THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 289, NO. 6, pp. 3547–3554, February 7, 2014

Rhes, a Striatal-selective Protein Implicated in Huntington Disease, Binds Beclin-1 and Activates Autophagy*

Received for publication, November 20, 2013 Published, JBC Papers in Press, December 9, 2013, DOI 10.1074/jbc.M113.536912

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Background: The striatal-specific protein Rhes is implicated in the selective pathology of HD.
Results: Rhes binds Beclin-1 and activates autophagy, a lysosomal degradation pathway critical in aging and neurodegeneration.
Conclusion: Rhes-induced autophagy occurs independent of mTOR and JNK-1 signaling and is inhibited by huntingtin.
Significance: The restricted expression of Rhes and its effect on autophagy may explain the selective striatal pathology and delayed onset of HD.

The protein mutated in Huntington disease (HD), mutant huntingtin (mHtt), is expressed throughout the brain and body. However, the pathology of HD is characterized by early and dramatic destruction selectively of the striatum. We previously reported that the striatal-specific protein Rhes binds mHtt and enhances its cytotoxicity. Moreover, Rhes-deleted mice are dramatically protected from neurodegeneration and motor dysfunction in mouse models of HD. We now report a function of Rhes in autophagy, a lysosomal degradation pathway implicated in aging and HD neurodegeneration. In PC12 cells, deletion of endogenous Rhes decreases autophagy, whereas Rhes overexpression activates autophagy. These effects are independent of mTOR and opposite in the direction predicted by the known activation of mTOR by Rhes. Rhes robustly binds the autophagy regulator Beclin-1, decreasing its inhibitory interaction with Bcl-2 independent of JNK-1 signaling. Finally, co-expression of mHtt blocks Rhes-induced autophagy activation. Thus, the isolated pathology and delayed onset of HD may reflect the striatal-selective expression and changes in autophagic activity of Rhes.

Huntington disease (HD)[4] is an autosomal dominant lethal neurodegenerative disease caused by an expansion of glutamine residues in the protein huntingtin (1). HD is characterized by selective and profound destruction of the corpus striatum, a brain region important in movement, emotion, and higher brain function. Thus, the cardinal symptoms of HD include a choreiform movement disorder, together with psychiatric and cognitive dysfunction (2). Despite the regional selectivity of HD, huntingtin (wtHtt) and mutant huntingtin (mHtt) are expressed uniformly throughout the brain and many body tissues (3). The distinct pathology of HD may reflect the interaction between mHtt and the striatal-selective G-protein Rhes (4), a topic that we reviewed recently (5). Rhes binds mHtt and acts as a SUMO (Small Ubiquitin-like MOdifier) E3 ligase to stimulate sumoylation of mHtt, a post-translation modification known to augment mHtt toxicity (4, 6). Rhes also physiologically regulates sumoylation and enhances a process we have termed "cross-sumoylation" (7). Independent work by other groups have confirmed the importance of Rhes in mHtt cytotoxicty using primary neuron and stem cell models of HD (8, 9). We found that deletion of Rhes dramatically reduces striatal degeneration and motor dysfunction in a toxin model of HD (10). Rhes-deleted mice also have delayed onset of symptomatology in a genetic model of HD (11).

The restricted expression pattern of Rhes explains the striatal-selective pathology of HD but not the delay in symptom onset, which typically occurs in late adult life. Conceivably, delayed onset is linked to macroautophagy (hereafter autophagy), a lysosomal degradation pathway implicated in aging and neurodegeneration (12–14). mHtt is a well established substrate of autophagy, and activating the autophagy pathway is protective in both cell and animal models of HD (15–18). Multiple findings further illustrate the importance of autophagy in HD, including diminished loading of autophagic vesicles (19), increased autophagosome levels in human HD lymphoblasts (20), and polyglutamine-dependent changes in neuronal autophagy (21).

Regulation of autophagy is a complex process, integrating signals from many different pathways (22). One important regulator of autophagy is mTOR, whose activation classically inhibits autophagy (23). We recently discovered that Rhes shares with Rheb the ability to bind and activate mTOR (24). Additionally, Rhes-deleted mice have markedly reduced L-DOPA-induced dyskinesia, a side effect of chronic L-DOPA (L-3,4-dihydroxyphenylalanine) treatment caused by aberrant mTOR signaling in the striatum (24, 25). Thus, Rhes plays a physiologic role in striatal mTOR activation and would be predicted to inhibit autophagy secondary to mTOR activation. A direct link between mHtt and mTOR has also been demonstrated, as Rubinsztein and co-workers (26) showed that mHtt

* This work was supported, in whole or in part, by National Institutes of Health Grant MH18501 from USPHS (to S. H. S.) and a grant from the Cure Huntington's Disease Initiative (to S. H. S.).
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[4] The abbreviations used are: HD, Huntington disease; mHtt, mutant huntingtin; wtHtt, wild-type huntingtin; mTOR, mammalian target of rapamycin.

aggregates sequester mTOR, leading to decreased mTOR kinase activity and enhanced autophagy. In this study, we have explored influences of Rhes on autophagy to evaluate its significance in HD pathophysiology.

EXPERIMENTAL PROCEDURES

Reagents, Plasmids, and Antibodies—Unless otherwise noted, reagents were obtained from Sigma. Antibodies for phospho-S6K (Thr-389), phospho-S6 (Ser-235/236), Beclin-1, LC3B, DARPP-32, and Myc-HRP were from Cell Signaling Technology; antibodies for LC3, GST, and FLAG were from Sigma; GAPDH antibody was from CalBiochem; Myc-M2 antibody was from Roche Applied Science; tubulin antibody was from Millipore. ER-Red (glibenclamide BODIPY-TR) and Golgi red (BODIPY-TR ceramide) were from Invitrogen and used as directed by the manufacturer. Plasmids for GST-Rhes, Myc-Rhes, and FLAG-Htt (N171)-18Q/82Q were described previously (4). AsRed-Beclin-1 was a gift from Dr. Zhenyu Yue at Mount Sinai School of Medicine. FLAG-Beclin-1 and Myc-Bcl-2 were gifts from Dr. Beth Levine at the University of Texas Southwestern Medical Center. GFP-Rhes was cloned into pEGFP-C1, with GFP fused to the N terminus of Rhes. FLAG-Beclin-1, Myc-Bcl-2, and Beclin-1 domain constructs were purchased from Addgene and described previously (27). Beclin-1 151–451 was generated using PCR.

PC12 Transfection with Rhes siRNA—PC12 cells were plated on poly-D-lysine-coated plates and transfected with Lipofectamine 2000 on two separate days with either scrambled siRNA (Sigma 4390849) or siRNA specific for Rhes (siRNA-1, Sigma s139719, and siRNA-2, Sigma s139720). GAPDH siRNA (Sigma 4390849) was used as a positive control to determine siRNA transfection efficacy. Forty-eight hours after the second transfection, cells were harvested and analyzed for changes in Rhes RNA expression with rat primers (forward, TGTTCCCAGCCCAGAGCCATGA; reverse, GCGCATGGCAGGGAACGGAT) using the SYBR Green PCR-CT system from Applied Biosystems. Actin and GAPDH RNA were measured as positive controls.

Recombinant Protein Production— Protein production was performed as described previously (7).

In Vitro Binding—Striatal lysates from wild-type mice were homogenized in Tris IP buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100, and 10% glycerol) plus protease inhibitors. Lysate (0.1 mg) was incubated with 5 μg of purified GST or GST-Rhes and 30 μl of glutathione-Sepharose beads (a total 0.5-ml reaction volume) at 4 °C overnight, followed by washing three times in Tris IP buffer and eluted with 30 μl of 2× LDS buffer (Invitrogen). FLAG and Myc immunoprecipitations were performed with a similar protocol using EZ View-Red affinity gel (Sigma). For Beclin-1/Bcl-2 binding, a primary Myc-HRP from Roche Applied Science was used to avoid cross-reactivity with immunoprecipitating beads.

Cell Culture and Western Blotting—HEK293 and HeLa cells were grown in DMEM + 10% FBS, penicillin/streptomycin, and L-glutamine, and were transfected with polyfect reagent (Qiagen). PC12 cells were grown in DMEM + 10% horse serum, 5% FBS, penicillin/streptomycin, and L-glutamine, and were transfected with Lipofectamine 2000 (Invitrogen). Cells were lysed in Tris IP buffer (described above) and analyzed by Western blot on 4–12% SDS NuPage gels (Invitrogen) for most experiments or 12% SDS NuPage gels to resolve LC3-I from LC3-II. Starvation medium was Earle's balanced salt solution plus Ca^{2+} and Mg^{2+}. Rapamycin was employed at 0.2 mM diluted in DMSO, and chloroquine added at 50 μM.

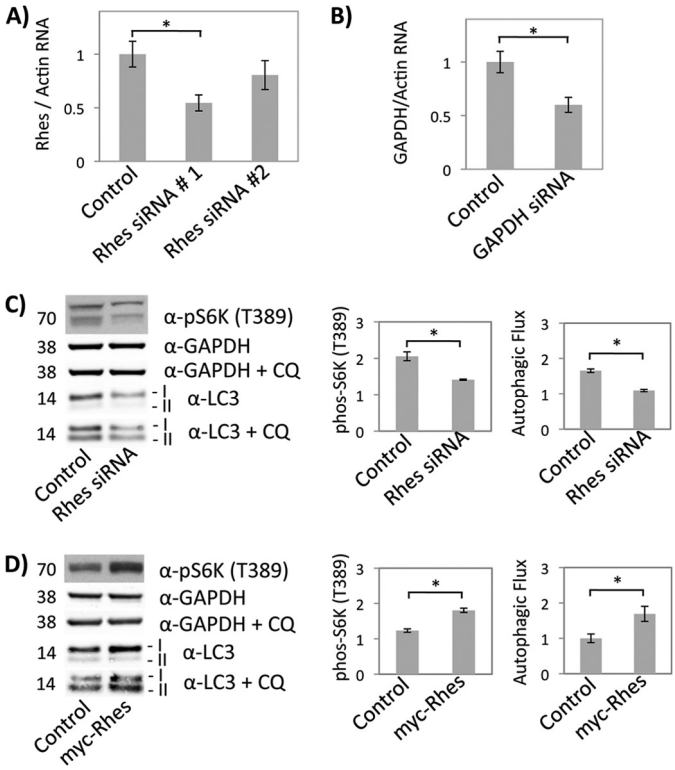

FIGURE 1. **Rhes regulates autophagy in PC12 cells.** *A*, RT-PCR analysis of Rhes RNA levels in PC12 cells transfected with serial siRNA transfection protocol. Transfection with Rhes siRNA resulted in a 50% reduction of Rhes RNA compared with scrambled (control) siRNA. *B*, RT-PCR analysis of GAPDH RNA levels in PC12 cells using a validated GAPDH siRNA, illustrating a similar reduction compared with Rhes, demonstrating that our ability to reduce RNA expression is a function of PC12 transfection efficiency. *C*, Rhes depletion in PC12 cells results in decreased mTOR activity as well as decreased autophagic flux. Cells were treated with scrambled (control) siRNA or siRNA to deplete endogenous Rhes and placed in medium ± chloroquine (*CQ*) for 90 min prior to lysis. *D*, Rhes overexpression in PC12 cells results in mTOR activation and increased autophagic flux. Cells were transfected with Myc- or Myc-Rhes for 48 h and then placed in medium ± chloroquine for 90 min. Quantification is shown at right. mTOR activity is determined by phosphorylation of S6K at Thr-389. Autophagic flux is measured as the amount of LC3-II in the presence of chloroquine minus the amount of LC3-II without chloroquine. *, $p < 0.05$.

Autophagic Flux—Autophagic flux was calculated as the amount of LC3-II in the presence of chloroquine minus the amount of LC3-II in the absence of chloroquine. Quantification was performed using fluorescently labeled secondary antibodies and the LiCOR Odyssey Imaging system, which is fully quantitative, and allows analysis to be performed between samples after normalization to the amount of GAPDH loading control per sample. This eliminates potential error using semi-quantitative chemiluminescent techniques and the requirement to analyze LC3-II levels from the same blot. The value obtained for autophagic flux represents the amount of autophagic membrane, or autophagosomes, delivered to lysosomes during the treatment time with chloroquine (28).

GFP-LC3 Puncta Analysis—GFP-LC3 puncta analysis was performed using HeLa cells stably expressing GFP-LC3 and were

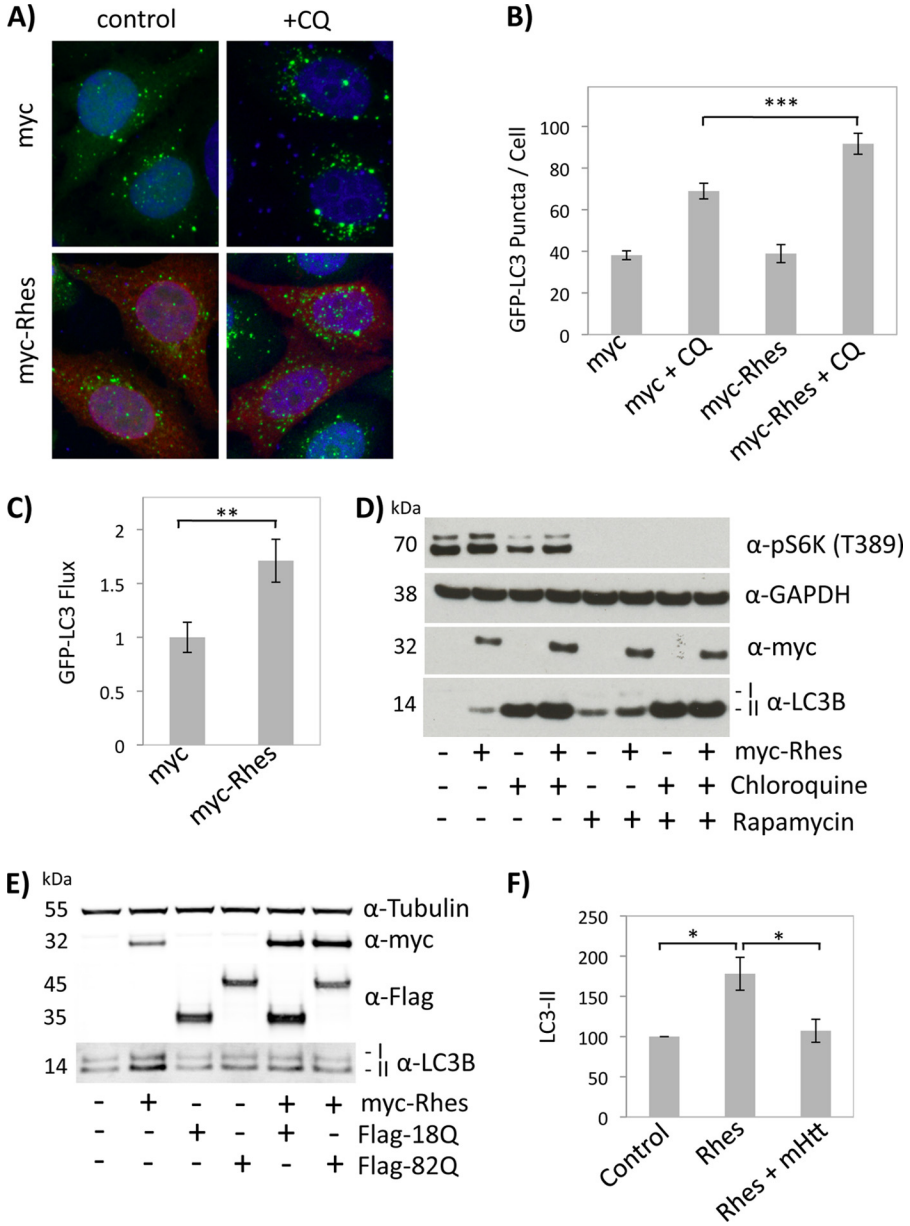

FIGURE 2. **Rhes activates autophagy in HeLa cells independent of mTOR.** *A*, HeLa cells stably expressing GFP-LC3 were transfected with Myc or Myc-Rhes for 24 h, transferred to low autophagy medium for 24 h, and then treated with or without chloroquine (*CQ*) for 2 h. Representative confocal images show GFP-LC3 (*green*), anti-Myc immunofluorescence (*red*), and Hoescht 33324 nuclear stain (*blue*). *B*, GPF-LC3 puncta analysis from HeLa cells in *A* demonstrates that Rhes increases the number of GFP-LC3 puncta following chloroquine treatment, consistent with increased autophagic flux. *C*, GFP-LC3 flux following 2 h of chloroquine treatment in cells transfected with Myc or Myc-Rhes, calculated as the number of GFP-LC3 puncta in the presence of CQ minus the number of GFP-LC3 puncta without CQ. *D*, Overexpression of Myc-Rhes in HEK293 cells increases LC3-II levels in the presence of rapamycin. Cells were transfected for 48 h and then placed in medium ± CQ and ± rapamycin for 90 min. *E*, huntingtin prevents Rhes-induced autophagy. HEK293 cells were transfected with control vectors or Myc-Rhes and wtHtt (FLAG-Htt-N171–18Q) or mHtt (FLAG-Htt-N171–82Q) for 24 h. *F*, quantification of LC3-II levels in HEK293 cells expressing control vector, Rhes alone, or both Rhes and mHtt from three separate experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

a gift from Dr. Beth Levine. Cells were transfected using polyfect with either Myc-Rhes or Myc-empty vector in normal DMEM +10% FBS. After 24 h, cells were transferred to low-autophagy medium (Opti-MEM + 5% FBS + 50 μg/ml G418). After an additional 24 h, cells were treated with or without 50 μM chloroquine for 2 h and then fixed with 4% paraformaldehyde, and immunofluorescence for Myc was performed using standard protocols. Cells were imaged as Z-section stacks with a 40× objective on a Leica SP8 MP confocal microscope, and the average number of GFP-LC3 puncta per cell were analyzed using the Puncta Analyzer plug-in for Image J2, written by Barry Wark. GFP-LC3 flux is the number of GFP-LC3 puncta after 2 h in the presence of chloroquine minus the number of GFP-LC3 puncta in the absence of chloroquine, normalized to control Myc-transfected cells (28).

Statistical Analysis—Statistical analysis was performed using Student's *t* test with results being considered significant if $p < 0.05$. Data are expressed as means ± S.E. Experiments were performed in triplicate and repeated a minimum of two times.

RESULTS

PC12 cells display multiple neuronal attributes and are one of the few cell lines that express endogenous Rhes (29). We

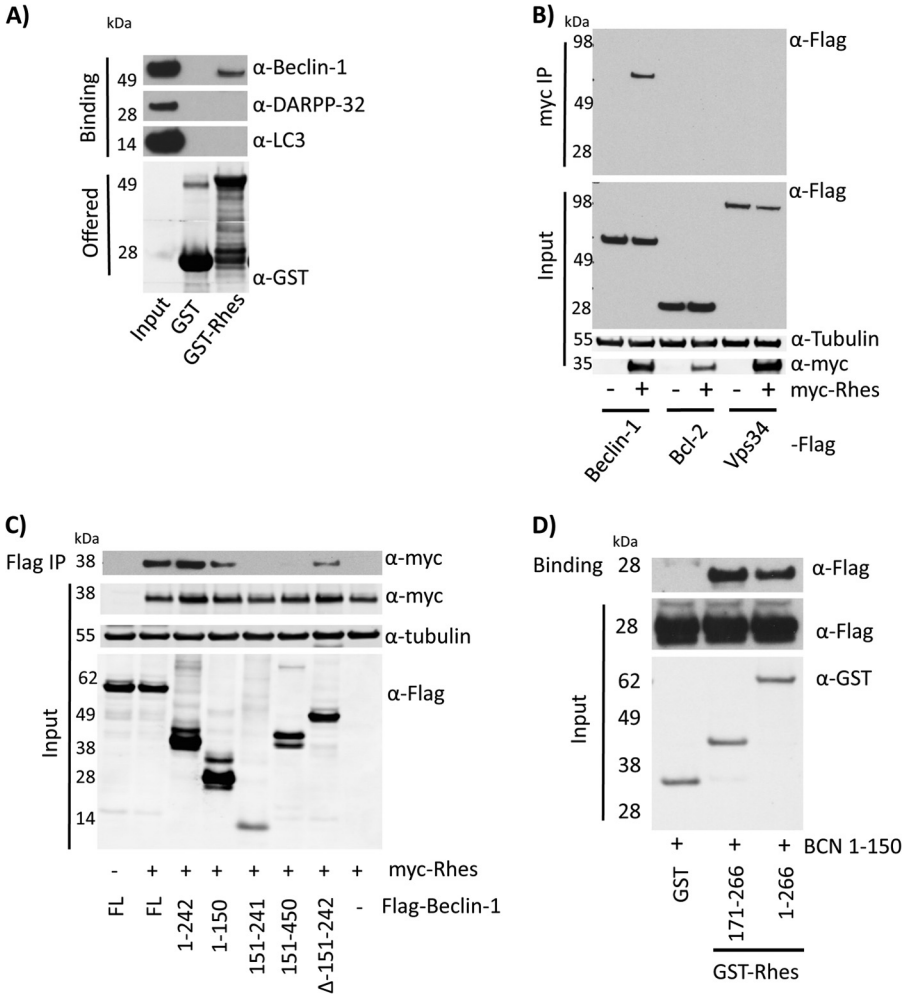

FIGURE 3. **Rhes binds the autophagy regulator Beclin-1.** *A*, recombinant GST-Rhes interacts with Beclin-1 from striatal lysates. Bacterially purified GST fusion protein was incubated with mouse striatal lysate, and bound proteins were precipitated with glutathione-Sepharose and eluted for Western analysis. *B*, Rhes binds Beclin-1 but not Bcl-2 or Vps34, known Beclin-1 interactors. cDNA for Myc-Rhes, FLAG-Beclin-1, FLAG-Bcl-2, and FLAG-Vps34 were transfected in HEK293 cells and precipitated with Myc affinity beads after 48 h. *C*, Rhes binds the N-terminal domain of Beclin-1 (amino acids 1–150), which contains the BH3 domain responsible for interactions with BH3 domain containing proteins such as Bcl-2. cDNA for Myc-Rhes and fragments of FLAG-Beclin-1 were transfected in HEK293 cells and precipitated with FLAG affinity beads. *D*, the C terminus of Rhes (amino acids 177–266) can bind the N terminus of Beclin-1 with comparable affinity as full-length Rhes. GST, GST-Rhes, and FLAG-Beclin-1 (amino acids 1–151) were expressed in HEK293 cells followed by glutathione-Sepharose precipitation.

employed siRNA to deplete Rhes in PC12 cells. Following optimization, this procedure reduces Rhes RNA levels by ~45% (Fig. 1*A*), comparable with a validated GAPDH siRNA (Fig. 1*B*), illustrating that our ability to reduce mRNA expression is limited by the transfection efficiency of PC12 cells, which is ~60% using GFP (data not shown). As Rhes siRNA-1 showed the greatest reduction in Rhes RNA, it was used in subsequent studies. Treatment with Rhes siRNA-1 is associated with a 40% decline of S6K phosphorylation, corroborating our earlier findings that Rhes is a major determinant of mTOR signaling (Fig. 1*C*). Activation of mTOR is known to inhibit autophagy (23). Autophagic flux is commonly measured by the conversion of LC3-I to LC3-II (distinguished by molecular weight on Western blot) in the presence of the lysosomal inhibitor chloroquine (28). Using a fluorescent secondary antibody to LC3-II and the fully quantitative LiCOR imaging system, we analyzed LC3-II levels normalized to total protein in control and Rhes-depleted cells. Surprisingly, Rhes depletion elicits a 40% decrease in autophagic flux following 90 min of chloroquine treatment, despite decreased mTOR signaling (Fig. 1*C*).

We next explored the influence of exogenous Rhes in PC12 cells (Fig. 1*D*). Rhes overexpression elicits a 50% increase in phospho-S6K as well as a 60% increase in autophagic flux, measured after 90 min of chloroquine treatment. This observation, coupled with the decline in autophagy following Rhes depletion, implies that Rhes stimulates both autophagy and mTOR signaling. As mTOR kinase activity normally inhibits autophagy, the regulation of autophagy by Rhes in these cells appears to be independent of mTOR.

To confirm that Rhes activates autophagic flux, HeLa cells that stably express GFP-LC3 were transfected with Rhes or control vector (Fig. 2*A*). The number of GFP-LC3 puncta generated during treatment with the lysosomal inhibitor chloroquine indicates the number of autophagosomes generated during that time, or the autophagic flux (28). Compared with controls, no difference was observed in the average number of

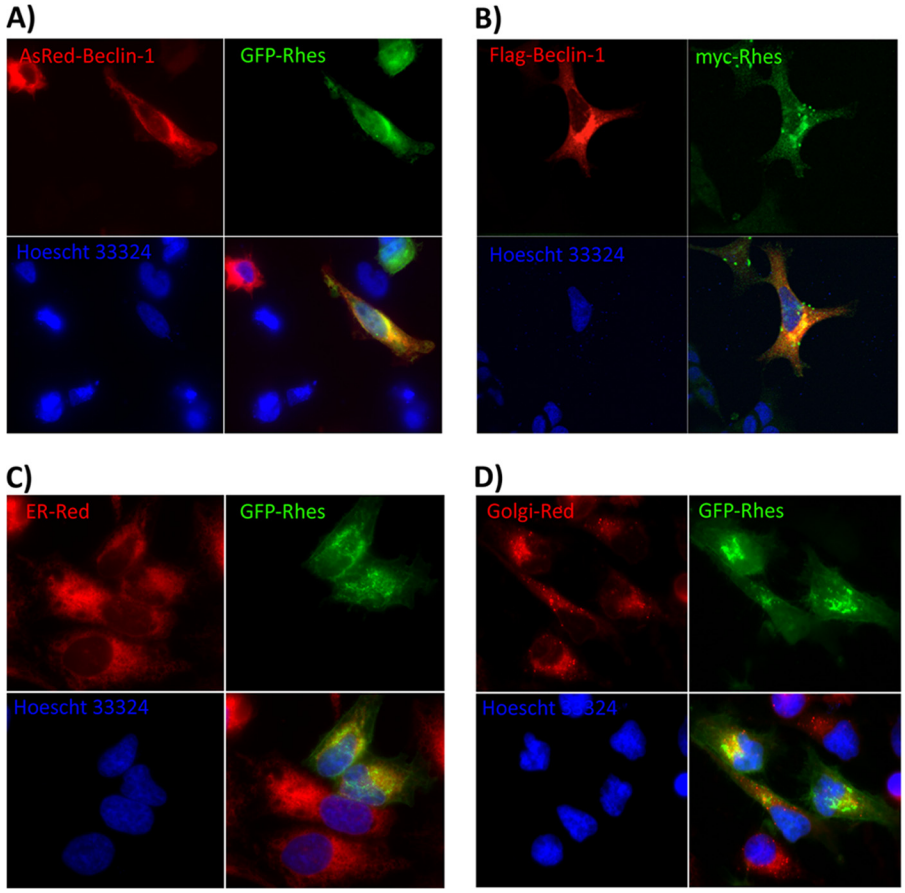

FIGURE 4. **Rhes co-localizes with Beclin-1.** *A*, cDNA for AsRed-Beclin-1 and GFP-Rhes were transfected into HEK293 cells and expressed for 24 h and then fixed with 4% paraformaldehyde and imaged using fluorescence microscopy. *B*, FLAG-Beclin-1 and Myc-Rhes were transfected into HEK293 cells and expressed for 24 h and then fixed with 4% paraformaldehyde and imaged following immunofluorescence labeling with primary antibodies for the indicated epitope tag and fluorescently labeled secondary antibodies. ER-Red (glibenclamide BODIPY-TR) (*C*) and Golgi-Red (BODIPY-TR ceramide) (*D*) were added to HeLa cells transfected with GFP-Rhes as recommended by Invitrogen, and live cells were imaged in PBS. Expression in the endoplasmic reticulum (ER) and trans-Golgi network (TGN) is consistent with the previously reported localization of Beclin-1. Nuclei are stained with Hoescht 33324.

GFP-LC3 puncta per cell under basal conditions in Rhes-transfected cells. However, after 2 h of treatment with chloroquine, cells expressing Rhes had an increase of 50 GFP-LC3 puncta per cell, significantly different from an increase of only 30 GFP-LC3 puncta in control cells ($p < 0.001$) (Fig. 2*B*). This represents an increase in GFP-LC3 autophagic flux by 170% in cells transfected with Rhes compared with controls ($p < 0.01$) (Fig. 2*C*).

Rapamycin is a potent inhibitor of mTOR and known inducer of autophagy. Overexpression of Rhes in HEK293 cells increases LC3-II levels in the presence and absence of chloroquine (comparing *lane 1 versus 2*, and *lane 3 versus 4*), again consistent with increased autophagic flux (Fig. 2*D*). The increase in LC3-II persists in the presence of rapamycin (*lane 5 versus 6*), supporting independence from mTOR signaling. There is no obvious difference in LC3-II levels in Rhes-expressing cells treated with both chloroquine and rapamycin (*lane 7 versus 8*), possibly because the cells have saturated their ability to generate LC3-II under such conditions.

We previously demonstrated that Rhes binds mHtt protein to enhance its cytotoxicity (4). To evaluate whether the interaction between Rhes and mHtt affects autophagy activation, we overexpressed Rhes in HEK293 cells in the presence or absence of huntingtin protein. Overexpressing mHtt, as well as wild-type Htt, abolishes the increase in LC3-II elicited by Rhes (Fig. 2, *E* and *F*). Expression of wtHtt alone or mHtt alone has no effect on the level of LC3-II.

We next sought to determine whether Rhes is capable of binding proteins other than mTOR that affect autophagy. In striatal lysates, bacterially purified GST-Rhes binds avidly to endogenous Beclin-1, a protein critical for the induction of autophagy (Fig. 3*A*). Rhes does not interact with the autophagic protein LC3 or DARPP-32, a striatal-enriched protein involved in dopamine signaling. When co-expressed in HEK293 cells, Rhes robustly binds Beclin-1 but fails to interact with Bcl-2 or Vps34, other proteins of the Beclin-1 signaling complex, demonstrating the specificity of the Rhes/Beclin-1 interaction (Fig. 3*B*).

A physiologic association of Rhes with Beclin-1 is supported by the co-localization of overexpressed Rhes and Beclin-1 in HeLa cells with both fluorescent protein tags (Fig. 4*A*) and small epitope tags (Fig. 4*B*). Using live-cell dyes specific for the endoplasmic reticulum (Fig. 4*C*) and trans-Golgi network (Fig. 3*D*), GFP-tagged Rhes colocalizes with these perinuclear structures, consistent with the known localization of Beclin-1 (30).

Specific domains of Beclin-1 mediate its interaction with various proteins in the coordination of autophagy (31). Activated Beclin-1 binds Vps34 through both its central coiled-coil domain and C-terminal evolutionarily conserved domain to

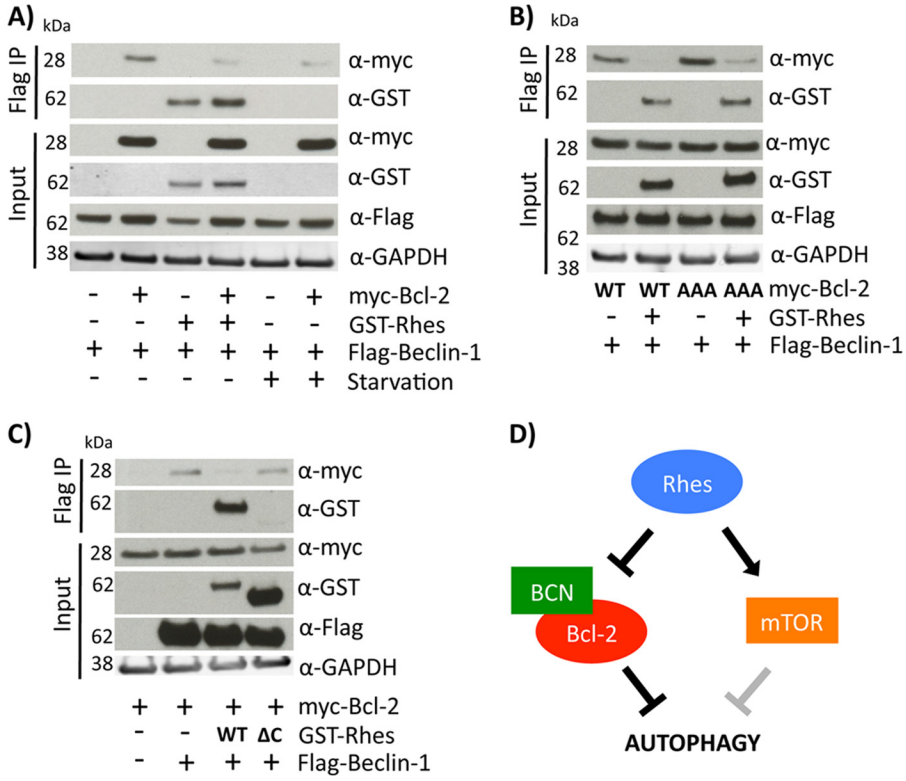

FIGURE 5. **Rhes decreases the inhibitory interaction between Beclin-1 and Bcl-2.** *A*, HeLa cells were transfected with the indicated combination of Rhes, Beclin-1, Bcl-2, or control empty vector for 24 h and then placed in either normal or starvation medium for 90 min where indicated. Binding of GST Rhes and Myc-Bcl-2 to FLAG-Beclin-1 was determined after FLAG immunoprecipitation. *B*, an equivalent reduction in Beclin-1/Bcl-2 binding by Rhes is seen with the phospho-null mutant of Bcl-2 (AAA), indicating that Rhes inhibits the interaction of Beclin-1/Bcl-2 independent of JNK-1 phosphorylation of Bcl-2. HeLa cells were transfected with indicated plasmids for 24 h prior to FLAG immunoprecipitation. *C*, Rhes 1–181 (ΔC), which lacks the C-terminal domain required to interact with Beclin-1, does not affect the interaction between Beclin-1 and Bcl-2. HeLa cells were transfected with indicated plamids for 24 h prior to FLAG immunoprecipitation. *D*, Rhes affects two independent pathways to regulate autophagy. Rhes can bind and activate mTOR, which normally inhibits autophagy. However, in cells with robust autophagy, Rhes activates autophagy independent of mTOR by displacing the inhibitory binding of Bcl-2 to Beclin-1 (BCN).

form a complex critical for autophagy. Binding of the apoptosis regulator Bcl-2 to the BH3 domain in the N terminus of Beclin-1 inhibits autophagy activation, whereas decreasing the interaction between Beclin-1/Bcl-2 activates autophagy (32). We mapped the interaction of Rhes to the N-terminal 150 amino acids of Beclin-1, as a fragment with only amino acids 1–150 binds Rhes, whereas a fragment lacking this region fails to bind (Fig. 4*C*). The unique C terminus of Rhes, not present in other Ras-like proteins except the closest relative of Rhes, DexRas1 (in which it is only 50% homologous), appears to mediate the binding with Beclin-1 (Fig. 4*D*). A fragment containing only the C-terminal 95 amino acids of Rhes binds as well as full-length Rhes to the N terminus of Beclin-1.

As both Rhes and Bcl-2 bind the N-terminal region of Beclin-1, we explored the influence of Rhes on the interaction between Beclin-1 and Bcl-2. Starvation stimulates autophagy by increasing JNK-1-mediated phosphorylation of Bcl-2, preventing the inhibitory binding of Bcl-2 to Beclin-1 (32, 33). Accordingly, when Beclin-1 is free from Bcl-2, it can stimulate autophagy. Overexpression of Rhes substantially decreases Beclin-1/Bcl-2 binding, comparable with the reduction of Beclin-1/Bcl-2 binding caused by starvation (Fig. 5*A*). To confirm that Rhes exerts its autophagy activating effects through binding of Beclin-1 and not through changes in JNK-1 mediated signaling, we expressed a mutant of Bcl-2 (AAA) that cannot be phosphorylated by JNK-1 (33). Thus, if Rhes simply affected upstream JNK-1 signaling it would have no effect on the interaction of Beclin-1 with the phospho-null mutant of Bcl-2 (AAA). Rhes decreases the interaction of Beclin-1 and Bcl-2 AAA to an equivalent extent as wild-type Bcl-2 (Fig. 5*B*). Furthermore, a Rhes mutant lacking the C-terminal region of Rhes (ΔC) that fails to bind Beclin-1 does not interrupt Beclin-1/Bcl-2 binding (Fig. 5*C*). However, residue Phe-123 in the BH3 domain of Beclin-1, which although required for Bcl-2 interaction, is not required for Rhes binding, suggesting that although Rhes and Bcl-2 bind the same general region of Beclin-1, they require different amino acids (data not shown). These findings suggest that Rhes competitively displaces Bcl-2 from Beclin-1 to activate autophagy independent of JNK-1 and mTOR signaling.

DISCUSSION

Although mHtt is present throughout the body, the expression pattern of Rhes mirrors the known pathology of HD. Previous work in our laboratory demonstrated that Rhes directly interacts with mHtt and enhances cytotoxicity through increased sumoylation of mHtt, providing a potential explanation for the striatal selectivity of HD (4). However, both Rhes and mHtt are expressed within the striatum for years, long before neuronal dysfunction and degeneration is evident. In the

present study, we demonstrate that Rhes activates autophagy, which may explain the delay of symptom onset in HD, which is linked to autophagy.

In cells with robust autophagy, overexpression of Rhes results in marked autophagic activation, whereas depletion of endogenous Rhes leads to diminished autophagic activation. The mechanism for autophagic enhancement reflects Rhes' sequestration of Beclin-1 from the inhibitory binding of Bcl-2. This effect seems to be prevented by the expression of huntingtin protein, possibly related to its own binding of Rhes. Competitive inhibition of Beclin-1/Bcl-2 binding occurs with many other important regulators of autophagy, including HMGB1, UVRAG, and Atg14L/Barkor (31). Rhes-induced autophagy is independent of mTOR, as the effect is not inhibited by rapamycin and occurs in the opposite direction as what would be predicted based our previous finding that Rhes activates mTOR (24, 34). Thus, Rhes can affect autophagy through two independent pathways (Fig. 5*D*). Other small G-proteins also impact autophagy with similar effects. For instance, RalB activates starvation-induced autophagy independent of mTOR but requires Beclin-1 (34). Ras induces autophagy and can do so despite activated mTOR (35, 36).

The relationship between mTOR and autophagy in the clearance of mHtt is complex. mTOR inhibition by drugs such as rapamycin, which induce autophagy, are clearly beneficial in HD models (17). However, the clearance of mHtt by autophagy can occur in the presence of elevated mTOR but depends on Beclin-1 (37), and separate studies demonstrate that mHtt accumulation is regulated by Beclin-1 (38). Furthermore, mHtt aggregates sequester mTOR, leading to decreased kinase activity and autophagy activation, presumably as a compensatory mechanism to cope with mHtt toxicity (26). A compensatory mechanism has been proposed to exist for Rhes; a meta-analysis of striatal gene expression found that Rhes is consistently down-regulated in HD and replacing Rhes expression augments toxicity (8). Rhes has also been implicated in other signaling pathways that may impact autophagy aside from mTOR. For example, recent studies have shown that Rhes can affect AKT signaling (39, 40). Rhes can also interact with PAP7/ACBD3 to regulate iron homeostasis as well as enhance mHtt toxicity (41, 42).

The gradual accumulation of mHtt insults and changes in the cellular processes that normally deal with such burdens are likely responsible for the delayed onset of HD. Early in life when autophagy is not required, proteosomal degradation is robust and capable of preventing cytotoxicity. With advancing age, proteasomal function becomes compromised, forcing cells to rely more heavily on the autophagic pathway (43). Autophagic capacity eventually decreases, which potentially leads to both normal aging and the pathologic aging seen in neurodegeneration (14). Accordingly, in most tissues, the cytotoxic influences of mHtt are initially minimized by the proteasome before autophagy is required. By contrast, in the striatum, Rhes/mHtt interactions may augment cytotoxicity as well as diminish the autophagic capacity of the neuron, which eventually accounts for the striatal selectivity and delayed onset of HD, respectively. Although we find that wtHtt appears to decrease the increase in LC3-II elicited by Rhes comparable with mHtt, this is likely of little consequence; we have previously shown that Rhes does not enhance cytotoxicity or sumoylation in the presence of wtHtt despite being capable of interacting, albeit with lower affinity, when compared with mHtt (4). We suspect that huntingtin inhibits the Rhes-induced increase in LC3-II by preventing the interaction of Rhes with Beclin-1.

In summary, Rhes displays multiple physiologic and pathophysiologic activities in the striatum. Rhes activates autophagy by competitively displacing the inhibitory binding of Bcl-2 to Beclin-1. Rhes enhances cross-sumoylation and regulates sumoylation in the striatum (7). Mice lacking Rhes have decreased L-DOPA-induced dyskinesia, demonstrating that Rhes physiologically activates mTOR in the striatum (24). We hypothesize that striatal shrinkage in HD stems from the sequestration of Rhes by mHtt, such that less is available to maintain trophic support through mTOR in the striatum (44). Rhes binds mHtt and enhances cytotoxicity, suggesting that deletion of Rhes should diminish HD pathophysiology (4). This notion is supported by our recent findings that the selective striatal damage and motor dysfunction elicited by 3-nitropropionic acid, a well established and striatal-specific model of HD neurotoxicity, is profoundly reduced in Rhes-deleted mice (10). Rhes-deleted mice also have decreased motor dysfunction in the R6/1 genetic model of HD, supporting our hypothesis that Rhes is responsible for the striatal selectivity of HD (11). Finally, the regulation of autophagy by Rhes may account for the delayed onset of HD neuropathology.

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