Huntingtin contains a highly conserved nuclear export signal

Jianrun Xia, Denise H. Lee, Jillian Taylor, Mark Vandelft and Ray Truant*

Department of Biochemistry, McMaster University, Health Sciences Centre Rm 4H45, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5

Received February 4, 2003; Revised and Accepted April 21, 2003

Huntington’s disease (HD), is a genetic neurodegenerative disease characterized by a DNA CAG triplet repeat expansion in the first exon of the disease gene, HD. CAG DNA expansion results in a polyglutamine tract expansion in mutant huntingtin protein. Wild-type and mutant full-length huntingtin have been detected in the nucleus, but elevated levels of mutant huntingtin and huntingtin amino-terminal proteolytic fragments are seen to accumulate in the nuclei of HD-affected neurons. The presence of huntingtin in both the nucleus and the cytoplasm suggested that huntingtin may be dynamic between these compartments. By live cell time-lapse video microscopy, we have been able to visualize polyglutamine-mediated aggregation and the transient nuclear localization of huntingtin over time in a striatal cell line. A classical nuclear localization signal could not be detected in huntingtin, but we have discovered a nuclear export signal (NES) in the carboxy-terminus of huntingtin. Leptomycin B treatment of clonal striatal cells enhanced the nuclear localization of huntingtin, and a mutant NES huntingtin displayed increased nuclear localization, indicating that huntingtin can shuttle to and from the nucleus. The huntingtin NES is strictly conserved among all huntingtin proteins from diverse species. This export signal may be important in Huntington’s disease because this fragment of huntingtin is proteolytically cleaved away during HD. The huntingtin NES therefore defines a potential role for huntingtin as a member of a nucleocytoplasmic dynamic protein complex.

INTRODUCTION

Huntington’s disease (HD) is an inherited neurodegenerative disease resulting in cognitive and severe movement disorders. The affected gene, HD, causes one of eight genetic diseases that is the result of a DNA CAG triplet repeat expansion in the open reading frame resulting in expanded tracts of polyglutamine in the huntingtin protein (reviewed in 1). Dominant huntingtin-induced phenotypes can be seen in transgenic mouse models in which only the first exon of huntingtin, containing the mutant polyglutamine expansion, is expressed in trans (2). This exon-1(R/6) mouse model manifests a much earlier and more severe disease phenotype than mouse models expressing full-length huntingtin as a gene knock-in (3–6) or as a YAC transgenic mouse (7). One effect of this polyglutamine expansion at the sub-cellular level in both HD brain cells (8), and in huntingtin transgenic mouse models, is aggregated huntingtin that has been found in both the cytoplasm and in the nuclei of neurons (9–11), but the relevance of these aggregates to Huntington’s disease is controversial (11,12). Wild-type huntingtin is a 350 kDa essential protein that localizes primarily to the cytoplasm by immunofluorescence studies (9), but full-length huntingtin has also been detected in the nucleus by immunofluorescence and biochemical fractionation (8,13–15). Huntingtin has also been visualized at the perinucleus and in sub-nuclear structures such as speckles, promyelocytic leukemia (PML) bodies and nucleoli (14–16).

The presence of mutant huntingtin fragments in the nucleus has been associated with neuronal toxicity (reviewed in 17), due to the presence of mutant huntingtin in nuclear inclusions in affected neurons (8,18). Neuronal toxicity of huntingtin can be increased by the addition of exogenous nuclear localization signals (NLS), or decreased by the addition of exogenous nuclear export signals (NES) (11,19). While nuclear accumulation huntingtin fragments has been seen in HD neurons, there has been some discordance between the presence of nuclear aggregates in neuronal sub-types and the specific brain regions affected in HD (12,20). In another polyglutamine expansion disease, spinocerebellar ataxia type 1 (SCA1), there is little correlation between large aggregate formation and pathogenesis, but a direct correlation between nuclear entry of ataxin-1 and disease (21). Huntingtin does not appear to have a classical NLS (22), but this is controversial (23), and large polyglutamine tracts do not appear to enter the nucleus without an added NLS (24).

*To whom correspondence should be addressed. Tel: +1 9055259140, ext 22450; Fax: +1 9055229033; Email: truantr@mcmaster.ca
We designed experiments using specific in vivo NLS and NES assays to span the entire wild-type and mutant huntingtin open reading frame for the presence of any nucleocytoplasmic transport signals. Using both human epithelial-derived HeLa cells and a mouse striatum-derived ST Hdh cell line (25), we spanned the entire sequence of huntingtin in overlapping fragments by an established in vivo nuclear import assay (26), to uncover any potential NLS activity by a novel signal, as NLS signals can be very diverse (27,28). By live cell video microscopy, we have observed the transient nuclear entry of huntingtin just prior to cell death. To look for possible export signals in huntingtin, we visually scanned the entire huntingtin amino acid sequence for any basic regions that may contain NLS activity. A putative nuclear localization signal in huntingtin (23), as well as two other basic sequences within huntingtin, were analyzed in the context of an established in vivo assay (26) in human HeLa epithelial cells (Fig. 1) and ST Hdh mouse clonal striatal cells (Fig. 2). This is essentially a binary assay, where NLS sequences led to nuclear localization of most of the eGFP-beta-galactosidase fusion (Fig. 1B–D) and lack of NLS activity reported as mostly cytoplasmic fluorescence (Fig. 1A). Potential NLS signals were expressed as enhanced green fluorescent protein (eGFP) and beta-galactosidase triple fusion proteins to ensure the presence of a NES in the 350 kDa huntingtin protein, and the increased nuclear localization of NES mutant huntingtin, demonstrated that huntingtin could shuttle to and from the nucleus. The lack of a classical NLS in huntingtin suggests that huntingtin may shuttle in complex with associated factors or directly enter the nucleus via an unconventional pathway.

RESULTS

Huntingtin does not contain a classic nuclear localization signal

We designed experiments to examine the entire huntingtin protein for the presence of any nucleocytoplasmic transport signals in the context of established nuclear localization or export signal assays. We visually scanned the huntingtin amino acid sequence for any basic regions that may contain NLS activity. A putative nuclear localization signal in huntingtin (23), as well as two other basic sequences within huntingtin, were analyzed in the context of an established in vivo assay (26) in human HeLa epithelial cells (Fig. 1) and ST Hdh mouse clonal striatal cells (Fig. 2). This is essentially a binary assay, where NLS sequences led to nuclear localization of most of the eGFP-beta-galactosidase fusion (Fig. 1B–D) and lack of NLS activity reported as mostly cytoplasmic fluorescence (Fig. 1A). Potential NLS signals were expressed as enhanced green fluorescent protein (eGFP) and beta-galactosidase triple fusion proteins to ensure...
that the signal activity was being assayed in the context of an inert cargo of at least 130 kDa in size, far above the diffusion limit for the mammalian nuclear pore complex (reviewed in 30). All triple fusions were compared with a negative control of eGFP–β-galactosidase (Fig. 1A), and positive controls of eGFP-SV40TagNLS–β-galactosidase (Fig. 1B) (26), the direct importin beta-dependent HIV-1 Tat protein NLS (31) (Fig. 1C), and the hnRNPA1 M9 transportin-dependent NLS (32) (Fig. 1D) to ensure that our cell assays could detect nuclear entry through a variety of pathways. The controversial huntingtin NLS, BS3, 1182-PIRRGKEK-1190 (22, 23), was not active in this assay (Fig. 1G), nor was it active in an in vitro nuclear uptake assay in digitonin permeabilized cells (data not shown). In addition, we did not see any NLS activity in the other two basic sequences in huntingtin (Fig. 1E and F), nor could we detect NLS activity of either mutant (138 glutamine) or wild-type (15 glutamine)
huntingtin exon 1 (Fig. 1H and I) after 18 h of expression. These assays were additionally performed in ST Hdh clonal striatal cells with similar results (data not shown). Cells expressing the triple fusion protein were also stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) for β-galactosidase activity (33) to ensure that stable, full-length protein is present in the assay (data not shown). We then assayed these basic sequences in the context of the polyglutamine expansion in case there was any synergy between these basic sequences and polyglutamine tracts, and then proceeded to scan the entire open reading frame of huntingtin in /C24600 amino acid overlapping fragments to detect any NLS activity in clonal striatal ST Hdh cells (Fig. 2) or HeLa cells (data not shown). We used fragments of huntingtin in excess of the largest described NLS signals to date (27,28,32). We could not detect nuclear localization in any of these fragments (all >80% cytoplasmic). Representative fluorescent laser confocal images of these expression constructs expressed in ST Hdh striatal cells and three control NLSs, are shown in Figure 2.

Huntingtin localizes to the nucleus prior to cell death

In order to try to understand how huntingtin has been observed in the nucleus, despite not having a classical NLS, we fused eGFP to a 1–588 amino acid Q138 mutant huntingtin (juvenile onset clinical isolate) cDNA fragment and expressed this protein in ST Hdh clonal striatal cells to observe any potential temporal differences in huntingtin sub-cellular localization. This fragment of huntingtin includes the expanded polyglutamine tract and the adjacent proline rich domain which has been seen to mediate protein–protein interactions (34). We observed live transfected striatal cells with an inverted fluorescence microscope over a 24 h time period at 33°C. At about 8 h post-transfection, we observed eGFP–huntingtin fluorescence. At about 16–18 h we visualized the onset of protein aggregation in both the cytoplasm and the nucleus (Fig. 3A–D; Supplementary Material, video 1). We saw that aggregates of polyglutamine expanded huntingtin protein formed at several locations simultaneously throughout the cell cytoplasm (Fig. 3A–D). We also observed that nuclear inclusions of huntingtin protein appeared to form from protein already present in the nucleus (Fig. 3, arrows in C and D), and not by the translocation of an aggregate across the nuclear envelope. We noted eGFP–huntingtin accumulating in the nucleus 40–60 min prior to cell death (Fig. 3E–H; Supplementary Material, video 2). Nuclear huntingtin accumulation was seen only after protein aggregates had formed in the cytoplasm (Fig. 3G). This data suggested that there was a temporal event related to toxicity or expression level that allowed huntingtin to enter the nucleus.

Identification of a nuclear export signal in huntingtin

While studying the potential NLS activity within huntingtin, we also assayed for the presence of an NES within huntingtin (8,13–15). It has been previously shown that the nuclear export inhibitor, leptomycin B, affects the sub-cellular distribution of endogenous huntingtin in ST Hdh cells, suggesting that huntingtin may have an NES (14). We used a version of an established in vivo NES assay (35) that relies on the fact that active nuclear transport is faster than the rate of diffusion of eGFP protein across the nuclear envelope. eGFP protein can
diffuse freely across the nuclear pore of mammalian cells due to its relatively small size of 26 kDa, below the diffusion limit for the nuclear pore (30) (Fig. 4A). A 50 amino acid fragment of HIV-1 Rev protein containing a known NES sequence (36), was fused to eGFP. eGFP–Rev NES fluorescence distribution is observed to be clearly towards the cytoplasm in all cells observed (Fig. 4B). This effect is due to active transport from the nucleus, and not due to increased size of the test protein because the same 50 amino acid fragment with a point mutation in the NES, a critical leucine residue mutated to serine (37), behaves like eGFP alone (Fig. 4C).

The entire human huntingtin amino acid sequence was examined for leucine-rich sequences that could be aligned with the loose consensus known for HIV-1 Rev-like NESs (LX2–3LXL) (29). From this sequence comparison, five potential NES sequences were determined in huntingtin and functionally tested the sequences in the context of 50 amino acid huntingtin fragments fused to eGFP. Results are indicative of 100 cells observed for each construct. Potential leucine-rich NES sequences (LS1-5) in the huntingtin fragments assayed are listed. Scale bar is ~10 μm.

Figure 4. Identification of the huntingtin nuclear export signal. Laser confocal images of transfected Hela cells for the NES assay. (A) NES assay, negative control of eGFP expression alone. (B) Fluorescence pattern of eGFP–RevC, containing a functional NES. (C) Same Rev fragment in panel B, except that NES function was abrogated by a leucine to serine point mutation. (D-I) Assay for activity of potential NESs 1–4 in the context of 50 amino acid fusions to eGFP. (H) NES5, a functional NES as compared with the positive control (B). (I) Same huntingtin 50 amino acids fragment as in (H), with a L2404S mutation in the leucine 2404 residue predicted to be important for NES activity. Results are indicative of 100 cells observed for each construct. Potential leucine-rich NES sequences (LS1-5) in the huntingtin fragments assayed are listed. Scale bar is ~10 μm.

Figure 5. The huntingtin nuclear export signal is highly conserved. BLAST sequence analysis of known NESs and the huntingtin NES across diverse species. (A) NES amino acid sequences from several described NESs and the consensus of leucine spacing. (B) Sequence comparison of the Human huntingtin NES with that of other known vertebrate species of huntingtin and location of the L2404S inactivating point mutation in the huntingtin NES.

The table below shows the amino acid sequences for the NESs tested:

<table>
<thead>
<tr>
<th>Species</th>
<th>NES Sequence</th>
<th>Consensus NES</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 Rev</td>
<td>LPPLERLTL</td>
<td>LX2–3LXL</td>
</tr>
<tr>
<td>TFIIA</td>
<td>LPVLENLTL</td>
<td></td>
</tr>
<tr>
<td>PKI</td>
<td>LALKLAGLDI</td>
<td></td>
</tr>
<tr>
<td>MEK1</td>
<td>LQKKEEEL</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>FRELNEALEL</td>
<td></td>
</tr>
<tr>
<td>hAPC NES2</td>
<td>LTKRIDSLPL</td>
<td></td>
</tr>
<tr>
<td>Consensus</td>
<td>LX2–3LXL</td>
<td></td>
</tr>
</tbody>
</table>

The entire human huntingtin amino acid sequence was examined for leucine-rich sequences that could be aligned with the loose consensus known for HIV-1 Rev-like NESs (LX2–3LXL) (29). From this sequence comparison, five potential NES sequences were determined in huntingtin and functionally tested the sequences in the context of 50 amino acid huntingtin fragments fused to eGFP. As seen in Fig. 4D–H, only one of the huntingtin sequences displayed NES function in vivo, LS5 (Fig. 4H), similar to the Rev NES positive control (Fig. 4B), while all other sequences (Fig. 4D–G) showed diffuse fluorescence across the nucleus and cytoplasm in a manner similar to our negative controls of eGFP alone or eGFP–Rev mutant NES (Fig. 4A and C, respectively). To determine if this activity was actually due to the leucine sequence that was predicted to have NES activity, a predicted critical leucine was mutated in a manner analogous to the Rev NES mutant (37). This leucine to serine mutation at position 2404 (Fig. 5B) completely abrogated the NES activity of this fragment (Fig. 4I). From this data, it was concluded that huntingtin has a leucine-type Rev-like NES that follows the consensus of active NES signals by critical leucine spacing.
This NES is located in the carboxy-terminal third of huntingtin protein, and by BLAST sequence alignment shows significant sequence similarity to known NESs in other human proteins (Fig. 5A). (B) Triple fusion of eGFP-SV40T NLS and the huntingtin NES, showing predominantly cytoplasmic distribution. (C) Similar construct as in (B), except that a L2404S point mutation in the huntingtin NES was used. (D and E) Leptomycin B treatment of eGFP-NLS–huntingtin NES before (D) and after (E) 2 h of 10 nM leptomycin B treatment in striatal ST Hdh cells. (F) Quantitation of nuclear fluorescence in experiment outlined in (D) and (E) displaying mean nuclear fluorescence as a percentage of mean total cell fluorescence, with standard deviations across three experiments. (G and H) Leptomycin B treatment of ST Hdh cells expressing eGFP–huntingtin full-length wild-type before (G) and after 90 min of 8 nM leptomycin B (H). (I) Quantitation of nuclear fluorescence of eGFP–nuclear huntingtin over a 90 min time-course of leptomycin B treatment displaying mean nuclear fluorescence as a percent of mean total cell fluorescence, with standard deviations across three experiments. Scale bar is ~10 μm.

Figure 6. The huntingtin NES is leptomycin B sensitive and functional in the full-length protein. Fluorescence images of transient transfections of eGFP fusions in HeLa cells (A–E) or striatal ST Hdh cells (G and H). (A) eGFP–NLS from SV40 T antigen. (B) Triple fusion of eGFP-SV40T NLS and the huntingtin NES, showing predominantly cytoplasmic distribution. (C) Similar construct as in (B), except that a L2404S point mutation in the huntingtin NES was used. (D and E) Leptomycin B treatment of eGFP-NLS–huntingtin NES before (D) and after (E) 2 h of 10 nM leptomycin B treatment in striatal ST Hdh cells. (F) Quantitation of nuclear fluorescence in experiment outlined in (D) and (E) displaying mean nuclear fluorescence as a percentage of mean total cell fluorescence, with standard deviations across three experiments. (G and H) Leptomycin B treatment of ST Hdh cells expressing eGFP–huntingtin full-length wild-type before (G) and after 90 min of 8 nM leptomycin B (H). (I) Quantitation of nuclear fluorescence of eGFP–nuclear huntingtin over a 90 min time-course of leptomycin B treatment displaying mean nuclear fluorescence as a percent of mean total cell fluorescence, with standard deviations across three experiments. Scale bar is ~10 μm.

To address the possible nuclear export pathway utilized by the huntingtin NES, we treated the eGFP–NLS–huntingtin NES expressing cells with leptomycin B (39), a potent nuclear export inhibitor of proteins that utilize the CRM-1/exportin export pathway (40). Leptomycin B specifically inhibits exportin-mediated nuclear export by directly binding to and modifying exportin/Crm-1 (41). As seen in Fig. 6D and E, addition of leptomycin B to cells expressing eGFP–NLS–huntingtin NES caused the fluorescence pattern to shift from the cytoplasm (Fig. 6D) to the nucleus (Fig. 6E), shifting nuclear fluorescence from ~5 to 40% after Leptomycin B treatment (Fig. 6F). This data confirmed the results in Fig. 6B and C, that this eGFP–NLS–huntingtin NES molecule was dynamic despite its apparent cytoplasmic localization.

In order to assess whether the identified NES sequence in huntingtin was active in the full-length huntingtin protein, ST Hdh cells expressing eGFP–wild-type huntingtin full-length were treated with leptomycin B. We observed partial nuclear accumulation of huntingtin after 90 min of treatment with

(Fig. 5A).
that comparing wild-type and mutant huntingtin, it was observed (Fig. 7A versus B) was observed. By laser confocal microscopy phenotype of nuclear accumulation of the L2404S mutant huntingtin has the ability to shuttle in and out of the nucleus.

Functional domain within full-length huntingtin and that NES (Fig. 7B versus C). These data indicated that the NES is a distribution to an eGFP construct lacking the L2404S NES mutant displayed a similar nuclear/cytoplasmic localization of the huntingtin in vivo (55). A HIV-1 Rev-like leucine-type nuclear export signal has been discovered in the carboxy terminal region of the huntingtin protein. The presence of this signal was predicted by others after observing endogenous huntingtin immunofluorescence shifting to the nucleus in leptomycin B treated ST Hdh cells (14). Like other NESs of this type, this signal is sensitive to the drug leptomycin B (41). By leptomycin B treatment of eGFP–huntingtin expressing cells, and by phenotypic analysis of a huntingtin NES point mutant, we concluded that a significant portion of normal huntingtin protein can shuttle between the nucleus and the cytoplasm.

In small fragments of huntingtin in nuclei as has been seen with a small fragments of huntingtin in nuclei of transiently transfected cos7 cells (52). The transient nuclear localization of huntingtin that was seen by video microscopy in this study (Supplementary Material, video 2) may have been due to the disruption of nuclear pore complexes. Huntingtin protein has some sequence similarity in its HEAT repeat domains with importins/karyopherins (53), and nucleoporin proteins (Nups) have been detected in purified synthetic polyglutamine aggregates (54). Huntingtin may be able to directly interact with the nuclear pore complex in manner similar to importin family proteins, rather than as a cargo protein targeted for nuclear entry.

Some proteins found in both the cytoplasmic and nuclear compartments of the cell contain a nuclear export signal as well as an import signal and shuttle dynamically to and from the nucleus (55). A HIV-1 Rev-like leucine-type nuclear export signal has been discovered in the carboxy terminal region of the huntingtin protein. The presence of this signal was predicted by others after observing endogenous huntingtin immunofluorescence shifting to the nucleus in leptomycin B treated ST Hdh cells (14). Like other NESs of this type, this signal is sensitive to the drug leptomycin B (41). By leptomycin B treatment of eGFP–huntingtin expressing cells, and by phenotypic analysis of a huntingtin NES point mutant, we concluded that a significant portion of normal huntingtin protein can shuttle between the nucleus and the cytoplasm.

In small fragments of huntingtin in nuclei as has been seen with a small fragments of huntingtin in nuclei of transiently transfected cos7 cells (52). The transient nuclear localization of huntingtin that was seen by video microscopy in this study (Supplementary Material, video 2) may have been due to the disruption of nuclear pore complexes. Huntingtin protein has some sequence similarity in its HEAT repeat domains with importins/karyopherins (53), and nucleoporin proteins (Nups) have been detected in purified synthetic polyglutamine aggregates (54). Huntingtin may be able to directly interact with the nuclear pore complex in manner similar to importin family proteins, rather than as a cargo protein targeted for nuclear entry.

DISCUSSION

It has been demonstrated by an in vivo nuclear import assay scanning the entire huntingtin protein that huntingtin does not contain a classic nuclear localization signal. Nuclear localization signals are defined classically, as protein sequences that can mediate the complete nuclear entry or exit of a heterologous protein away from the context of the original protein (42). Human epithelial carcinoma (HeLa) cells were used for these experiments, in addition to mouse striatal ST Hdh cells, for several reasons: HeLa cells are classically used for NLS assays (42); they known to express every isoform of the NLS-recognition nuclear import adapter protein, importin alpha, including the p-type found in brain cells (43); they have a consistent morphology suitable for the clear visualization of the nucleus by laser confocal microscopy; and they permit relatively low protein expression levels by transient transfection. These assays were duplicated in a clonal striatal cell line, ST Hdh, in order to assay for NLS activity in a cell line that was physiologically relevant to HD and could display clear mutant huntingtin-induced phenotypes (14,25,44). These results agree with what others have observed in different assays on a putative huntingtin NLS (22), and observations that polyglutamine alone cannot enter the nucleus without an added NLS (24).

In normal cell biology, there are nuclear proteins that contain polyglutamine tracts with as many as 38 repeats, such as the TATA box-binding protein, TBP (45), and the CREB binding protein, CBP (46), but these proteins enter the nucleus using classic, basic nuclear localization signals (47,48). Huntingtin may be entering the nucleus through interactions enhanced by expanded polyglutamine tracts with nuclear proteins such as CBP (46), CA150 (49), TBP (50), the HYP WW domain proteins (34,51), or by factors yet to be determined. Another possibility is that small huntingtin fragments can diffuse into the nucleus at some time point in huntingtin-induced toxicity, as has been seen with a small fragments of huntingtin in nuclei of transiently transfected cos7 cells (52). The transient nuclear localization of huntingtin that was seen by video microscopy in this study (Supplementary Material, video 2) may have been due to the disruption of nuclear pore complexes. Huntingtin protein has some sequence similarity in its HEAT repeat domains with importins/karyopherins (53), and nucleoporin proteins (Nups) have been detected in purified synthetic polyglutamine aggregates (54). Huntingtin may be able to directly interact with the nuclear pore complex in manner similar to importin family proteins, rather than as a cargo protein targeted for nuclear entry.

At 350 kDa, huntingtin is a large protein that may have multiple functional roles throughout the cell, possibly defined by several protein–protein interactions (58). We have observed that huntingtin nuclear aggregates form within the nucleus, and are not transported across the nuclear membrane as aggregated protein (Supplementary Material, video 1). These observations indicate that even a 65 kDa amino-terminal huntingtin fragment with an additional 26 kDa fluorescent fusion protein can exist in the nucleus, in local concentration levels high enough to induce polyglutamine-mediated aggregation.

The presence of an NES in huntingtin may hint at the unknown biological function of this protein as part of a nuclear-cytoplasmic shuttling protein complex. The strict evolutionary conservation of this export signal could underscore the importance of this signal to the biological function of huntingtin. This NES within huntingtin is strictly conserved in presence and position of the critical leucine residues, even within highly divergent species. Only conservative substitutions of isoleucine to valine are seen at the start of the NES sequences (Fig. 5B). Leucine, valine and isoleucine are typically interchangeable at these positions without affecting NES function (29).

The consequence of huntingtin-mediated export from the nucleus in a protein complex could suggest several roles for...
Huntingtin. Huntingtin could serve as a negative transcriptional regulator, analogous to IkBα in NFκB signal-mediated transcription regulation (59, 60), where huntingtin could mediate the export of a transcription factor, such as CBP (46) or HYP-B (61). Huntingtin protein is found in intranuclear aggregates and is ubiquitinated and truncated (18). The presence of an NES in huntingtin, as well as huntingtin’s presence in ubiquitin-positive aggregates (49), could suggest a regulatory role for huntingtin similar to the human adenoma-tous polyposis coli (APC) tumor suppressor protein on beta-catenin (62, 63).

Several huntingtin-associated proteins bind mutant huntingtin with greater affinity than wild-type huntingtin (34, 49, 64). Nuclear huntingtin localizes to RNase sensitive foci (15), speckles, nucleoli and PML bodies (16), sub-nuclear sites rich in RNA and messenger RNA processing factors (reviewed in 65). This suggests that one target molecule for huntingtin-mediated nuclear export may be RNA, an idea also recently proposed by others (16). The suggestion that huntingtin associates with RNA may be more relevant in the context of the discovery of the inherent nuclear export ability of huntingtin. Huntingtin could potentially have a role in mRNA nuclear export of critical mRNAs important for normal brain development (66–68).

In the Huntington’s disease situation, the protein dynamics of a polyglutamine expanded huntingtin-containing complex could change as a result of the enhanced binding to specific proteins or the proteolysis of huntingtin. In all studied profiles of huntingtin proteolysis in transgenic mouse models, HD brains and cellular models, the carboxy-terminal fragment of huntingtin containing its NES is cleaved away (69–71). This cleavage would result in an amino-terminal huntingtin fragment that can still enter the nucleus, but not exit. Huntingtin fragments generated in either the nucleus or cytoplasm would be able to compete for interactions with the wild-type huntingtin target(s) normally exported from the nucleus. This would exert a dominant effect and a gain of function by sequestering huntingtin associated proteins or RNA in the nucleus. In the absence of proteolytic cleavage, nuclear transport dynamics of mutant huntingtin could be affected by the slowed dynamics induced by expanded polyglutamine tracts, as demonstrated by others in live cell fluorescence recovery after photo bleaching (FRAP) studies of polyglutamine-expanded huntingtin aggregates (72, 73).

The huntingtin NES is the only functional localization signal in huntingtin described to date and has implications as to the possible biological function of this protein. The potential
factor(s) that can mediate the nuclear entry of huntingtin, and the nuclear export targets of huntingtin, are as yet unknown and will be the subject of further study.

MATERIALS AND METHODS

**In vivo nuclear localization signal assay**

The assay for NLS activity was based on the pHM830 eGFP–beta-galactosidase triple fusion plasmid construct and conducted as described (26). Fragments of wild-type (Q15) and mutant (Q138) huntingtin cDNA were PCR-amplified with nested SacII and XbaI restriction enzyme recognition sites in-frame and ligated into SacII- and XbaI-digested pHM830. Plasmid cDNAs for wild-type human huntingtin and mutant juvenile-onset patient cDNA were gifts of M. Hayden, UBC. Small basic sequences were constructed with synthetic DNA oligonucleotides from the McMaster Mobix Facility and ligated into SacII- and XbaI-digested pHM830. All enzymes related to plasmid construction were purchased from New England Biolabs. All constructs used in this report were confirmed by automated fluorescence sequencing at the McMaster Mobix facility.

The following amino acid sequences were expressed as a triple fusion in pHM830 and transfected by polyethylimine method (ExGen500, Fermentas) into human HeLa cells (ATCC, CCL-2) or clonal striatal ST HdhQ11 (wild-type huntingtin) cells (25): residues 1–86 (Q15); residues 1–86 (Q138); residues 1–109 (Q15); residues 1–109 (Q138); residues 1–452 (Q15); residues 1–452 (Q138); residues 81–452; residues 81–109 (BS1); residues 435–445 (BS2); residues 1182–1190 (BS3-putative NLS) (23); residues 423–520 (Q15); residues 423–520 (Q138); residues 86–423–520; residues 86–1208–1810; residues 86–1208–1810; residues 1775–2413; residues 2374–2985; and residues 2982–3144 (end not including stop codon). All numbering is in reference to human huntingtin cDNA sequence NCBI no. XM_003405. For each construct, 100 cells each were observed over three separate transfection and expression experiments.

**In vivo nuclear export signal assay**

This assay was done in a similar manner as previously described by others (35), except that 50 amino acid fusions to eGFP (Clontech) were used. The C-terminal 50 amino acid fragment of wild-type or M10 mutant (37) HIV-1 Rev protein, which includes only its NES, was fused to the C-terminus of eGFP by PCR amplification of the Rev fragment with nested EcoR1 and BamH1 restriction enzyme sites and ligated into EcoR1- and BamH1-digested peGFP-C1 (Clontech). Cells were fixed with 2% formaldehyde, inverted and mounted in 50% glycerol/PBS and observed for GFP fluorescence 12–16 h post-transfection by argon 488 nm laser confocal microscopy (Zeiss LSM510) at pinhole settings of 0.60 Airy units. Potential huntingtin NES signals were visually identified by consensus to the LX2-1LX3-1LXL motif (29) and fused in the context of 50 amino acid fragments of huntingtin protein to eGFP in a manner similar to Rev. The leucine sequences (LS) amplified and assayed included: LS1, residues 275–325 including sequence LLLGVLVLL; LS2, residues 347–397, including sequence LVQVYELTL; LS3, residues 814–864, including sequence LQLIDVTL; LS4, residues 2195–2245, including sequence VVSKLPSHL; and LS5, residues 2364–2414, including sequence IIISLARPL. The LS sequence was subsequently point mutated to IIISLARSPL (L2404S) by weave DNA oligo PCR site-directed mutagenesis. For each NES construct, 100 cells each were observed over three separate transfection and expression experiments.

**Mutant huntingtin NES characterization and live-cell video microscopy**

For *in vivo* huntingtin NES characterization, plasmid constructs encoding the huntingtin NES (LS5) or NES L2404S were modified by the addition of synthetic oligonucleotides corresponding to the cDNA sequence for the SV40 large T antigen NLS upstream of the NES fusion at the upstream BspE1 site. The resultant plasmids encoded a triple fusion of eGFP–SV40TNLS–huntingtin NES or NESL2404S. Full-length huntingtin NES mutant L2404S was made by Quickchange PCR (Stratagene) with a mutagenic oligonucleotide. Leptomycin B was a gift from M. Yoshida or purchased from Sigma chemical and prepared as per supplier’s instructions and applied for 2 h at 8 or 10 nM concentration.

All NLS and NES assays were performed in human HeLa epithelial carcinoma cell line (American Tissue and Cell Collection, ATCC, CCL-2) and an ST HdhQ11 clonal striatal (25) cell line generously provided by M.E. MacDonald (MGH/ Harvard). Striatal cells were clonally selected and grown under G418 drug selection at 33°C to ensure temperature sensitive selection with 5% CO2 as described elsewhere (25). NLS and NES assays were blinded by random letter assignment and visualized by laser confocal microscopy with a LSM510 confocal microscope (Zeiss) observing ~35 randomly chosen cells for each fragment over three separate transfection experiments (total of 100 cells). Leptomycin B treatment experiments, huntingtin NES mutants and time-lapse experiments were visualized at 33°C using a Nikon TE200 inverted fluorescence microscope with a Biotech Delta TC heated culture dish system (Biotech, PA, USA) and images were pixel quantitated using SimplePCI 5.0 software (Compix, PA, USA) or with Scion Image 1.6 (Scion Corp., PA, USA). Time-course animations and images were digitally de-convolved after quantitation using AutoDeblur 9.0 iterative two-dimensional algorithm (Autoquant Imaging, USA).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

We would like to thank M. Hayden and A. Hackam at the University of British Columbia for the prompt gift of Huntington’s disease patient cDNAs and cloning advice. We thank M. MacDonald at MGH/Harvard for the kind gift of ST HdhQ11 striatal cells. We would also like to thank M. Yoshida at the University of Tokyo for the gift of leptomycin B. We would like to acknowledge the grant support of this work by the Hereditary Disease Foundation Cure Initiative and the Canadian...
Institutes of Health Research. We would like to acknowledge major equipment funding support from the Canadian Foundation Institute and the Ontario Innovation Trust. R.T. is supported by a CIHR New Scientist Award.

REFERENCES


