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Leucine-Rich Repeat (in Flightless I) Interacting Protein-1 Regulates a Rapid Type I Interferon Response

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The cell autonomous response to viral infection is carefully regulated to induce type I interferons (IFNs), which in turn induce the establishment of an antiviral state. Leucine-rich repeat (in Flightless I) interacting protein-1 (LRRFIP1) and LRRFIP2 are 2 related proteins that have been identified as interacting with MyD88 and Flightless I homolog, a leucine-rich repeat protein. LRRFIP2 positively regulates NFκB and macrophage cytokine production after lipopolysaccharide, but less is known about LRRFIP1. We hypothesized that LRRFIP1 could be more important in antiviral responses, as overexpression led to type I IFN production in a pilot study. The induction of type I IFNs occurred even in the absence of virus, but was enhanced by the presence of virus. Conversely, knockdown of LRRFIP1 compromised IFN expression. We found that LRRFIP1 was rapidly recruited to influenza-containing early endosomes in a p38-dependent fashion. This was specific for virus-containing endosomes as there was almost no colocalization of LRRFIP1 with early endosomes in the absence of virus. Further, LRRFIP1 was recruited to RNA-containing vesicles. Taken together, these data suggest that LRRFIP1 participates in cell responses to virus at early time points and is important for type I IFN induction.

Introduction

Leucine-rich repeat (in Flightless I) interacting protein-1 (LRRFIP1) is a poorly characterized protein implicated in toll-like receptor (TLR) responses and the regulation of tumor necrosis factor-α. A short murine ortholog of LRRFIP1, called FLAP-1, has been identified as interacting with both MyD88 and the Flightless I homolog [Fliih] (Dai and others 2009). Its C-terminus and N-terminus are highly related to LRRFIP2, which has also been implicated in the positive regulation of TLR responses (Dai and others 2009). The divergent C-terminus has been described as having nucleic-acid-binding activity (Shibutani and others 1998; Wilson and others 1998), including the transactivating response region (TAR) hairpin of HIV (Wilson and others 1998). Little else is known about its function and it has little homology to other protein families. We wished to examine its role in the innate response to viral infections because we had observed overexpression of type I interferons (IFNs) in an array study of *LRRFIP1*-transfected cells.

Innate responses to virus represent a critical facet of overall host defenses. In most cell types, viral infection is sensed by cytoplasmic helicase proteins such as RIG-I and MDA5 that activate IRF3/7, leading to type I IFN expression (Yoneyama and others 2004; Kato and others 2005; Yoneyama and Fujita 2007). Additional proteins such as STING and NLRX1 have been implicated in antiviral responses, suggesting a complex interplay of proteins (Ishikawa and Barber 2008; Moore and others 2008). TLRs also function as innate sensors of viral infections, and TLRs 3, 7, 8, and 9 appear to be particularly important in sensing viral infections. TLRs signal through MyD88 or TRIF and activate NFκB, MAP kinases, and IRF molecules. TLR responses to virus are particularly important for antigen-presenting cells. Because of a limited range of expression, they have a less significant role outside of antigen-presenting cell responses. There remains much to learn regarding the interplay of the TLR and helicase systems and the regulation of the system by accessory signaling molecules.

To examine the potential role of LRRFIP1, we defined its ability to upregulate type I IFN expression. Our data indicate that LRRFIP1 can drive a type I IFN response. LRRFIP1 colocalizes with virus very early in influenza infection and colocalization is dependent on p38. It is phosphorylated in response to viral infection and this too is dependent on p38. Taken together, our data indicate that LRRFIP1 is an important regulator of type I IFN production.

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Materials and Methods

Viruses

The attenuated influenza virus (FluMist) used for the uptake studies was obtained from MedImmune, Inc. It was labeled with the Alexa Fluor 488 protein-labeling kit (Invitrogen). The nonattenuated influenza A/PR8/34 virus was purchased from Charles River Laboratories. The sindbis virus is a laboratory-adapted strain of sindbis (HRsp) that expresses GFP from a subgenomic promoter (Burnham and others 2007). In each case, a titration was performed to establish the optimal multiplicity of infection (MOI). Alexa Fluor 488 *Escherichia coli* and Alexa Fluor 488 Transferrin (Invitrogen) were used as controls.

Antibodies

Antibodies used were mouse anti-LRRFIP1 (BD Bioscience), rabbit anti-LRRFIP1 (Sigma), rabbit polyclonal anti-LRRFIP1 (Rikiyama and others 2003), anti-NP (Light Diagnostics, Millipore), rabbit anti-phospho-serine (Invitrogen), goat anti-EEA1, rabbit anti-p38, mouse IgM anti-phospho-p38, goat anti-PB1, goat anti-NS1, goat-anti-RIG-I, HRP-conjugated goat anti-mouse IgM, and bovine anti-goat TRITC (Santa Cruz Bio). Alexa Fluor 647 chicken anti-mouse was from Invitrogen.

Cells

3T3 cells were obtained from ATCC and cultured in Dulbecco's modified Eagle media (DMEM) with cosmic calf serum. Mouse embryonic fibroblasts (MEFs) were cultured in Roswell Park Memorial Institute (RPMI) with 10% fetal bovine serum. 3T3 cell transfections were performed with either Amaxa or Lipofectamine (Invitrogen) using 2–4 mg of DNA per transfection. The BLOCK-iT™ U6 RNAi Entery Vector (Invitrogen) was used for the shRNA transfections. Three different vectors expressing shRNA were generated by designing dsDNA oligonucleotides coding for a sense-loop-antisense sequence to 3 different regions of the *LRRFIP1* gene. The dsDNA oligonucleotides were designed using the Invitrogen BLOCK-iT Designer and the top 3 hits were selected. The levels of knockdown were verified by Western and the construct that was most effective was used for the experiments (top strand, 5′ caccgcaagagataactgccttagacgaatctaag gcagttatctcttgc 3′; bottom strand, 5′ aaaagcacaccgcaagagata actgccttagacgaatctaaggcagttatctcttgc 3′). Cells were transfected with the Amaxa® Cell Line Lonza Nucleofector® Kit and rested for 4 days, transfected again, and rested for 48 h before virus administration. The efficacy of every knockdown experiment was verified by Western blot, with the remaining LRRFIP1 protein levels approximately half of the starting levels.

Confocal analysis

Labeled virus was preincubated with cells at 4°C for 10 min. Cells were fixed at various time points subsequent to the 37° shift in 2% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 1% Triton X-100 SigmaUltra, and then blocked with 1% bovine serum albumin in PBS at room temperature. For the hemaglutinin (HA) beads, green fluorescent aldehyde sulfate beads (Molecular Probes) were crosslinked to purified HA (His-tagged H3N2 HA; MyBioSource). The HA content of the beads was confirmed by Western blot. For the RNA beads, amino-modified, Cy3-labeled RNA was crosslinked to fluorescent green carboxylate beads (Molecular Probes). The specific dsRNA phosphorothioate sequence was 5′ AACCUAAUAAUAAUUAUCAAAAUG 3′. An OLYMPUS Fluoview FV1000 model confocal microscope was used with an Argon laser (488 nm wavelength), a UV laser (405 nM), and a HeNe laser (543 and 633 nm wavelength). Z-sections at the depth of 0.25–0.45 μm were generated and the images were deconvoluted (AutoDeblur®; Media Cybernetics, Inc.). In some cases contrast and/or intensity was adjusted to improve comparison of different stains. When applied, these changes affected the entire panel.

VOLOCITY 4® (IMPROVISION®) was used for 3D reconstruction and colocalization analyses used an object-based identification method on the 3D reconstruction (Bolte and Cordelieres 2006). Structures were identified based on size, intensity, and wavelength. Centroid colocalization was used to define the coincidence of multiple wavelengths (Boutte and others 2006). For the influenza colocalization studies, the virion was used as the anchor for colocalization. For the LRRFIP1-EEA1 analyses, LRRFIP1 was used to anchor the colocalization analysis. Multiple frames were evaluated and the colocalization averaged.

Western blot and reverse transcription–polymerase chain reaction

The coimmunprecipitations utilized either protein A or protein A/G beads (Invitrogen and Santa Cruz, respectively). RNA was prepared using sequential TRIzol and RNeasy (Invitrogen and Qiagen) and reverse transcribed using Advantage RT-for-PCR (Clontech). Type I IFN messages were detected using specified primers/probes from Applied Biosystems run on a TaqMan SDS 7900HT. Message levels were normalized to 18S rRNA levels and calibrated to the indicated controls.

Results

LRRFIP1 structure

LRRFIP1 is an 80, 120, or 160 kDa protein that has been described as interacting with RNA species such as the TAR hairpin of HIV (Wilson and others 1998). The short 80 kDa LRRFIP1 murine isoform and the related LRRFIP2 were shown to bind MyD88 and Fliih (Wilson and others 1998; Rikiyama and others 2003; Dai and others 2009). There do not appear to be other family members and the domain structure of LRRFIP1 yields few clues as to its function (Fig. 1). Both LRRFIP1 and LRRFIP2 have RNA-binding and coiled-coil domains. They differ primarily in their C-terminus.

LRRFIP1 induces type I IFN expression

An array study performed on cells overexpressing LRRFIP1 revealed a large number of IFN-inducible genes that were upregulated (data not shown). On the basis of the array data and the reported modulation of TLR signaling (Dai and others 2009), we hypothesized that LRRFIP1 could be implicated in the innate responses to virus and initially sought to confirm the ability of LRRFIP1 to regulate type I IFN expression. We utilized influenza virus as a model.

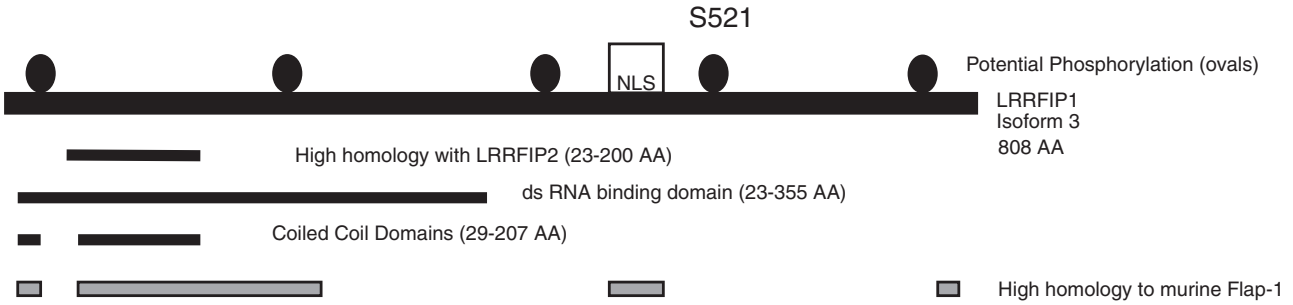

FIG. 1. LRRFIP1 structure. The C-terminus and N-terminus contain most of the identifiable domains. The dsRNA-binding domain is the largest conserved domain with 2 coiled-coil domains embedded within it. The C-terminus and N-terminus are also the region of homology with LRRFIP2 and the murine ortholog of LRRFIP1, Flap-1. The C-terminus is highly divergent. The NLS and the potential Ser521 phosphorylation site (oval) are indicated in the schematic diagram. LRRFIP1, leucine-rich repeat (in Flightless I) interacting protein-1; NLS, nuclear localization signal.

Influenza virus is an RNA virus of the Orthomyxoviridae family. Both TLRs and RIG-I are known to be important for the local defense against influenza virus (Pichlmair and others 2006; Le Goffic and others 2007).

We examined the effect of LRRFIP1 overexpression on the induction of type I IFN expression in 3T3 cells infected with influenza virus. 3T3 cells represent a model cell line capable of supporting a productive infection with influenza virus. 3T3 cells were transfected with pCMV-LRRFIP1 or the corresponding pCMV-GFP vector. Twenty-four hours later, cells were infected with PR8 influenza virus at an MOI of 10. Message levels of αIFN a2, αIFN a4, and βIFN were determined by quantitative reverse transcription (qRT)–polymerase chain reaction at various time points after infection. Overexpression of LRRFIP1 resulted in increased type I IFNs at baseline and increased responses to viral infection (Fig. 2). The type I IFN response to infection was more rapid and of greater magnitude in the LRRFIP1-overexpressing cells. These data suggest that LRRFIP1 can regulate type I IFNs independently of virus.

Wild-type influenza induces a very modest IFN response due to the action of the NS1 protein, which inhibits RIG-I and IRF3 (Donelan and others 2004; Kochs and others 2007; Mibayashi and others 2007; Shin and others 2007). To confirm our findings using a different RNA virus, we utilized sindbis virus. This virus has a limited ability to downmodulate IFN production (Frolova and others 2002). 3T3 cells were transfected with pCMV-LRRFIP1 or the corresponding GFP vector and infected 24 h later with sindbis virus at an MOI of 1. The LRRFIP1-overexpressing cells expressed significantly more type I IFN transcripts than the vector-transfected cells. Conversely, LRRFIP1 knockdown reduced type I IFN production (Fig. 2).

To directly test the effect of LRRFIP1 on viral replication, HEK293 (a replication-competent cell line) cells were transfected with pCMV-LRRFIP1 or vector. Twenty-four hours later, cells were infected with PR8 influenza at an MOI of 10. Supernatants were collected at 72 h and viral progeny titered on HEK293 cells (Fig. 2). LRRFIP1 overexpression led to decreased viral infection/replication ($P = 0.004$).

Induction of type I IFN expression by LRRFIP1 is dependent on RIG-I expression

In nondendritic cells, influenza virus is sensed primarily by the helicase protein, RIG-I (Loo and others 2008). To determine whether LRRFIP1 functioned in the RIG-I pathway, we transfected *LRRFIP1* into RIG-I knockout MEFs. We reasoned that if LRRFIP1 was acting distal to RIG-I or via an alternative pathway, then the knockout MEFs would have an intact response to LRRFIP1 overexpression. pCMV-LRRFIP1 was transfected into the knockout MEFs and heterozygous (Het) controls. Two days later the cells were infected with PR8 influenza virus at an MOI of 10. Type I IFN message was detected at 24 h after infection, which was the peak of the response. LRRFIP1 was not capable of inducing type I IFN expression in the RIG-I knockout MEFs either with or without viral infection, whereas a normal induction of type I IFNs was seen in the wild-type cells (Fig. 3). These data place LRRFIP1 in the same pathway as RIG-I. We noted that the effect of LRRFIP1 in the MEF cells was less robust than in the 3T3 cells. One significant difference is expression of TLR3 in 3T3 cells. MEF cells do not express TLR3. We therefore investigated the possible interaction of TLR3 and LRRFIP1.

LRRFIP1 interacts with TLR3 and is recruited to RNA-containing endosomes in vivo

LRRFIP1 and LRRFIP2 have both been identified as interacting with MyD88 and modulating downstream NFκB signaling. The conserved N-terminus mediates the physical interaction with MyD88 (Dai and others 2009). 3T3 cells also express TLR3 and we hypothesized that LRRFIP1 could interact with TLR3 after viral infection because of its known ability to interact with Fliih, which can regulate TLR3 responses (Wilson and others 1998; Wang and others 2006). To test this we performed a coimmunprecipitation (Fig. 4). TLR3 demonstrated a time-dependent association with LRRFIP1 after viral infection. The biphasic nature of the interaction was reproducibly seen and mimics somewhat the proposed interaction of LRRFIP2 with MyD88, which has been hypothesized to initially augment TLR signaling and then subsequently inhibit signaling (Dai and others 2009).

The presence of an RNA-binding domain and the interactions with TLR3 suggested that LRRFIP1 might bind nucleic acids *in vivo*. To test this, we incubated 3T3 cells with dsRNA-coated latex beads. A phosphorothioate oligonucleotide labeled with Cy3 was bound to fluorescent green carboxylate beads. Noncoated and HA-coated beads were used as controls. In this assay, LRRFIP1 was clearly associated with the RNA beads but not the HA or the noncoated beads (Fig. 4). These data suggest that LRRFIP1 can interact with nucleic-acid-containing endosomes *in vivo*.

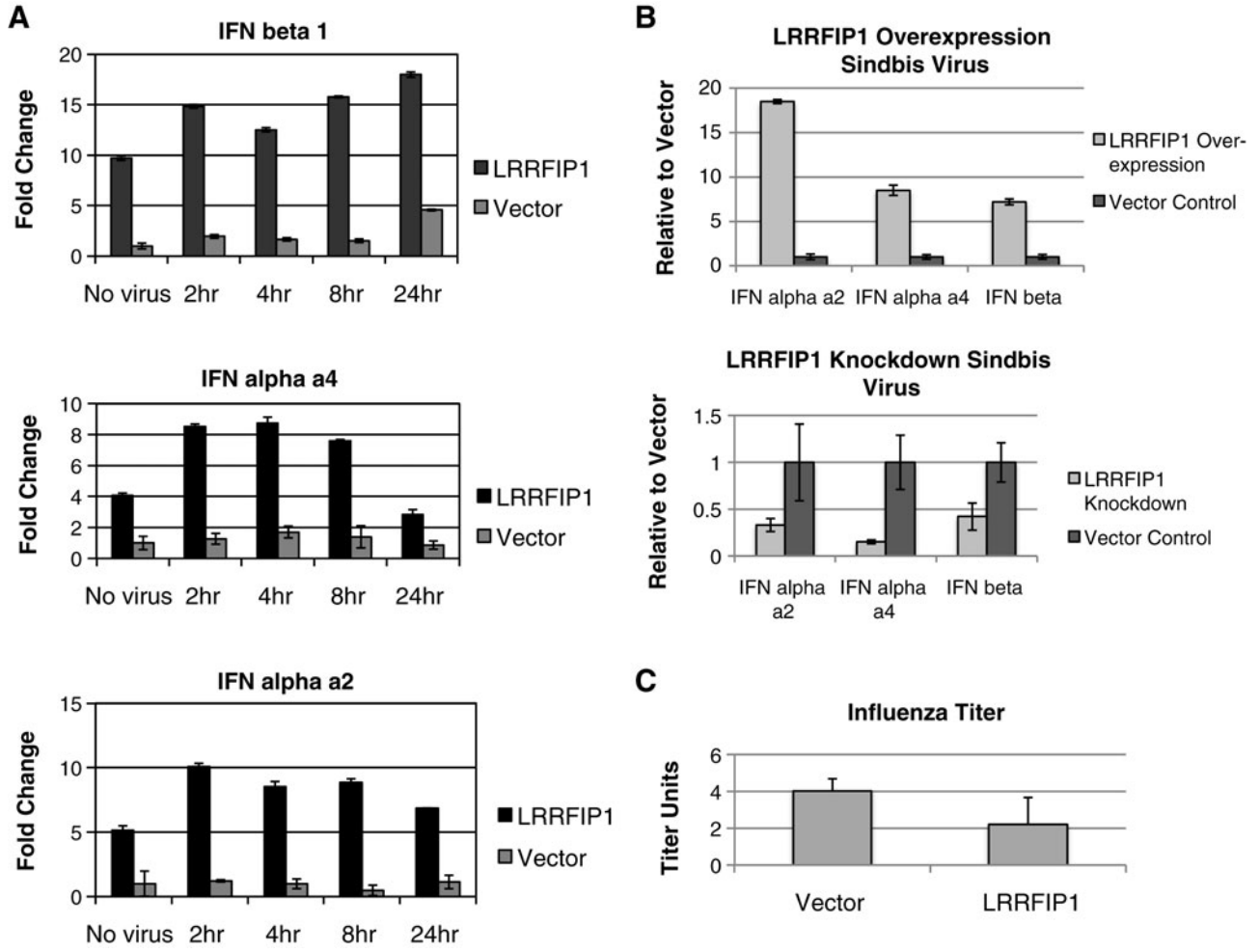

FIG. 2. LRRFIP1 overexpression leads to type I IFN expression. (**A**) 3T3 cells were transiently transfected with pCMV-LRRFIP1 or pCMV-GFP. Protein levels were confirmed with Western blot. Three type I IFNs were detected by qRT-PCR at various time points after infection with PR8 at an MOI of 10. IFN responses to wild-type influenza in the vector-transfected cells were minimal. LRRFIP1-transfected cells produce type I IFN at baseline and produce an increased response to influenza infection. The IFN signal was normalized to 18S rRNA and calibrated to uninfected, vector-transfected cells ($N = 5$). (**B**) 3T3 cells were transfected as above and also with an shRNA knockdown construct and infected with sindbis virus at an MOI of 1. Protein levels were confirmed with Western blots. Cells were harvested at 24 h and type I IFN messages were detected by qRT-PCR, normalized to the 18S rRNA signal. Type I IFN message levels were expressed relative to the vector for both overexpression and knockdown ($N = 3$). (**C**) HEK293 cells were transfected and 24 h later infected with PR8 at an MOI of 10. Seventy-two hours after infection, supernatants were titered on freshly passaged HEK293 cells. Viral counts were obtained by fixing cells 48 h after application of the supernatants and staining for NP. IFN, interferon; LRRFIP1, leucine-rich repeat (in Flightless I) interacting protein-1; PCR, polymerase chain reaction.

LRRFIP1 colocalizes with influenza virus at early time points

RIG-I translocates from the cytoplasm to mitochondria upon activation, whereas TLR3 translocates from the endoplasmic reticulum to the endosomes upon viral infection (Nishiya and others 2005; Seth and others 2005; Brinkmann and others 2007; Ohman and others 2009). We wished to define the cellular compartment where LRRFIP1 exists to better understand how it could interact with other proteins involved in the innate response to virus. In the absence of virus, LRRFIP1 was found largely in cytoplasmic granules. These granules did not costain with markers of lysosomes, or endosomes, nor did they colocalize with Unc93b, a TLR3-binding protein located on the endoplasmic reticulum (data not shown). To better understand how it could participate in responses to virus, we examined colocalization with influenza virus using confocal microscopy and quantitative analyses. Attenuated influenza virus was used for the uptake assays. Whole attenuated influenza virus (FluMist) was labeled with Alexa Fluor 488 and incubated with 3T3 cells at 4° to synchronize binding and then warmed. Influenza virus is known to enter predominantly via the endosomal compartment (Lakadamyali and others 2003; Sakai and others 2006). The virus then transits to the perinuclear region for uncoating. At various times, the cells were fixed and stained for EEA1, an early endosome marker, and LRRFIP1. By 3 min, it was possible to see some colocalization of LRRFIP1, EEA1, and influenza virus, although the majority of LRRFIP1 was not associated with EEA1 (Fig. 5). From 5 to

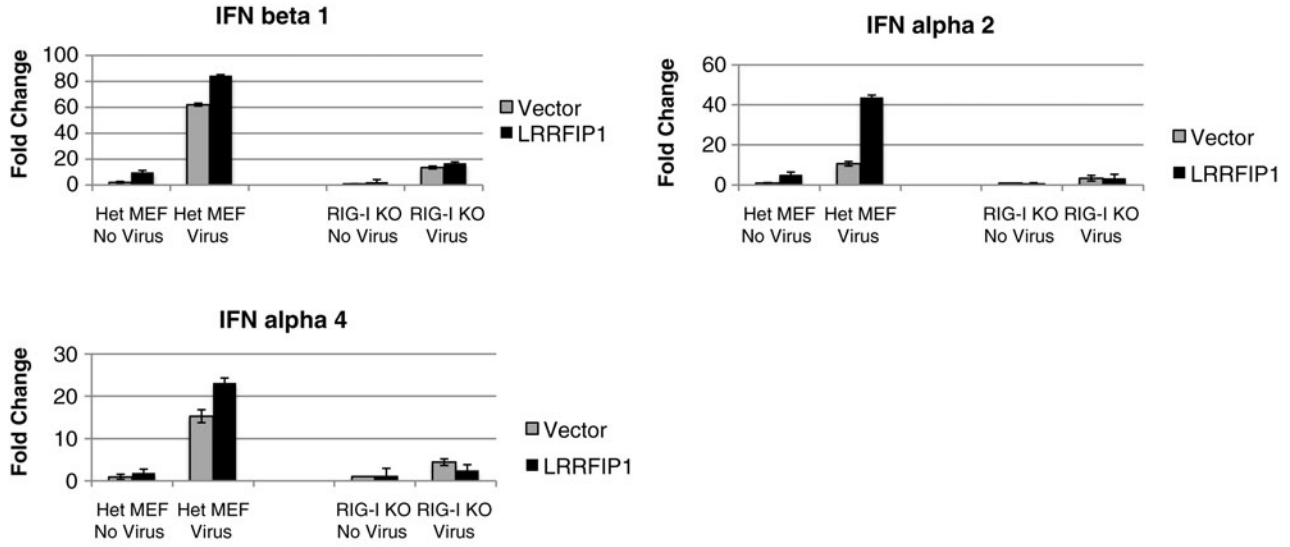

FIG. 3. LRRFIP1 induction of type I IFNs is dependent on RIG-I. RIG-I KO MEFs or heterozygous (Het) MEFs were transfected as before. The cells were infected with PR8 48 h later, and RNA was harvested 24 h after infection. Type I IFNs were detected as in Fig. 2. IFN, interferon; KO, knockout; LRRFIP1, leucine-rich repeat (in Flightless I) interacting protein-1; MEFs, mouse embryonic fibroblasts.

15 min, the majority of influenza particles were colocalized with both LRRFIP1 and EEA1. After 20–30 min, virions were no longer seen. LRRFIP1 and EEA1 did not colocalize in the absence of virus. Deconvolution and quantitation using Volocity was performed to clarify the degree of interaction between the 3 species (Collins and others 2002). Object-based analysis was used to identify objects based on size and wavelength (Bolte and Cordelieres 2006). As LRRFIP1 and EEA1 were present throughout the cytoplasm, quantitation was performed to ensure that the observed colocalization represented true recruitment to the endosome. In each slice, influenza virions were identified by wavelength and size and used as an anchor for the quantitation of EEA1 and LRRFIP1. Approximately 60%–70% of the influenza virions were associated with both EEA1 and LRRFIP1 within 5 min of viral entry. To address the degree of colocalization of LRRFIP1 and EEA1 in the absence of virus, we again deconvoluted and quantitated colocalization based on wavelength and size. For this analysis, LRRFIP1 was used as the anchor and only 5%–10% of LRRFIP1 granules were found to be associated with EEA1 in the absence of virus. The colocalization of influenza virus, EEA1, and LRRFIP1 was specific, as neither *E. coli*, transferrin, or latex beads induced colocalization. Transferrin exhibited time-dependent colocalization with EEA1 but was not found associated with LRRFIP1 at any time point (Fig. 6).

LRRFIP1 is dephosphorylated and rephosphorylated in response to influenza

Little is known about the biochemistry of LRRFIP1. Several potential tyrosine and serine phosphorylation sites were identified bioinformatically. Many proteins involved in innate responses to virus are phosphorylated in response to virus. We hypothesized that LRRFIP1 could be phosphorylated in response to viral infection and this could regulate its function. p38 phosphorylation regulates EEA1 and is important for IRF3 activation. Further, 2 MAP kinase phosphorylation sites at Ser16 and Ser521 were identified bioinformatically using 3 independent software systems: PPSP, GPS2.1, and NetPhosK 1.0. In addition, the ELM program identified a p38 docking motif in proximity to the Ser521. We therefore examined the potential of p38 to regulate LRRFIP1. We first examined the response of 3T3 cells to dsRNA. We observed a peak in LRRFIP1 serine phosphorylation at 2 h after transfection of RNA (Fig. 7A). This phosphorylation was abrogated in cells treated with the p38 inhibitor SB203580. To further examine the role of MAP kinases, we examined the phosphorylation of LRRFIP1 in the monocyte cell line J774.1. This cell line produces brisk inflammatory cytokine responses to influenza virus infection and LRRFIP1 has been implicated in modulating TLR responses in monocyte-lineage cells (Dai and others 2009). J774.1 cells do not support viral replication. In this setting, LRRFIP1 became dephosphorylated on serine after infection, subsequently regaining its phosphorylated state. Rephosphorylation of LRRFIP1 was compromised in SB203580-treated cells (Fig. 7B), suggesting that p38 might play a role in either the constitutive phosphorylation of LRRFIP1 or the serine re-phosphorylation in response to virus. We then examined the role of p38 in the recruitment of LRRFIP1 to the virus-containing endosomes in 3T3 cells, hypothesizing that if phosphorylation is important for function, then inhibition of LRRFIP1 phosphorylation would affect colocalization. SB203580 nearly abrogated colocalization of LRRFIP1 with virus-containing endosomes (Fig. 7C). In spite of this, there was no obvious decrement in the number of virions taken up in the presence of SB203580 by visual inspection (data not shown).

Discussion

The data supporting the role of LRRFIP1 in viral defense include the induction of type I IFN expression upon overexpression, diminished induction of type I IFN expression with knockdown, and colocalization with virus at very early

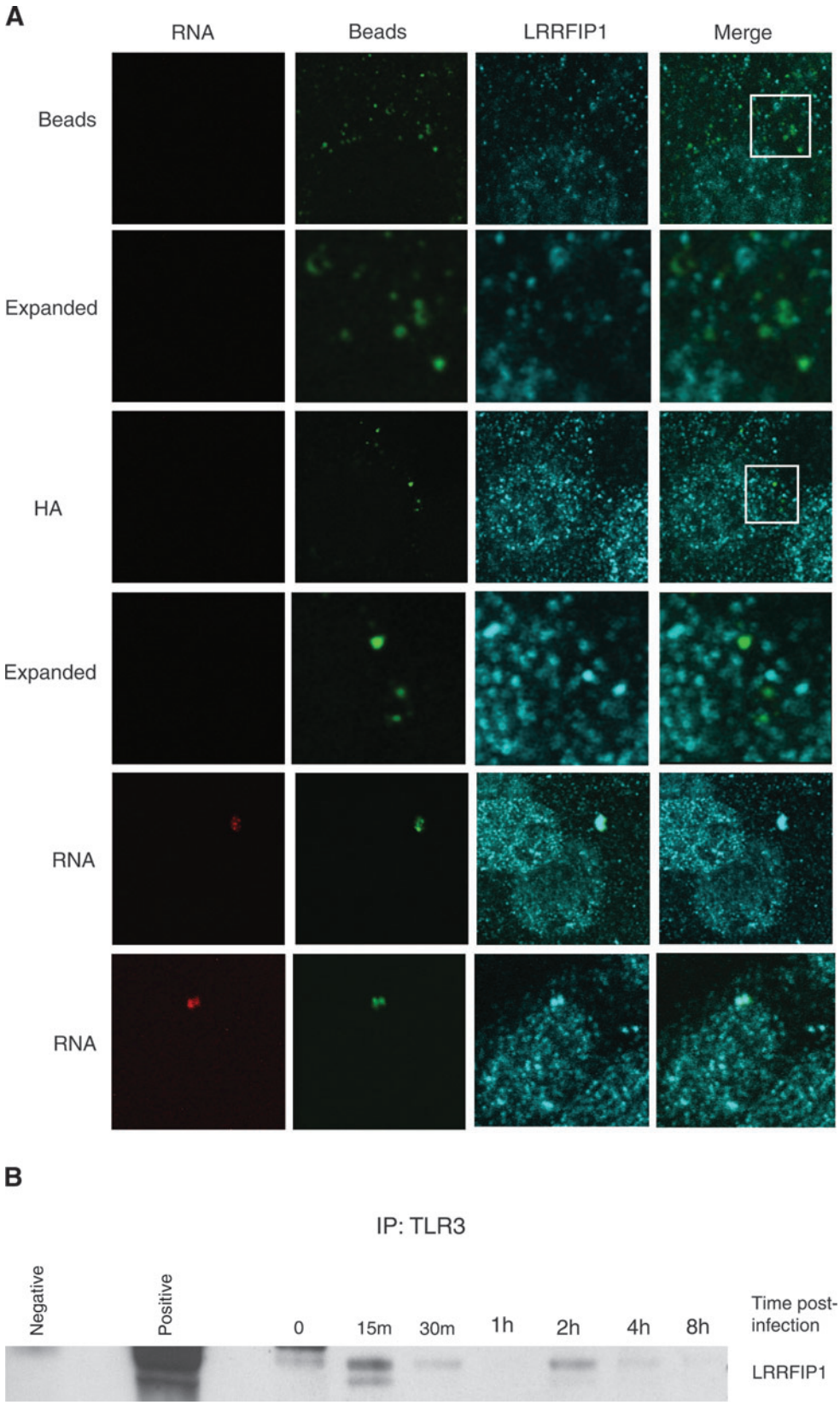

FIG. 4. LRRFIP1 colocalizes with RNA. (**A**) Green fluorescent beads (visible in the second column) were coated with either HA or dsRNA. The dsRNA was Cy3 labeled and may be seen in the first column. The RNA-coated beads induced a strong aggregation of LRRFIP1, whereas the plain beads and the HA-coated beads did not colocalize with LRRFIP1. An expanded view (boxed) of the plain beads and the HA-coated beads are shown beneath each row. (**B**) TLR3 was immunoprecipitated from 10 million 3T3 cells and LRRFIP1 was detected. The association of LRRFIP1 and TLR3 was time and virus dependent. The positive control utilized unmanipulated lysate from 3T3 cells and the negative control lane utilized an immunoprecipitation with no antibody. LRRFIP1, leucine-rich repeat (in Flightless I) interacting protein-1; TLR, toll-like receptor.

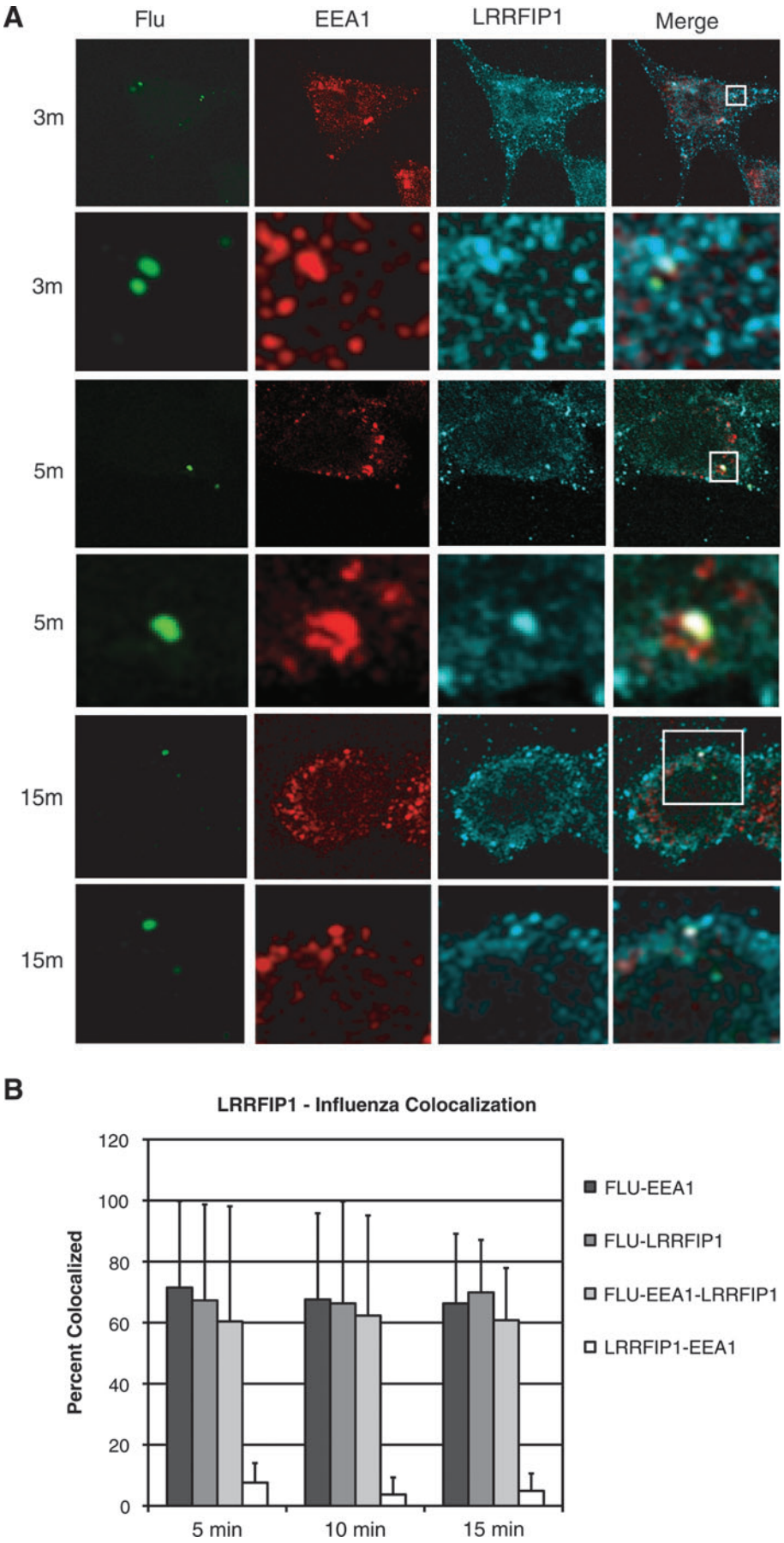

FIG. 5. LRRFIP1 colocalization with virus-containing endosomes. (**A**) 3T3 cells were incubated with labeled attenuated virus for the indicated times. Confocal analysis was performed on fixed cells. The whole-cell view is given and the expanded view of the boxed region is given below. At 3 min, approximately half of the virus-containing endosomes are colocalized with LRRFIP1. This association peaks at 15–30 min when ~65% of virus-containing endosomes colocalize with LRRFIP1. (**B**) LRRFIP1 and EEA1 colocalize specifically at virus-containing endosomes. The gray bars demonstrate colocalization with influenza as the anchor. The EEA1-LRRFIP1 colocalization used LRRFIP1 as the anchor. LRRFIP1, leucine-rich repeat (in Flightless I) interacting protein-1.

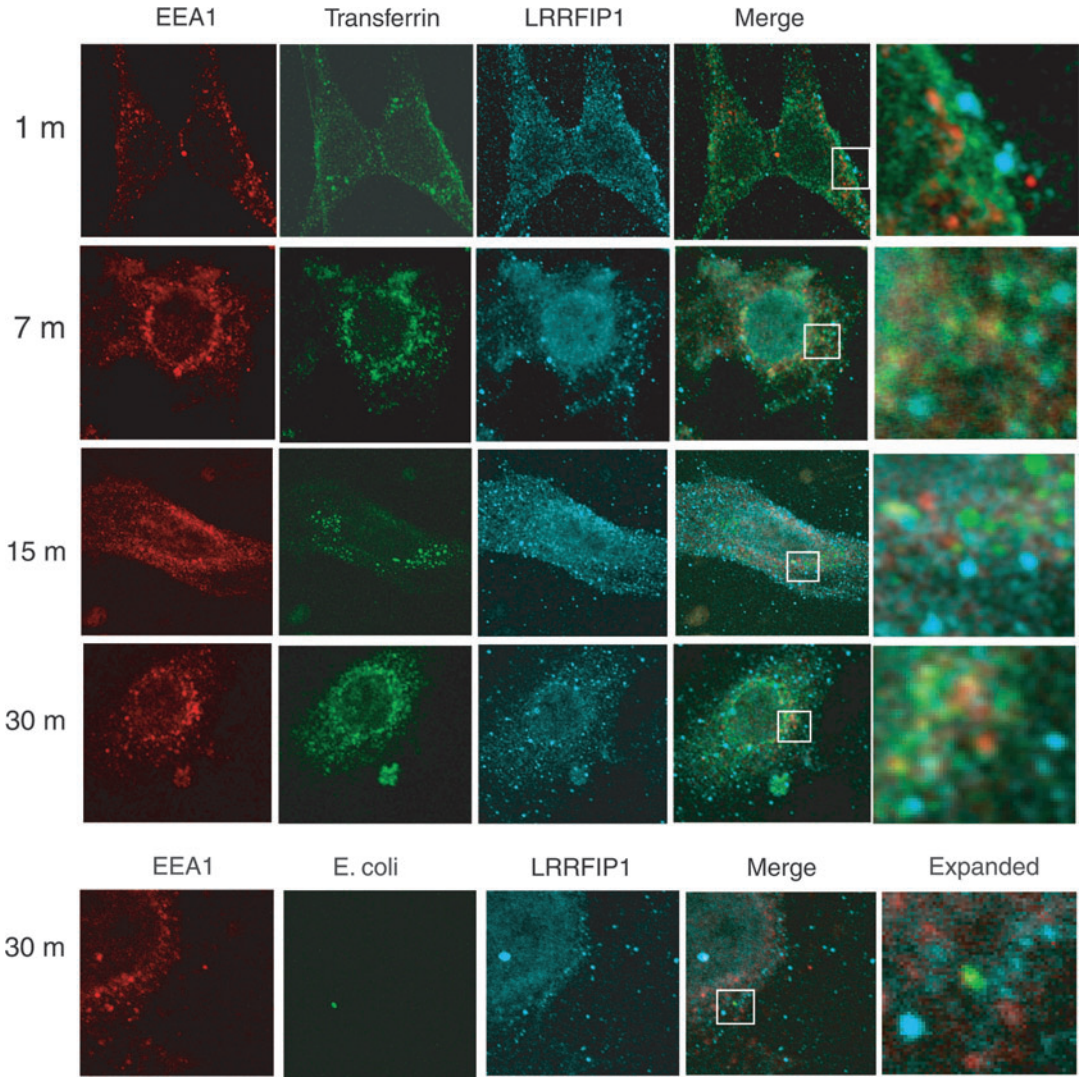

FIG. 6. LRRFIP1 does not colocalize with other endosomes. 3T3 cells were incubated with transferrin or *Escherichia coli* for the indicated times. From 7 min onward, transferrin is extensively associated with EEA1 (yellow color), but there is no colocalization with LRRFIP1. As *E. coli* are taken up poorly by 3T3 cells, care was taken to ensure that the bacteria were inside the cell by examining multiple slices. LRRFIP1, leucine-rich repeat (in Flightless I) interacting protein-1. The boxed regions are expanded in the last column.

time points after infection. The colocalization of LRRFIP1 with virus at early time points and its ability to drive a strong type I IFN response place it within the pathways related to innate viral responses. Colocalization with RNA-coated beads also supports a role in antiviral responses.

LRRFIP1 has been previously shown to bind dsRNA in preference to other nucleic acids (Wilson and others 1998). In this study, it could not be determined whether LRRFIP1 directly recognized RNA associated with the influenza virion or was recruited by another RNA-recognizing protein. The dependence on RIG-I and the association with TLR3 suggest that in spite of its RNA recognition domain, it is dependent on other proteins for recognition of virus within the endosomes.

Inactive influenza can induce IFN production, suggesting that responses to virus before replication can be biologically significant (Isaacs and Lindenmann 1957; Collins and others 2004; Hidmark and others 2005). The mechanism underlying this response is not fully understood, but this study implicates LRRFIP1. Transfection of 3T3 cells with LRRFIP1 led to a wave of type I IFN expression as early as 2 h postinfection, before significant replication. Early responses to influenza are critical in determining outcome (Kash and others 2006; Kobasa and others 2007) and an early wave of IFN may provide protection to surrounding cells and initiate a signal to the adaptive immune system (Wijburg and others 1997; Durbin and others 2000).

The choreography of proteins involved in innate responses to viruses may be important in segregating host nucleic acids from the central signaling complex or TRADDosome (Michallet and others 2008). Thus, the recruitment of LRRFIP1 to a compartment specific for virus may be an important aspect, as is true for TLR9 responses to exogenous DNA (Latz and others 2004). The role of p38 appears to actively regulate LRRFIP1 recruitment to the endosome. Ensuring that LRRFIP1 is restricted to appropriate sites by p38 may limit its interactions with endogenous RNAs. We hypothesize that recruitment of LRRFIP1 to the virus-containing endosomes serves to bridge the TLR3 signal to TRAF6 and the MAP kinase pathway.

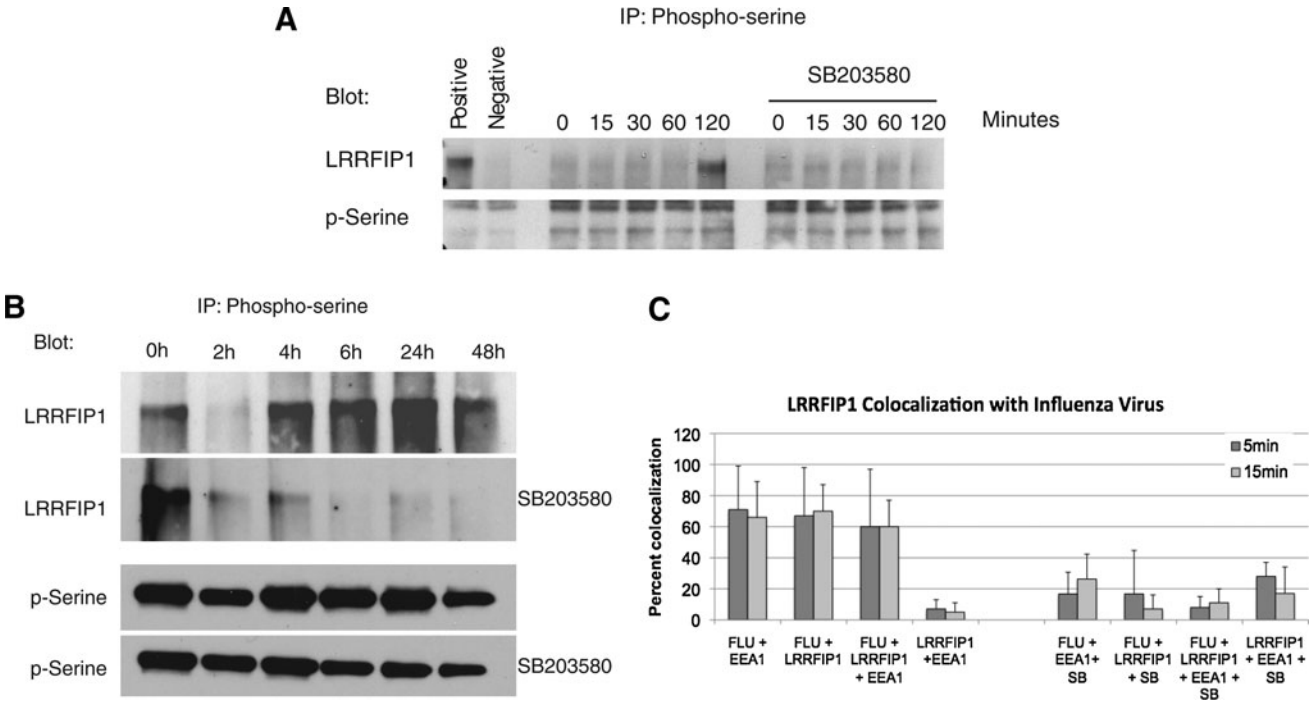

FIG. 7. SB203580, a p38 inhibitor, leads to diminished colocalization. (**A**) 3T3 cells were transfected with poly I:C and lipofectamine. Lysates were immunoprecipitated with antibody to phospho-serine and then blotted with antibody to LRRFIP1. The equivalency of the loading was confirmed by reprobing with anti-phospho-serine. The positive control was generated from whole lysates and the negative control was lysate immunoprecipitated with beads alone. (**B**) J774.1 cells were infected with PR8 virus at an MOI of 10 in the presence or absence of the p38 inhibitor, SB203580, and harvested at various times after infection. Lysates were immunoprecipitated with antibody to phospho-serine and then blotted with antibody to LRRFIP1 (*top panels*). To confirm equal loading, the blots were stripped and probed with anti-phospho-serine (*bottom panels*). (**C**) 3T3 cells were infected with labeled attenuated influenza and, at various times after infection, fixed for confocal analyses. The SB203580-treated cells received inhibitor 30 min before infection. Confocal analyses with 3D reconstruction and deconvolution were used to quantitate colocalization of EEA1, LRRFIP1, and influenza. Object-based identification was used to define colocalization. The average and standard deviation are shown in the graph for each combination of events. The differences between the SB203580 (SB)-treated cells and mock-treated cells are significant with $P < 0.05$ at all time points except the 15 min time point for Flu + EEA1. LRRFIP1, leucine-rich repeat (in Flightless I) interacting protein-1.

In summary, this study identified another protein implicated in the innate responses to virus. We examined its role in non-antigen-presenting cells and found that LRRFIP1 can induce type I IFNs and is recruited to influenza and RNA-containing endosomes. Although dependent on RIG-I in MEF cells, we found minimal association of RIG-I and LRRFIP1 by coimmunoprecipitation (not shown) and the effect was overall less robust in MEFs than 3T3 cells. In 3T3 cells, LRRFIP1 coimmunoprecipitated with TLR3 but not Unc93b (not shown), another protein recruited to endosomes after viral infection. We hypothesize that LRRFIP1 serves to modulate TLR pathway responses to virus, similar to LRRFIP2 modulation of TLR pathway responses to bacterial stimuli.

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Author Disclosure Statement

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