Identification of a post-translationally myristoylated autophagy-inducing domain released by caspase cleavage of Huntingtin

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Huntington disease (HD) is a debilitating neurodegenerative disease characterized by the loss of motor control and cognitive ability that ultimately leads to death. It is caused by the expansion of a polyglutamine tract in the huntingtin (HTT) protein, which leads to aggregation of the protein and eventually cellular death. Both the wild-type and mutant form of the protein are highly regulated by post-translational modifications including proteolysis, palmitoylation and phosphorylation. We now demonstrate the existence of a new post-translational modification of HTT: the addition of the 14 carbon fatty acid myristate to a glycine residue exposed on a caspase-3-cleaved fragment (post-translational myristoylation) and that myristoylation of this fragment is altered in a physiologically relevant model of mutant HTT. Myristoylated HTT553–585–EGFP, but not its non-myristoylated variant, initially localized to the ER, induced the formation of autophagosomes and accumulated in abnormally large autophagolysosomal/lysosomal structures in a variety of cell types, including neuronal cell lines under nutrient-rich conditions. Our results suggest that accumulation of myristoylated HTT553–586 in cells may alter the rate of production of autophagosomes and/or their clearance through the heterotypic autophagosomal/lysosomal fusion process. Overall, our novel observations establish a role for the post-translational myristoylation of a caspase-3-cleaved fragment of HTT, highly similar to the Barkor/ATG14L autophagosome-targeting sequence domain thought to sense, maintain and/or promote membrane curvature in the regulation of autophagy. Abnormal processing or production of this myristoylated HTT fragment might be involved in the pathophysiology of HD.

INTRODUCTION

Huntington disease (HD) is a progressive disease that leads to the dysfunction and death of neuronal cells. Cell death is most pronounced in the striatum of the brain, which plays a key role in initiating and controlling movements of the body, limbs and eyes (1–3). Consequently, the disease is characterized by a loss of cognitive ability and motor skills, eventually leading to dementia and changes in personality (1). HD is caused by a highly polymorphic CAG trinucleotide repeat expansion in the gene that encodes for huntingtin (HTT) (4). This leads to an extended stretch of glutamines (Q) in the N-terminal region of the protein that promotes aggregation of huntingtin (HTT) in cells and ultimately leads to cell death. The onset of HD is typically from 35 to 40 years, and the disease leads to death within 10–20 years after the appearance of the first diagnosable symptoms (2,3,5).

Both normal and mutant HTT (mHTT) undergo a myriad of different post-translational modifications including phosphorylation, SUMOylation, ubiquitination, acetylation, proteolytic cleavage and palmitoylation (5–9). In the presence of the HD mutation, some post-translational modifications are significantly altered and can result in changes in the disease phenotype in mouse models (5,10,11). In particular, palmitoylation involves the reversible attachment of the fatty acid palmitate to cysteines

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and is involved in trafficking and functional modulation of different membrane proteins including HTT (12). Previously, it has been demonstrated that mHTT is less palmitoylated owing to its reduced interaction with the palmitoyltransferase, huntingtin-interacting protein 14 (HIP14), leading to increased formation of inclusions and neuronal toxicity (13). These findings directly link defects in fatty acylation with neuronal dysfunction and neurodegeneration in HD.

HTT undergoes several proteolytic cleavages by caspases under basal and apoptotic conditions (14–16). There are two primary sites of caspase cleavage in HTT that map to locations associated with cellular toxicity: Asp552 and Asp586. Position 552 is cleaved by both caspase-2 and caspase-3 (referred to as the caspase-3 site herein) whereas position 586 is thought to be predominantly cleaved by caspase-6 (6,14). However, new studies have suggested that other proteases or caspases may also cleave HTT in the absence of caspase-6 (17,18). Regardless, inhibition of cleavage of mHTT at Asp586 in mice completely ameliorates toxicity of mHTT, demonstrating that cleavage of mHTT at Asp586 is critical for its cytotoxicity (16). Caspase cleavage at Asp552 and Asp586 results in the release of a 34-amino acid fragment of HTT (HTT553–586). Blocking the HD cleavage site at Asp552 did not appear to improve the HD phenotype in mice using a transgenic model (16). However, this proteolytic event can be detected in vivo and may play an important role following the cleavage at Asp586 (14,16,19).

Using a caspase-cleavable tandem fluorescent protein reporter assay, we reported that the glycine residue following the HTT caspase-3 cleavage site is post-translationally myristoylated (20). Myristoylation is another type of protein fatty acylation in which the 14 carbon fatty acid myristate is irreversibly added to an amino-terminal glycine either co-translationally following the removal of the initiator methionine or post-translationally following apoptosis-induced caspase cleavage (21–23). It is mediated by one of two N-myristoyltransferases (NMTs): NMT-1 and NMT-2 (22,24,25).

mHTT is known to be associated with defects in autophagy. HTT-labeled vacuoles display the same ultrastructural features of autophagosomes, but are larger and more abundant in cells expressing mHTT (26–28). Furthermore, in one instance, the treatment of HD lymphoblastic cells with staurosporine increased autophagic vacuole formation (28). This may be due, in part, to a decrease in autophagosome and lysosome fusion in the presence of the polyQ expansion, leading to an increase in the levels of autophagosomes and less clearance of autophagic markers, such as LC3-II (29). This can lead to the appearance of increased autophagy, when, in fact, it is due to less clearance. The presence of an increased number of autophagic vesicles has been demonstrated in several cell types expressing mHTT, and a link between mHTT and deficiency in cargo recognition in HD has recently been made (29,30). This deficiency in cargo recognition leads to inefficient engulfment of cytosolic components by autophagosomes and slower turnover, functional decay and accumulation of autophagosomes, thereby contributing to toxicity in HD (30).

Herein, we characterize the role of a post-translationally myristoylated 34-amino acid HTT fragment (myr-HTT553–586) released by caspase cleavage and demonstrate its involvement in the formation of autophagic vesicles, which provides another link between HTT and altered autophagy in HD. Because impaired autophagy results in the inefficient removal of damaged cytosolic proteins and organelles, myr-HTT553–586 could be, in part, responsible for some of the cellular dysfunctions in autophagy associated with HD.

RESULTS
Post-translational myristoylation of Gly553 is altered in the presence of the polyQ mutation

HTT is proteolytically processed by caspases at Asp552 and Asp586, respectively (Fig. 1A) (5,6). A screen looking for new post-translationally myristoylated proteins previously revealed that the first 10 amino acids downstream of the caspase-3 cleavage site of HTT (HTT553–562) were sufficient to confer post-translational myristoylation (20). Consequently, we sought to investigate whether the fragment released by caspase cleavage at Asp552 and Asp586 could be post-translationally myristoylated in cells. Post-translational myristoylation of HTT was confirmed using HTT1–588–YFP, a physiologically relevant longer construct of HTT that recapitulates disease phenotypes when overexpressed in mouse striatum (31,32). The HTT1–588–YFP construct contains both Asp552 and Asp586 caspase-cleavage sites (Fig. 1B). Cells transiently expressing HTT1–588–YFP were induced to undergo apoptosis with TNFα and cycloheximide in the presence of the myristate analog, alkyne-myristate (Alk-Myr). Caspase-cleaved YFP fragments were immunoprecipitated using anti-GFP antibodies and examined for levels of incorporation of the myristate analog, which results in biotinylated-myristoylated proteins detectable by NeutrAvidin linked to horse radish peroxidase (NA–HRP) and enhanced chemiluminescence (33). Alk-Myr was incorporated into the ~30-kDa caspase-3-cleaved HTT553–588–YFP fragment in untreated cells, and the amount of incorporation was increased upon TNFα treatment. The production of the caspase-3-cleaved HTT553–588–YFP fragment and the incorporation of Alk-Myr were both decreased in the presence of a general caspase inhibitor (z-VAD-fmk), suggesting the involvement of caspases in the process. Alk-Myr incorporation was completely abrogated in the presence of the NMT inhibitor 2-hydroxymyristic acid (HMA) (Fig. 1C) (34).

The fact that incorporation of Alk-Myr into Gly553 of HTT553–588–YFP fragment also occurred in the absence of apoptotic induction, albeit at a lower level, might reflect the natural processing of endogenous HTT seen in neuronal and HEK293 cells previously (19,35) or to the fact that 10–20% of transfected HeLa cells typically undergo cell death in culture at any given time (36).

The treatment of transiently transfected cells with TNFα appeared to result in a slight, reproducible increase in the production of HTT553–588–YFP in the mHTT (138Q) construct. However, the incorporation of Alk-Myr into HTT553–588–YFP decreased significantly, by nearly 70% (n = 3, P = 0.024), in the presence of the polyQ mutation (Fig. 1D). This suggests that the presence of the polyQ mutation also leads to a reduction post-translational modification of HTT by myristate at G553. This result parallels the reduction in HTT palmitoylation seen in mHTT (7).
Evaluation of post-translational myristoylation of caspase-truncated proteins with various reporter constructs is a common practice (37,38). As such, we generated a reporter protein bearing HTT553–585 appended to EGFP, including a hydrophilic linker and an initiator methionine to confirm myristoylation (Fig. 1E) and to study the localization of myristoylated HTT553–585 in cells. Asp586 was omitted from this construct to prevent further processing by caspases. When using HTT553–585–EGFP, incorporation of Alk-Myr occurred cotranslationally and was inhibited in the presence of the NMT inhibitor HMA (Fig. 1F, left) and completely abrogated when the essential Gly was mutated to Ala (G553A-HTT553–585–EGFP) (Fig. 1F, right). This confirms that caspase-cleaved HTT is myristoylated at G553.

**Myr-HTT553–585–EGFP induces the formation of vesicular structures reminiscent of autophagosomes**

HeLa cells transiently expressing HTT553–585–EGFP were visualized by live-cell fluorescence microscopy to investigate the localization of HTT553–585 and its impact on cells. Myr-HTT553–585–EGFP was localized to the endomembrane system in various cell lines and typically appeared first in a reticulated pattern reminiscent of the endoplasmic reticulum (Fig. 2A) (Supplementary Material, Video S1). We observed that the ectopically expressed myr-HTT553–585–EGFP not only localized to the endomembrane system (Fig. 2A) but also appeared to be localized to and to promote the time-dependent formation of vesicular/tubular structures observed using time-lapse video microscopy (Supplementary Material, Videos S1–S3) and shown in Figures 2A–C and 4B and Supplementary Material, Figure S4. Of note, the large vesicular structures containing myr-HTT553–585–EGFP were also found in cells with the lowest detectable levels of EGFP fluorescence, suggesting this phenomenon is not necessarily an artifact of overexpression (data not shown). Further analysis of cells expressing myr-HTT553–585–EGFP using time-lapse imaging allowed us to visualize the detailed formation of the vesicular structures induced by myr-HTT553–585–EGFP expression (Fig. 2B and C). In particular, the inset in Figure 2C (Supplementary Material, Video S2) shows the nucleation, elongation and recircularization...
Figure 2. Expression of myr-HTT553–585–EGFP in HeLa cells promotes the formation of vesicular structures reminiscent of autophagosomes. (A) HeLa cells were transiently transfected for 22 h and imaged using live-cell confocal spinning-disk microscopy. In A, myr-HTT553–585–EGFP localized to the endomembrane system in a reticulated pattern. Over time, the formation of small vesicles was observed (Supplementary Material, Video S1). (B) The formation of vesicular structures (0.5–2 μm) as observed over time and (C) the formation of one vesicle from the boxed region in B (Supplementary Material, Video S2). In D, cells were transfected for 18 h and treated with either 1 mM sodium myristic acid (NaMA) (left) or 1 mM HMA (right) for an additional day. Images were acquired by wide-field microscopy and deconvolved using Huygens SV1. Scale bar represents 20 μm (A, C and D) or 2.5 μm (B).
of the elongated tubule leading to the formation of one of the ves-
cular/tubular structures seen in cells expressing myr-HTT553–
585–EGFP. This sequence of events is reminiscent of the forma-
tion of an autophagosome from an omegasome (39).

Interestingly, the localization of HTT553–585–EGFP to a
select set of endomembranes and its ability to promote the forma-
tion of vesicular structures was completely abrogated in the pres-
ence of the NMT inhibitor HMA (Fig.2D, right panel) and in the
G553A-HTT553–585–EGFP mutant (Figs 3–7, Supplementary
Material, Figs S1–S3 and S5). This is consistent with myristoy-
ation as a requirement for membrane localization and function
of HTT553–585–EGFP.

Myr-HTT553–585–EGFP promotes the formation
of autophagic vesicles

Because the formation of large vesicular structures containing
myr-HTT553–585–EGFP was reminiscent of the formation of
autophagosomes (Fig. 2C), we further investigated the
possibility that myr-HTT553–585–EGFP was localized to and
promoted the formation of autophagic vesicles including autop-
hagosomes and autophagolysosomes using immunogold electron
microscopy and fluorescence microscopy. As shown in
Figure 3A, myr-HTT553–585–EGFP was localized to a tubular
network reminiscent of the ER and to vesicular structures that
appeared to have double membranes and contained electron
dense material, two distinctive morphological features of autop-
hagosomes. Live-cell fluorescence microscopy was used to iden-
tify some of the content of the myr-HTT553–585–
EGFP-containing vesicles. In cells expressing myr-HTT553–
585–EGFP and the mitochondrial marker protein pyruvate de-
hydrogenase (PDH)-mCherry, we demonstrate the presence of
mitochondria engulfed in the large vesicles containing
myr-HTT553–585–EGFP in HeLa cells (Fig.3B and C). This ob-
servation was corroborated in HeLa cells and primordial striatal
mouse cells expressing myr-HTT553–585–EGFP stained with
Mitotracker® (Supplementary Material, Fig. S1), but not in
cells expressing non-myristoylatable G553A-HTT553–585–EGFP.

Figure 3. Expression of myr-HTT553–585–EGFP induces the formation of autophagocytic vesicles. HeLa cells transiently expressing HTT553–585–EGFP for 24 h were fixed and prepared for immunogold EM (A) using rabbit anti-GFP antibodies and donkey anti-rabbit conjugated to 18nm colloidal gold particles. The ‘inset’ illustrates an expanded view of the boxed area in myr-HTT553–585–EGFP panel showing a vesicle originating from the ER. Arrowheads indicate gold beads and ‘N’ marks nucleus. (B) HeLa cells co-expressing PDH-mCherry and myr-HTT553–585–EGFP. Images were acquired by wide-field live-cell microscopy and decon-
volved using SVI Huygens deconvolution software. Images are presented as 3D z-stacks. Scale bars are representative of 10 μm. (C) A 3D reconstruction of the boxed region of inset from B. Arrowheads indicate the presence of mitochondria within the myr-HTT553–585–EGFP-containing large vesicular structures.
Interestingly, mitochondria were often found juxtaposed to large autophagocytic structures containing myr-HTT 553–585–EGFP (e.g. Fig. 3C and Supplementary Material, Fig. S1B and D). Altogether, these results suggest that myr-HTT 553–585–EGFP localizes to the ER where it induces the formation of vesicles as part of the autophagy pathway.

Like autophagosomes, the autophagic vesicles induced by the expression of myr-HTT 553–585–EGFP arise from the ER

To corroborate the immunogold electron microscopy findings showing gold particles marking myr-HTT 553–585–EGFP and the ER marker dsRed-ER had highly similar localization patterns in cells prior to the formation of vesicles (Fig. 4A, top row) and that the fluorescent signals originating from both proteins nearly completely overlapped after vesicle formation (Fig. 4A, second row). In addition, we further demonstrate that the fluorescent signals originating from myr-HTT 553–585–EGFP extensively overlapped with those from two other ER markers, ER-tracker™ and concanavalin A (Supplementary Material, Fig. S2A and B, respectively). Altogether, our results denote an extensive localization of myr-HTT 553–585–EGFP at the ER and that the localization of HTT 553–585–EGFP to the ER required its myristoylation because the non-myristoylable G553A-HTT 553–585–EGFP was found to be diffused throughout the cytoplasm like EGFP itself (Fig. 4A, bottom two rows). It is also noteworthy to mention that the vesicles containing myr-HTT 553–585–EGFP did not appear to colocalize with endosomes, clathrin or Golgi apparatus (Supplementary Material, Fig. S3).

Myr-HTT 553–585–mApple and GFP-Rab32 localize to the same vesicles

The overexpression of myr-HTT 553–585–EGFP appears to induce the formation of autophagic vesicles in the absence of starvation or other common inducers of autophagy. This is reminiscent to what is observed when other proteins involved in the initiation and formation of autophagosomes such as DFCP1 (39), ATG14L (40) and Rab32 (41) are overexpressed. To test whether myr-HTT 553–585 induces the formation of autophagosomes similar to those produced when Rab32 is overexpressed, we co-transfected HeLa cells with myr-HTT 553–585–mApple and GFP-Rab32 (Fig. 5). Myr-HTT 553–585–mApple and GFP-Rab32 appeared to be distributed to the same vesicular structures and showed a high degree of overlap (Fig. 5, inset). These data suggest that myr-HTT 553–585–EGFP/mApple induce the formation of autophagic vesicles in a manner that may be mechanistically related to those induced by the...
expression of a protein like Rab32, which is also responsible for the initiation of autophagy.

**Myr-HTT\textsubscript{553 – 585}–EGFP accumulates in autophagolysosomes and lysosomes in HeLa cells**

LC3 is a critical component of autophagy and is often used as a marker to track the induction of autophagy (42,43). LC3 is important for the generation of autophagosomes and remains with the autophagosomes until they fuse with lysosomes where it is degraded or recycled and re-used on nascent autophagosomes (42). To further confirm that myr-HTT\textsubscript{553 – 585}–EGFP associates within vesicles that are a part of the autophagocytic pathway under steady state conditions, we assessed the extent of co-distribution of RFP-LC3 and myr-HTT\textsubscript{553 – 585}–EGFP in cells treated or not with bafilomycin A1, a specific inhibitor for vacuolar-type proton (V-H\textsuperscript{+}) pump known to reduce the acidification of lysosomes (44). As a result, bafilomycin A1 inhibits the fusion between the autophagosomes and lysosomes (44). In untreated cells, only a small portion of the RFP-LC3 signal was found to be co-localized with the myr-HTT\textsubscript{553 – 585}–EGFP-associated signal (Fig. 6A and Supplementary Material, Fig. S4). As seen previously with the mitochondrial markers, we also occasionally detected RFP-LC3 inside some myr-HTT\textsubscript{553 – 585}–EGFP vesicles (Supplementary Material, Fig. S4), further suggesting that these vesicles are part of the autophagocytic pathway. In contrast, cells treated with bafilomycin A1 showed a marked increase in the overlap of the signals originating from myr-HTT\textsubscript{553 – 585}–EGFP-containing vesicles and RFP-LC3-containing autophagosomes (Fig. 6A inset). Because bafilomycin

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**Figure 5.** Myr-HTT\textsubscript{553 – 585}–mApple and EGFP-Rab32 localize to similar autophagic vesicles. (A) Myr-HTT\textsubscript{553 – 585} and G553A-HTT\textsubscript{553 – 585} were appended to the monomeric fluorescent protein mApple and co-transiently transfected with EGFP-Rab32 in HeLa cells for 24 h. Images were acquired by wide-field live-cell microscopy and deconvolved using SVI Huygens Deconvolution software. Images are presented as z-stacks. Scale bar is representative of 10 μm.
A1 blocks the fusion of autophagosomes with lysosomes, this suggests that myr-HTT \textsuperscript{553–585}–EGFP-induced vesicles are autophagosomes that ultimately fuse with lysosomes.

Interestingly, the formation of abnormally large lysosomal structures containing myr-HTT \textsuperscript{553–585}–EGFP and Cathepsin B-BFP (Fig. 6A) detected in untreated cells was abrogated by the bafilomycin A1 treatment. These results suggest, once again, that the expression of myr-HTT \textsuperscript{553–585}–EGFP induces the formation of autophagic vesicles and that these would eventually fuse with lysosomes leading to the formation or expansion of large lysosomes or autophagolysosomes. Because bafilomycin A1 prevented the formation of the abnormally large lysosomal structures and prevents the fusion between autophagosomes and lysosomes, we can conclude that under steady-state conditions, the expression of myr-HTT \textsuperscript{553–585}–EGFP somehow leads to the expansion of the lysosomal compartment after the fusion step between autophagosomes and lysosomes.

For its proper activity and localization, native LC3 (LC3-I) is post-translationally modified by the addition of phosphatidylethanolamine on its C-terminus resulting in the ‘active’ LC3-II form in autophagy (45). Blocking the fusion between the autophagosomes and lysosomes with bafilomycin A1 prevents the degradation of LC3-II, which accumulates and therefore can act as an indicator of autophagy (44). To confirm the activation of autophagy, we compared the levels of LC3-II in cells expressing HTT \textsuperscript{553–585}–EGFP or EGFP in the presence or absence of bafilomycin A1. In untreated cells, LC3-II levels were highest in cells expressing myr-HTT \textsuperscript{553–585}–EGFP.
The addition of bafilomycin A1 also significantly increased the levels of LC3-II. The effect of the treatment with bafilomycin A1 on LC3-II levels assessed by two-way ANOVA was highly significant ($P = 0.0041$), demonstrating the likelihood of the involvement of an autophagic event (Fig. 6B and C). Of note, the levels of LC3-II in the presence of non-myristoylated HTT553-585–EGFP consistently appeared to be lower than those found in cells expressing myr-HTT553-585–EGFP, but higher than EGFP or mock-transfected cells, suggesting that the amino acids between 553 and 585 may provide a secondary autophagy-inducing signal that is potentiated by myristoylation. Altogether, our data strongly suggest that myr-HTT553-585–EGFP promotes the formation of autophagosomes that accumulate in abnormally large autophagolysosomal/lysosomal structures.

DISCUSSION

Using a tandem fluorescent protein reporter assay, we previously identified the N-terminal glycine residue that is exposed following caspase-3 cleavage of HTT as a substrate for post-translational
myristoylation (20). Herein, we confirmed that Gly553 of HTT is post-translationally myristoylated following caspase cleavage (Fig. 1C). This was dependent on both caspase cleavage and active NMT as shown by the loss of myristoylation of HTT in cells treated with either a general caspase inhibitor or the myristoylation inhibitor HMA, respectively. Post-translational myristoylation of HTT553–586 assessed using a physiologically relevant construct of HTT that recapitulates disease phenotypes when overexpressed in mouse striatum (31,32), HTT1–588-YFP, was significantly reduced in the mutant construct containing the polyQ expansion in transiently transfected HeLa cells (Fig. 1D and E). This result is similar to what was observed with palmitoylation of HTT, which is decreased in mHTT (7). Whether the reduction in myristoylation occurs in HD brains remains to be established. It is also not known whether the reduction in myristoylation is specific to mHTT in general or owing to the inhibition of NMTs by mHTT or one of its fragments. Interestingly, a reduction in the myristoylation efficiency of not only HTT553–586 but also of numerous other proteins could potentially have detrimental effects for the cell and would likely have an impact on the ontology of HD. The expression of myristoylated HTT553–585–EGFP, but not the G553A myristoylation-deficient mHTT553–585–EGFP, in a variety of cell lines, induced the formation of autophagosomal vesicles originating from the ER. Although there is evidence to support that autophagosomes can originate from mitochondria (47) or the plasma membrane (48), our results support that the ER and possibly ER–mitochondria contact points (39,49) are the main membrane donors for the production of myr-HTT553–585–EGFP-containing autophagic vesicles. The expression of myr-HTT553–585–EGFP resulted in the accumulation of abnormally large (>3 μm) autophagolysosomal/lysosomal structures that were observed by electron microscopy (Fig. 3) and fluorescence microscopy (Figs 2–7 and Supplementary Material, Figs S1–S5). This accumulation might be due to an over-production of autophagosomes in cells expressing myr-HTT553–585–EGFP that could overwhelm the lysosomal degradation system and impedes the proper clearance of the autophagosomes containing myr-HTT553–585–EGFP. In addition, myr-HTT553–585–EGFP might also have fusogenic properties that may promote the rate of heterotypic fusion between autophagosomes and lysosomes resulting in enlarged autophagolysosomes under steady-state conditions. Alternatively, because bafilomycin A1 prevented the formation of the large lysosomal structures containing myr-HTT553–585–EGFP and promoted their accumulation in the autophagosome compartment (as indicated by the increased extent of overlap with the RFP-LC3 fluorescent signals), the expression of myr-HTT553–585–EGFP could actually lead to the expansion of the autophagolysosomal/lysosomal compartment after the fusion step between the autophagosomes and lysosomes by altering some key properties of the autophagolysosomal/lysosomal compartment, perhaps including acidification. This latter possibility is supported by the fact that the large autophagolysosomal or lysosomal vesicular structures containing myr-HTT553–585–EGFP are only weakly stained by the acidotropic dye Lyso-Tracker® (Fig. 7) and are thus more alkaline. Of note, because the levels of myr-HTT553–585–EGFP do not appear to be altered in the presence of bafilomycin, unlike LC3-II, this suggests that it is not degraded by the lysosome. Blocking myristoylation of HTT553–585–EGFP completely abrogated the formation of autophagic vesicles as seen using fluorescence and electron microscopy. It is important to note that appending reporter proteins such as EGFP to the C-terminus of myristoylated and/or palmitoylated peptides (i.e. Fyn, Lck and Src) is common practice and does not lead to the formation of vesicular structures like those seen in cells expressing myr-HTT553–585 (38,50,51). This suggests that there are elements within the amino acid sequence of HTT553–586 that are required for the formation of these autophagic vesicular structures. Interestingly, there is a short stretch of amino acids in the caspase-cleaved HTT553–586 that aligns with the Barkor/ATG14L autophagosome-targeting sequence (BATS) domain of ATG14L. The BATS domain of ATG14L is thought to sense and maintain membrane curvature while recruiting ATG proteins to the isolation membrane (52). It is postulated to also promote membrane curvature (52). ATG14L is required for the membrane localization of the PI3-kinase complex consisting of Beclin-1 and Vps34, which is essential for initiating the formation of autophagosomes (40,53). The fact that myr-HTT553–585 shares sequence homology with a common domain, localization and perhaps function with ATG14L is an exciting observation that could explain how myr-HTT promotes the formation of autophagic vesicles even under nutrient-rich conditions. In fact, cells expressing myr-HTT553–585–EGFP look almost identical to cells over expressing gEGFP-BATS [compare Supplementary Material, Video S3 with Fig. S3C–E and Movie S2 of Fan et al. (52)]. Moreover and interestingly, it has been suggested that HTT may have evolved from the autophagy pathway in yeast and, consequently, HTT may have a regulatory role in autophagy and protein clearance (54). Our data suggest that we have identified a cryptic autophagy-inducing domain within HTT that is released by caspase cleavage. Typically, membrane curvature sensing proteins contain amphipathic α-helices (AH) that insert themselves into the membrane (55). Those that are implicated in inducing membrane curvature generally require electrostatic interactions between the polar face of the helix with phospholipid head groups. However, there are examples wherein the AH is enriched in Ser/Thr as in the BATS domain and possibly myr-HTT553–586 (52,55). It has been demonstrated that alkyl chains of fatty acids such as those found in myristate and palmitate have similar membrane curvature ‘sensing’ selectivity (56). Therefore, we postulate that the myristate moiety of HTT553–586 may act as a membrane curvature sensor or promoter, whereas the amino acid sequence may act to stabilize the increased membrane curvature and/or recruit other autophagy factors to promote membrane curvature and induce the formation of primordial autophagosomes. The fact that non-myristoylated G553A-HTT553–585–EGFP increased LC3-II suggests that there may be an autophagy domain within this sequence that is augmented by myristoylation. The sequence of amino acids between, and including, the caspase-cleavage sites at Asp552 and Asp586 (HTT549–586) shares nearly 100% sequence identity among mammalian species including mouse, cow and monkey (Supplementary Material, Table S1). This suggests that both the caspase-3 cleavage and post-translational myristoylation of HTT at the newly exposed glycine residue are well conserved among mammals and may be suggestive of a critical cellular function. In addition,
over a third of the 34-amino acid sequence is comprised of either Ser or Thr residues, thereby serving as ideal possible candidates for phosphorylation. In fact, several of these Ser and Thr residues are predicted to be phosphorylated within HTT553–586 (Supplementary Material, Fig. S6).

Overall, our data reveal a novel post-translational modification of HTT via the post-translational myristoylation of one of its caspase-cleaved fragments (HTT53–586). Myr-HTT53–585–EGFP induced the formation of autophagosomes and accumulated in abnormally large autophagolysosomes and/or lysosomes in a variety of cell types in cell culture models. Of particular note, the presence of larger and more abundant autophagosomes has been documented in lymphoblasts of HD patients and that both the number and size of these structures were increased by the treatment with staurosporine (28), which is an established inducer of apoptosis that promotes activation of several caspases (57) that could alter the production of HTT553–586. Abnormal processing or production of myristoylated HTT53–586 fragment might be involved in the pathophysiology of HD by altering the flux of production of autophagosomes and/or their clearance with possible deleterious consequences in the brains of HD patients. Therefore, this initial characterization of a post-translationally myristoylated caspase-cleaved HTT fragment provides a new link between HTT and autophagy with possible implications in HD.

MATERIALS AND METHODS

Myr-HTT53–585–EGFP was generated as described previously (20). Briefly, phosphorylated oligonucleotides correlating to the full-length 34-amino acid fragment of HTT, excluding the asparagine from the caspase-6 site and including an initiator methionine, were ligated into the pTRAMPP vector using SalI and HindIII. Subsequently, the HTT53–585–EGFP portion was shuttled into the pEGFP-NI vector using BglII and NotI. Phosphorylated oligonucleotides corresponding to G553A-HTT53–585–EGFP were ligated into myr-HTT53–585–EGFP using BglII and BmtI, thereby substituting the Gly to Ala. LAMP1-RFP and RFP-LC3 were purchased from Addgene (Addgene plasmid 1817 and Addgene plasmid 21075, respectively). HTT1–588–YFP was provided by Dr Ray Truant (32), whereas mApple-N1 and Cathepsin-BFP were from the Davidson laboratory stocks.

Cell culture and transfection

HeLa cells were maintained in DMEM at 37°C and 5% CO2 in a humidified incubator. All maintenance media was supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 mg/ml streptomycin and 2 mM L-Glutamine. Cells were transiently transfected with the indicated constructs using FuGene 6 transfection reagent per manufacturer’s instructions in DMEM supplemented with 10% FBS and 100 U/ml penicillin and 0.1 mg/ml streptomycin and 2 mM L-glutamine unless otherwise indicated. Typically, transfection reagent–DNA complex was left in the medium for the duration of the experiments.

Detection of α-alkynyl-fatty acid-labeled proteins using click chemistry

Transiently transfected cells were incubated with 20 μM Alk-Myr as described previously (33). HMA was prepared as described previously (33). GFP immunoprecipitates were adjusted to 1% SDS and incubated with 100 μM Tris-(benzyltriazolylmethyl)amine, 1 mM CuSO4, 1 mM Tris-carboxyethylphosphate and 100 μM azido-biotin at 37°C in darkness for 30 min. Alk-myristoylated and biotinylated proteins were detected by western blot analysis using NA–HRP.

Immunoprecipitation

Cells were washed with cold PBS, harvested and lysed with cold EDTA-free RIPA buffer [0.1% SDS, 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% Igepal CA-630, 0.5% sodium-deoxycholate, 2 mM MgCl2, EDTA-free complete protease inhibitor ([Roche]) by rocking for 15 min at 4°C. When click chemistry was to be performed following immunoprecipitation, the Tris–HCl was replaced with 50 mM HEPES pH 7.4 in RIPA buffer. Cell lysates were centrifuged at 16 000 × g for 10 min at 4°C, and the post-nuclear supernatants were collected. GFP fusion proteins were immunoprecipitated with affinity purified goat anti-GFP cross-linked to sepharose beads (www.eusera.com) by rocking for 2 h or overnight at 4°C. The beads were extensively washed with 0.1% SDS–RIPA, resuspended in 50 mM HEPES pH 7.4 with 1% SDS and heated for 15 min at 80°C. The supernatants containing the fusion proteins were collected.

Microscopy

Live-cell microscopy

For live-cell microscopy, cells were plated on Poly-d-Lysine-coated 35-mm glass-bottom dishes (MatTek Corporation, Ashland, MA, USA) and transiently transfected with the indicated fluorescently tagged proteins using Fugene6 as recommended by the suppliers.

Wide-field live-cell microscopy

Z-stacks of observed cells were acquired by 0.26–0.3 μm step size using a Zeiss Observer Z1 microscope and Axiovision software (Axiovision, version 4.8). Cells were illuminated by a Kublan HXP 120 C lamp using bright-field illumination with GFP and Rhodamine filter cubes. Cells were maintained at 37°C and 5% CO2 using both a Pecon CO2 module S1 and Tempmodule S1.

When viewing HTT53–585–EGFP, cells were transfected for 24 or 48 h as indicated. Mitochondrial potential was detected with MitoTracker Red CM-H2XRos (1 μM) as recommended by the supplier. Lysosomes were stained using (1 μM) LysoTracker Red (Life Technologies).

Spinning-disk live-cell confocal microscopy

Spinning-disk confocal microscopy was performed on an UltraView Vox Confocal Imaging System (PerkinElmer, Woodbridge, ON, Canada) attached to a Leica DMI6000B microscope. Images were acquired on an ImagEM camera (Hamamatsu Corporation, Bridgewater, NJ, USA). Excitation radiation was provided by a 488-nm laser line paired with a W55 527-nm emission filter (PerkinElmer). The growth...
environment was maintained inside of a Universal ASI Stage Water Jacketed Incubator (Okolab, Ottaviano, NA, Italy). The atmosphere was kept at 19% O2 and 5% CO2 by paired DGT02BX and DGTc02BX gas mixers (Okolab). Temperature was measured by a TP00–1 thermometer (Okolab), maintained by an Ecoline Star edition E103 water bath (LAUDA, Lauda-Königshofen, Germany) controlled by TempControl Basic software (Okolab).

Where indicated, cells co-expressing HTT{553–585}{–EGFP and dsRed-ER [a construct that contains the N-terminal of calreticulin ER localization signal and a C-terminal KDEL ER retention signal attached to dsRed fluorescent protein (Clontech Laboratories, Inc.)] for 20 h were starved by replacing DMEM media with HBSS media for an additional 4 h. Furthermore, in HeLa cells transiently expressing HTT{553–585}{–EGFP, RFP-LC3 and Cathepsin B-BFP, 0.5 μM Baf was added for an additional 24 h after 24-h post-transfection to inhibit autophagosome fusion with lysosomes.

**Deconvolution**

Deconvolution of microscopic Z-stacks was performed using Huygens Professional software (Scientific Volume Imaging B.V., Hilversum, Netherlands). The software used a theoretical point spread function, an estimated signal-to-noise ratio, automatic background estimation, automatic bleaching correction, automatic brick mode, optimized iteration mode, automatic padding mode and a quality change threshold of 0.1%, for a maximum of 40 during CMLE. Where indicated, deconvolved images were reconstructed into 3D renderings using Bitplane’s Imaris (Imaris 64, version 6.4.2).

**Immunoelectron microscopy**

HeLa cells transiently expressing HTT{553–585}{–EGFP or EGFP alone for 24 h were prepared for immunoelectron microscopy as described previously. The cells were pelleted and fixed in a mixture of 2% glutaraldehyde and 2% paraformaldehyde in Dulbecco-PBS (D-PBS) for 20 min at 37°C. The fixed cells were rinsed in D-PBS and then dehydrated with alcohol series (30, 50, 70 and 80% ethylalcohol) and infiltrated with LR White (London Resin, Berkshire, United Kingdom). The infiltrated cells were embedded into gelatin capsules and polymerized under UV for 24 h at 4°C. Ultrathin sections of 60 nm were cut and loaded on a 300-mesh nickel grid without coating. Immunolabeling with anti-GFP IgG was performed. The ultrathin sections on the grid were initially blocked with 5% bovine serum albumin in D-PBS for 10 min and incubated with the first primary rabbit antibody, anti-GFP IgG (1:10,000), for 2 h. The secondary antibody bearing 18 nm colloidal gold-conjugated donkey anti-rabbit IgG (1:10) was also incubated for 1.5 h each. All antibodies were diluted with bovine serum albumin (1% final volume) in D-PBS, and the staining was performed at room temperature. After immunolabeling, ultrathin sections were contrasted with 2% aqueous uranyl acetate for 15 min. The grids were examined in a Philips 410 (Mahwah, NJ, USA) transmission electron microscope, at 80 kV equipped with a charge-coupled device camera (MegaView III, Soft Imaging System, Olympus,Melville, NY, USA).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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