Tumor Antigen LRRC15 Impedes Adenoviral Infection: Implications for Virus-Based Cancer Therapy

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Adenoviruses for gene or oncolytic therapy are under development. Notable among these strategies is adenoviral delivery of the tumor suppressor p53. Since all therapeutics have limitations in certain settings, we have undertaken retroviral suppressor screens to identify genes conferring resistance to adenovirus-delivered p53. These studies identified the tumor antigen LRRC15, which is frequently over-expressed in multiple tumor types, as a repressor of cell death due to adenoviral p53. LRRC15, however, does not impede p53 function per se but impedes adenoviral infection. Specifically, LRRC15 causes redistribution of the coxsackievirus-adenovirus receptor away from the cell surface. This effect is manifested in less adenoviral binding to the surfaces of LRRC15-expressing cells. This discovery, therefore, not only is important for understanding adenoviral biology but also has potentially important implications for adenovirus-based anticancer therapeutics.

MATERIALS AND METHODS

Plasmids. Plasmids involved in the generation of ERM (enhanced retroviral mutagenesis) virus-infected cells were kindly provided by Zhou Songyang (Baylor College of Medicine, Houston, TX) and have been described previously (9). pWZLNeo-EcoR and pcDNA3-LRRC15-hemagglutinin (HA) were kind gifts from Gordon Peters (Cancer Research UK—London Research Institute) and Daniel Haber (Harvard Medical School), respectively. pBabePuro-LRRC15-HA was generated by digestion of pcDNA-LRRC15-HA with HindIII and BamHI. The fragment was then blunt-ended and cloned into the SnaBI site of pBabe-Puro. Expression vectors for p53 (pCB6+p53) and Mdm2 (pCHDM21A) have been described previously (3, 11). The green fluorescent protein (GFP) expression plasmid EGFP-N1 is from BD Biosciences.

Cell culture and generation of cell lines. Saos2-EcoR, A549-EcoR, and HCT-116-EcoR cells were generated by amphotropic retroviral infection of the respective parental cell lines with pWZLNeo-EcoR as previously described (16). The cells were then infected using an ecotropic packaging system with either pBabePuro-LRRC15-HA or an empty pBabePuro vector as a control. Saos2-ERM cells were generated as previously described (9). All lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and incubated at 37°C under an atmosphere of 5% CO2 in air. Transgene expression was induced by addition of 1 μg/ml doxycycline (Dox) (Sigma). For p53 degradation assays, Saos2 cells were transiently transfected by calcium phosphate precipitation as described elsewhere (2). After 16 h, cells were washed and then incubated for a further 24 h before harvesting for Western blotting.

Generation of replication-defective adenoviruses. Plasmid EGFp-N1-p53 was made by inserting wild-type p53 sequences into the EcoRI and BamHI restriction sites of pEGFP-N1 (Clontech). pShuttleCMV-GFP and pShuttleCMV-p53 were made by inserting the GFP and WTp53-GFP fragments from plasmids EGFP-N1 and pcDNA3-LRRC15-hemagglutinin into pShuttleCMV (Stratagene) using BglII and NotI restriction sites. Linearized “Shuttle” plasmids were electroporated into BJ5183-AD-1

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electrocompetent cells (Stratagene) containing the Adeasy-1 adenoviral construct
(Stratagene). Recombinant plasmids were then amplified in XL10-Gold
cells (Stratagene) and transfected into HEK293 cells after restriction digestion
with PacI. Following infectious adenovirus amplification in 293 cells, purified
virus was isolated by freeze-thaw extraction and titered using the BD Biosciences
Adeno-X Rapid Titer kit. Adenoviruses were added to cell cultures at the
multiplicities of infection indicated in the figures and legends.

Western blotting. Cells were lysed in a 2× Western northern dodecyl sulfate
sample buffer and transferred to nitrocellulose membranes as previously de-
scribed (2). Membranes were probed using standard immunoblotting techniques
with antibodies that recognize LRRC15 (horse-radish peroxidase-conjugated
HRP; Roche), p53 (DO-1; Pharmingen), GFP (Covance), Hdm2 (Ab1; Oncogene
Science), the coxsackievirus-adenovirus receptor (CAR) (RmcB; ATCC), and
actin (clone 1A4; Sigma).

Flow cytometry and cell death assays. Total populations of cells, including
floating and adherent cells, were processed for flow cytometric analysis (FACScan;
Becton Dickinson) as described previously (17). (17). The percentage of
cells with a sub-G1 DNA content was taken as a measure of the extent
of apoptosis in the cell population at that time.

Identification of ERM clones. Pools of cells infected with ERM viruses were
challenged with three rounds of infection with adenoviral p53. Each round of
infection was separated by a period of 7 to 10 days. RNA was isolated from
surviving clones and subjected to seminested reverse transcription-PCRs (RT-
PCRs) using the following primers and conditions. The RNAs were reverse
transcribed using a random primer, RT3 (5′-CCGTAATGGACTCTATAG
GGATCCNNNNSTGG-3′), and the GeneAmp RNA PCR core kit (Applied
Biosystems). Thirty cycles of PCR amplification were carried out using Proof-
start polymerase (Qiagen) and PCR primers HAI1 (5′-CCACAGGGCCGGCC
AAGCACTATCCGATGA-3′) and TT (5′-GGGATATTACCTCCTAATA
GGG-3′) according to the manufacturer’s manual. PCR conditions (MI
Research DNA Engine PTC-200) were as follows: 95°C for 5 min; 30 cycles of
94°C for 30 s, 60°C for 1 min, and 72°C for 1 min; and finally 72°C for 2 min.
Products from these reactions were sequenced and integration sites identified
using the “Ensembl” genome browser (http://www.ensembl.org) and the NCBI

qPCR. DNA and RNA were prepared using Trizol reagent (Invitrogen).
Quantitative PCR (qPCR) analysis was carried using the DyNamo Sybr green
2-step qRT-PCR kit (Finzymes). Data were collected using a Chromo4 real-
time PCR detector and were analyzed with Opticon Monitor 3. Primers for
LRRC15 were as follows: forward, GGCCTGTAATGGACTCTATACT
(TCC) reverse, GGATAATGCCATTTCAGTGGT. Primers for GFP were from Qiagen
and were purchased from Invitrogen. Cells were pelleted and washed three times
with ice-cold fluorescence-activated cell sorter (FACS) buffer (PBS, 2% fetal
bovine serum, 0.5 mM EDTA [pH 8.0]). Cells were spun down, resus-
pended in ice-cold FACS buffer containing 5% fetal bovine serum and a
monoclonal antibody (2 μg/ml), and incubated on ice for 60 min. Antibodies
to CAR (RmcB; ATCC), integrin αv (ab16821; Abcam), integrin β1 (catalog no.
555752; BD Pharmingen), and integrin αv, β3 (MAR2019Z; Chemicon) were used.
Cells were pelleted and washed three times with FACS buffer. Cells were
then resuspended in FACS buffer containing goat anti-
mouse immunoglobulin G conjugated with fluorescein isothiocyanate (cate-
log no. 0479; Dako) and incubated at room temperature for 30 min. Cells
were pelleted and again washed three times with FACS buffer, and the
expression of CAR or integrins on the cell surface was determined using a
Becton Dickinson FACScanExpert flow cytometer.

Immunofluorescence. Cells were seeded on glass coverslips at 50% conflu-
ence. The following day, cells were infected with adenovirus (90 min on ice); washed
three times, for 5 min each time, with PBS; and fixed in PBS containing 4%
parafomaldehyde. For staining, coverslips were rinsed in PBS-0.1% Triton
X-100, incubated in blocking solution (PBS, 5% milk, 0.1% Triton X-100) for
30 min at room temperature, washed in blocking solution,
and then incubated with anti-adenovirus type 5 antibody (ab982; Abcam) for
60 min at 37°C. The coverslips were washed in PBS-0.1%
Triton X-100, incubated in a goat anti-rabbit secondary antibody conjugated with
Alexa Fluor 488 (A11008; Molecular Probes) for 30 min at 37°C, washed in
PBS-0.1% Triton X-100, and refixed in PBS-4% paraformaldehyde for 20 min at
room temperature. Cells were then washed again and mounted on slides.
Microscopy was carried out using a Zeiss Axioplan 2IE microscope and ISIS
software.

RESULTS AND DISCUSSION

A screen for factors causing resistance to adenoviral p53
identifies LRRC15. In order to identify genes that cause resis-
tance to adenoviral p53, we utilized the ERM system, which
has been described previously (9, 10). In brief, this system
comprises tetracycline-responsive exon trap retroviruses (in
three reading frames) that integrate randomly throughout
the target cell genome when they are used to infect cells. Gene
fusions occur between exons of endogenous genes and a se-
quence tag on the retrovirus (ERM tag) via a splice donor site
in the viral vector. This causes up-regulation of expression
of the endogenous gene due to the enhancer effect of the viral
long terminal repeat (Fig. 1a). The ERM tag can then be used
to facilitate identification of the viral integration site by PCR.

Large pools of Saos2 cells, which are null for endogenous
p53, were infected with this viral system as previously described
(9, 16) in order to attain a high diversity of viral integration.
Pools of these cells were challenged with a regimen of infection
with adenoviral p53 that is just sufficient to kill an entire pop-
ulation of Saos2 cells without ERM (data not shown). Follow-
ing adenoviral infection of ERM cells, however, a number of
surviving colonies were found to emerge. Sequencing of one of
these clones using the viral tag as previously described (9)
revealed an in-frame integration at the beginning of exon 2 of
The LRRC15 gene (Fig. 1b). Since LRRC15 has a noncoding first exon, this integration involved the entire reading frame of LRRC15.

LRRC15 (also known as hLib) belongs to the leucine-rich repeat superfamily, members of which are involved in cell-cell and cell-extracellular matrix interactions. While it was first identified as a protein induced by β-amyloid (19), subsequent studies have indicated that LRRC15 is frequently overexpressed in various tumor types and, in particular, is associated with high-grade, aggressive breast and prostate tumors (14, 18, 20, 23). This association with tumor development caused us to examine further the clones of cells with an integration in LRRC15. First, we retested to determine whether the cells were resistant to death by adenoviral p53 and, since expression from ERM viruses is tetracycline responsive, whether resistance to cell death was dependent on a viral integration. For this purpose, cells were incubated with the tetracycline analog Dox (1 µg/ml for 48 h) to switch off ERM integrations (the ERM constructs are “Tet-Off” regulated). This caused a marked reduction in the levels of both ERM-tagged LRRC15 and total LRRC15 (Fig. 2a). Cells were then challenged with adenoviral p53 and assessed for cell death induction after 48 h by flow cytometry as previously described (17). This revealed clearly that an ERM integration was causing resistance to the effects of adenoviral p53. In the absence of Dox, the amount of cell death from adenoviral p53 was similar to that in control cells. Following Dox treatment, however, cell death from adenoviral p53 was markedly increased (Fig. 2b). Interestingly, this increase in cell death from adenoviral p53 when LRRC15 was switched off occurred concomitantly with an increase in the levels of p53 protein (Fig. 2b).

Since the possibility remained that the ERM-LRRC15 cells contained more than one viral integration, such that the effects on cell death and p53 levels were independent of LRRC15, we...
LRRC15 does not affect p53 stability. Saos2 cells were transiently transfected with the combinations of plasmids indicated. After 24 h, cells were harvested and analyzed for protein expression by Western blotting. Equal amounts of total protein were added to each lane. The results shown are representative of five separate experiments. GFP was included in each transfection as a control for transfection efficiency.

FIG. 3. LRRC15 does not affect p53 stability. Saos2 cells were transiently transfected with the combinations of plasmids indicated. After 24 h, cells were harvested and analyzed for protein expression by Western blotting. Equal amounts of total protein were added to each lane. The results shown are representative of five separate experiments. GFP was included in each transfection as a control for transfection efficiency.

LRRC15 reestablished a cell line that constitutively expresses LRRC15 from a heterologous promoter. Cells were infected with a retroviral construct that had been generated using a cDNA for LRRC15 that was previously described (14) (Fig. 2c). These cells (Saos2-LRRC15) and empty vector control cells (Saos2-pBabe) were infected with adenoviral p53 and analyzed for cell death induction and p53 protein levels. As with the ERM-LRRC15 cells in the absence of Dox, the presence of LRRC15 caused a marked reduction in the amount of cell death seen following infection with adenoviral p53 (Fig. 2d), confirming that the decreased level of cell death seen in ERM-LRRC15 cells is attributable to the viral integration in the LRRC15 locus. When the levels of p53 protein were analyzed following adenoviral infection, the result was also similar to that observed for ERM-LRRC15 cells: less p53 was detectable in LRRC15-expressing cells than in vector-only controls (Fig. 2d).

LRRC15 impedes adenoviral infection. The lower levels of p53 in LRRC15-expressing cells caused us to consider whether LRRC15 was impeding p53 function, in a manner similar to that of Mdm2, by causing p53 degradation. To test this, Saos2 cells were transiently transfected as previously described (2) with p53 in combination with either Mdm2, LRRC15, or an empty vector as a control. In line with previous studies (7, 8), coexpression of Mdm2 caused a marked reduction in p53 levels (Fig. 3). In contrast, coexpression of LRRC15 had no effect on p53 protein levels, indicating that LRRC15 does not promote p53 degradation (Fig. 3).

Since we observed that expression of LRRC15 had no effect on the levels or death-inducing activity of p53 following transfection of cells by calcium phosphate precipitation (data not shown), we next considered whether LRRC15 may affect either the efficiency of infection or transgene expression by the adenoviral vector. If either of these were possible, we would expect to see similar effects on the expression of any adenovirally delivered transgene. Saos2-LRRC15 cells, Saos2-pBabe cells, and ERM-LRRC15 cells (with or without Dox) were therefore infected with a previously described adenovirus containing a transgene that expresses GFP (1). As was observed with p53, GFP levels were indeed lower in Saos2-LRRC15 cells and ERM-LRRC15 cells than in Saos2-pBabe cells or Dox-treated ERM-LRRC15 cells, respectively (the ERM-driven integration of LRRC15 in these cells is “Tet-Off” and hence is switched off by treatment with Dox) (Fig. 4a and b). These results therefore indicate that LRRC15 impedes the efficacy of adenoviral gene transfer per se, since the effects we originally saw are not specific to p53. Since LRRC15 is a plasma membrane protein, we considered it most likely that LRRC15 impedes the infectivity of the adenoviral particle as opposed to having an effect on, for example, transgene expression. If the latter were the case, we would expect fewer adenoviral genomes in LRRC15-expressing cells, which would result in lower levels of transgene expression at the protein level. As a reflection of the number of GFP-containing adenoviral genomes in an infected cell population, we analyzed GFP gene levels in adenovirus-infected Saos2-LRRC15 cells, Saos2-pBabe cells, and ERM-LRRC15 cells (with or without Dox) by qPCR as previously described (5). This analysis indeed revealed that LRRC15-expressing cells contain fewer copies of the GFP transgene than control cells (Fig. 4c and d), and since the adenoviral vectors used are replication defective, this finding confirms that LRRC15 impedes adenoviral infection.

To test the generality of this observation, we extended our study to two other cell lines from different tissues of origin—HCT-116 and A549, which are derived from human colonic and lung tumors, respectively. These cell lines were infected in an identical manner to Saos2 cells with either a retrovirus expressing LRRC15 or an empty viral vector as a control (Fig. 4e). In both cases, as was observed for Saos2 cells (Fig. 4a to d), the presence of LRRC15 reduced both the amount of GFP expressed in cells (Fig. 4f and g) and the number of GFP transgenes upon transduction with adenovirus relative to those observed for the controls (Fig. 4h and i).

LRRC15 affects surface CAR expression and cell surface adenoviral binding. We were interested in understanding how LRRC15 was affecting the delivery of adenovirus to cells. Since changes in cellular morphology and cell cycle stage have been reported to affect adenoviral infectivity (21), we first explored whether LRRC15 was affecting either of these cellular phenotypes, but no significant differences were observed between cells expressing LRRC15 and those containing an empty viral vector as a control (see Fig. S1 in the supplemental material). A number of cell surface proteins have also been shown to affect different stages of adenoviral infectivity. CAR is known to mediate adenoviral attachment to the cell surface, while integrins αvβ3 and αvβ5 control internalization (24). Since we observed the most robust effects of LRRC15 expression in A549 cells, we chose to use these cells to explore these cellular parameters of adenoviral infection in the context of LRRC15. Surface expression of integrins and CAR was therefore analyzed by flow cytometry in A549 cells expressing either LRRC15 or an empty viral vector. This revealed that while the expression of LRRC15 had limited effects on the surface expression of integrins αvβ3 and αvβ5, surface expression of CAR was markedly reduced in cells expressing LRRC15 relative to that in controls (Fig. 5a). Interestingly, total levels of CAR were not affected by the expression of LRRC15 (Fig. 5b),
indicating that CAR must be redistributed away from the cell surface in LRRC15-expressing cells. The destination(s) of the relocalized CAR, however, has yet to be determined.

Since CAR is involved in the attachment of adenoviral particles to the cell surface, we postulated that LRRC15 may directly affect this process. To test this, LRRC15-expressing cells and controls were incubated on ice in order to permit adenoviral attachment, but not internalization, such that attachment could be assessed exclusively (24). Cells were then incubated with the adenovirus that contains the GFP transgene. Since the GFP is expressed only when the adenovirus is within the host cells, cells were subsequently fixed and stained with an antibody raised against whole adenovirus (type 5). This clearly revealed, as would be predicted by the decrease in

FIG. 4. LRRC15 impedes adenoviral infection. (a and b) After 48 h of pretreatment with Dox (to switch off LRRC15, which is driven by the ERM integration), ERM-LRRC15 cells were infected with an adenovirus containing a GFP transgene (Ad-GFP). Twenty-four hours postinfection, GFP and GFP gene levels were determined by Western blotting (a) and qPCR (b), respectively. MOI, multiplicity of infection (number of virus particles per cell as determined by plaque assay). (c and d) Saos2-LRRC15 cells and control (Saos2-Cont) cells were infected with an adenovirus expressing GFP. Twenty-four hours postinfection, GFP and GFP gene levels were determined by Western blotting (c) and qPCR (d), respectively. (e) HCT-116 and A549 cells were infected either with a retrovirus expressing HA-tagged LRRC15 or with an empty viral vector as a control. The expression of HA-tagged LRRC15 was assessed and compared to that in Saos2-LRRC15 cells by Western blotting. (f and g) HCT-116-LRRC15 and HCT-116-Cont cells were infected with an adenovirus expressing GFP. Twenty-four hours postinfection, GFP and GFP gene levels were determined by Western blotting (f) and qPCR (g), respectively. (h and i) A549-LRRC15 and A549-Cont cells were infected with an adenovirus expressing GFP. Twenty-four hours postinfection, GFP and GFP gene levels were determined by Western blotting (h) and qPCR (i), respectively.
surface CAR expression that we had observed, that LRRC15 markedly reduced the extent of attachment of adenoviral particles to the surfaces of cells (Fig. 6).

We believe that our findings, taken together with the fact that LRRC15 is overexpressed in multiple tumor types, report a new factor that not only is involved in the mediation of adenoviral infectivity but also may have important implications for the successful application of adenoviruses for either gene therapy or oncolytic viral therapy. The further analysis of LRRC15 in other cancers will undoubtedly determine how far-reaching this effect of LRRC15 may be and whether it is a factor for consideration in treating multiple tumor types. Identification of high levels of LRRC15 in a tumor could be used as a criterion in decisions relating to the selective use, or the strategy of administration, of an adenovirus-based therapy in any individual case. Moreover, future studies to further explore the mechanism of action of LRRC15 will also be rewarding and may lead to strategies to enhance adenoviral infectivity in cases where LRRC15 is either at normal levels or overexpressed.

FIG. 5. LRRC15 decreases cell surface, but not total, CAR levels. (a) A549-LRRC15 and A549 control (Cont) cells were analyzed for surface expression of the following parameters, which are known to affect adenoviral transduction: CAR, integrin αv, integrin β3, and integrin β5. Surface expression was quantified by flow cytometry, and the percentage of cells showing a change in surface expression in A549-LRRC15 cells is shown relative to that observed in A549-Cont cells. (b) Total CAR levels in A549-LRRC15 and A549-Cont cells were measured by Western blotting. A lysate from 293 cells is included as a positive-control guideline for detection of CAR expression.

FIG. 6. LRRC15 impedes adenoviral attachment. (a and b) A549-LRRC15 (b) and A549 control (Cont) (a) cells were incubated with Ad-GFP for 90 min on ice to permit adenoviral attachment but not internalization. Cells were subsequently stained with an anti-adenovirus type 5 (Ad5) antibody and 4',6'-diamidino-2-phenylindole (DAPI) before being visualized by fluorescent microscopy.
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