK1, mutant MuV-V (Vc189a) was able to bind to RACK1 but poorly to STAT-1 (Fig. 5C). These results are consistent with evidence showing that mutant MuV-V could not promote the degradation of STAT-1 (21a).

DISCUSSION

Evidence derived from the suppression of IFN-stimulated gene expression by MuV highlights the possibility of modulation or dysfunction of the IFN signal transduction pathway (5, 6). Indeed, MuV inhibits the establishment of the IFN-induced antiviral state and the expression of various IFN-regulated genes by blocking of IFN signaling. The blocking is caused by a decrease in the constitutive level of STAT-1 (7, 21). MuV-V or MuV-Vsp plays a critical role in the decrease in STAT-1

production or the proteosome-mediated degradation of STAT-1 (13).

In this study, we identified the intracellular target protein of MuV-V as RACK1 by using a yeast two-hybrid screening system with a cDNA library of K562 cells. MuV-V interacted with RACK1 with a high affinity. This is the first report to identify the target protein of MuV-V. RACK1 has a molecular mass of 36,000 Da and contains seven repeats of tryptophan and aspartic acid (WD) motifs that resemble the structure of the β subunit of G protein (16, 17). It is clear that RACK1 acts as an adaptor protein to modulate signaling from protein kinase C, Src tyrosine kinase, and β-integrin in various systems (1, 14, 16). Furthermore, RACK1 interacts with various virus proteins or components. Smith et al. reported that A73 protein translated from CST (BART BARF0) transcript RNA of Epstein-Barr virus is able to interact with cellular RACK1 and modulate signaling from protein kinase C and Src tyrosine kinase to regulate cancer growth, such as nasopharyngeal carcinoma (19). It has also been noted that RACK1 acts as a human immunodeficiency virus Nef intracellular docking site, bringing Nef and protein kinase C together (a Nef-protein kinase C adaptor) (8). Sang et al. demonstrated that RACK1 physically interacts with adenovirus E1A and acts as a repressor of E1A, possibly by antagonizing the effects of E1A on host gene transcription (18).

Recently, Croze et al and Usacheva et al. revealed that RACK1 is able to interact with the IFN receptor and STAT-1 (2, 20). RACK1 specifically binds to the cytoplasmic domain of IFNαRβL in a ligand-independent manner without tyrosine phosphorylation. The minimum site of the receptor for RACK1 binding was mapped to aa 300 to 346, close to the proposed Jak-1 binding site. RACK1 binding to IFNαRβL did not require two WD domains, WD1 and WD2 (Fig. 1B). Furthermore, it has also been demonstrated that the interaction between the human IFN-α receptor and STAT-1 is not direct but is mediated by RACK1.

In the early steps of the IFN signal transduction pathway, after binding of IFNs to their receptors, members of the JAK family are activated to phosphorylate STAT-2 and STAT-1. Therefore, STAT-1 activation and IFN signaling are mainly dependent on the establishment of the association between the IFN receptor and STAT-1. Usacheva et al. reported that RACK1 constitutively interacts with nonphosphorylated STAT-1 and functions as an adaptor for this factor and IFNαRβL (20). Therefore, disruption of the complex formed by these three components, probably by release of RACK1 from the IFN receptor, abolishes not only IFN-induced tyrosine phosphorylation of STAT-1 but also activation of STAT-2.

We demonstrate here that MuV-V is able to interact with RACK1 with a high affinity, but not with IFNαRβL. The RACK1 site for MuV-V binding is estimated to be in the region including WD3 to WD7, which is the same region involved in binding to the IFN receptor. It is possible that the same site or region in RACK1 is competitively recognized by MuV-V and IFNαRβL. In other words, MuV-V competes with the IFN receptor for an interaction with RACK1. As a result of MuV-V binding to RACK1, RACK1 is dissociated from the IFN receptor. Furthermore, Fig. 5A showed that MuV-V competed with RACK1 for an association with STAT-1, and the capacity of STAT-1 for binding to RACK1 was significantly diminished (Fig. 5). We therefore suggest that the function of RACK1 as an adaptor for STAT-1 and the IFN receptor is inhibited by the binding of MuV-V to RACK1. It was also indicated, as shown in Fig. 5C, that STAT-1 was able to interact with MuV-V instead of RACK1. This result is consistent with the reports of Parisien et al. (15) and Yokosawa et al. (21a). It was also recently found that the ubiquitination and degradation of STAT-1 are correlated with complex formation between MuV-V and STAT-1 (21a). Taken together, these data suggest that, in the presence of MuV-V, STAT-1 is not able to interact with the IFN receptor and RACK1 and subsequently becomes an unstable molecule. This event is followed by complex formation by STAT-1 and MuV-V (Fig. 5C) and by ubiquitination through the proteosome pathway. These events may be the reason for the decrease in STAT-1 production in MuV-infected cells.

At least two cysteine residues (positions 189 and 207) in MuV-Vsp did not contribute to the efficiency of the interaction with RACK1 (Table 1 and Fig. 5). However, one of the single amino acid substitutions of a cysteine residue with alanine in the Cys-rich region, Vc189a, drastically reduced the level of proteosome-mediated degradation of STAT-1, and the mutation in the V protein reduced the efficiency of its binding to STAT-1 in a cellular system (21a) and in an in vitro mixture experiment (Fig. 5C). The interaction between MuV-V and STAT-1 is undetectable in the absence of a proteosome inhibitor because of the disappearance of STAT-1 in virus-infected cells. These results suggest the possibility that MuV-V interacts with RACK1 at a site other than Cys-rich region but that the interaction between MuV-V and STAT-1 is, at least in part, dependent on the Cys-rich region. In other words, cysteine residues in the Cys-rich region may be necessary for the formation of a more stable complex of MuV-V and STAT-1. Furthermore, in the absence of RACK1, MuV-V and mutant MuV-V are able to interact with STAT-1 to the same extents (data not shown), although a poor association between Vc189a and STAT-1 was demonstrated in the presence of RACK1 (Fig. 5C). The efficiency of binding of mutant MuV-V to STAT-1 is affected by the presence of RACK1. However, the reason for this finding awaits further studies.

From the evidence described here, we suggest that the formation of a complex consisting of RACK1, STAT-1, and MuV-V is rather unexpected because both the RACK1 and the STAT-1 binding sites for MuV-V (MuV-Vsp) are close to each other. Finally, the complex formed from nonphosphorylated STAT-1 and MuV-V could be ubquitinated and degraded through the proteosome degradation pathway.

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