表1　偽ウイルス感染実験に用いた細胞株

	Designation	Organism	Source		Morphology
			Organ	Disease	
1	Vero E6	Cercopithecus aethiops (monkey, African green)	Kidney	normal	epithelial
2	HepG2	Homo sapiens (human)	liver	hepatocellular carcinoma	epithelial
3	HuH-7	Homo sapiens (human)	liver	Highly differentiated, hepatocelluler carcinoma	Epithelial-like
4	HEp-2	Homo sapiens (human)	Larynx	Epidermoid carcinoma	Epithelial-like
5	Sq-1	Homo sapiens (human)	Lung	Lung squamous cell carcinoma	Half epithelial-like,
6	S2	Homo sapiens (human)	Lung	Human small cell carcinoma	Epithelial-like
7	LU65	Homo sapiens (human)	Lung	Small cell carcinoma	Epithelial-like , partly floating.
8	11-18	Homo sapiens (human)	Lung	Adenocarcinoma	Epithelial-like
9	86-2	Homo sapiens (human)	Lung	Large cell carcinoma	Epithelial-like
10	LK-2 (JCRB)	Homo sapiens (human)	Lung	Squamous cell carcinoma of lung	Epithelial-like , island-forming .
11	OBA-LK-1	Homo sapiens (human)	Lung	Lung cancer cell line (large cell carcinoma)	Epihelial-like

表２　　SARS ウイルス感染に及ぼす漢方エキスの影響

The name of drug	The highest concentration μg/ml	The inhibition rate% by MTT	The inhibition rate% by CPE
黄耆	500	11.14	20
连翘	125	0	0
补中益气汤	500	18.22	28.88
清新莲子饮	125	4	2.22
柴胡	500	17.97	11.11
黄芩	125	16.37	20
桂皮	62.5	0	0
甘草	125	0.5	2.22
丁子	62.5	2	11.11
柴胡桂枝汤	500	0	2.22
小青龟汤	250	0	0
葛根汤	500	7	2.22

ＩＩＩ．刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍名	出版社名	出版地	出版年	ページ
該当なし							

雑誌

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服部俊夫	SARSを含む新興感染症	日本内科学会雑誌			印刷中

Ⅳ．研究成果の刊行物

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Human Parvovirus B19 nonstructural protein transactivates the p21/WAF1 through Sp1☆

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Abstract

The expression of human Parvovirus B19 nonstructural protein 1 (NS1) induces cell cycle arrest at the G1 phase and is accompanied by increased expression of the cyclin-dependent kinase inhibitor, p21/WAF1. Here, we provide direct evidence that NS1 mediates the transactivation of p21/WAF1. Up-regulation of p21/WAF1 by wild-type NS1 but not an NS1 mutant deleted of its NTP binding motif was observed. We also demonstrated that the wild-type NS1 is unable to induce G1 arrest in p21-deficient cells. Using reporter plasmids containing various mutants of the p21/WAF1 promoter, luciferase assay further revealed that the binding sites of the promoter to the transcription factor Sp1 are critical for NS1-mediated transactivation. Indeed Sp1 interacts only with the wild-type NS1 but not the NS1 mutant. These results indicate a cooperative contribution of NS1 and Sp1 to the transactivation of p21/WAF1, which leads to G1 arrest.

Keywords: Parvovirus B19; Nonstructural protein; Cell cycle arrest

Introduction

Parvovirus B19 is the only pathogenic member of the *Parvoviridae* in humans. There are five major clinical manifestations related to B19 virus infection: aplastic crisis in patients with hemolytic anemia, erythema infectiosum, arthralgia/arthritis, persistent infections manifesting as chronic anemia in immunocompromised patients, and after maternal infection, nonimmune hydrops fetalis, which can lead to intrauterine fetal death (Anderson et al., 1983, 1988; Bradley et al., 1987; Brown et al., 1994; Kurtzman et al., 1987). B19 virus is a potent inhibitor of erythroid cell differentiation and is cytotoxic for erythroid precursor cells (Ozawa et al., 1987), and most B19-associated clinical manifestations are closely related to damage of erythroid precursor cells (Brown et al., 1994).

A nonstructural protein 1 (NS1) is encoded by the left side of the B19 genome and appears on Western blots as a doublet with apparent molecular weights of 70 and 77 kDa (Morita et al., 2001). NS1 is synthesized from a strong left side promoter at map unit 6 (p6), which is the only functional promoter in the B19 genome. NS1 is cytotoxic, and NS1-transfected cells and B19 virus-infected cells have ultrastructural features typical of apoptosis (Morey et al., 1993; Yaegashi et al., 1999). NS1 is also essential for the replication of viral DNA and the activation of the B19 p6 promoter (Brown et al., 1994) and has a positive feedback effect on the activity of the p6 promoter (Doerig et al., 1990). Such effects of NS1 on p6 promoter activity have been suggested to be mediated by a multicomponent complex that combines NS1 with ATF, NF-κB/cRel, and cellular GC-box binding factors (Gareus et al., 1998).

In another Parvovirus, the minute virus of mice (MVM) transactivation by NS1 is dependent on the presence of

☆ Activation of p21/WAF1 by B19 NS1.

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functional TATA and GC box sequences in the MVM p38 promoter (Ahn et al., 1992). Sp1, a transcriptional factor that is known to bind GC box elements, plays a key role in the activation of a variety of viral and cellular gene promoters (Suske, 1999). In this context, an interaction between MVM NS1 and Sp1 is reportedly required to stimulate transcription of the p38 promoter, which contains an Sp1 binding site (Krady and Ward, 1995).

Nonstructural protein genes are highly homologous among the Parvoviruses, including the B19 virus, MVM, and adeno-associated virus (AAV). MVM and AAV each express nonstructural protein, NS1 and Rep, which are homologous proteins, respectively, that are required for viral DNA replication and transcriptional regulation (Im and Muzyczka, 1990; Jindal et al., 1994). Moreover, the genes that encode these proteins contain the A-type consensus sequence [(G/A) XXXXGK (S/T)], referred to as a nucleoside triphosphate (NTP) binding motif, which was originally identified in the large T antigens of Polyomaviruses and E1 proteins of Papillomaviruses (Bradley et al., 1987). Mutations in the NTP binding motifs of MVM NS1 and AAV Rep affect their functions, which include ATP binding, ATPase and helicase enzymatic activities, transactivation, and DNA replication (Horer et al., 1995; Jindal et al., 1994). The DNA- and RNA-dependent nucleoside triphosphatases are key enzymes in the biochemical pathways of replication, repair, recombination, transcription, and translation of a variety of cellular and viral genes (Momoeda et al., 1994). B19 virus NS1 also contains an NTP binding motif in the middle of the sequence, which has been shown to harbor at least a helicase activity (Astell et al., 1987; Raab et al., 2002). Furthermore, an NS1 mutant in which lysine 334 in the NTP binding motif is converted to glutamate loses its cytotoxic activity (Momoeda et al., 1994).

Infection by Parvoviruses such as B19, AAV, and MVM induces cell cycle arrest of their target cells (De Beeck et al., 2001; Morita et al., 2001, 2003; Winocour et al., 1988). In AAV, the growth of virus-infected cells is inhibited by the accumulation of G2-phase cells, in which the AAV hairpin DNA plays a critical role (Raj et al., 2001). MVM-infected cells are known to be arrested at the G1, S, and G2 phases (De Beeck et al., 2001; Op De Beeck and Caillet-Fauquet, 1997). We have also demonstrated that B19 virus infection induces cell cycle arrest at the G1 and G2 phases; the G1 arrest is mediated by NS1, and the G2 arrest is mediated by a different mechanism since it can be induced by an inactivated B19 virus that cannot express NS1 (Morita et al., 2001, 2003). NS1-transfected cells that arrest at the G1 phase express p21/WAF1, a cyclin-dependent kinase (CDK) inhibitor (Morita et al., 2003). p21/WAF1, which was initially identified as a target gene for p53 transactivation (el-Deiry et al., 1993), is involved in the suppression of G1 to S-phase cell cycle progression through its binding to G_1 cyclin/CDK complexes (Harper et al., 1993). However, there is accumulating evidence for a p53-independent transcriptional activation of p21/WAF1, in which the proximal region of the p21/WAF1 promoter (base pairs −210 to +1) is a major responsive element containing several GC-rich motifs that serve as binding sites for members of the Sp1 family of transcription factors (Nakano et al., 1997). Here we demonstrate that a complex consisting of NS1 and Sp1 transactivates p21/WAF1, resulting in cell cycle arrest at the G1 phase.

Results

An NTP-binding-defective mutant of NS1 is unable to induce cell cycle arrest at the G1 phase or expression of p21/WAF1

We previously reported that in some cell lines treated with the mitotic inhibitor, paclitaxel, the transfection of NS1, as well as B19 virus infection induce cell cycle arrest at the G1 phase as well as the expression of p21/WAF1 (Morita et al., 2003). To address the functional significance of NS1 in cell cycle arrest and p21/WAF1 expression, we constructed an NS1 mutant expression vector, pMP6-NS1-KE, that has a single amino acid substitution in the NTP binding motif. The percentages of 293T cells arrested in the G1 phase were 40.0%, 16.3%, and 16.3% for cells transfected with the wild-type NS1, NS1-KE, and the control vector, respectively (Fig. 1A). Similar results were obtained using UT7/Epo-S1 cells (Fig. 1B). These results indicated that the NS1-KE mutant lost its ability to induce cell cycle arrest at the G1 phase. Next, we examined the expression levels of NS1 and p21/WAF1 by Western blotting. The expression level of NS1-KE was similar to that of the wild-type NS1; however, the expression levels of p21/WAF1 were clearly different in the cells transfected with the wild-type versus the mutant NS1: the NS1-KE mutant suppressed by approximately 40% the expression of p21/WAF1, as compared to wild-type NS1 (Fig. 1C). The transfection efficiencies of the wild-type NS1, NS1-KE, and the control vectors ranged from 60% to 80% (data not shown). These results suggest that the intact NTP binding motif of NS1 is essential to induce G1 arrest and the expression of p21/WAF1.

Small interfering RNAs (siRNAs) to p21/WAF1 impair G1 arrest induced by NS1

To investigate the role of p21/WAF1 in NS1-induced G1 arrest, we examined the effect of pMACS–$H2K^k$–siRNA–p21, an siRNA vector, which suppresses p21/WAF expression, on the G1 arrest. Following introduction of the siRNA vector into 293T cells, enrichment of $H2K^k$-positive cells was done by AutoMACS machine. There was no significant bias of cell cycle distribution upon siRNA–p21 transfection (data not shown). $H2K^k$-positive 293T cells were further transfected with NS1 or control vectors followed by

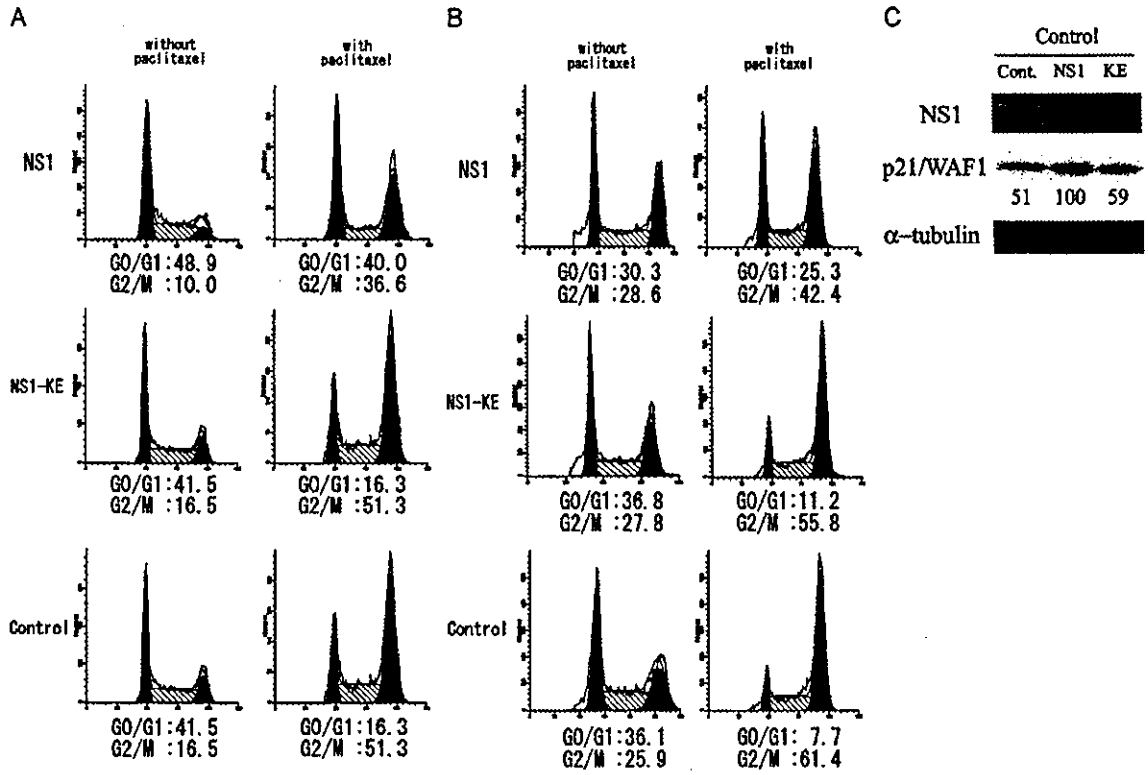

Fig. 1. The NS1-KE mutant did not induce cell cycle arrest at the G1 phase. (A and B) The 293T cells (A) and UT7/Epo-S1 cells (B) were transfected with NS1 (upper panels), NS1-KE (middle panels), or control vector (lower panels) and incubated for 48 and 24 h, respectively. They were then treated (right panels) or not treated (left panels) with paclitaxel, a mitotic inhibitor, for 24 h. Subsequently, they were stained with PI to detect the DNA content and loaded on a FACS caliber. (C) The expressions of NS1, NS1-KE, and control vector were detected by Western blotting with anti-NS1, and p21/WAF1 and α-tubulin were detected with their specific antibodies in the lysates of 293T cells transfected as described above. The percentage of p21/WAF1 expression level is shown below the middle panel.

paclitaxel treatment. We observed a significant inhibition of p21/WAF1 expression in $H2K^k$-positive 293T cells (Fig. 2A, approximately 50% suppression). Furthermore, G1 arrests of NS1-transfected cells showed 25.6% in siRNA–p21-transfected cells and 39.5% in siRNA–control-transfected cells (Fig. 2B). We obtained similar results in triplicate experiments (Fig. 2C). These results suggest that the down-regulation of p21/WAF expression by the siRNA leads to a decrease of the NS1-induced G1 arrest.

NS1-mediated p21/WAF1 promoter activation

NS1 transactivates the p21/WAF1 promoter, for which the Sp1 binding sites of the promoter are indispensable. We next investigated the mechanism by which NS1-induced G1 arrest through p21/WAF1. Hence, the effects of NS1 on p21/WAF1 promoter activity were examined by transient transfection using a luciferase assay. Forty-eight hours after the transfection, NS1 had induced a 3.8-fold increase in the luciferase activity of the full-length wild-type p21/WAF1 promoter, pWWP, as compared with the control vector (Fig. 3B), indicating that NS1 transactivates the p21/WAF1 promoter. We next determined the NS1 responsive element(s) in the p21/WAF1 promoter using a series of p21/WAF1 promoter mutants: pWP124, in which the two p53 binding sites were deleted but all six Sp1 binding sites remained; pWP101, in which the Sp1-1 and Sp1-2 sites were deleted; and pWPdel–SmaI, in which the Sp1-1 to Sp1-4 sites were deleted (Fig. 3A). NS1 induced 4.6-, 3.5-, and 1.6-fold increases in the luciferase activities of pWP124, pWP101, and pWPdel–SmaI, respectively, as compared with the control vector (Fig. 3B). The NS1-KE mutant showed negligible transactivation of any p21/WAF1 promoter (data not shown). The basal promoter activities of pWP124, pWP101, and pWWP were not significantly different, whereas pWPdel–SmaI showed a decreased promoter activity at 13.4% of pWWP (Fig. 3B). Taken together, these results indicated that NS1-mediated p21/WAF1 promoter activation requires the region containing the Sp1-3, Sp1-4, and Sp1-5-6 sites and the TATA element.

pWP101 was further mutated at the Sp1 sites and the TATA element. These constructs were termed pWP101-mtSp1-3, pWP101-mtSp1-4, pWP101-mtSp1-5-6, and pWP101-mtTATA, respectively (Fig. 3C). In the NS1-induced luciferase assays, pWP101-mtSp1-3 showed an activation of only 1.1-fold of control levels, compared with the 3.5-fold activation of pWP101, and the basal activity of pWP101-mtSp1-3 was 8.2% of that of pWP101 (Fig.

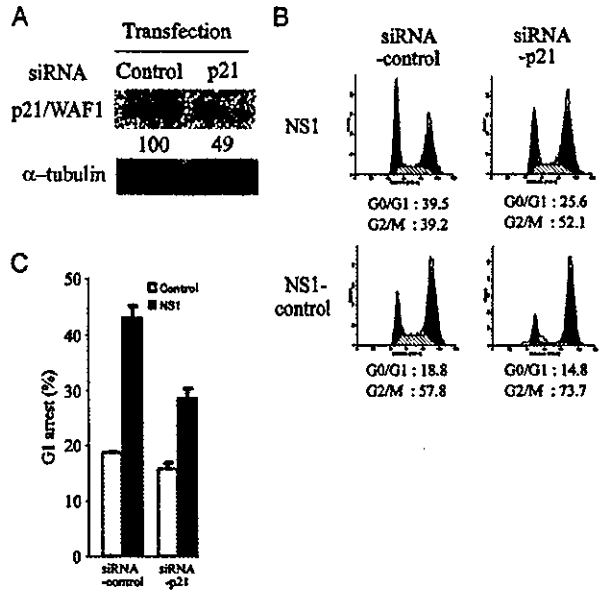

Fig. 2. Small interfering RNAs (siRNAs) to p21/WAF1 impair G1 arrest induced by NS1. The 293T cells were transfected with pMACS-H2K^k–siRNA–p21 (p21/WAF1 siRNA) or pMACS-H2K^k–siRNA–control (empty vector). H2K^k-positive cells were transfected with NS1 or control vector as described under Materials and methods. (A) The p21/WAF1 protein level in nuclear extracts from NS1-transfected cells were detected using anti-p21/WAF1 antibody, cells transfected with control vector (left), or siRNA–p21 (right). (B) The 293T cells transfected with siRNA vectors were transfected with NS1 (upper panels) or control vector of NS1 (lower panels) and incubated for 24 h, respectively. They were then treated with paclitaxel for another 24 h. Cells were stained with PI to detect the DNA content by a FACS caliber. (C) Percentages of the cells at the G1 phase in panel B were counted.

3C). pWP101-mtSp1-4 had a basal activity that was 33.1% of pWP101; however, the NS1-induced luciferase activity was 2.8-fold of control levels (Fig. 3C). In contrast, the basal activities of pWP101-mtSp1-5-6 and pWP101-mtTATA were 0.1% that of pWP101, that is, background level, and the NS1-induced activation was entirely abolished in these constructs (Fig. 3C). From these results, we concluded that the Sp1-3 site but not the Sp1-4 site is the main NS1-responsive element for regulating transactivation, and that the Sp1-5-6 site and TATA element are indispensable for the basal promoter activity of the p21/WAF1 promoter.

Since the two Sp1 sites play critical roles in the NS1-mediated transactivation, we performed an EMSA using probes for wild-type Sp1-3, Sp1-3wt, and a mutant Sp1-3, Sp1-3mt, and nuclear extracts from 293T cells transfected with NS1, NS1-KE, and control vector (Fig. 4). The EMSA with the Sp1-3wt probe revealed three specific bands that we refer to as the fast, slow, and slowest bands. In EMSAs with nuclear extracts of cells transfected with NS1 and NS1-KE, the intensities of the three bands were very similar to those obtained using nuclear extracts from cells transfected with the control vector (Fig. 4, lanes 1–3). When the nuclear extracts were pretreated with the anti-Sp1 antibody, the intensity of the slowest band decreased significantly and a supershifted band became detectable, but the slow and fast bands changed little (Fig. 4, lanes 4–6). In contrast, when the nuclear extracts were pretreated with the anti-Sp3 antibody, the slow and fast bands decreased and faint supershifted bands were detectable, but the slowest band changed little (Fig. 4, lanes 7–9). Pretreatment with both anti-Sp1 and anti-Sp3 antibodies induced significant reductions in all three bands (Fig. 4, lanes 10–12); the three bands were undetectable when Sp1-3mt was used as a probe (Fig. 4, lanes 13–15). Pretreatment with the anti-NS1 antibody had no effect on the three bands (data not shown). These results suggest that the slowest bands corresponded to the probes bound to Sp1, and the slow and fast bands corresponded to probes bound to Sp3, and that NS1 does not affect the Sp1 binding affinity to the Sp1-3 site in the p21/WAF1 promoter. We also confirmed that NS1 has no effect on the binding of Sp1 and Sp3 to the Sp1-5-6 site (data not shown).

NS1-mediated transactivation of the p21/WAF1 promoter is up-regulated by Sp1 and CBP

Since the Sp1-3 and Sp1-5-6 sites played important roles in the p21/WAF1 promoter activation induced by NS1, we examined whether Sp1 and Sp3 are involved in the NS1-mediated transactivation of the p21/WAF1 promoter. The coexpression of Sp1 with NS1 significantly enhanced the expression of the wild-type p21/WAF1 reporter plasmid, pWWP, in a dose-dependent manner (Fig. 5A). In contrast, the activities of pWWP were unchanged by varying doses of Sp3. Furthermore, the NS1-mediated transactivation of the p21/WAF1 promoter was also inhibited by Sp3 (Fig. 5B). These results suggest that Sp1 but not Sp3 is a critical transcription factor in the p21/WAF1 promoter activation mediated by NS1 in 293T cells.

p300/CBP has been shown to interact functionally with a number of transcriptional activators including Sp1, CREB, NF-κB, p53, and others (Chan and La Thangue, 2001; Fang and Lu, 2002). We next addressed the possible involvement of CBP in the NS1-mediated p21/WAF1 promoter activation. The coexpression of CBP with NS1 significantly enhanced the expression of pWWP-Luc in a dose-dependent manner (Fig. 5C). We further examined the effects of CBP on the p21/WAF1 mutant reporter plasmids, pWP124 and pWPdel–SmaI. The luciferase assay results of pWP124 were similar to those of pWWP (Fig. 5D), whereas neither NS1 nor CBP induced expression of pWPdel–SmaI (data not shown). These results suggest that CBP contributes to the NS1-mediated activation of p21/WAF1 transcription, for which the Sp1 sites are also required.

To determine whether Sp1 acts as a mediator of the NS1-induced transactivation, we used a system in which Sp1 was fused to the DNA binding domain of the bacterial transcriptional factor GAL4. Consequently, the ability of the

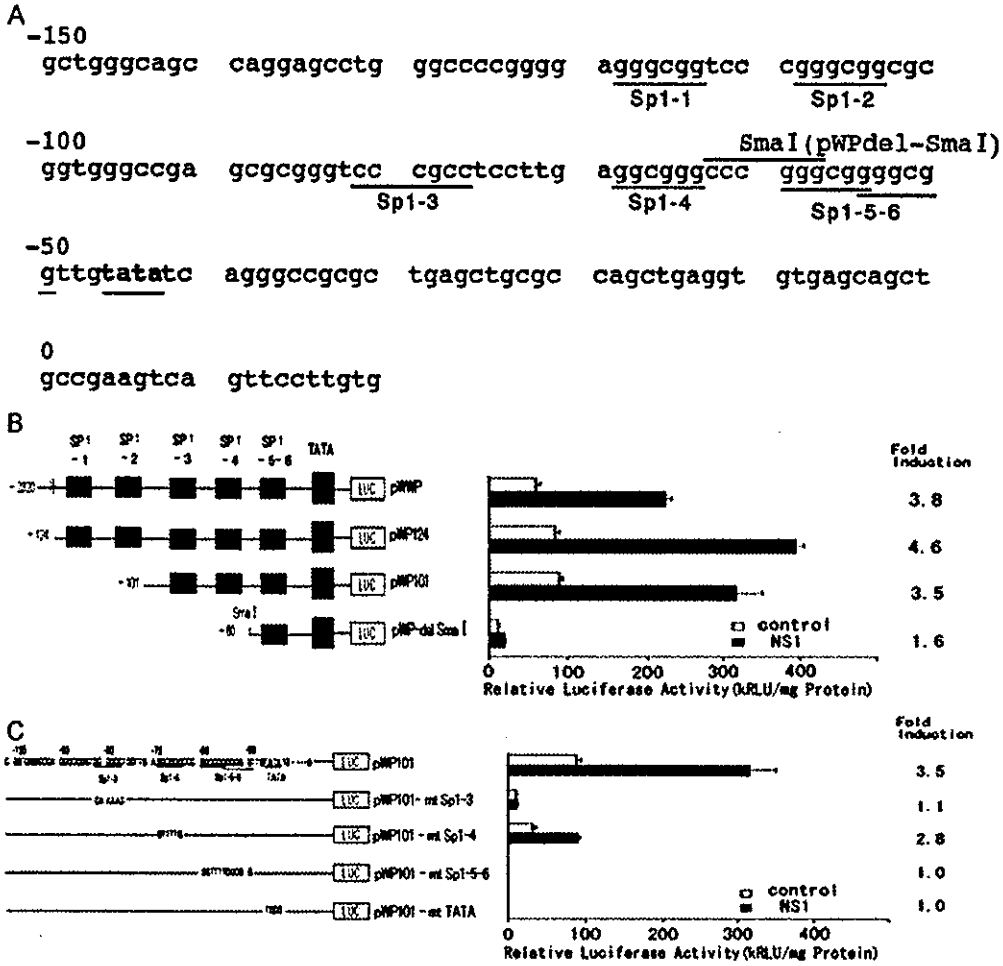

Fig. 3. Sp1 binding sites are critical for NS1-mediated p21/WAF1 promoter activation. (A) Genomic sequence of the p21/WAF1 promoter region. The transcription start site is indicated by the number 0 above the sequence. The multiple Sp1 binding sites termed Sp1-1, Sp1-2, Sp1-3, Sp1-4, and Sp1-5-6 are indicated by underlining. The TATA box is indicated by bold letters and underlining. The enzyme site of pWPdel–SmaI is indicated. (B) Luciferase analyses with a series of 5′deletion mutants of the p21/WAF1 promoter. The 293T cells were transiently cotransfected with the constructed plasmids, pWWP, pWP124, pWP101, and pWPdel–SmaI, or the control vector (white bar), or NS1 (black bar), and the luciferase activities were analyzed after incubating the transfected cells for 48 h. Relative luciferase activities are shown as the raw light units (RLU) in cell lysates per 1 mg of protein, and the fold inductions of luciferase activities were calculated in comparison to the luciferase activity induced by the control vector. (C) Luciferase analyses with mutants containing point mutations in the Sp1 sites on the p21/WAF1 promoter. As shown on the left, four mutants are identical to the wild-type pWP101 except for the mutations indicated. Luciferase activities were analyzed in a similar manner to panel B.

fusion protein to activate transcription from the consensus GAL4 DNA binding site in both the presence and absence of NS1 could be measured. In this way, Sp1 up-regulates a luciferase reporter plasmid, pG5-Luc, which has five consensus GAL4 DNA binding sites, in a dose-dependent manner. Furthermore, the coexpression of Sp1 with NS1 significantly enhanced the activity of the luciferase reporter plasmid (Fig. 5E). This data suggested that the transcriptional activity of Sp1 is generally enhanced by coexpression of NS1.

To explore whether there is a cooperative function of Sp1 and CBP in the NS1-mediated p21/WAF1 promoter activation, we carried out p21/WAF1 luciferase assays using 293T cells transfected with NS1, Sp1, Sp3, and CBP. The combination of NS1, Sp1, and CBP showed the highest activity, 9.2-fold, and the combination of NS1, Sp3, and CBP showed a lower activity, 2.7-fold, than the previous combination (Fig. 5F, lanes 13 and 14). The addition of Sp3 to the combination of NS1 and Sp1 did not enhance the luciferase activity further (Fig. 5F, lanes 6 and 12). These data indicated that CBP and Sp1 but not Sp3 synergistically participate in the NS1-mediated p21/WAF1 transcriptional activation.

Direct binding of NS1 to Sp1

Since NS1, Sp1, and CBP are cooperatively involved in the transactivation of the p21/WAF1 promoter through a minimal region containing the Sp1 sites, we examined complex formation among the three molecules. The 293T

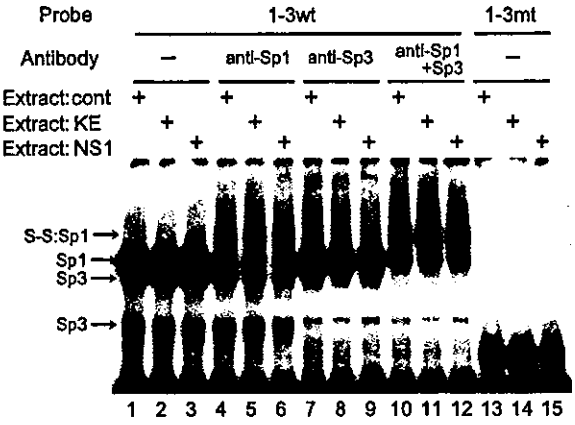

Fig. 4. EMSAs for the Sp1 binding sites on p21/WAF1. EMSAs were carried out with nuclear extracts of 293T cells transfected with NS1, NS1-KE, or control vector. (A) End-labeled oligonucleotides were used for the Sp1-3wt probe (lanes 1–12) and Sp1-3 mutant probe (lanes 13–15). No antibody (lanes 1–3 and 13–15), the anti-Sp1 antibody (lanes 4–6), and the anti-Sp3 (lanes 7–9) and anti-Sp1+ anti-Sp3 (lanes 10–12) antibodies were used. The positions of Sp1 and Sp3 are indicated on the left. S-S: Sp1 indicates supershifted band of Sp1.

cells were transiently cotransfected with NS1, Sp1, Sp3, and CBP, and their nuclear extracts were immunoprecipitated and immunoblotted with each antibody. In cotransfected cells, NS1 and Sp1 were co-immunoprecipitated (Fig. 6A), indicating a direct interaction between NS1 and Sp1. A small amount of NS1 was co-immunoprecipitated with Sp1 in lysates from cells transfected with NS1 alone (Fig. 6A), suggesting an interaction between NS1 and endogenous Sp1. On the other hand, the co-immunoprecipitation of NS1 and Sp3 was not seen in lysates from cells cotransfected with both constructs (Fig. 6B). NS1 and CBP did not co-immunoprecipitate with each other either (data not shown). These results showed that NS1 interacts with Sp1, but not Sp3 or CBP. We also examined whether the NS1-KE mutant interacts with Sp1. In contrast to wild-type NS1, the NS1-KE mutant did not co-immunoprecipi-

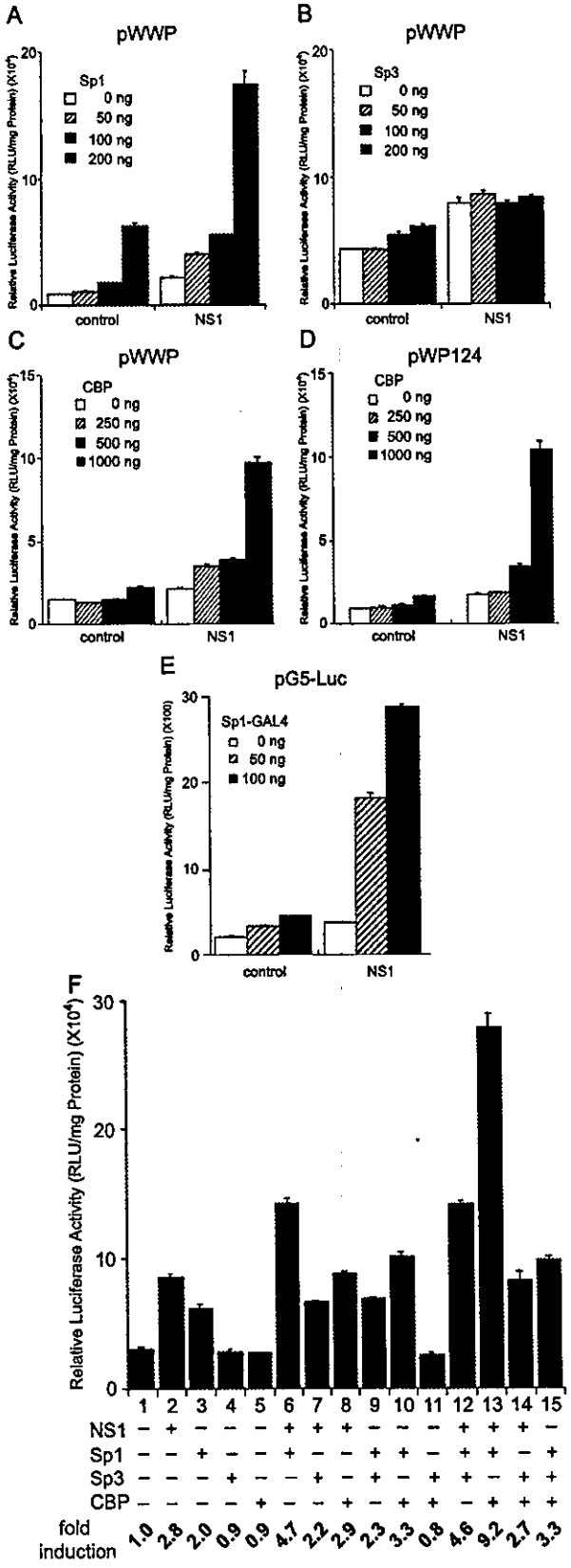

Fig. 5. NS1-mdiated activation of the p21 promoter in luciferase assays. (A and B) The 293T cells were cotransfected with the indicated amounts of the Sp1 (pEVR2/Sp1) or Sp3 (pRc/CMV/Sp3) expression construct and the p21/WAF1 luciferase reporter plasmid (pWWP) and pMP6-NS1 (NS1) or pMP6 (control). (C and D) The indicated amounts of HA-tagged CBP expression construct (pRc/RSV/CBP) were cotransfected with pWWP or pWP124 containing all six Sp1 binding sites and pMP6-NS1 or pMP6. (E) The 293T cells were cotransfected with the indicated amounts of the pM-Sp1 (GAL4-Sp1) expression construct, the luciferase reporter plasmid containing five consensus GAL4 DNA binding sites (pG5-Luc), and pMP6-NS1 or pMP6. (F) According to the combinations of the indicated plasmids, 293T cells were transfected with 10 ng of pWWP, 1 μg of pMP6-NS1, 200 ng of pEVR2/Sp1, 200 ng of pRc/CMV/Sp3, and/or 1 μg of pRc/RSV/CBP. The total amounts of plasmid were adjusted by adding empty control vectors. Luciferase activities were analyzed after incubation for 48 h. The relative luciferase activities are shown as raw light units (RLU) in cell lysates per 1 mg of protein, and fold inductions that are compared with the four transfected control vectors (lane 1) are indicated below.

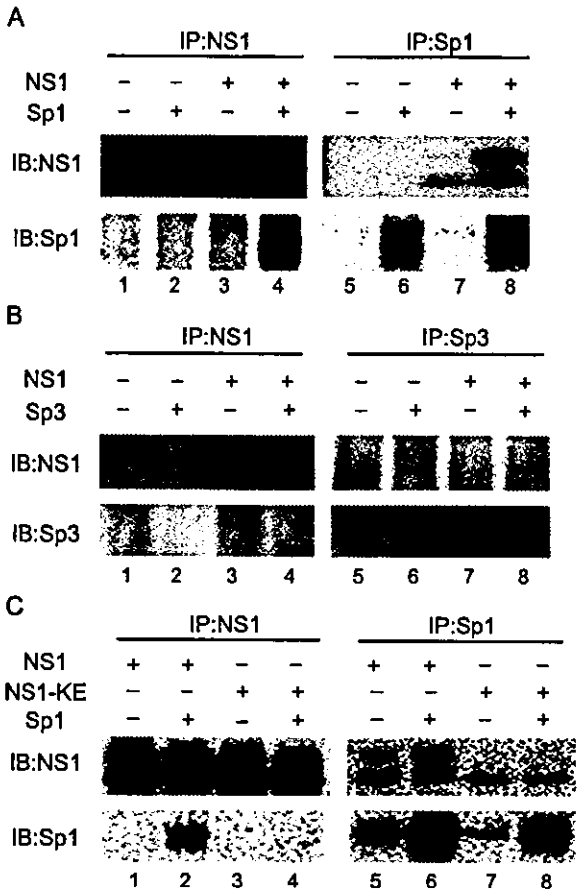

Fig. 6. Co-immunoprecipitation of NS1 and Sp1. Nuclear extracts of 293T cells transfected with the indicated combinations of NS1, NS1-KE, and Sp1 were immunoprecipitated then immunoblotted with antibodies against NS1 and Sp1.

tate with Sp1, or vice-versa, even in lysates from cotransfected cells (Fig. 6C). These results implied that the NS1-KE mutant, in which the NTP binding motif was deleted, could not interact directly with Sp1, suggesting that the formation of a complex containing NS1 and Sp1 is indispensable for the transactivation of the p21/WAF1 promoter.

p21/WAF1 is indispensable to the NS1-induced G1 arrest

The luciferase assay showed that NS1-induced transactivation of the p21/WAF1 promoter requires the presence of Sp1 binding sites, Sp1-3 and Sp1-5-6, but not p53 binding sites (Fig. 3B). To confirm the indispensable role of the NS1 and Sp1 complex in induction of p21/WAF1, we first established a p53 knock-down and p21-deleted cell line, HCT116$^{p53KD,\ p21-/-}$, by using pRNAT-U6.1/Neo-p53-1. The NS1-induced G1 arrest was 33.2% in HCT116$^{p53KD,\ p21+/+}$ cells, and 13.9% in HCT116$^{p53KD,\ p21-/-}$, respectively (Fig. 7A). The cell population in the G1-phase of HCT116$^{p53KD,\ p21-/-}$ cells significantly decreased in a time-dependent manner, but little in HCT116$^{p53KD,\ p21+/+}$ cells (Fig. 7B). We further showed that the G1 arrest in HCT116$^{p53KD,\ p21-/-}$ cells is mediated by the wild-type NS1, but not NS1-KE mutant or control vector (Fig. 7C). The accumulation of p21/WAF1 expression in HCT116$^{p53KD,\ p21+/+}$ cells transfected with NS1 was confirmed to be much higher than those transfected with control or NS1-KE (data not shown). Thus, p21/WAF1 plays a critical role in the NS1-mediated induction of G1 arrest.

Discussion

We previously reported that B19 virus can induce not only G2 arrest but also G1 arrest, and NS1, a nonstructural protein of B19 virus, plays an important role in the induction of G1 arrest accompanied by the expression of p21/WAF1, a CDK inhibitor (Morita et al., 2003). However, it remained unclear whether NS1 directly contributes to the expression of p21/WAF1. In the present study, we first showed that the NS1-KE mutant, which has a point mutation in the NTP binding motif, does not induce G1 arrest or the accumulation of p21/WAF1. Second, wild-type NS1 transactivated the p21/WAF1 promoter via a minimal

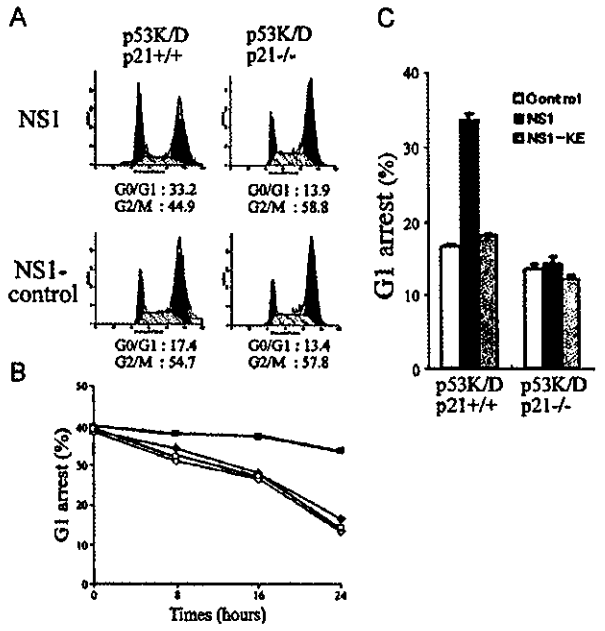

Fig. 7. The NS1-induced G1 arrest is completely abrogated in p21-deficient cells. (A) HCT116$^{p53KD,\ p21+/+}$ cells or HCT116$^{p53KD,\ p21-/-}$ cells were transfected with NS1 (upper panels) or control vector of NS1 (lower panels) and incubated for 24 h, respectively. They were then treated with paclitaxel for 24 h. Subsequently, they were stained with PI to detect the DNA content and loaded on a FACS caliber. (B) At the indicated times after paclitaxel treatment, percentages of the cell at the G1 phase were counted. Cell lines were HCT116$^{p53KD,\ p21+/+}$ cells transfected with control vector (closed diamonds), or with NS1 vector (closed squares), and HCT116$^{p53KD,\ p21-/-}$ cells transfected with control vector (open diamonds), or with NS1 vector (open squares). (C) Percentages of the cells at the G1 phase in panel A were counted.

region consisting of the Sp1 binding sites and the TATA element, but the NS1-KE mutant, had no such transactivating activity. NS1 but not the NS1-KE mutant bound directly to Sp1. Finally, NS1-induced G1 arrest was abrogated in a p21-deficient cell line. Since the accumulation of p21/WAF1 is generally known to induce G1 arrest (Harper et al., 1993), we conclude that Sp1 combining with NS1 contributes to the activation of the p21/WAF1 promoter, which results in the accumulation of p21/WAF1 to induce cell cycle arrest at the G1 phase. Moreover, these suggest that the NTP binding motif of NS1 is indispensable for its interaction with Sp1 and its transactivation of the p21/WAF1 promoter.

Transfection of NS1 reportedly induces a cytotoxicity, but not of NS1-KE mutant (Momoeda et al., 1994). We have shown that NS1 has a potent ability to induce G1 arrest, resulting in growth arrest (Morita et al., 2003). In this study, an NS1-KE mutant was unable to undergo cell cycle arrest, which may reflect the abolishment of the cytotoxicity. These results suggest that the NTP binding motif of NS1 is important for the induction of cell cycle arrest at the G1 phase, which may be responsible for its cytotoxic effect.

The functional significance of NS1 has been investigated in the transactivation of the viral p6 promoter; NS1 transactivates the p6 promoter, and the binding sites for ATF/CREB, NF-κB/cRel, and GC-box are critically involved in this transactivation (Gareus et al., 1998). Since the GC-boxes in the p6 promoter correspond to Sp1 binding sites (Dynan and Tjian, 1983), a direct interaction between NS1 and Sp1 is also thought to be important for the p6 transactivation. In the regulation of cellular gene expression, Sp1 is also known to exert its transcriptional properties by direct interaction with basal transcriptional factors and transcriptional activators (Suske, 1999). Hence, the cellular target genes for Sp1 are expected to be activated by NS1. We demonstrated here that a complex of NS1 and Sp1 contributes to the expression of a cellular gene, p21/WAF1. With respect to binding of NS1 to Sp1 family members, not only Sp1 but also Sp3 was revealed to bind NS1 in a previous report by using an ELISA assay (Raab et al., 2002). However, we here demonstrate that NS1 is able to be co-immunoprecipitated with Sp1 but not Sp3, suggesting that Sp1 is preferentially involved in NS1-induced p21/WAF1 expression. This discrepancy of NS1 binding to the Sp1 family members may be due to the difference in assay systems. Since the co-immunoprecipitation assay reflects quantitative rather than qualitative binding as compared with the ELISA assay, our present study indicates more precise binding affinities between NS1 and Sp1 family members. In addition, an NS1-KE mutant, which is unable to undergo cell cycle arrest and to transactivate p21/WAF1 promoter, did not bind to Sp1. In fact, cotransfection of NS1 with Sp1 induced a significant level of p21/WAF1 promoter activation but not with Sp3. Similarly, Sp1 involvement was shown in promoter activation of p21/WAF1 and type II procollagen gene (Billon et al., 1999; Chadjichristos et al., 2002; Kardassis et al., 1999). Taken together, the direct binding of NS1 to Sp1 is critical for transactivation of p21/WAF1 promoter.

We demonstrated that an Sp1-3wt probe containing the TCCCGCCT motif is the main NS1-responsive element for transactivation of p21/WAF1, and that NS1 does not alter the binding of Sp1 and Sp3 to the motif. Though we could not detect NS1 binding to the Sp1-3wt probe, it may be due to a possibility that our NS1 mAb, which recognizes the C-terminal region of NS1, is unable to bind NS1, which preforms a complex with Sp1 on p21 promoter. It is still unclear how the complex of NS1 and Sp1 affects the p21/WAF1 promoter. In this context, B19 NS1 is known to interact directly with the region D of its own p6 promoter, and the region D contains the entirely same motif as Sp1-3wt probe, but NS1 does not inhibit the binding of Sp1 to the motif (Raab et al., 2002). On the other hand, MVM NS1 reportedly required Sp1 for its binding to the own viral promoter (Krady and Ward, 1995). Taken together, we speculate that not only Sp1 but also NS1 binds to Sp1-3 element of the p21/WAF1 promoter, but the NS1 binding to the element may be too faint to be detected in our EMSA assay. Alternatively, NS1 may interact with Sp1-3wt element through binding to Sp1.

CBP and p300 are transcriptional cofactors, which contain highly conserved amino acid sequences and appear to regulate a variety of gene expressions (Goodman and Smolik, 2000). p300 is known to contribute to the Sp1-induced activation of p21/WAF1 gene expression (Billon et al., 1999; Xiao et al., 2000), and both p300 and CBP have histone acetyltransferase (HAT) activity, which is generally important for the transcriptional regulation of cellular genes (Chan and La Thangue, 2001). We revealed here that the NS1-mediated transactivation of p21/WAF1 promoter is enhanced by CBP. Since we did not detect the direct interactions between CBP and NS1, or between CBP and Sp1 in the presence of NS1 (data not shown), the mechanism remains unknown but one can speculate that CBP may be involved in the NS1-mediated transactivation of p21/WAF1 promoter through activation of Sp1. Alternatively, since Sp1 is known to interact with the hTAFII 130 protein, a cofactor in the TFIID complex that also has HAT activity (Tanese et al., 1996), the hTAFII 130 protein may be replaced by overexpressed CBP in our assay system for the NS1-mediated transactivation of p21/WAF1 promoter. Nevertheless, NS1 is predicted to initiate transcription of p21/WAF1 gene through interaction with Sp1 that in turn possibly activates HAT.

Since the deletion mutants of the carboxy-terminus of MVM NS1, which have an intact NTP binding motif, are unable to transactivate its own p38 promoter (Legendre and Rommelaere, 1994), both the NTP binding motif and the carboxy-terminus of MVM NS1 play critical roles in this transactivation. A similar pattern seen in MVM NS1 can be addressed to B19 NS1, so that not only the NTP binding motif but also the carboxy-terminus region may be

indispensable for NS1-mediated transactivation of a cellular target gene, p21/WAF1. Defining molecule(s) that interact with the carboxy-terminus region of NS1 may provide critical information about the functional significance of NS1 in G1 arrest and the cytotoxicity of B19 virus-infected cells.

We previously revealed that inactivated B19, which expresses little NS1, can induce G2 arrest but not G1 arrest (Morita et al., 2003), and here that NS1 has inducing ability for G1 arrest but not G2 arrest. These observations suggest that NS1 and other viral factors including hairpin structures have distinct roles in regulation of cell cycle arrests. Furthermore, we demonstrated the significant role of p21/WAF1 in the NS1-induced G1 arrest using a siRNA vector and a p21-deficient cell line. In addition, MVM NS1-induced G2 arrest depends on p21/WAF1 (De Beeck et al., 2001), and the sustenance of the G2 arrest induced by DNA damage requires p21/WAF1 (Bunz et al., 1998). These observations suggest the possibility that p21/WAF1 plays important roles in induction and maintenance of cell cycle arrests at G2 as well as G1 in B19-infected cells.

Materials and methods

Cell lines and transfection

HCT116 cells and HCT116 p21(−/−) cells (a kind gift from Dr. B. Vogelstein, Johns Hopkins University School of Medicine, Baltimore, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and incubated at 37°C. UT7/Epo-S1 cells, which are highly susceptible to B19 virus infection (Morita et al., 2001), were propagated in Isocove's modified Dulbecco's medium containing 10% fetal calf serum and 2 U/ml recombinant erythropoietin (Epo) (a gift from Kirin Brewery Pharmaceutical Research Laboratory, Tokyo, Japan). The 293T cells were grown at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For luciferase assays and cell cycle analyses, subconfluent cells were transfected with the indicated plasmids using FuGENE 6 (Roche Diagnostics, Indianapolis, Ind.). The amounts of reporter and effector plasmids used for the luciferase assays are described in the text. For cell cycle analyses, transient transfection of UT7/Epo-S1 cells was performed with the Amaxa electroporation gene transfer system (Amaxa GmbH, Berlin, Germany).

Plasmids and site-directed mutagenesis

The wild-type NS1 expression plasmid, pMP6-NS1, and the control vector plasmid, pMP6, were described previously (Morita et al., 2003). The human wild-type p21/WAF1/Cip1 promoter luciferase fusion plasmid, pWWP-Luc, was a gift from Dr. B. Vogelstein (el-Deiry et al., 1993). A series of p21/WAF1/Cip1 promoter luciferase plasmids, pM-Sp1, pM-Sp3, and pG5-Luc were gifts from Dr. T. Sakai (Nakano et al., 1997). pEVR2/Sp1 and pRC/CMV/Sp3 are expression vectors for Sp1 and Sp3, respectively, provided by Dr. G. Suske (Hagen et al., 1994). pRc/RSV/CBP-HA is a CBP expression vector provided by Dr. T. Nakajima (St. Marianna University School of Medicine, Kanagawa, Japan).

The QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to engineer a lysine-to-glutamate (K334E) mutation into the wild-type NS1 expression vector, pMP6-NS1, with the following oligonucleotide primers: 5′-CCGCCAAGTACAGGAGAAACAAACTTGGCAATG-3′ (forward primer) and 5′-CATTGCCAAGTTTGTTTCTCCTGTACTTGGCGG-3′ (reverse primer) for K334E. We named this construct pMP6-NS1-KE. Successful mutagenesis of the plasmids was verified by sequencing.

Cell cycle analysis

Cell cycle analyses were performed as described previously (Darzynkiewicz et al., 1992). In brief, cells were transfected with plasmids and cultured for the indicated times. After the cells were washed twice with phosphate-buffered saline (PBS), they were suspended in propidium iodide (PI) solution (50 μg/ml PI, 0.1% sodium citrate, 0.2% NP-40, 0.05 mg/ml RNase), then incubated for 30 min at 4°C. PI-positive cells in the G_0/G_1 and G_2/M fractions were counted with a FACScan fluorescence-activated cell sorter (FACS; BD Biosciences, Franklin Lakes, NJ). To detect G_1-arrested cells, the cells were treated with 20 μM paclitaxel, a mitotic inhibitor (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 24 h before staining with PI.

Western blotting

Western blot analyses were carried out as described elsewhere (Asao et al., 1997). In brief, cells were lysed in an aliquot of whole-cell extraction buffer [10 mM $NaHPO_4$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 400 mM KCl, 10% glycerol, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μM pepstatin, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 1 mM $Na3VO_4$]. After one freeze-and-thaw cycles, cell lysates were spun in a microcentrifuge at 14,000 rpm for 20 min to remove cell debris. Ten micrograms of protein from each supernatant was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 10–12% polyacrylamide, and the proteins were transferred to nitrocellulose membrane (Millipore, Billerica, MA) by electroblotting for 1.5 h at 25 V in an ATTO semidry blotting system. The membrane was then incubated for 1 h in blocking buffer [Tris-buffered saline (TBS) solution containing 1% Tween 20 and 5% bovine serum albumin] and further incubated for 3 h at room temperature with the following antibodies: anti-NS1 monoclonal antibody

(MAb), ParC-NS1, specific for the NS1 C-terminal half of B19 virus (Morita et al., 2003), anti-α-tubulin MAb (Sigma-Aldrich Fine Chemicals, St. Louis, Mo.), and anti-p21 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibodies were labeled with horseradish peroxidase-conjugated anti-mouse antibody, washed extensively, then visualized with a sensitive enhanced chemiluminescence detection system (ECL detection kit; Amersham Bioscience Corp., Piscataway, NJ).

Luciferase assay

Cells were seeded into 6-well plates at a density of 3×10^5cells/well, 1 day before transfection. The 293T cells were transfected with 10 ng of promoter–reporter plasmids, 2 ng of pRL-null control vector (Promega Corp., Madison, WI), the indicated amounts of effector constructs, and/or empty control vectors to adjust the total amount of plasmid DNA so that it was equal in each well. After 2 days of transfection, total cell lysates were assayed for luciferase activity using a luminometer and dual luciferase assay kit (Promega Corp.). The luciferase activities were normalized against the activity of the control vector and the protein concentration. Each experiment was performed at least three times.

Immunoprecipitation

The 293T cells were transfected with an expression construct for NS1, NS1-KE, or the pMP6 control vector together with pEVR2/Sp1 or pRC/CMV/Sp3. Forty-eight hours after transfection, the cells were washed, scraped from the dish, and collected. Their nuclear extracts were prepared as described previously (Andrews and Faller, 1991). Samples were incubated with protein A-Sepharose beads for 1 h at 4°C, followed by incubation with protein A-Sepharose beads conjugated with anti-NS1 MAb, anti-Sp1, or anti-Sp3 antibody (Santa Cruz Biotechnology) for 6 h at 4°C. The beads were washed five times with buffer containing 25 mM Tris–HCl pH 7.5, 140 mM NaCl, 1% NP-40, 1 mM EDTA, and 10 mM $Na_4P_2O_7$. The immune complexes were then eluted by boiling for 5 min and separated by SDS–PAGE. Aliquots of the nuclear extracts were directly subjected to SDS–PAGE without immunoprecipitation. The subsequent procedures were as described above in the section on Western blotting.

Electrophoretic mobility shift assay (EMSA)

Single-stranded oligonucleotides were synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan), and the complementary sense and antisense strands were then annealed into double-stranded DNAs and used for EMSA. The double-stranded DNAs were end-labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP. Subsequently, 0.1–0.2 ng of probes (approximately 2×10^5 cpm) were incubated with 6 μg of nuclear extract on ice for 20 min in 15 μl of binding reaction mixture that contained 10 mM Hepes (pH 7.9), 25 mM NaCl, 1 mM EDTA, 0.25 mM dithiothreitol, 10% glycerol, 0.5 mM magnesium spermidine, and 30 ng/μl poly(dI-dC). The reaction was stopped by adding gel loading buffer, and the mixture was immediately loaded onto a 5% acrylamide–bisacrylamide (79:1) and 0.5× TBE nondenaturing gel at 15 V/cm. The run was stopped when the bromophenol blue reached the lower margin of the gel. The antibodies used for the gel supershift assays were anti-Sp1, anti-Sp3, and anti-NS1 antibodies; 2 μg/μl antibody was added to the binding reaction mixture, which was incubated on ice for an additional 1 h before loading. The oligonucleotide probes, Sp1-3wt and Sp1-5-6wt, corresponded to the sequences from −92 to −70 and −69 to −47 of the wild-type p21/WAF1 promoter, respectively, and their mutants were Sp1-3mt and Sp1-5-6mt, respectively (Fig. 3A). Their sequences were as follows: Sp1-3wt probe: 5′-GAGCGCGGGTCCCG-CCTCCTTGA-3′; Sp1-3mt probe: 5′-GAGCGCGGGT-CCCGAATCCTTGA-3′; Sp1-5-6wt probe: 5′-GGCGGGCCCGGGCGGGGCGGTTG-3′; Sp1-5-6mt probe: 5′-GGCGGGCCCGTTCGGGGCGGTTG-3′.

Small interfering RNAs

The mammalian expression vector, piGENE hU6 (iGENE Therapeutics, Inc., Ibaraki, Japan), was used for expression of siRNA in 293T cells. The gene-specific insert specifies a 21-nucleotide sequence corresponding to nucleotides 950–970 downstream of the transcription start site (gtaaacagatggcactttgaa) of p21/WAF1. A control vector, pU6–siRNA–control, was constructed using a 21-nucleotide sequence (gtgcgctgctggtgccaaccc) with specific homology to firefly luciferase gene sequence and therefore serves as a negative control. We constructed these vectors according to the manufacturer's instructions. The purified vectors were digested with *Eco*RI and *Hin*dIII and the fragments were ligated into the *Eco*RI and *Hin*dIII cloning site in the pBluescript KS II (+) (Stratagene), respectively. Subsequently, the *Eco*RI and *Sal*I fragments in the pBluescript KS II (+) were ligated into pMACS K^k.II (Miltenyi Biotec, Gladbach, Germany). DNA sequence analysis confirmed the correct sequence. These vectors were referred to as pMACS–H2K^k–siRNA–p21 and pMACS–H2K^k–siRNA–control, respectively. Transient transfections to 293T cells were performed with FuGene 6 (Roche Diagnostics). Cells were trypsinized 48 h after transfection, resuspended in PBE (PBS supplemented with 0.5% bovine serum albumin and 5 mM EDTA) buffer, and incubated with MACSelect K^k MicroBeads (Miltenyi Biotec). H2K^k-positive cells were enriched using the AutoMACS machine (Miltenyi Biotec) and the enrichment rates ranged from 70% to 80% (data not shown). The enriched cell fractions were then cultured again for second transfection.

The p53 gene-specific sequence (gactccagtggtaatctac) and nonspecific sequence (gaattcatctcagcgcgta) were

inserted into pRNAT-U6.1/Neo (GenScript Corp., Piscataway, NJ) (Brummelkamp et al., 2002). We created siRNA expression vector against p53, pRNAT-U6.1/Neo-p53-1. These vectors were transiently transfected into HCT116 cells with Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA). After trypsinization, cells, which the GFP positive, were obtained by using a BD FACSAria cell sorter (BD Biosciences). Western blotting showed that the specific inhibition of p53 expression was approximately 75% in cells transfected with pRNAT-U6.1/Neo-p53-1, as compared with control vector (data not shown).

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Hrs, a Mammalian Master Molecule in Vesicular Transport and Protein Sorting, Suppresses the Degradation of ESCRT Proteins Signal Transducing Adaptor Molecule 1 and 2*

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The degradation and sorting of cytoplasmic and cell-surface proteins are crucial steps in the control of cellular functions. We previously identified three mammalian Vps (vacuolar protein sorting) proteins, Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and signal transducing adaptor molecule (STAM) 1 and -2, which are tyrosine-phosphorylated upon cytokine/growth factor stimulation. Hrs and the STAMs each contain a ubiquitin-interacting motif and through formation of a complex are involved in the vesicle transport of early endosomes. To explore the mechanism and cellular function of this complex in mammalian cells, we established an Hrs-defective fibroblastoid cell line ($hrs^{-/-}$); embryos with this genotype died *in utero*. In the $hrs^{-/-}$ cells only trace amounts of STAM1 and STAM2 were detected. Introduction of wild-type Hrs or an Hrs mutant with an intact STAM binding domain (Hrs-dFYVE) fully restored STAM1 and STAM2 expression, whereas mutants with no STAM binding ability (Hrs-dC2, Hrs-dM) failed to express the STAMs. This regulated control of STAM expression by Hrs was independent of transcription. Interestingly, STAM1 degradation was mediated by proteasomes and was partially dependent on the ubiquitin-interacting motif of STAM1. Revertant Hrs expression in $hrs^{-/-}$ cells not only led to the accumulation of ubiquitinated proteins, including intracytoplasmic vesicles, but also restored STAM1 levels in early endosomes and eliminated the enlarged endosome phenotype caused by the absence of Hrs. These results suggest that Hrs is a master molecule that controls in part the degradation of STAM1 and the accumulation of ubiquitinated proteins.

Membrane traffic is a dynamic process that is responsible for the maintenance of cellular metabolism, stress response, down-regulation of cell-surface molecules, and signal transduction. Cell-surface molecules, including growth factor receptors that bind to specific ligands or macromolecules, are internalized from the cell surface into membrane compartments called endosomes. Although some cargos are pinched off in recycling endosomes to be sent back to the cell surface, other receptors and molecules are pinched off into endosomes that form multivesicular bodies (MVB).[1] As the maturation step proceeds, early endosomes become acidic to form late endosomes, which then fuse with acidic compartments called lysosomes, which contain many acidic proteases. Although it has not been clear how receptors that have captured their ligands are recognized and sequestered into the endocytic vesicles, a growing body of evidence suggests the involvement of monoubiquitin modification and implicates the intracytoplasmic region of the receptors (1).

The identification of vacuolar protein sorting (Vps) mutants has permitted genetic analyses in yeast to identify the proteins that make up the core machinery for MVB formation. The functional loss of Vps proteins results in deformed MVBs with unusually enlarged vesicles, called "class E" compartments, and defective vesicular trafficking. At present, 17 Vps genes are known; they are genetically categorized into four kinds of endosomal sorting complexes required for transport (ESCRT), ESCRT-0, I, II, and III. One of the ESCRT proteins involved in MVB formation is yeast Vps27. Vps27, along with Hse1, acts in an ESCRT-0 complex upstream of ESCRT-I to assist in protein sorting (2).

In studies to find molecules involved in cytokine/growth factor signaling, we identified Hrs, STAM1, and STAM2 (3–5). Hrs is a mammalian orthologue of yeast Vps27, and STAM1 and STAM2 are mammalian counterparts of yeast Hse1. We as well as others previously reported that cytokines and growth factors such as interleukin 2, granulocyte-macrophage colony-stimulating factor, hepatocyte growth factor, platelet-derived growth factor, and epidermal growth factor induce the tyrosine phosphorylation of Hrs and the STAMs (3, 4, 6). The rapid kinetics of their phosphorylations prompted us to look for the functions of these molecules, which are immediately downstream of the receptors. Hrs possesses several functional domains, including a FYVE finger domain, which binds specifi-

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[1] The abbreviations used are: MVB, multivesicular bodies; Vps, vacuolar protein sorting; Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate; STAM1 and STAM2, signal transducing adaptor molecule 1 and 2; UIM, ubiquitin-interacting motif; ESCRT, endosomal sorting complexes required for transport; VHS, Vps27-Hrs-STAM; ITAM, immunoreceptor tyrosine-based activation motif; RT, reverse transcription; MEF, mouse embryonic fibroblastoid; HA, hemagglutinin; E3, ubiquitin ligase; Ub, ubiquitin.

cally to phosphatidylinositol 3-phosphate and is important for membrane anchoring, and a clathrin binding domain, required for membrane-coated vesicle binding (7–11). Hrs also contains an N-terminal VHS (Vps27-Hrs-STAM) domain and a ubiquitin-interacting motif (UIM). The overexpression of Hrs, but not of a UIM deletion mutant, inhibits the recycling of a ubiquitin-fused transferrin receptor, suggesting a role for Hrs-UIM in vesicular transport (12). Furthermore, an accumulation of ligand-activated epidermal growth factor receptors within early endosomes is observed in cells overexpressing Hrs, suggesting the involvement of Hrs in ubiquitinated protein sorting (11, 13–15).

After the identification of STAM1 and STAM2 (EAST/Hbp), two closely related molecules, we showed that their roles in intracellular cytokine/growth factor signaling included induction of the proto-oncogene *c-myc* (3, 5, 16–18). The STAMs have a unique structure, including a VHS, a UIM, an Src homology 3 (SH3) domain, and an ITAM (immunoreceptor tyrosine-based activation motif) (3, 5). We and others reported that the ITAMs of STAM1 and STAM2 associate with Hrs through its second coiled-coil (CC2) domain (4). Like its binding partner, STAM2 binds directly to ubiquitins (19). Both Hrs and the STAMs bind Eps15, and therefore, all these molecules seem to participate in the sorting of ubiquitinated proteins into the MVB pathway (19).

To further characterize the function of the mammalian STAM-Hrs complexes in MVB formation and/or protein sorting, we established Hrs-deficient fibroblastoid cell lines. The results of introducing wild-type or a mutant Hrs into these cells suggested a novel function for Hrs in regulating the fate of ubiquitinated proteins. We demonstrate here that Hrs controls the STAM protein stability. Our demonstration of the STAM relationship with ubiquitinated proteins provides insight into how their UIM domain functions in protein sorting.

EXPERIMENTAL PROCEDURES

Targeted Disruption of hrs in Vivo—Genomic clones of the mouse *hrs* gene were isolated from a λFixIImouse 129/Sv genomic library (Stratagene). A targeting construct for *hrs* was designed to replace a 0.6-kilobase (HindIII-HindIII) genomic fragment encompassing exon 6, flanked by 3.2-kilobase (XbaI-XbaI) and 3.0-kilobase (EcoRI-XbaI) genomic sequences (Fig. 1*A*). A pGK-neo cassette flanked by a pair of loxP sequences and a diphtheria toxin A-chain (DT) gene cassette without a polyadenylation site were inserted into the construct to allow further positive/negative selection. The construct was linearized and transferred into 129/Sv-derived J1 ES cells by electroporation, and G418-resistant colonies were picked (20–22). The occurrence of homologous recombination events for the *hrs* allele was examined by Southern hybridization using a 1.8-kilobase *hrs* genomic fragment (XbaI-BamHI) as the probe (Fig. 1*B*). The targeted *hrs* loxP-flanked (floxP) ES clone was confirmed to have a normal number of chromosomes and was injected into C57BL/6 blastocysts. The blastocysts were then transferred into foster mothers to obtain chimeric mice. By crossing the chimeras with C57BL/6 mice we obtained F_1 heterozygous mice carrying the floxP allele. The genotypes were confirmed by both genomic PCR and Southern blot analyses (data not shown). The F_1 heterozygous mice were intercrossed, but the genotyping of more than 100 offspring showed that no viable $hrs^{floxP/floxP}$ mice were produced. To identify $hrs^{floxP/floxP}$ embryos *in utero*, the genotypes of living embryos were determined using yolk sac DNA (23). The following oligonucleotide primers were used: Hrs-1650F (primer A), 5′-GAGTGAGGAGGCGGTGTTCCCTAAACCTTG-3′; Hrs-1953R (primer B), 5′-AACATATACTGCTGGCAAAGCATCCATACA-3′; Hrs-CKO1787R (primer C), 5′-TATAGCATACATTATACGAAGTTATGTCGA-3′. PCR was carried out by initial denaturation at 94 °C for 5 min followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C. The wild-type and mutant alleles gave rise to PCR-amplified fragments of 330 and 160 bp, respectively (Fig. 1*C*).

Establishment of hrs-deficient and hrs-mutant Embryonic Fibroblastoid Cell Lines—Primary fibroblastoid cells derived from E9.5 $hrs^{floxP/floxP}$ mouse embryos were obtained through timed-breeding experiments, as described previously (24). Cells were stably immortalized by transfecting them with pEF321-T, an SV40 large T-antigen expression plasmid. Deletion of the floxP fragment was performed by transient transfection with pCXN2-Cre, a Cre-recombinase expression construct. The presence in cells of the *hrs* deletion ($hrs\Delta/\delta$) was verified by PCR. The following oligonucleotide primers were used: neo2760F (primer D), 5′-CTTGGGTGGAGAGGCTATTC-3′; neo3030R (primer E), 5′-AGGTGAGATGACAGGAGATC-3′; Hrs-1650F (primer A), 5′-GAGTGAGGAGGCGGTGTTCCCTAAACCTTG-3′; Hrs-CKO4108R (primer F), 5′-ATCACATTTCCCTCCCCAGTGCTTTCAAAC-3′. Limiting dilution and subsequent cloning were used to establish HRSd, an immortalized fibroblastoid cell line carrying the $hrs^{\delta/\delta}$ genotype (Fig. 1*E*). HRSw, HRSdC2, HRSdFYVE, and HRSdM were HRSd sublines stably transformed with expression vectors for wild-type Hrs and for the dC2, dFYVE, and dM Hrs mutants, respectively. The expression of the wild-type or mutated Hrs proteins in these clones was confirmed by Western blotting.

Reverse-transcription (RT)-PCR—RT-PCR was carried out with the total RNA derived from HRSd and MEFw cells. The total RNA was prepared with TRIzol Reagent (Invitrogen) and subjected to first-strand synthesis using the SuperScript III first-strand synthesis system (Invitrogen). PCRs were performed with a series of 3-fold-diluted templates to amplify the *hrs* and *β-actin* cDNAs. The primer set for *hrs* was designed to amplify a coding region within the N-terminal VHS domain. The primers used were as follows: Hrs-Exon 1 sense, 5′-TTCGAGCGTCTCCTAGACAAAGC-3′; Hrs-Exon 3 antisense, 5′-CATATTTTGCTTGTGTGTCCCCC-3′; β-actin sense, 5′-CCACTGCCACATCCTCTTC-3′; β-actin antisense: 5′-CATCTGCTGGAAGGTGGAC-3′

Plasmids—pCXN2-Hrs, pCXN2-STAM1-V5, and pCXN2-STAM2-V5 were expression plasmids for wild-type Hrs, STAM1, and STAM2, respectively. Expression vectors for the mutated Hrs, Hrs-dC2, Hrs-dFYVE, and Hrs-dM were described previously (4, 5, 16, 25, 26). An expression plasmid for DsRed1-fused Eps15 (DsRed1-Eps15) was generated by ligating a PCR-amplified human Eps15 cDNA into pDSRed1-C1 (Clontech). A newly developed expression plasmid for HrsL265E (Hrs-mUIM) was generated using a PCR fragment of human Hrs carrying a point mutation at amino acid 265 (27) ligated into pCXN2. The expression plasmid for V5-tagged STAM1 with point mutations at leucines 176, 182, and 184 that caused them to be substituted with alanine (STAM1-mUIM-V5) was generated by ligating the PCR fragment of wild-type human STAM1 with point mutations at amino acids 176, 182, and 184 into pCXN2. The SV40 large T-antigen expression plasmid, pEF321-T, was a kind gift from Dr. M. Obinata (Tohoku University), and the expression plasmid for ubiquitin, pcDNA3-HA-Ub, was a kind gift from Dr. K. Miyazono (Tokyo University) (28). FLAG-tagged ubiquitin, p3XFLAG-Ub, was generated by PCR-based cloning of human ubiquitin into the p3XFLAG-CMV-10 vector (Sigma).

Cell Culture—HRSd and HRSd sublines transfected with wild-type Hrs or Hrs mutants were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics under 7% CO_2 in a humidified incubator. To transfect the cells with plasmid DNAs, FuGENE™ 6 transfection reagent (Roche Applied Science) was used according to the manufacturer's protocol. Where indicated lactacystin (Kyowa Medex) and E-64-d (Peptide Institute, Inc.) were added to the cell culture to a final concentration of 10 and 100 μM, respectively.

Immunoprecipitation and Immunoblots—Immunoprecipitation and immunoblotting were carried out as described previously (4). In brief, cells were lysed in Nonidet P-40 lysis buffer (1% Nonidet P-40, 40 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin). The cell lysates were precleared of cellular debris by centrifugation (10,000 × *g*) for 20 min at 4 °C and were then subjected to immunoprecipitation with antibodies immobilized on protein A-Sepharose beads (Amersham Biosciences) at 4 °C overnight. For this assay rabbit anti-Hrs polyclonal antibodies (4) and anti-ubiquitin (Santa Cruz), anti-STAM1 (TUS-1) (29), anti-STAM2 (ST2–2) (5), and anti-V5 monoclonal antibodies (Invitrogen) were used. The immunoprecipitates were then separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). After being blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20, the membranes were probed with the indicated primary antibodies. After another wash, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling). Signals were visualized by the ECL detection system (Amersham Biosciences), and digital images were collected by a Lumi-Imager F1 (Roche Applied Science). For degradation assays, the cells were cultured in the presence of 25 μg/ml cycloheximide (Wako) for the period indicated, and the cell lysates from these cultures were analyzed by Western blotting.

Northern Blots—Northern blot analyses were performed as described