武田薬品における麻しん、風しん及びおたふくかぜワクチン株のシードウイルスの設定

分担研究者　仁田義弘　武田薬品工業（株）生物製剤部 部長
協力研究者　末原章宏　武田薬品工業（株）同 生物技術グループ マネジャー
協力研究者　渡辺秀夫　武田薬品工業（株）同 生物第一グループ マネジャー

研究要旨　麻しんワクチン、風しんワクチン及びおたふくかぜワクチン製造用ウイルス株の「シードロットシステム」導入のため、マスターシードウイルス及びワーキングシードウイルスの設定について検討を行った。マスターシードウイルス、ワーキングシードウイルス及びワーキングシードウイルスを 1 代継代して製造する原液は、いずれのウイルス株についてもオリジナルワクチンから 5 代以内の継代歴で製造が可能であるが、市場への製品供給の観点から、「シードロットシステム」移行までの措置として、マスターシードウイルスから 1 代継代して製造した原液の一部をワーキングシードウイルスとして確保することが現実的と考えられる。また、マスターシードウイルス及びワーキングシードウイルスの品質管理法などについては今後の検討課題となる。

A．研究目的

生ワクチンの製造に用いるウイルス株は、生物学的製剤基準に準じて、製造承認ウイルスから 4 代までをワーキングシードウイルスとして使用するよう管理しているが、ワーキングシードが消費された段階で、1 代継代培養して次のワーキングシードウイルスを作製していることを昨年度報告した。

「シードロット導入」を実現するため、長期的に実行可能なマスターシードロット及びワーキングシードロットを設定することを、今年度の研究目的とした。

B．研究方法

生物学的製剤基準で規定されている継代数を超えない範囲でワーキングシードウイルスの再作製が必要となるが、シードロットシステムを構築するため、WHO の勧告及び製造に用いるウシ血清の規制(薬食発第0218004 号)を踏まえ、マスターシードウイルス及びワーキングシードウイルスの製造、保管量並びに運用方法について検討した。

C．研究結果

1．マスターシードウイルス、ワーキングシードウイルス及び原液の継代歴

麻しんウイルス（シュワルツ FF-8 株）

オリジナルワクチンより 3 代継代して作製するウイルス液をマスターシードとし、4 代継代して作製するウイルス液がワーキングシードとして設定することとなり、原液はオリジナルワクチンから 5 代以内の継代となる。

風しんウイルス（TO-336 株）

オリジナルワクチンより 2 代継代して作製するウイルス液をマスターシードとし、3 代継代して作製するウイルス液がワーキングシードとして設定されることになり、原液はオリ

ジナルワクチンから 5 代以内の継代となる。

おたふくかぜウイルス（鳥居株）

オリジナルワクチンより 1 代継代して作製するウイルス液をマスターシードとし、2 代継代して作製するウイルス液がワーキングシードとして設定されることになり、原液はオリジナルワクチンから 5 代以内の継代となる。

2. マスターシードウイルス及びワーキングシードウイルスの保管量及び運用方法

現在、ウシ血清の規制(薬食発第0218004 号)に伴い、区分 C に分類される製造用種ウイルスを BSE 発生以前のものを使用して、ワクチン原液の製造を行っている。麻しんウイルス株及び風しんウイルス株については、これらワクチン原液の一部をマスターシードウイルスとして既に確保している。マスターシードウイルスの保管量は、麻しんウイルス株で 4000L 以上、風しんウイルス株で 1800L 以上のワーキングシードウイルスを作製可能とするものである。また、おたふくかぜウイルス株については、マスターシードウイルスとして作製を行った。その保管量は、2000L 以上のワーキングシードウイルスを作製可能とするものである。

ワーキングシードウイルスについては、いずれのワクチン株も未だ確保していないが、シードロットへ移行するまでは上述したウシ血清規制及び市場への製品供給の観点から、マスターシードウイルスと同様の考え方に基づき、マスターシードロットから製造したワクチン原液の一部を、第 1 ワーキングシードウイルスのロットとして確保することを想定している。

D．考察

シードロット導入のため、麻しんワクチンウイルス株(シュワルツ FF-8 株)、風しんワクチンウイルス株(TO-336 株)及びおたふくかぜワクチンウイルス株(鳥居株)のマスターシードウイルス並びにワーキングシードウイルス設定の検討を行った。

いずれのウイルス株についてもオリジナルワクチンから 5 代以内の継代歴でワクチン原液製造が可能となり、マスターシードウイルスに関しては、いずれのワクチン株についても確保を完了した。

WHO の勧告のようにワーキングシードを単一な構成とし大量に調製すると、製造に用いる生物由来原料などの変更が生じた場合、保管している大量のワーキングシードウイルスの廃棄などが懸念される。このようなリスクを回避するためには、第 1 番目のワーキングシードウイルスが消費された段階で、マスターシードウイルスから新たに第 2 番目のワーキングシードウイルスを作製することが、現実的であると考えられる。

シードウイルスの品質管理として、ワーキングシードウイルスについては、現在、国家検定に合格したワクチン原液の一部を採取して保管することを想定しているが、シードロット導入後は、サル接種試験を除く生物学的製剤基準で規定されている品質項目を自家試験で確認することで問題ないのか等、今後の検討課題となる。

E．結論

・麻しん、風しん及びおたふくかぜワクチン株のマスターシードウイルスを確保した。
・ワーキングシードウイルスの確保は、シードロット移行時期を見据

えて確保する予定である。

F．健康危険情報

特になし。

G．研究発表

1．論文発表
なし。

2．学会発表
なし。

H．知的財産権の出願・登録状況
（予定を含む。）

1．特許取得
なし。

2．実用新案登録
なし。

3．その他
なし。

シードロットシステムに基づく ワクチン製造株のマスターシード設定について

分担研究者　李　富雄　　北里研究所　生物製剤研究所　部門長
協力研究者　佐々木 学　　北里研究所　生物製剤研究所　部門長
協力研究者　服部 信章　　北里研究所　生物製剤研究所　部門長

研究要旨　弱毒生ワクチン製造用株のシードロットシステム導入のためにワクチン製造株の樹立、製造承認株、製造用株の継代歴及び保管状況、現在までのワクチン製造について調査し、マスターシードの設定を検討した。その結果、麻しんワクチン AIK-C 株は製造承認株から１代継代、風しんワクチン高橋株は２代継代、おたふくかぜワクチン星野株は２代継代のシードウイルスをマスターシードとして設定する。今後、これらのマスターシードから１代継代してワーキングシードを作製し、これら製造用株の管理方法を検討する。

A．研究目的

本研究では、製造用株から製造される弱毒生ワクチンが一定品質のものが安定的に供給されるために、日本の生物学的製剤基準のもとで、WHO 生物製剤基準のシードロットシステムの考えを導入したものを基準として、マスターシードの設定を検討した。また、ワーキングシードの設定及び継代管理について検討を加えた。

B．研究方法

製造用株の名称及び内容は、１）オリジナルワクチンは臨床試験で有効性と安全性を確認した試作ワクチンで、ワクチン製造承認株、２）マスターシードは製造承認株から作製される、３）ワーキングシードはマスターシードから１代で作製される、４）ワクチン原液はワーキングシードから１代で作製され、製造承認株からの継代は５代以内とするとの定義（厚生労働省科学研究費補助金「ワクチンの製造株の品質管理に関する研究」平成１６年度研究報告書）に基づき、且つ、以下の条件を満たすものをマスターシードと設定する。

１）製造承認株からの継代数（３代以内の範囲）ができるだけ少ないシードウイルス
２）これまで製造用株として十分な使用実績のあるシードウイルス
３）神経毒力試験（弱毒確認試験）を含む原液の試験に適合したシードウイルス
４）ウイルス残量が多量に保管されているシードウイルス

次に、同品質のワクチンを安定的に供給するために、上記の条件を満たすマスターシードを用いてワーキングシードを作製し、その製造管理を検討する。

C. 研究結果と考察

１．マスターシードの設定

１）麻しんワクチン AIK-C 株

麻しんワクチン AIK-C 株は、治験ワクチン Lot #TV-12（製造承認株）がオリジナルワクチンであり、それから１代継代して作製した原液をマスターシード Lot #0-1 と設定した。

このシードウイルスは、原液の試験（弱毒確認試験を含む）に適合したウイルス液であり、これまでワーキングシードとしても使用され、残量も多く保管されている。今後、このマスターシードから１代継代してウイルス液を作製し、ワーキングシードとして設定する。ワーキングシードから１代で作製するワクチン原液は製造承認株から３代となり、この継代数は、過去に出荷したワクチンの継代範囲内であり、最小限の継代数でワクチン製造が可能となる。

２）風しんワクチン高橋株

風しんワクチン高橋株は、治験ワクチン Lot #TV-5（製造承認株）がオリジナルワクチンであり、それから２代継代して作製した原液をマスターシード Lot #0-2 と設定した。このシードウイルスは、原液の試験（神経毒力試験を含む）に適合したウイルス液であり、これまでワーキングシードとしても使用され、残量も多く保管されている。今後、このマスターシードから１代継代してウイルス液を作製し、ワーキングシードとして設定する。ワーキングシードから１代で作製するワクチン原液は製造承認株から４代となり、この継代数は、過去に出荷したワクチンの継代範囲内であることから、これまでの製剤と同等の品質のワクチンが製造されるものと考えられる。

３）おたふくかぜワクチン星野株

おたふくかぜワクチン星野株は、治験ワクチン Lot #TV-3（製造承認株）がオリジナルワクチンであり、それから２代継代して作製した原液をマスターシード Lot #0-2 と設定した。このシードウイルスは原液の試験（神経毒力試験を含む）に適合したウイルス液であり、これまでワーキングシードとしても使用され、残量も多く保管されている。今後、このマスターシードを１代継代してウイルス液を作製し、ワーキングシードとして設定する。ワーキングシードから１代で作製するワクチン原液は製造承認株から４代となり、この継代数は、過去に出荷したワクチンよりも１代進むことになるが、生物製剤基準の５代の範囲内である。シードウイルスの継代数が１代進むことにより、継代による性状の変化がないこと、プラークサイズの確認が必要と考えられる。

２．ワーキングシードの作製

設定するマスターシードを用いて、ワーキングシードを作製する計画が進められる。そのワーキングシード作製は以下のとおりに予定している。

これまでのシードウイルスは、製造用株を用いて作製したワクチン原液の一部をワーキングシードとして使用することがあった。この原液は、原液の試験に適合し、ワクチン原液として使用され、且つその一部はワーキングシードとして使用された。しかし、今後作製するワーキングシードは、ワクチン製造用のシードウイルスとして使用する。ワーキングシード作製は、ワクチン原液製造と同一の方法で行い、作製量は１バッチ分の原液製造量が適当である。このワーキングシード量は、数十年分以上の製剤を安定供給できる量である。

ワーキングシードの品質試験は、生物製剤基準で規定されている各ワクチンにおける個体別培養細胞の試験、ウイルス浮遊液の試験、原液の試験を適用する予定であるが、原液の試験に含まれる神経毒力試験（弱毒確認試験）は、麻しんワクチン AIK-C 株、風しんワクチン高橋株、おたふ

くかぜワクチン星野株とも連続５回のワクチンにおいて確認されていること、また、設定するマスターシードが本試験に適合しているシードウイルスであることから、神経毒力試験（弱毒確認試験）を省くことが可能と思われる。今後、一定品質の製剤を恒常的に製造するために、シードウイルスの管理方法（品質試験）を検討する必要がある。

D. 結論

WHO のシードロットシステムの基準、並びに生物学的製剤基準の継代数の規格を満たし、且つ、一定継代数の製品を長期に供給できるマスターシードの設定を確認できた。

E. 健康危険情報

特になし。

F. 研究発表

１．論文発表
なし。

２．学会発表
なし。

G. 知的財産権の出願・登録状況
（予定を含む。）

１．特許取得
なし。

２．実用新案登録
なし。

３．その他
なし。

弱毒生ウイルスワクチンのシードロットシステム移行における研究

分担研究者　大隈 邦夫　（財）化学及血清療法研究所 品質管理部 部長
協力研究者　倉永 雅彦　（財）化学及血清療法研究所 第一製造部第二課 課長
協力研究者　上田 謙二　（財）化学及血清療法研究所 第一製造部 上級技術員

研究要旨　弊所の弱毒生ウイルスワクチン（風しん、おたふくかぜ）について、シードロットシステム導入についての課題を検証し、感染研（加藤主任研究者）との協議のもと、マスターシード及びワーキングシードの定義から具体的な継代株の指定を行い、今後の評価及び検証項目及び日程計画を提示した。今後も各課題への対応を継続して実施する。

A. 研究目的

弱毒生ウイルスワクチンの品質保証においては、シードロットシステムが重要であることは公知である。弊所の生産する弱毒生ウイルスウイルスワクチンでは、乾燥弱毒生風しんワクチン（松葉株）、乾燥弱毒生おふくかぜワクチン（宮原株）及び乾燥細胞培養痘そうワクチン（LC16m8 株）が対象である。本研究では風しん及びおたふくかぜについて、シードロットシステム導入の為の課題を検証し、マスターシード及びワーキングシードの定義及び具体的な評価項目の立案を行う。

B. 研究方法

弊所の松葉株は 1969 年の開発であり、宮原株は 1970 年の開発である。開発当時の成績及び資料から、現在までの当該生ワクチン生産・出荷の実績を確認し、研究班会議（2005 年 9 月 27 日及び 2006 年 1 月 26 日）での加藤篤主任研究者らとの協議を踏まえて、具体的な評価及び検証項目を立案した。以下の４項目についての具体的な計画を立案した。

1）マスターシード及びワーキングシードの定義・指定、2）製造株のバンキング、3）ウイルス継代条件、4）安定性の評価（品質試験）

C. 研究結果

1）　マスターシード及びワーキングシードの定義及び具体的指定

開発当時の成績及び資料から、現在までの当該生ワクチン生産・出荷の実績を踏まえ、更に、研究班会議での提案を踏まえて、オリジナルワクチン、オリジナルシードウイルス、マスターシード及びワーキングシードについて指定した。製造承認事項などの機密事項にも関することから、詳細は別途に加藤主任研究者に提出した。概要は下記の通り。

（1）風しん（松葉株）

オリジナルワクチン（治験に用いたワクチン）の継代歴は初代ウサギ腎細胞(RK)12 代である。従って、班会議での提案（WHO 基準など）に照らして、RK12 代がオリジナルワクチンとなる。

オリジナルシードウイルスは RK11 代、マスターシード（1 次シードロット）は RK12 代とし、ワーキングシード（2 次シードロット、製造用シード）は RK13 代、ワクチン原液は RK14 代となる。

この結果は弊所がこれまで出荷した製剤の継代数の範囲に相当し、使用実績の面でも問題はないと考える。

（2）おたふくかぜ（宮原株）

オリジナルワクチン（治験に用いたワクチン）の継代歴は初代ニワトリ胚細胞(CE)27 代である。従って、班会議での提案（WHO 基準など）に照らして、CE27 代がオリジナルワクチンとなる。

オリジナルシードウイルスは CE26 代、マスターシード（1 次シードロット）は CE27 代とし、ワーキングシード（2 次シードロット、製

造用シード）はCE28代、ワクチン原液はCE29代となる。

この結果は弊所がこれまで出荷した製剤の継代数から1代追加となるが、従前の定義で継代した場合でも4代となり、基準である5代以内も満たしている。

2）　製造株のバンキング

前記1）の具体的な指定に基づき、現行の継代方法（培養温度、期間など）に従ったワーキングシードを作製する。そのワクチン製造株という特殊性から、事故などの対応のため、リスク分散の観点からもバンキングの必要性には賛同する。今後は、保管する体制やルール（保管する機関、必要な量及び種類など）の整備が必要と考える。

3）　ウイルス継代条件

製造承認事項に基づき、従来から生産方法と同一の条件での、継代を実施してきている。今後は、m.o.i、培養温度や期間などの明記などの整備が必要と考える。前記2）にも示したとおり、培養方法の詳細な明示も含めて、恒常的な生産方法のSOP化（数値化及び文書化）の充実を図る。

4）　安定性の評価（品質試験）

新規に作製したワーキングシード及び適切な継代数での、生物学的製剤の規定に従った品質管理試験を実施する。更に、温度感受性、プラークサイズあるいは塩基レベル解析を実施する。

D. 考察

開発当時の成績及び資料から、現在までの当該生ワクチン生産・出荷の実績を確認し、研究班会議での加藤篤主任研究者との協議を踏まえて、以下の4項目についての検証の具体的な計画を立案した。

1）マスターシード及びワーキングシードの定義及び具体的な指定、2）製造株のバンキング、3）ウイルス継代条件、4）安定性の評価（品質試験）

今後は、別途に提出した日程計画に従い、前述した対応を継続する。

E. 結論

本研究の成果により、弊所の生ウイルスワクチン（風しん、おたふくかぜ）のシードロットシステム移行の為の具体的なマスターシード及びワーキングシードの指定及び検証項目等の確認を達成した。

F. 健康危険情報

特になし。

G. 研究発表

1. 論文発表
   なし。
2. 学会発表
   なし。

H. 知的財産の出願・登録状況

1．特許取得
   なし。
2．実用新案登録
   なし。
3．その他
   なし。

シードロットシステム導入後のワクチンの品質管理に関する臨床・疫学的研究

分担研究者：岡部信彦　国立感染症研究所　感染症情報センター
協力研究者：多屋馨子　国立感染症研究所　感染症情報センター
研究協力者：上野久美　国立感染症研究所　感染症情報センター
研究協力者：佐藤 弘　国立感染症研究所　感染症情報センター

研究要旨 シードロットシステム導入後の品質管理に関する臨床・疫学的検討方法として、今年度は、血清疫学調査を用いて検討した。現在、国として血清疫学調査が実施されていない任意予防接種対象疾患を選択し、今年度は水痘の血清疫学調査を実施した。対象は、分与許可が得られた血清銀行に保管された2002～2004年の検体を用いて、EIA 法で水痘 IgG 抗体価を測定した。0 歳では 0%であった抗体陽性率は、1 歳で 31.8%に上昇し、幼稚園入園時期と重なる 4 歳以上群で急上昇し、4-6 歳群で 88.9%の抗体保有率を示した。その後は、なだらかに上下しつつ推移し、25 歳以上で 100%の陽性率となった。地域間に若干の相違はあるものの、概ね年齢毎の抗体獲得の傾向は変わらなかった。シードロットシステム導入後の臨床・疫学的調査として、本方法を用いることにより、免疫獲得状況を把握し、ワクチンの品質管理に用いることが可能と考えられた。

A. 研究目的

シードロットシステム導入後の品質管理に関する臨床・疫学的検討方法について、昨年度は、予防接種後副反応報告に関して、必要な検索が容易に実施可能なデータベースを作成し報告した。今年度は、ワクチンの品質管理の評価としては、当該疾病の患者発生状況、血清疫学調査が必要と考えられたので、その中で、血清疫学調査について検討した。

定期接種対象疾患においては、厚生労働省の事業である感染症流行予測調査事業において、経年的に国民の抗体保有状況が調査されているが、任意接種対象疾患については、調査は実施されていない。そのため、今年度は、任意接種対象疾患の内、1987 年以降 1 歳以上を対象に導入されている水痘ワクチンに関して検討することを目的に、水痘の血清疫学調査を実施した。

B. 研究方法

対象： 2002～2004 年の 0-1 歳、2-3 歳、4-6 歳、7-9 歳、10-14 歳、15-19 歳、20-24 歳、25-29 歳、30-39 歳、40-49 歳、50-59 歳、60 歳以上の 12 年齢群について、各年齢群 27 検体、計 324 検体を対象とした。全国を北海道/東北/関東地方、中部/近畿地方、中国/四国/九州地方の 3 地域に分類し、人口比に基づいて 4：3：2 の比率で国内血清銀行より血清の分与を申請し、審査の上、分与許可が得られた血清を対象とした。

方法：水痘-帯状疱疹ウイルス（以下 VZV）に対する抗体価を EIA 法により測定し、VZV に対する年齢群別抗体保有率を調査した。抗体価測定にはデンカ生研製ウイルス抗体 EIA「生研」水痘 IgG を用い、duplicate で測定し、その平均値を解析に用いた。

(倫理面での配慮)

本研究における調査は、国内血清銀行に保管された血清を用いての調査であるため、年齢、性別、居住地、採血年の情報のみしか得られず、個人情報は全く含まれていない。また、研究内容は、国立感染症研究所に設置された倫理委員会に申請し、承認が得られている。

C. 研究結果

1 検体についての測定結果は、1 回目と 2 回目で相関係数 0.959 と良好な相関が認められた。0 歳群は n=5 と少数であったが、移行抗体の残存を考慮し、1 歳群（n=22）と分けて検討した。

年齢群別抗体保有率を図 1 に示した。0 歳では 0%であった抗体陽性率は、1 歳で 31.8%に上昇し、幼稚園入園時期と重なる 4 歳以上群で急上昇し、4-6 歳群で 88.9%の抗体保有率を示した。その後は、なだらかに上下しつつ推移し、25 歳以上で 100%の陽性率となった。

図 2 に地域別に抗体陽性率を示した。地域間

急上昇し、4-6 歳群で 88.9%の抗体保有率を示した。その後は、なだらかに上下しつつ推移し、25 歳以上で 100%の陽性率となった。

図２に地域別に抗体陽性率を示した。地域間に若干の相違はあるものの、概ね抗体獲得の傾向は変わらず、中国・四国・九州地域では 10 歳以上で 100%、その他の地域では 25 歳以上で 100%となった。

図1　年齢群別水痘抗体陽性率

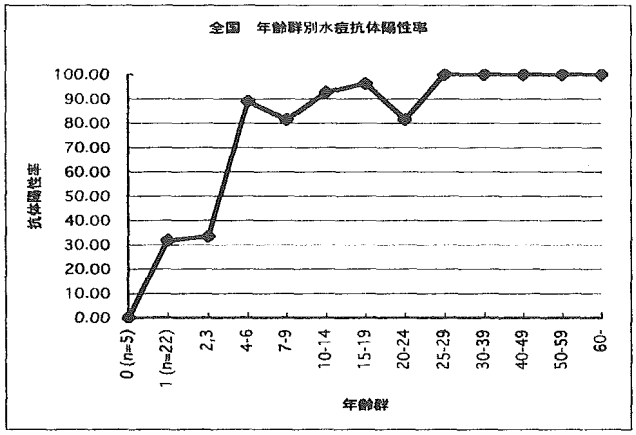
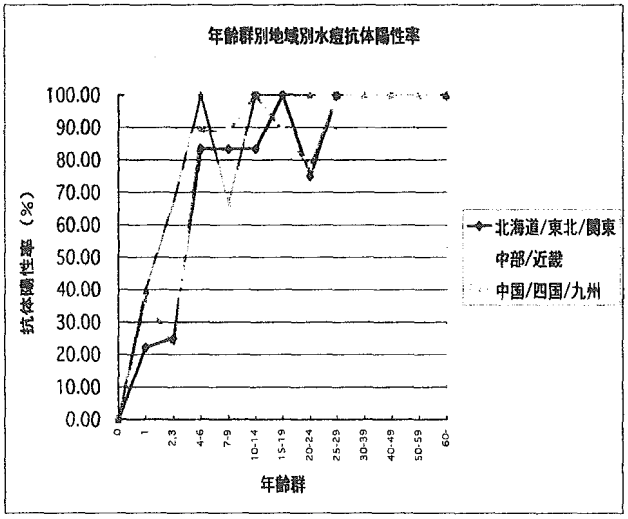


図2　年齢別地域別水痘抗体陽性率



D. 考察

任意接種の枠組みでは小児の予防接種率は低く推移することが示されているが、国として正確な予防接種率は把握されていない。

自治体を対象とした水痘の罹患歴、予防接種歴アンケート調査（馬場ら）によると、水痘ワクチンの接種率は 10-20%程度と低く、抗体獲得はほとんどが水痘罹患により得られていると考えられる。小学校入学前までに概ね 90%程度の小児が水痘に罹患済みあるいは予防接種済みであることが示されており、本調査結果はその結果とも一致した結果が得られている。多数の検体の抗体価を迅速に測定するには、EIA 法は適した方法であり、今回の調査結果が、上記アンケート調査結果とも一致していることから、本方法を用いることで、免疫獲得状況を把握することは可能であると考える。

今後、シードロットシステムが導入された場合は、ロット No を含めたワクチン接種歴の調査を実施し、接種されたワクチンロット（シードロット）毎に抗体陽性率を比較検討し、ワクチンの品質管理の資料として用いることが可能であると考えられた。

E. 結論

シードロットシステム導入後の臨床・疫学的調査として、本方法を用いることにより、免疫獲得状況を把握し、ワクチンの品質管理に用いることが可能と考えられた。

F. 健康危険情報

特になし

G. 研究発表

1. 論文発表
   現時点でなし
2. 学会発表
   現時点でなし

H. 知的財産の出願・登録状況

1．特許取得
   なし。
2．実用新案登録
   なし。
3．その他
   なし。

III. 研究成果の刊行に関する一覧表

<邦文>

1. T. Kubota, N. Yokosawa, S. Yokota, N. Fujii, M. Tashiro, and A. Kato. Mumps virus V protein antagonizes interferon without the complete degradation of STAT1. *J Virol.* **79**:4451-4459 (2005).
2. Numazaki K. Human cytomegalovirus infections in premature infants by breastfeeding. *Afr J Biotechnol*, **4**:867-872 (2005).
3. Tanaka K, Numazaki K, Tsutsumi H.: Human cytomegalovirus genetic variability in strains isolated from Japanese children during 1983-2003. *J Med Virol*, **76**:356-360 (2005).

<和文>

1. 沼崎 啓. 感染制御と教育、市民(親や子ども)の教育/啓蒙・コミュニケーション一麻疹根絶に向けての取り組みを中心に一、小児科臨、**58**:2575-2583 (2005)
2. 沼崎 啓. 冬の院内ウイルス感染対策. 感染と抗菌薬、**8**:413-415 (2005).

JOURNAL OF VIROLOGY, Apr. 2005, p. 4451–4459 Vol. 79, No. 7
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.7.4451–4459.2005

Mumps Virus V Protein Antagonizes Interferon without the Complete Degradation of STAT1

Toru Kubota,[1]* Noriko Yokosawa,[2] Shin-ichi Yokota,[2] Nobuhiro Fujii,[2] Masato Tashiro,[1] and Atsushi Kato[1]

Department of Virology III, National Institute of Infectious Diseases, Musashi-Murayama, Tokyo,[1] and Department of Microbiology, School of Medicine, Sapporo Medical University, Sapporo, Hokkaido,[2] Japan

Received 5 September 2004/Accepted 5 November 2004

Mumps virus (MuV) has been shown to antagonize the antiviral effects of interferon (IFN) through proteasome-mediated complete degradation of STAT1 by using the viral V protein (T. Kubota et al., Biochem. Biophys. Res. Commun. 283:255–259, 2001). However, we found that MuV could inhibit IFN signaling and the generation of a subsequent antiviral state long before the complete degradation of cellular STAT1 in infected cells. In MuV-infected cells, nuclear translocation and phosphorylation of STAT1 and STAT2 tyrosine residue (Y) at 701 and 689, respectively, by IFN-β were significantly inhibited but the phosphorylation of Jak1 and Tyk2 was not inhibited. The transiently expressed MuV V protein also inhibited IFN-β-induced Y^{701}-STAT1 and Y^{689}-STAT2 phosphorylation, suggesting that the V protein could block IFN-β-induced signal transduction without the aid of other viral components. Finally, a substitution of an alanine residue in place of a cysteine residue in the C-terminal V-unique region known to be required for STAT1 degradation and inhibition of anti-IFN signaling resulted in the loss of V protein function to inhibit the Y^{701}-STAT1 and Y^{689}-STAT2 phosphorylation.

The antiviral activity of interferon (IFN) is a major host defense mechanism generated during the early phase of viral infection. Antiviral activity is induced through an IFN signaling process called the Jak-STAT pathway. Briefly, the binding of IFN-α/β to the cell surface type I IFN receptor activates the two receptor-bound kinases Jak1 and Tyk2, which subsequently phosphorylate the tyrosine residues (Y) of STAT1 and STAT2 at positions 701 and 689, respectively. The transcriptional activator, the ISGF3 complex, composed of Y^{701}-phosphorylated STAT1 (pY^{701}-STAT1), Y^{689}-phosphorylated STAT2 (pY^{689}-STAT2), and IRF9, is once formed and translocated to the nuclei. The ISGF3 complex then activates IFN-stimulated genes (ISGs). Typical ISG products such as 2′,5′-oligoadenylate synthetase (2-5AS), RNA-dependent protein kinase (PKR), and Mx protein are known to exert antiviral activities (21).

However, it was previously reported that some viruses evolve to acquire the ability of antagonizing IFN functions through the suppression of the IFN signal transduction pathway (3, 14, 25–27, 49). Among these viruses, the members of the family *Paramyxoviridae*, which includes many important human and animal pathogens as well as emerging viruses such as *Hendra virus* and *Nipah virus*, have been found to antagonize IFNs (6, 7, 9, 15, 28, 31, 35, 36, 40, 46, 49). The individual paramyxoviruses have their own properties, which are obtained by coevolution with their respective hosts, but these viruses share common features regarding genome structure and function. They are enveloped viruses with a linear, nonsegmented negative-sense RNA genome of 15 to 18 kb. On the genome, basically six genes, the nucleocapsid protein, phospho (P) protein, matrix protein, fusion glycoprotein, attachment glycoprotein (HN, H, or G), and large (L) protein genes, are carried in that order from the 3′ to 5′ ends (20). The P and L proteins form RNA-dependent RNA polymerase. Among these genes, more than one protein is exceptionally produced from the P gene and at least one of these proteins is involved in the anti-IFN activities of the paramyxoviruses. The P gene gives rise to P and V mRNAs by a mechanism known as RNA editing. From these two mRNAs, P and V proteins are respectively translated in almost all paramyxoviruses, but in some paramyxoviruses, the C protein is additionally translated by use of an overlapping reading frame.

The carboxyl terminus of the V protein has seven cysteine residues and is highly conserved among the paramyxoviruses. *Simian virus 5* (SV5), *Simian virus 41* (SV41), *Mumps virus* (MuV), and *Human parainfluenza virus type 2* (hPIV2) belonging to the *Rubulavirus* genus and *Newcastle disease virus* (NDV) belonging to the *Avulavirus* genus have the P and V proteins, but not the C protein in the P gene, and all of these viruses have been shown to antagonize IFNs by using the V protein (1, 12, 18, 28, 31, 33). The viruses of the *Morbillivirus* and *Henipavirus* genera have the P, V, and C proteins in the P gene and have also been shown to counteract IFNs by using V protein (33, 35, 36, 40, 49). Among the viruses in these genera, non-negligible anti-IFN activity was also reported to be associated with the C protein of the Nipah and measles viruses (33, 37). Although the P gene of *Sendai virus* (SeV) of the *Respirovirus* genus codes P, V, and C proteins, the SeV C protein does counteract IFNs in the signaling process but V protein does not (15, 16).

The means by which such viral proteins inhibit the Jak-STAT pathway differ among the paramyxoviruses (9). For example, the V protein of MuV, SV5, SV41, and NDV induces

* Corresponding author. Mailing address: Department of Virology III, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-Murayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771. Fax: 81-42-567-5631. E-mail: kubota@nih.go.jp.

the degradation of STAT1 (1, 12, 18, 28) and the V protein of hPIV2 induces the degradation of STAT2 (1, 28, 31). On the other hand, the V proteins of measles, Nipah, and Hendra viruses generate anti-IFN activity without STAT degradation (30, 35, 36, 40). In this latter case, instead of STAT degradation, IFN-induced phosphorylation and nuclear localization of STAT1 and STAT2 are inhibited.

The degradation of STATs found in members of the *Rubulavirus* and *Avulavirus* genera was originally demonstrated in persistently infected cells and by using a plasmid-based V expression system (6, 18, 28, 31). The importance of the V-unique carboxyl-terminal region for degradation was subsequently indicated by several V protein expression studies (12, 18, 28). Such observations have been confirmed in the context of viral replication by using recombinant hPIV2, SV5, and NDV lacking carboxyl-terminal V-unique regions (11, 12, 17). However, it is noteworthy that a spontaneous SV5 mutant with mutations in the P/V common domain showed no anti-IFN activity, indicating the contribution of the P/V common domain for generating anti-IFN activity (4, 45, 50). The degradation of these STAT proteins is thought to be the result of an ubiquitin-proteasome pathway because the amount of STAT mRNA does not change following viral infection; in addition, a proteasome inhibitor, MG132, recovers the STAT level, although the recoveries are partial (7, 47).

The interaction of V protein with cellular proteins was examined using glutathione *S*-transferase-V fusion protein and a yeast two-hybrid system. In those studies, p127 UV-damage-specific DNA binding protein 1 (DDB1) and receptor for activated C kinase 1 (RACK1) were identified as cellular counterparts of SV5 V and MuV V proteins, respectively (19, 22). The carboxyl-terminal V-unique region is necessary for both interactions. Copurification experiments revealed that the V protein of SV5 and hPIV2 forms a complex with STAT1, STAT2, DDB1, and Cul4A (31, 41). The good correlation between the binding of SV5 V to DDB1 and its ability to degrade STAT1 has been well demonstrated (2). As Cul4A is one of the components of cellular ubiquitin ligase, STAT1 degradation by the V protein was estimated to occur through an ubiquitin-proteasome pathway by using DDB1 and Cul4A. The ubiquitination of STAT1 has been demonstrated in MuV-infected cells (42, 47). On the other hand, the biological significance of RACK1 remains unclear. The V proteins of *Rubulavirus* have also been reported to bind to both STAT1 and STAT2 at their carboxyl termini, and these interactions are thought to be necessary for the ubiquitination and degradation of STATs (29, 32).

In this study, we demonstrated that the antiviral activity of IFN could be established in MuV-infected cells before the degradation of STAT1. Our observations therefore indicate that the complete degradation of STAT1 is not required for generating IFN antagonism of MuV.

MATERIALS AND METHODS

Cells, viruses, and IFN. Simian-kidney-derived CV1 and Vero cells were grown in Dulbecco's modified Eagle's medium and in Eagle's minimal essential medium in the presence of 10% fetal bovine serum and 1% penicillin-streptomycin solution (Invitrogen, Carlsbad, Calif.), respectively. The RW strain of MuV used throughout the present study was kindly supplied by J. S. Wolinsky, the Department of Neurology at Johns Hopkins University School of Medicine. The virus was grown in Vero cells and was titrated by plaque assay. Human IFN-β was purchased from Toray Industries, Inc. (Tokyo, Japan).

Plasmids and antibodies. Four plasmids encoding MuV V protein of the Torii strain (pTM-V) and three mutant V proteins in which the cysteine residue at amino acid position 189, 207, or 214 was replaced by an alanine residue (pTM-Vc189a, pTM-Vc207a, or pTM-Vc214a, respectively) were used. These plasmids were constructed as described previously (19, 48). Polyclonal antibodies against human STAT1 p84/p91 (E-23), STAT2 p133 (C-20), and STAT1 phosphorylated at Y^{701} were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif.). Rabbit antibodies against Jak1, Tyk2, and STAT2 phosphorylated at Y^{689} were obtained from Upstate, Inc. (Charlottesville, Va.). Antibody to Jak1 phosphorylated at Y^{1022} and Y^{1023} [anti-Jak1pYpY(1022/1023) antibody] was obtained from BioSource International (Camarillo, Calif.). Antibody to Tyk2 phosphorylated at Y^{1054} and Y^{1055} [anti-Tyk2pYpY(1054/1055) antibody] was obtained from New England Biolabs, Inc. (Cambridge, Mass.). Monospecific polyclonal sera against MuV P, V (39), and HN, prepared at our institute, were also used. Labeled anti-mouse immunoglobulin G conjugates and anti-rabbit immunoglobulin G conjugates, used for the immunofluorescence experiments, were purchased from Molecular Probes, Inc. (Eugene, Oreg.).

Assay for antiviral activity. Vero cells were plated 2 days prior to virus infection in 12-well or 48-well plates. Before the IFN treatment, monolayered Vero cells were inoculated with the RW strain of MuV at a multiplicity of infection (MOI) of 1 (Fig. 1) or that indicated in the text (Fig. 2). At 72 h (Fig. 1) or 24 h (Fig. 2) postinfection, the cells were treated with several concentrations of IFN-β as indicated in the text and were cultured for 24 h. Then, the cells were challenged with vesicular stomatitis virus (VSV) for 24 h and were fixed and stained with naftol blue black solution (0.1% naftol blue black, 0.1% sodium acetate, 9% acetate). Extracts from both attached and detached cells in parallel samples were prepared and analyzed by immunoblotting.

Immunoblotting. MuV-infected and mock-infected cells were washed with phosphate-buffered saline (PBS) once just before harvesting, and the cells were lysed in a dish or plate on ice with cell lysis buffer (50 mM HEPES [pH 7.5], 4 mM EDTA, 150 mM NaCl, and 1% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride, 2 mg of aprotinin/ml, and 0.1 mM Na_3VO_4. The cell lysates were centrifuged at 10,000 × *g* for 5 min, and the supernatants were used for the subsequent experiments.

For detection of the MuV proteins STAT1, pY^{701}-STAT1, STAT2, pY^{689}-STAT2, Jak1, and Tyk2, the soluble supernatants of cell lysates were subjected to NuPAGE (Invitrogen), electrotransferred onto nitrocellulose membranes, and immunoblotted with the specific antibody. For the detection of tyrosine-phosphorylated Jak1 at positions 1022 and 1023 and Tyk2 at positions 1054 and 1055, the soluble supernatants were first mixed with 5 μg of anti-Jak1 or anti-Tyk2 antibody and were incubated at 4°C for 1 h. Then, 20 μg of protein A-Sepharose 4B (Amersham Pharmacia Biotech, Inc., Uppsala, Sweden) was added to the cultures, which were then incubated at 4°C for 3 h. The respective proteins trapped by antibody were subjected to NuPAGE, electrotransferred, and then probed with anti-phospho Jak1 or anti-phospho Tyk2 antibodies.

Immunofluorescence. An indirect-immunofluorescence assay was carried out to observe the protein localization in the cells. CV1 cells grown to 60% confluence on chamber slides (Nalge Nunc International, Naperville, Ill.) were inoculated with the RW strain of MuV at an MOI of 0.05. At 12 h postinfection, the cells were treated with IFN-β at 0 or 1,000 IU/ml for 30 min. The cells were then fixed with 10% formaldehyde in PBS for 10 min. After washing four times with PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and were incubated with primary antibody against MuV NH and STAT1 for 1 h. The cells were then washed five times with PBS and were incubated with secondary antibody for 1 h. After being washed three times with PBS, immunofluorescence-stained cells were observed using a fluorescence microscope, Axiovert 135 (Carl Zeiss Japan, Tokyo, Japan).

Plasmid transfection. CV1 and Vero cells grown on 24-well plates to approximately 70 to 80% confluence were transfected with pTM-V, pTM-Vc189a, pTM-Vc207a, or pTM-Vc214a by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Forty-eight hours later, the cells were then incubated with or without IFN-β at 1,000 IU/ml at the time indicated in Fig. 5 and 6, and the transiently expressed protein was analyzed by immunoblotting.

RESULTS

MuV antagonizes the antiviral effects of IFN-β. The RW strain of MuV is able to infect several cell lines, including human and monkey cell lines, without manifesting obvious

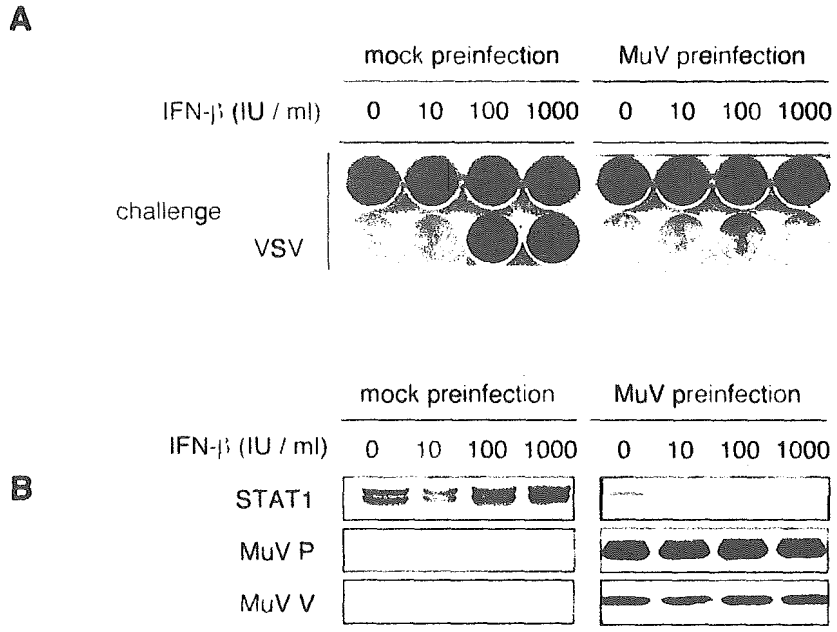


FIG. 1. Anti-IFN activity of the MuV RW strain. Cells were preinfected or mock-infected with the RW strain of MuV. The dose of IFN (IU/ml) used for the treatment of cells is indicated at the tops of panels A and B. (A) Cells which survived the VSV challenge infection are stained in black. (B) The MuV P, MuV V, and cellular STAT1 in samples parallel to those in panel A are shown.

cytopathic effects (23, 24). The final virus yield of the RW strain is almost the same as that of the other MuV strains that lead to the formation of giant cells by cell fusion. We used the nonapparent cytopathic character of the MuV RW strain to see how MuV infection inhibited the establishment of the antiviral state induced by IFNs. When the cells were incubated with IFN-β for 24 h at 0, 10, 100, or 1,000 IU/ml, MuV-preinfected cells were detached from the plate following the VSV challenge at any concentration of IFN used while the mock preinfected cells treated with IFN-β at 100 or 1,000 IU/ml attached to the plate even after the VSV challenge (Fig. 1A). The RW strain of MuV has thus been

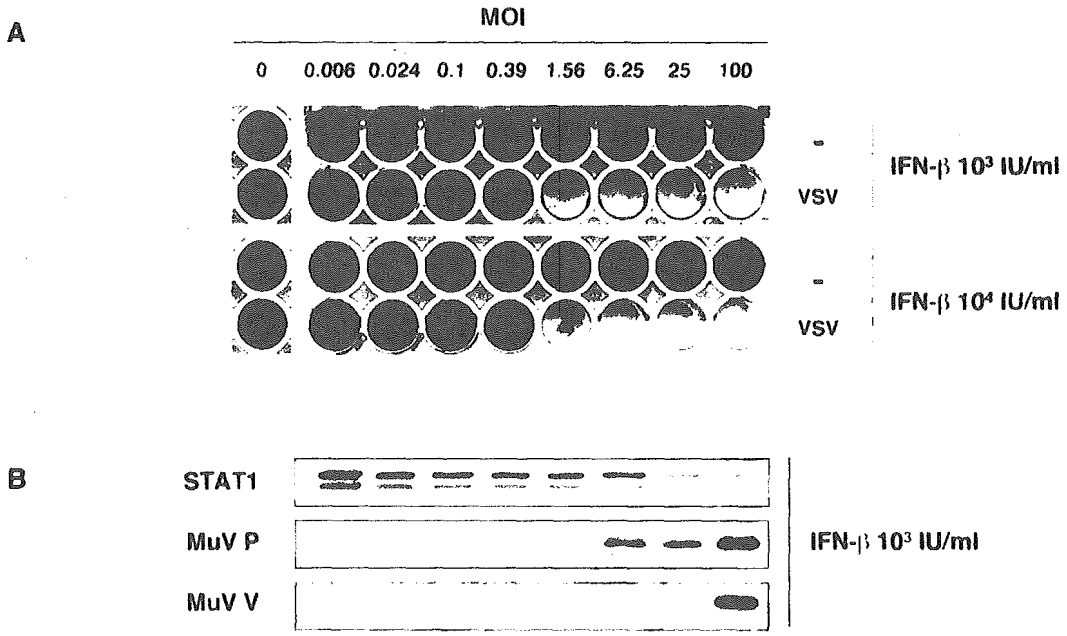


FIG. 2. Correlation between STAT degradation and antagonism of the antiviral activity of MuV. The preinfected MuV dose is shown at the top. The IFN doses used for inducing the antiviral state are shown at right. (A) Cells which survived the VSV challenge infection are stained in black. (B) MuV P, MuV V, and cellular STAT1 in samples parallel to those in panel A are shown. The faint band at MuV P at an MOI of 0.006 is nonspecific due to the use of antiserum mixture.

confirmed to antagonize the antiviral activity of IFN, as shown in the case of other MuV strains (18).

Until recently, the V protein of MuV has been reported to degrade STAT1, a key molecule of IFN-α/β signal transduction, and to thereby antagonize the antiviral activity of IFN (18, 42). To observe the degradation of STAT1, we prepared whole-cell extracts of both attached and detached cells at 24 h post-VSV challenge following IFN-β treatment (Fig. 1B). The STAT1 levels in MuV-infected cells were certainly lower than those in uninfected cells, indicating the degradation of STAT1, but STAT1 was still detected and the degradation of STAT1 was also found to be incomplete (Fig. 1B). Viral P and V proteins in MuV-infected cells showed that MuV had actually grown in these cells.

These results suggested that anti-VSV activity induced by IFN was generated even in the presence of STAT1 in MuV-infected cells. However, it is also possible that the remaining STAT1 proteins were derived from cells that could not have been infected with MuV under these experimental conditions. To clarify the relationship between the MOI of the input MuV and the degradation of STAT1, we then investigated the correlation between the inhibition of IFN-β-induced antiviral activity and cellular STAT1 levels under conditions with a relatively high MOI. MuV-infected cells at different MOI doses were subsequently incubated with IFN-β at 0, 10^3, or 10^4 IU/ml for 24 h and were challenged with VSV (Fig. 2).

In MuV-uninfected cells preincubated with IFN-β at both 10^3 and 10^4 IU/ml induced resistance against VSV infection by the antiviral activity of IFN, as shown in Fig. 1, while in MuV-infected cells at an MOI of 1.56 or higher, no IFN-β antiviral activity was generated and those cells detached completely from the plate (Fig. 2A). However, the antiviral activity was generated in the MuV-infected cells at an MOI of 0.39 or lower, as was the case in MuV-uninfected cells, and those cells were attached to the bottom of the culture plate. These results indicated that MuV infection at an MOI of 1.0, in which almost all of the cells were expected to be infected by one virus, was a boundary point determining whether or not IFN-β led cells to an antiviral state, thus preventing VSV multiplication.

In these experimental conditions, the MuV P protein was detected in cells infected at an MOI of 1.56 or higher and the MuV V protein was detected at an MOI of 6.25 or higher (Fig. 2B). The discrepancy between the results obtained with these two antibodies was probably due to the differences in the intracellular viral protein levels and the antibody titers. The STAT1 level in the cells decreased in accordance with the increase in the MOI. There were no critical differences in the STAT1 levels in the cells at an MOI of 0.39 and an MOI of 1.56; the former resisted VSV infection, and the latter did not. Interestingly, even in the extract from cells that were infected with MuV at an MOI of 100, STAT1 was only incompletely degraded and was still present under these conditions. In addition, in the separate experiment designed as detailed above, the infected cells could be immunostained fluorescently with anti-MuV serum in proportion to the MOI by using fluorescence microscopy. The cells infected with an MOI of 1.56 or more were stained wholly (data not shown). These results clearly indicated that the remaining STAT1 was not preceded by cells that were not infected with MuV and that the inhibition of antiviral activity induced by IFNs occurred even in the presence of STAT1. The complete degradation of STAT1 is thus shown to be nonessential for antagonizing IFN.

Inhibition of IFN-β-induced STAT1 nuclear translocation in MuV-infected cells. In MuV-infected cells, the antiviral effect of IFN-β could be blocked long before the complete degradation of STAT1. In virus-uninfected cells, IFN-β stimulation led to the nuclear translocation of STAT1, together with that of STAT2 and IRF9, thereby forming a multiprotein complex that functions as a transcriptional activator, ISGF3. To determine which step of IFN-α/β signaling was inhibited, the IFN-β-induced nuclear translocation of STAT1 was examined by indirect immunofluorescence assay. As shown in Fig. 3, without IFN-β treatment, cellular STAT1 was located in the cytoplasms of uninfected cells. However, treatment with IFN-β for 30 min led to the accumulation of STAT1 in the nuclei. These nuclear translocations were also observed when anti-STAT2 antibody was used instead of anti-STAT1 antibody (data not shown). In the MuV-infected cells, no nuclear translocation of STAT1 (Fig. 3) and STAT2 (data not shown) was observed, even in the presence of IFN-β. These results indicated that the MuV infection inhibited IFN-β signaling, at least at the nuclear translocation step of STAT1 and STAT2 proteins, which is required for the activation of ISG transcription.

Effects of MuV infection on the IFN-β-induced phosphorylation of signaling molecules. The binding of IFN-α/β to the cell surface receptor triggers IFN signaling. The stimulation of IFN-β is transduced from the surface receptor to the nucleus by the sequential phosphorylation of signaling molecules such as the type I IFN receptors Jak1, Tyk2, STAT1, and STAT2 at a tyrosine residue. To determine why STAT1 and STAT2 were retained in the cytoplasms of MuV-infected cells regardless of IFN-α/β treatment, the IFN-β-induced phosphorylation of STAT1 and STAT2 was initially examined in MuV-infected cells. MuV-infected and mock-infected cells were incubated with IFN-β for 30 min. Then, pY^{701}-STAT1 levels, as well as total STAT1 levels, were respectively measured by immunoblot analysis using specific antibodies (Fig. 4A). In mock-infected cells, pY^{701}-STAT1 was not detected without IFN-β but pY^{701}-STAT1 was detected at 30 min following incubation with IFN-β (Fig. 4A). Within this short incubation period, the total STAT1 levels did not change significantly. On the other hand, in MuV-infected cells, the pY^{701}-STAT1 level was significantly reduced after IFN-β treatment in comparison with that in mock-infected cells, thus indicating the inhibition of STAT1 phosphorylation by MuV. Likewise, the respective pY^{689}-STAT2 levels were measured using the specific antibodies (Fig. 4B). Although the levels of STAT2 did not change significantly during this short period of incubation with IFN-β, pY^{689}-STAT2 was detected in the mock-infected cells after 30 min of IFN-β incubation. pY^{689}-STAT2 was not detected either before or after the IFN-β treatment in MuV-infected cells. This result was consistent with the previous finding obtained by using MuV-infected FL cells (47).

We then examined the IFN-β-induced phosphorylation of Jak1 and Tyk2, which are located upstream of STAT1 and STAT2 phosphorylation in the IFN signaling pathway. In the mock-infected cells, Jak1 and Tyk2 phosphorylation was detected after 15 min of IFN-β treatment (Fig. 4C and D). Under the same conditions, Jak1 and Tyk2 in MuV-infected cells were

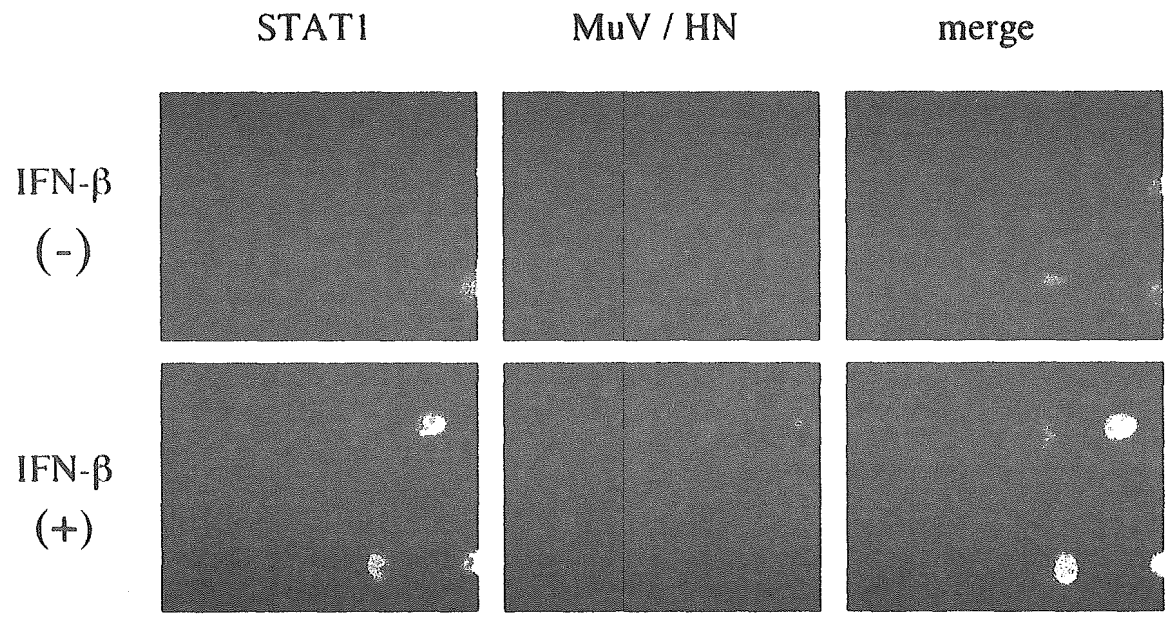


FIG. 3. Inhibition of IFN-β-induced STAT1 nuclear translocation by MuV. MuV-infected CV1 cells were incubated with 0 or 1,000 IU of IFN-β/ml. STAT1 was detected with rabbit polyclonal antibody (left panels), and the MuV HN was detected with mouse monoclonal antibody (middle panels). The merged images are shown in the right panels. Identical microscopic fields of view are shown, and the MuV HN and STAT1 are shown in red and green, respectively.

phosphorylated at levels comparable to those in the uninfected cells. These results indicated that MuV infection did not affect Jak1 and Tyk2 phosphorylation but that it did inhibit STAT1 and STAT2 phosphorylation.

The MuV V protein inhibits the IFN-β-induced phosphorylation of both Y^{701}-STAT1 and Y^{689}-STAT2. It was previously reported that the MuV V protein inhibits host IFN signaling by degrading STAT1 protein (18, 42). We then investigated

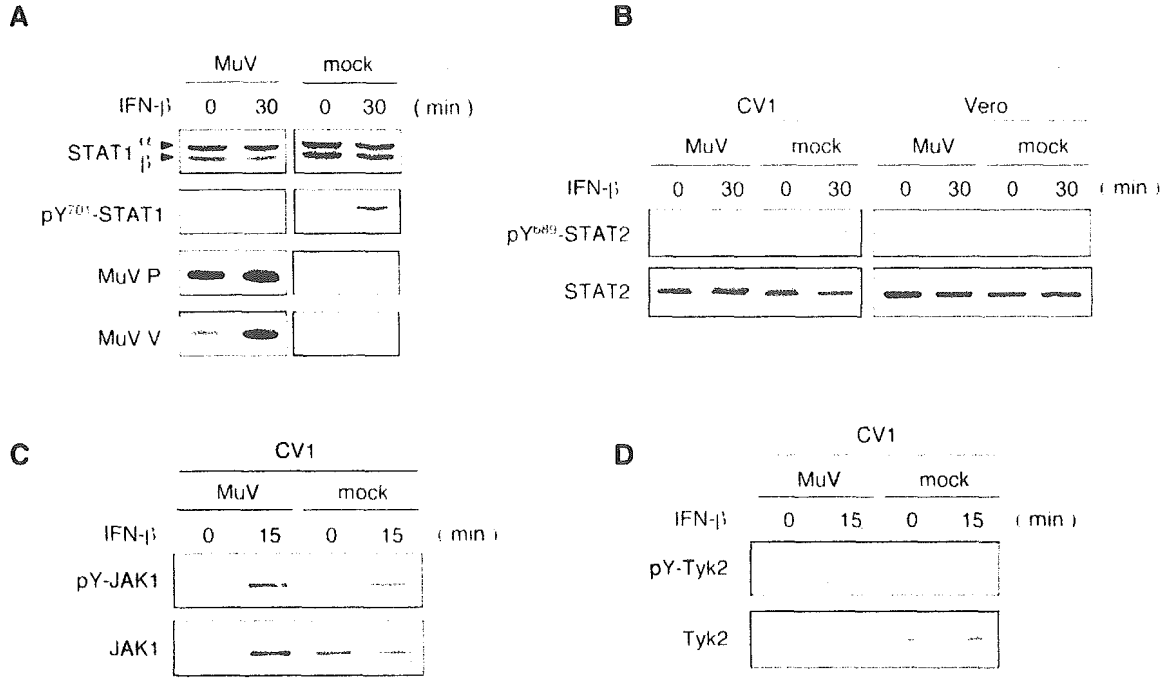


FIG. 4. The effect of MuV infection on IFN-β-induced phosphorylation of signaling molecules. Cells infected with MuV at an MOI of 5.0 and uninfected cells were treated with 0 or 1,000 IU of IFN-β/ml at 48 h postinfection. The MuV P, MuV V, STAT1, and pY^{701}-STAT1 are shown (A); STAT2 and pY^{609}-STAT2 (B), Jak1 and pY-Jak1 (C), and Tyk2 and pY-Tyk2 (D) are also shown.

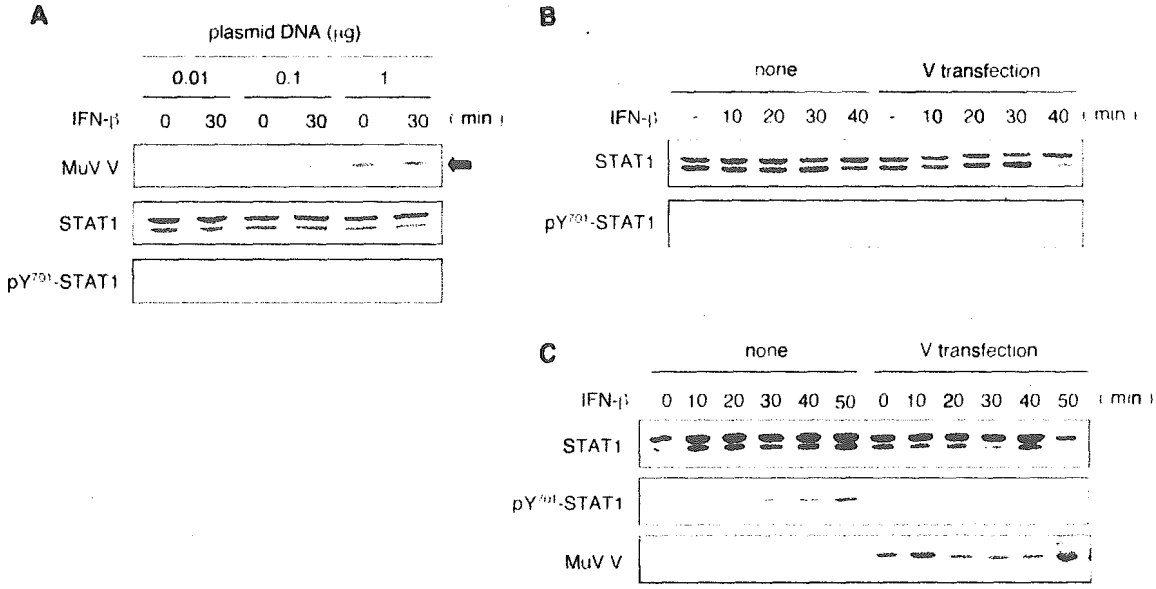


FIG. 5. Inhibition of IFN-β-induced Y^{701}-STAT1 phosphorylation by MuV V protein. The MuV V of the Torii strain expressing plasmid DNA (pTM-V) transiently transfected to Vero cells is shown at the top (A). The MuV V, STAT1, and pY^{701}-STAT1 detected in the cells treated with 1,000 IU of IFN-β/ml for 0 or 30 min are shown. Vero cells (B) or CV1 cells (C) transiently transfected with 0 μg (none) or 1 μg (V transfection) of pTM-V were subsequently treated with 1,000 IU of IFN-β/ml for the indicated times. STAT1 and pY^{701}-STAT1 detected in the cells are shown.

whether or not V protein was also involved in the inhibition of the IFN-β-induced phosphorylation of STAT1 on Y^{701}. Plasmid DNA that expressed the V protein of MuV was transfected and was transiently expressed in these cells (Fig. 5). After 48 h of transfection, both nontransfected and V-expressing plasmid-transfected cells were treated with IFN-β for 0 or 30 min. The cells expressed the V protein in a manner that depended on the amount (0.01, 0.1, or 1 μg) of V-expressing plasmid used for the transfection (Fig. 5A). The total STAT1 levels were nearly equal in these cells, independent of the amount of transfected plasmid and also independent of the duration of IFN-β treatment. In 0.01 or 0.1 μg of plasmid-transfected cells, pY^{701}-STAT1 was detected after 30 min of IFN-β treatment, as was observed in the case of the nontransfected cells (data not shown). However, in 1 μg of plasmid-transfected cells, IFN-β-induced STAT1 phosphorylation on Y^{701} was significantly inhibited.

To more clearly observe this type of inhibition, a time course study was carried out to examine IFN-β-induced STAT1 phosphorylation on Y^{701} in both V-expressing plasmid-transfected cells and nontransfected cells (Fig. 5B and C). In the nontransfected cells, the phosphorylation of STAT1 on Y^{701} was not seen at 0 min of incubation but was detected starting at 10 min. This trend was observed to increase linearly in Vero cells until 40 min had passed (Fig. 5B) and in CV1 cells until 50 min had passed (Fig. 5C) under the condition involving IFN-β treatment. In the V-expressing plasmid-transfected cells, although the phosphorylation of STAT1 on Y^{701} also increased linearly, the pY^{701}-STAT1 level at each time point examined in these cells was significantly lower than that observed in the nontransfected cells (Fig. 5B and C). Since the total STAT1 levels were almost identical in the nontransfected cells and in the V-transfected cells during the incubation period, these results indicated that V protein is able to inhibit IFN-β-induced STAT1 phosphorylation on Y^{701}. In the MuV-infected cells, phosphorylation of both STAT1 and STAT2 on tyrosines 701 and 689, respectively, was inhibited.

The effect of V protein expression on the tyrosine phosphorylation of STAT2 at 689 was then examined (Fig. 6). In the nontransfected cells, the phosphorylation of both STAT1 on 701 and STAT2 on 689 was not found in the IFN-β-untreated cells (0 min) but found in treated cells (30 min). The total STAT1 and STAT2 levels did not significantly change during the IFN-β incubation. In the same experimental condition but in the MuV V-protein-expressing cells, none of the pY^{701}-STAT1 or pY^{689}-STAT2 was found even after the incubation of IFN-β. Since the total STAT1 and STAT2 levels were not changed before and after the IFN-β treatment, the phosphorylation of STAT2 on Y^{689} was thus found to be inhibited by the

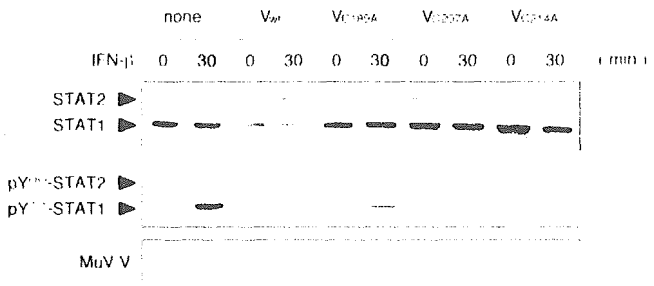


FIG. 6. Involvement of C-terminal cysteine residues of the MuV V protein for the inhibition of IFN-β-induced STAT1 phosphorylation. The plasmid DNAs able to express parental V protein of the Torii strain (V_{wt}) or three mutant V proteins (Vc189a, Vc207a, and Vc214a) were transiently transfected to CV1 cells. The cells were incubated with 1,000 IU of IFN-β/ml for the indicated times shown above the gels. STAT1, pY^{701}-STAT1, STAT2, pY^{689}-STAT2, and MuV V detected in the cells are shown.

V protein. These findings suggested that the MuV V protein alone can antagonize IFN-β by inhibiting the tyrosine phosphorylation of both STAT1 and STAT2.

Importance of the conserved C-terminal cysteine-rich region of MuV V protein for the inhibition of IFN-β-induced STAT1 and STAT2 phosphorylation. The cysteine-rich region within the carboxyl terminus of the V protein has been shown to contribute to STAT1 degradation in MuV and SV5 (11, 18, 47), and this region also appears to contribute to STAT2 degradation in hPIV2 (17, 28) because artificially mutated V proteins lacking this region or those with a substitution at relevant cysteine were unable to degrade each STAT when they were expressed intracellularly (2, 18, 28, 47). To determine whether or not these cysteine residues are also involved in the inhibition of IFN-β-induced STAT1 and STAT2 phosphorylation, plasmids were transfected to cells that expressed mutated V proteins in which the cysteine residue at position 189, 207, or 214 was replaced with an alanine (Vc189a, Vc207a, or Vc214a, respectively) (Fig. 6). In the cells examined, the total STAT1 and STAT2 levels did not change significantly. In nontransfected cells, pY^{701}-STAT1 and pY^{689}-STAT2 were not detected before the incubation of IFN-β but were detected after the incubation (30 min) (Fig. 6).

Though in the V protein-expressing plasmid-transfected cells, pY^{701}-STAT1 and pY^{689}-STAT2 were not found, in three plasmid-transfected mutant cell lines expressing Vc189a, Vc207a, and Vc214a, pY^{701}-STAT1 and pY^{689}-STAT2 were again detected at the same levels observed in nontransfected cells. Since these three mutant V proteins and wild-type V protein were expressed equally in these transfected cells (Fig. 6), the normal appearance of pY^{701}-STAT1 and pY^{689}-STAT2 was not thought to be caused by indirect effects such as low expression of or instability of the mutated V protein but instead was attributed to the direct effects of amino acid substitution. Namely, these mutations rendered the V protein nonfunctional in terms of the inhibition of STAT1 and STAT2 phosphorylation on Y^{701} and Y^{689}, respectively.

DISCUSSION

Among natural immunity engaged during early periods of viral infection, the IFN system plays a principal role in the antiviral response that is dedicated to fighting viruses. However, infection by numerous viruses belonging to the subfamily *Paramyxovirinae* has acquired the ability to alter IFN susceptibility in host cells (6, 7, 9, 15, 28, 31, 33, 35, 36, 40, 46, 49). Among these IFN antagonisms observed in paramyxoviruses, viruses belonging to the genera *Rubulavirus* and *Avulavirus* have been reported to inhibit the establishment of the antiviral state via the degradation of STAT1 or STAT2 through an ubiquitin-proteasome pathway (7, 47). The viral factor concerning STAT degradation is the V protein, which is able to degrade STAT1 or STAT2 without the aid of other viral elements (6, 18, 28, 31). Expression of the carboxyl-terminal V region of MuV and NDV degrades STAT1, and that of hPIV2 degrades STAT2 (12, 18, 28). The V proteins of MeV, the genus *Morbillivirus*, the Nipah and Hendra viruses, and the genus *Henipavirus* also antagonize IFN-induced host antiviral effects (30, 35, 36, 38, 49). Even though the carboxyl terminus of the V protein is the most highly conserved region among the proteins of the paramyxoviruses, IFN antagonisms of these viral V proteins do not induce the degradation of any STAT proteins but do inhibit the signaling step leading to the ISGF3 complex formation. These findings revealed that the means of antagonizing IFN-induced antiviral activity are diverse, in spite of the structural conservation of viral V proteins.

As was demonstrated in the present study, the RW strain of MuV antagonizes IFN by its V protein, not only via STAT1 degradation, but also by the inhibition of STAT1 and STAT2 phosphorylation. These two antagonizing functions are not RW strain specific, though the RW strain of MuV leads to few cytopathic effects in infected cells compared to that of other strains. Because there is no amino acid difference in the carboxyl terminus of V protein which is important for degradation between the RW and Torii strains, a representative of other strains causes the degradation of STAT1. In addition, the fact that transfection of a plasmid expressing the V protein of the Torii strain resulted in a reduction of IFN-β-induced STAT1 and STAT2 phosphorylation (Fig. 5 and 6) can generalize the two anti-IFN functions of MuV V proteins, degradation of STAT1 and inhibition of STAT1 and STAT2 phosphorylation. The RW strain is thus not unique among MuV strains with respect to IFN antagonism.

Our present and previous studies (47) have demonstrated that cysteine residues in the carboxyl terminus of the MuV V protein are required for the inhibition of both STAT1 and STAT2 phosphorylation (Fig. 6) and the induction of STAT1 degradation. However, it remains unknown whether or not both abilities were generated via the mediation of the same host molecule that binds to the cysteine residues. For example, we previously found that host RACK1 binds to the carboxyl-terminal region of V protein (19). RACK1 has seven WD (tryptophan-aspartic acid) repeat motifs that function as a protein-protein interaction domain and are known to interact with many signaling molecules, such as STAT1, β-chain of type I IFN receptor, Jak1, Jak2, and Tyk2 (5, 10, 44). It is of note that RACK1 interacts with STAT1 and mediates the binding of STAT1 to the IFN receptor because this binding regulates Jak-mediated phosphorylation of STAT1 in response to IFN (43). DDB1 is another host protein known to interact with V protein (22). However, the contribution of DDB1 protein to the inhibition of STAT1 phosphorylation remains unclear at present.

STAT degradation by the rubulavirus V proteins, including MuV, was demonstrated in present and previous studies. Whereas the interruption of IFN signaling must occur as early as possible in the viral infection process, the degradation of STAT protein does not appear to occur so quickly. SV5 infection led to a decrease in the STAT1 level from around 4 h postinfection and to a complete loss of STAT1 by 8 h postinfection in 2fTGH cells (7), and it resulted in the disappearance of STAT1 by at least 12 h postinfection in Vero cells (34). In addition, hPIV2 infection led to a nearly complete loss of STAT2 at 9 h postinfection in CV1 cells (31). In MuV-infected CV1 and Vero cells, STAT1 degradation progresses more slowly. For example, when the RW strain of MuV was used to infect CV1 cells at an MOI of 10, 64.3% of the STAT1 remained in the cells at 24 h postinfection compared to the level in uninfected cells (Fig. 4A). Taking into consideration the fact that IFN signaling can be triggered at a very early stage of

infection, the complete degradation of STAT was found to take place too late to interrupt the generation of the antiviral activity of IFN. Thus, it is quite natural that rubulaviruses, and in particular MuV, have mechanisms other than protein degradation to achieve the inhibition of IFN signaling that functions prior to the activation of host IFN signaling.

The coauthors of this study, Yokosawa et al., previously described the complete degradation of STAT1 in MuV-Torii-infected FL cells (47). In our separate experiment, we observed that STAT1 disappears in the MuV-Torii-infected FL cells but not in the MuV-RW-infected Vero and CV1 cells. In addition, if the Torii strain was inoculated to the Vero and CV1 cells, the complete degradation of STAT1 did not occur within our experimental conditions. On the other hand, if the RW strain was inoculated to the HeLa cells, the degradation of STAT1 occurred earlier and more extensively (data not shown). By these results, it is possible to say that the difference in the degradation kinetics of STAT1 is due to the cell types rather than virus strains. Regarding the STAT degradation pathway driven by rubulavirus V proteins, the presence of STAT1, STAT2, and DDB1 was at least thought to be essential (2, 32, 41). Since the additional unknown factors may be involved in the degradation process, levels of these known and unknown factors in cells would affect the degradation level of STAT1 during the MuV infection.

In a previous study, the coauthors of this study, Yokosawa et al., demonstrated that treatment with the proteasome inhibitor MG132 partially recovers cellular STAT1 levels as well as sensitivity to IFN-α, at least at certain levels (47). This previous finding was inconsistent with the notion that IFN signaling was inhibited independent of STAT1 degradation in the cells examined. However, since the observed increases in IFN-α-induced IRF1 and 2-5AS mRNA levels, as measured by reverse transcription-PCR, were significantly lower in MG132-treated MuV-infected cells than in the corresponding MuV-uninfected cells, the recovery of IFN sensitivity following MG132 treatment would not be sufficient to establish an antiviral state. Moreover, treatment with proteasome inhibitors such as MG132 and lactacystin has a side effect on several cellular regulatory mechanisms and alters the profiles of cellular proteins that are involved in signal transduction, transcription, and translation (13). It therefore remains difficult to precisely determine the cytokine effects resulting from MG132 treatment.

Our present results clearly indicated that MuV infection reduces IFN-β-induced STAT1 phosphorylation at Y^{701} and STAT2 phosphorylation at Y^{689} as well as STAT1 level for inhibiting the establishment of an antiviral state by using the carboxyl-terminal region of the viral V protein. The mechanism by which MuV V protein induces these two effects remains to be clarified in future studies.

ACKNOWLEDGMENTS

This work was partially supported by research grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

We thank Minoru Kidokoro, Naoko Miyajima, and Sakura Saito.

REFERENCES

1. **Andrejeva, J., D. F. Young, S. Goodbourn, and R. E. Randall.** 2002. Degradation of STAT1 and STAT2 by the V proteins of simian virus 5 and human parainfluenza virus type 2, respectively: consequences for virus replication in the presence of alpha/beta and gamma interferons. J. Virol. **76**:2159–2167.
2. **Andrejeva, J., E. Poole, D. F. Young, S. Goodbourn, and R. E. Randall.** 2002. The p127 subunit (DDB1) of the UV-DNA damage repair binding protein is essential for the targeted degradation of STAT1 by the V protein of the paramyxovirus simian virus 5. J. Virol. **76**:11379–11386.
3. **Barnard, P., and N. A. McMillan.** 1999. The human papillomavirus E7 oncoprotein abrogates signaling mediated by interferon-α. Virology **259**: 305–313.
4. **Chatziandreou, N., D. Young, J. Andrejeva, S. Goodbourn, and R. E. Randall.** 2002. Differences in interferon sensitivity and biological properties of two related isolates of simian virus 5: a model for virus persistence. Virology **293**:234–242.
5. **Croze, E., A. Usacheva, D. Asarnow, R. D. Minshall, H. D. Perez, and O. Colamonici.** 2000. Receptor for activated C-kinase (RACK-1), a WD motif-containing protein, specifically associates with the human type I IFN receptor. J. Immunol. **165**:5127–5132.
6. **Didcock, L., D. F. Young, S. Goodbourn, and R. E. Randall.** 1999. Sendai virus and simian virus 5 block activation of interferon-responsive genes: importance for virus pathogenesis. J. Virol. **73**:3125–3133.
7. **Didcock, L., D. F. Young, S. Goodbourn, and R. E. Randall.** 1999. The V protein of simian virus 5 inhibits interferon signaling by targeting STAT1 for proteasome-mediated degradation. J. Virol. **73**:9928–9933.
8. **Fujii, N., N. Yokosawa, and S. Shirakawa.** 1999. Suppression of interferon response gene expression in cells persistently infected with mumps virus, and restoration from its suppression by treatment with ribavirin. Virus Res. **65**:175–185.
9. **Gotoh, B., T. Komatsu, K. Takeuchi, and J. Yokoo.** 2001. Paramyxovirus accessory proteins as interferon antagonists. Microbiol. Immunol. **45**:787–800.
10. **Haro, T., K. Shimoda, H. Kakumitsu, K. Kamezaki, A. Numata, F. Ishikawa, Y. Sekine, R. Muromoto, T. Matsuda, and M. Harada.** 2004. Tyrosine kinase 2 interacts with and phosphorylates receptor for activated C kinase-1, a WD motif-containing protein. J. Immunol. **173**:1151–1157.
11. **He, B., R. G. Paterson, N. Stock, J. E. Durbin, R. K. Durbin, S. Goodbourn, R. E. Randall, and R. A. Lamb.** 2002. Recovery of paramyxovirus simian virus 5 with a V protein lacking the conserved cysteine-rich domain: the multifunctional V protein blocks both interferon-beta induction and interferon signaling. Virology **303**:15–32.
12. **Huang, Z., S. Krishnamurthy, A. Panda, and S. K. Samal.** 2003. Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. J. Virol. **77**:8676–8685.
13. **Jin, B. F., K. He, H. X. Wang, J. Wang, T. Zhou, Y. Lan, M. R. Hu, K. H. Wei, S. C. Yang, B. F. Shen, and X. M. Zhang.** 2003. Proteomic analysis of ubiquitin-proteasome effects: insight into the function of eukaryotic initiation factor 5A. Oncogene **22**:4819–4830.
14. **Joseph, T. D., and D. C. Look.** 2001. Specific inhibition of interferon signal transduction pathways by adenovirus infection. J. Biol. Chem. **276**:47136–47142.
15. **Kato, A., Y. Ohnishi, M. Kohase, S. Saito, M. Tashiro, and Y. Nagai.** 2001. Y2, the smallest of the Sendai virus C proteins, is fully capable of both counteracting the antiviral action of interferons and inhibiting viral RNA synthesis. J. Virol. **75**:3802–3810.
16. **Kato, A., C. Cortese-Grogan, S. A. Moyer, F. Sugahara, T. Sakaguchi, T. Kubota, N. Otsuki, M. Kohase, M. Tashiro, and Y. Nagai.** 2004. Characterization of the amino acid residues of Sendai virus C protein that are critically involved in its interferon antagonism and RNA synthesis down-regulation. J. Virol. **78**:7443–7454.
17. **Kawano, M., M. Kaito, Y. Kozuka, H. Komada, N. Noda, K. Nanba, M. Tsurudome, M. Ito, M. Nishio, and Y. Ito.** 2001. Recovery of infectious human parainfluenza type 2 virus from cDNA clones and properties of the defective virus without V-specific cysteine-rich domain. Virology **284**:99–112.
18. **Kubota, T., N. Yokosawa, S. Yokota, and N. Fujii.** 2001. C terminal CYS-RICH region of mumps virus structural V protein correlates with block of interferon alpha and gamma signal transduction pathway through decrease of STAT 1-alpha. Biochem. Biophys. Res. Commun. **283**:255–259.
19. **Kubota, T., N. Yokosawa, S.-I. Yokota, and N. Fujii.** 2002. Association of mumps virus V protein with RACK1 results in dissociation of STAT-1 from the alpha interferon receptor complex. J. Virol. **76**:12676–12682.
20. **Lamb, R. A., and D. Kolakofsky.** 2001. Paramyxoviridae: the viruses and their replication, p. 1305–1340. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed. Lippincott, Williams and Wilkins Press, Philadelphia, Pa.
21. **Levy, D. E., and J. E. Darnell, Jr.** 2002. Stats: transcriptional control and biological impact. Nat. Rev. Mol. Cell Biol. **3**:651–662.
22. **Lin, G. Y., R. G. Paterson, C. D. Richardson, and R. A. Lamb.** 1998. The V protein of the paramyxovirus SV5 interacts with damage-specific DNA binding protein. Virology **249**:189–200.
23. **McCarthy, M., B. Jubelt, D. B. Fay, and R. T. Johnson.** 1980. Comparative studies of five strains of mumps virus in vitro and in neonatal hamsters: evaluation of growth, cytopathogenicity, and neurovirulence. J. Med. Virol. **5**:1–15.
24. **Merz, D. C., A. C. Server, M. N. Waxham, and J. S. Wolinsky.** 1983. Biosynthesis of mumps virus F glycoprotein: non-fusion strains efficiently cleave the F glycoprotein precursor. J. Gen. Virol. **64**:1457–1467.

25. **Miller, D. M., Y. Zhang, B. M. Rahill, W. J. Waldam, and D. D. Sedmak.** 1999. Human cytomegalovirus inhibits IFN-α-stimulated antiviral and immunoregulatory responses by blocking multiple levels of IFN-α signal transduction. J. Immunol. **162:**6107–6113.
26. **Morrison, T. E., A. Mauser, A. Wong, J. P.-Y. Ting, and S. C. Kenney.** 2001. Inhibition of IFN-γ signaling by an Epstein-Barr virus immediate-early protein. Immunity **15:**787–799.
27. **Munoz-Jordan, J. L., G. G. Sanchez-Burgos, M. Laurent-Rolle, and A. Garcia-Sastre.** 2003. Inhibition of interferon signaling by dengue virus. Proc. Natl. Acad. Sci. USA **100:**14333–14338.
28. **Nishio, M., M. Tsurudome, M. Ito, M. Kawano, H. Komada, and Y. Ito.** 2001. High resistance human parainfluenza type 2 virus protein-expressing cells to the antiviral and anti-cell proliferative activities of alpha/beta interferons: cysteine-rich V-specific domain is required for high resistance to the interferons. J. Virol. **75:**9165–9176.
29. **Nishio, M., D. Garcin, V. Simonet, and D. Kolakofsky.** 2002. The carboxyl segment of the mumps virus V protein associates with Stat proteins in vitro via a tryptophan-rich motif. Virology **300:**92–99.
30. **Palosaari, H., J.-P. Parisien, J. J. Rodriguez, C. M. Ulane, and C. M. Horvath.** 2003. STAT protein interference and suppression of cytokine signal transduction by measles virus V protein. J. Virol. **77:**7635–7644.
31. **Parisien, J.-P., J. F. Lau, J. J. Rodriguez, B. M. Sullivan, A. Moscona, G. D. Parks, R. A. Lamb, and C. M. Horvath.** 2001. The V protein of human parainfluenza virus 2 antagonizes type I interferon responses by destabilizing signal transducer and activator of transcription 2. Virology **283:**230–239.
32. **Parisien, J.-P., J. F. Lau, J. J. Rodriguez, C. M. Ulane, and C. M. Horvath.** 2002. Selective STAT protein degradation induced by paramyxoviruses requires both STAT1 and STAT2 but is independent of alpha/beta interferon signal transduction. J. Virol. **76:**4190–4198.
33. **Park, M.-S., M. L. Shaw, J. Muñoz-Jordan, J. F. Cros, T. Nakaya, N. Bouvier, P. Palese, A. García-Sastre, and C. F. Basler.** 2003. Newcastle disease virus (NDV)-based assay demonstrates interferon-antagonist activity for the NDV V protein and the Nipah virus V, W, and C proteins. J. Virol. **77:**1501–1511.
34. **Poole, E., B. He, R. A. Lamb, R. E. Randall, and S. Goodbourn.** 2002. The V proteins of simian virus 5 and other paramyxoviruses inhibit induction of interferon-β. Virology **303:**33–46.
35. **Rodriguez, J. J., J.-P. Parisien, and C. M. Horvath.** 2002. Nipah virus V protein evades alpha and gamma interferons by preventing STAT1 and STAT2 activation and nuclear accumulation. J. Virol. **76:**11476–11483.
36. **Rodriguez, J. J., L.-F. Wang, and C. M. Horvath.** 2003. Hendra virus V protein inhibits interferon signaling by preventing STAT1 and STAT2 nuclear accumulation. J. Virol. **77:**11842–11845.
37. **Shaffer, J. A., W. J. Bellini, and P. A. Rota.** 2003. The C protein of measles virus inhibit the type I interferon response. Virology **315:**389–397.
38. **Shaw, M. L., A. García-Sastre, P. Palese, and C. F. Basler.** 2004. Nipah virus V and W proteins have a common STAT1-binding domain yet inhibit STAT1 activation from the cytoplasmic and nuclear compartments, respectively. J. Virol. **78:**5633–5641.
39. **Takeuchi, K., K. Tanabayashi, M. Hishiyama, Y. K. Yamada, A. Yamada, and A. Sugiura.** 1990. Detection and characterization of mumps virus V protein. Virology **178:**247–253.
40. **Takeuchi, K., S. I. Kadota, M. Takeda, N. Miyajima, and K. Nagata.** 2003. Measles virus V protein blocks interferon (IFN)-alpha/beta but not IFN-gamma signaling by inhibiting STAT1 and STAT2 phosphorylation. FEBS Lett. **545:**177–182.
41. **Ulane, C. M., and C. M. Horvath.** 2002. Paramyxovirused SV5 and HPIV2 assemble STAT protein ubiquitin ligase complexes from cellular components. Virology **304:**160–166.
42. **Ulane, C. M., J. J. Rodriguez, J.-P. Parisien, and C. M. Horvath.** 2003. STAT3 ubiquitination and degradation by mumps virus suppress cytokine and oncogene signaling. J. Virol. **77:**6385–6393.
43. **Usacheva, A., R. Smith, R. Marshall, G. Baida, S. Seng, E. Cruz, and O. R. Colamonici.** 2001. The WD motif-containing protein receptor for activated protein kinase C (RACK1) is required for recruitment and activation of signal transducer and activator of transcription 1 through the type I interferon receptor. J. Biol. Chem. **276:**22948–22953.
44. **Usacheva, A., X. Tian, R. Sandoval, D. Salvi, D. Levy, and O. R. Colamonici.** 2003. The WD motif-containing protein RACK-1 functions as a scaffold protein within the type I IFN receptor-signaling complex. J. Immunol. **171:**2989–2994.
45. **Wansley, E. K., and G. D. Parks.** 2002. Naturally occurring substitutions in the P/V gene convert the noncytopathic paramyxovirus simian virus 5 into a virus that induces alpha/beta interferon synthesis and cell death. J. Virol. **76:**10109–10121.
46. **Yokosawa, N., T. Kubota, and N. Fujii.** 1998. Poor induction of interferon-induced 2′,5′-oligoadenylate synthetase (2-5 AS) in cells persistently infected with mumps virus is caused by decrease of STAT-1 alpha. Arch. Virol. **143:**1985–1992.
47. **Yokosawa, N., S.-I. Yokota, T. Kubota, and N. Fujii.** 2002. C-terminal region of STAT-1α is not necessary for its ubiquitination and degradation caused by mumps virus V protein. J. Virol. **76:**12683–12690.
48. **Yokota, S., N. Yokosawa, T. Kubota, T. Suzutani, I. Yoshida, S. Miura, K. Jimbow, and N. Fujii.** 2001. Herpes simplex virus type I suppresses the interferon signaling pathway by inhibiting phosphorylation of STATs and janus kinases during an early infection stage. Virology **286:**119–124.
49. **Yokota, S., H. Saito, T. Kubota, N. Yokosawa, K. Amano, and N. Fujii.** 2003. Measles virus suppresses interferon-alpha signaling pathway: suppression of Jak1 phosphorylation and association of viral accessory proteins, C and V, with interferon-alpha receptor complex. Virology **306:**135–146.
50. **Young, D. F., N. Chatziandreou, B. He, S. Goodbourn, R. A. Lamb, and R. E. Randall.** 2001. Single amino acid substitution in the V protein of simian virus 5 differentiates its ability to block interferon signaling in human and murine cells. J. Virol. **75:**3363–3370.