We reported previously that normal Huntingtin is associated with epidermal growth factor receptor (EGF) signaling complex (Liu, Y. F., Deth, C. R., and Devys, D. (1997) J. Biol. Chem. 272, 8121–8124). To investigate the potential role of normal and polyglutamine-expanded Huntingtin in the regulation of growth factor receptor-mediated cellular signaling and biological function, we stably transfected full-length Huntingtin containing 16, 48, or 89 polyglutamine repeats into PC12 cells where cellular signaling mechanisms, mediated by nerve growth factor (NGF) or EGF receptors, are well characterized. Expression of polyglutamine-expanded Huntingtin, but not normal Huntingtin, leads to a dramatic morphological change. In clones carrying the mutated Huntingtin, both NGF and EGF receptor-mediated activation of mitogen-activated protein kinase, c-Jun N-terminal kinase, and Akt are significantly attenuated, and NGF receptor-mediated neurite outgrowth is blocked. Co-immunoprecipitation studies show that the associations of NGF or EGF receptors with growth factor receptor-binding protein 2 (Grb2) and phosphoinositide 3-kinase are significantly inhibited. NGF-induced tyrosine phosphorylation of NGF receptors (TrkA) is also consistently suppressed. Our data demonstrate that polyglutamine-expanded Huntingtin disrupts cellular signaling mediated by both EGF and NGF receptors in PC12 cells. It is known that Huntington’s disease patients exhibit an extremely low incidence of a variety of cancers and are deficient in glucose metabolism. Thus, our results may reflect an important molecular mechanism for the pathogenesis of the disease.

Huntington’s disease (HD) is a dominant inherited neurodegenerative disorder, characterized by choreiform movement, psychiatric disturbance, and cognitive decline (2). The HD gene encodes a 350-kDa protein designated as Huntingtin (3), which is ubiquitously expressed in all tissues (4, 5). Huntington in the regulation of growth factor receptor-mediated signaling and function mediated by growth factor receptors, we stably transfected full-length Huntingtin containing 16, 48, or 89 polyglutamine repeats into PC12 cells. We report that expression of polyglutamine-expanded Huntingtin leads to disruption of cellular signaling and biological function mediated by both EGF and NGF receptors.

MATERIALS AND METHODS

Cell Culture and Stable Transfection—PC12 cells were maintained in DMEM supplemented with 10% horse serum and 5% fetal bovine serum (full DMEM). Different full-length Huntingtin constructs containing 16, 48, or 89 CAG repeats (13) were separately co-transfected with PY-3, a plasmid containing the hygromycin B-resistant gene, into PC12 cells using Lipofectin as per instructions by the manufacturer. Multiple 16F, 48F, or 89F clones were selected using full DMEM containing 250 μg/ml hygromycin B. These selected PC12 clones were maintained in full DMEM containing 150 μg/ml hygromycin B.
Kinase Assays—Two to 3 days prior to the experiments, multiple 16F, 48F, or 89F clones were cultured in regular DMEM without hygromycin B to remove inhibition of protein synthesis by the antibiotic. Wild-type PC12 cells and multiple 16F, 48F, or 89F clones were serum-starved for 18–24 h. Cells were treated with NGF (10 ng/ml) for 15 min or EGF (100 ng/ml) for 5 min at 37°C and then lysed with 1% Triton X-100 buffer (13). Cell lysates (30 μg of protein) were resolved in a 10% SDS gel and transferred. The blots were analyzed by an anti-phospho-MAPK, anti-phospho-JNK, or anti-phospho-Akt antibody (Cell Signaling) to detect activated MAPK, JNK, or Akt. The blots were then stripped and re-probed with an anti-MAPK, anti-JNK, or anti-Akt antibody to detect total MAPK, JNK, or Akt.

FIG. 1. Characteristics of PC12 subclones carrying full-length normal or polyglutamine-expanded Huntingtin construct. A, the levels of expressed Huntingtin in multiple PC12 subclones. Cell lysates from PC12 cells or multiple clones were resolved in a 5% SDS gel. The blot was probed with 437. Data represent a typical experiment that has been repeated once with similar results. NHtt, normal Huntingtin; MHtt, polyglutamine-expanded Huntingtin. B, phase contrast shows morphological changes of clones carrying normal or the mutated Huntingtin.

RESULTS

Full-length Huntingtin expression vector containing 16, 48, or 89 CAG repeats was stably transfected into PC12 cells. The positive clones were identified by Western blot analysis using 437, an anti-Huntingtin antibody that has been characterized previously (1). As shown in Fig. 1A, in clones carrying mutated Huntingtin constructs with either 48 or 89 CAG repeats (48F or 89F clones), the amount of expressed mutated Huntingtin is about equivalent to the amount of endogenously expressed Huntingtin in PC12 cells. In clones carrying normal Huntingtin construct with 16 CAG repeats, the amount of Huntingtin is about doubled (Fig. 1A). To avoid variations of different PC12 subclones, all experiments were simultaneously conducted with two different clones carrying the same construct, and the results were further verified using another subclone. Because the results are similar, we only present data from one or two clones; and the data from other clones are mentioned in the figure legends.

Expression of full-length normal Huntingtin with 16 polyglutamine repeats (16F clones) did not alter the morphology of...
PC12 cells. Expression of full-length mutated Huntingtin with either 48 or 89 polyglutamine repeats (48F or 89F clones) led to a significant morphological change. As shown in Fig. 1B, both 48F-22 and 89F-21 clones exhibit epithelial-like morphology. They were well attached to the plate and therefore look much bigger than PC12 or 16F-1 cells. They also proliferated much faster, and the doubling time was about one-third that of wild-type PC12 cells.

It is known that in PC12 cells, stimulation of NGF and EGF receptors causes activation of MAPK, JNK, and Akt (17, 18). We next examined whether expression of full-length normal or polyglutamine-expanded Huntingtin affects NGF- or EGF-induced activation of these kinases. In wild-type PC12 cells stimulation with NGF or EGF led to an elevation of MAPK activity (Fig. 2A). In 16F-1 clones, stimulation with NGF or EGF also increased MAPK activation that was comparable with that in PC12 cells (Fig. 2A). In multiple 48F and 89F clones, NGF- or EGF-induced MAPK activity was reduced by 85–95%. This inhibition was not due to down-regulation of total MAPK because the amount of total MAPK in either 48F or 89F clones was similar to that in PC12 cells (Fig. 2A). Similarly, expression of normal Huntingtin did not alter JNK activation induced by NGF or EGF, although expression of the mutated Huntingtin with either 48 or 89 polyglutamine repeats significantly inhibited JNK activation. As shown in Fig. 2B, JNK activity induced by NGF or EGF was reduced by 80–90% in multiple 48F or 89F clones. Decrease in total JNK may partially account for inhibition of JNK activation by the mutated Huntingtin because the amount of total MAPK in either 48F or 89F clones was similar to that in PC12 cells (Fig. 2A). Similarly, expression of normal Huntingtin did not alter JNK activation induced by NGF or EGF, although expression of the mutated Huntingtin with either 48 or 89 polyglutamine repeats significantly inhibited JNK activation. As shown in Fig. 2B, JNK activity induced by NGF or EGF was reduced by 80–90% in multiple 48F or 89F clones. Decrease in total JNK may partially account for inhibition of JNK activation by the mutated Huntingtin because total JNK was reduced by ~30% in these 48F and 89F clones (Fig. 2B). In PC12 cells, stimulation with NGF or EGF caused an ~10-fold increase of Akt activity (Fig. 2C). In 16F-1 clones carrying normal Huntingtin, the basal level of Akt was increased by 2–3-fold and stimulation with NGF or EGF further increased Akt activity (Fig. 2C). In multiple 48F and 89F clones NGF- or EGF-induced Akt activation was significantly attenuated or nearly completely blocked (Fig. 2C). This inhibition of Akt activation by the mutated Huntingtin is not the result of a decrease of total Akt because the level of total Akt in these 48F or 89F clones is similar to that in PC12 cells.

In PC12 cells stimulation with NGF leads to activation and auto-phosphorylation of TrkA which, in turn, interacts with Src homology collagen-Grb2 and leads to activation of the Ras-dependent signaling pathway and p85 of PI 3-kinase and resultant activation of the Akt and Rac-JNK signaling pathways (17–19). To explore molecular mechanisms by which polyglutamine-expanded Huntingtin inhibits activation of MAPK, JNK, and Akt mediated by NGF or EGF receptors, we examined the association of Grb2 or p85 of PI 3-kinase with TrkA by co-immunoprecipitation. Stimulation with NGF significantly increased the association of GRB2 (Fig. 3A) and p85 (Fig. 3B) with TrkA in both PC12 cells and 16F-1 clones. In 48F-22 clones, the association of Grb2 or p85 with TrkA is significantly inhibited. This may explain why, in 48F clones, NGF-induced activation of MAPK, JNK, or Akt is suppressed. To explore why the mutated Huntingtin inhibits assembly of the TrkA signal-complex in 48F or 89F clones, data in this figure represent a typical experiment that has been repeated at least three times using both the same and different 16F, 48F, or 89F clones with similar results. A and B, PC12 cells and multiple 16F, 48F, or 89F clones were serum-starved overnight and stimulated with NGF. Cell lysates were precipitated with an anti-Grb2 (A) or anti-p85 antibody (B). The blots were probed with an anti-TrkA antibody. C, PC12 cells, multiple 16F, 48F, or 89F clones were serum-starved overnight and stimulated with NGF. The blot was probed with an anti-phosphotyrosine antibody (cell signaling). Tyrosine-phosphorylated TrkA (140K) are clearly seen in the blot. D, PC12 cells and multiple 16F, 48F, or 89F clones were serum-starved overnight and stimulated with NGF. Cell lysates were precipitated an anti-TrkA antibody, and the blot was probed with 437, an anti-Huntingtin antibody. HDIP, a positive control; PC12 cell lysates, immunoprecipitated (IP) with 437; NHtt, normal Huntingtin.
ing complexes, we examined whether stimulation with NGF induces tyrosine phosphorylation of TrkA. As shown in Fig. 3C, NGF induced tyrosine phosphorylation of TrkA in both PC12 and 16F-1 cells. In both 48F and 89F clones TrkA remains tyrosine-phosphorylated after serum starvation for 24 h. Stimulation with NGF did not cause a significant increase of the amount of tyrosine-phosphorylated TrkA (Fig. 3C). We also examined the potential association of Huntingtin with TrkA by co-immunoprecipitation. As shown in Fig. 3D, in both PC12 and 16F clones, normal Huntingtin was detectable in TrkA immunoprecipitates. In 48F and 89F clones, both normal and polyglutamine-expanded Huntingtin was not present in TrkA immunoprecipitates, suggesting that the mutated Huntingtin may act as a dominant negative mutant and inhibit normal Huntingtin to interact with TrkA signaling complex. These data are consistent with our previous observations that normal Huntingtin inhibits the mutated Huntingtin to bind to PSD-95 (16) and the dominant inherited phenotype of the disease.

It is known that activation of TrkA by NGF in PC12 cells induces neuronal differentiation (18, 19), so we next examined how expression of normal or polyglutamine-expanded Huntingtin influences NGF-induced neurite outgrowth. As shown in Fig. 4, treatment of PC12 cells with NGF for 4 days resulted in neurite outgrowth. In 16F clones treatment of NGF also induced neurites outgrowth, but the number of these neurites are fewer, and the length of these neurites are shorter. In both 48F and 89F clones, NGF failed to stop switch these cells from proliferation to neuronal differentiation, and both 48F and 89F cells appeared to be overgrown after 4 days (Fig. 4, middle panel). We separately plated 48F and 89F cells in a very low density. Again, both 48F and 89F clones failed to differentiate in the presence of NGF (Fig. 4, lower panel). These data are consistent with inhibition of NGF-induced activation of MAPK and Akt in these clones which, in turn, mediate neurite outgrowth in PC12 cells (18, 19).

DISCUSSION

In the present study, we demonstrated that expression of full-length polyglutamine-expanded Huntingtin, but not normal Huntingtin, disrupts cellular signaling mediated by NGF and EGF receptors in PC12 cells. NGF-induced neuronal differentiation was also blocked. Further studies show that the mutated Huntingtin may, constitutively, cause activation of these growth factor receptors and inhibit assembly of the receptor-associated signaling complex. These interesting phenotypes are not due to overexpression of the mutated Huntingtin because the level of expressed mutated proteins is equal to the level of endogenously expressed Huntingtin. In other words, these clones are “heterozygous” cells of HD. Also, these changes do not result from variation of different subclones of PC12 because we observed similar phenotypes in three different clones carrying the same gene. Because the only difference between 16F clones and 48F or 89F clones is the length of the polyglutamine repeat, these distinct phenotypes, observed in both 48F or 89F clones, are due to expansion of the polyglutamine repeat in Huntingtin.

Although some studies have suggested that a significantly lower incidence of a variety of cancers among HD patients may result from an apoptotic effect of polyglutamine-expanded Huntingtin (11), many studies have shown that mutated Huntington selectively induces apoptotic cell death of certain types of neurons; and many cells, including some neuronal cells, are resistant to toxicity mediated by the mutated protein (13, 20, 21). Because the lower incidence of cancer in HD patients is not restricted in one to two types of cancer but almost all cancer types investigated (11), it is very likely that a more general mechanism that regulates cell proliferation is involved. Without question, growth factor receptor-mediated cellular signaling, particularly the Ras-MAPK pathway, provides the most important and general mechanism for the regulation of cell proliferation in a mammalian system. Assembly of Grb2-signaling complexes is required for activation of the Ras-MAPK pathway mediated by many growth factor receptors (22). Because polyglutamine-expanded Huntington interferes with both NGF and EGF receptors and the inhibition occurs at assembly of signaling complexes associated with these activated receptors, it is possible that the mutated Huntingtin may also inhibit cellular signaling mediated by other receptors. If mutated Huntingtin attenuates cellular signaling, cell proliferation mediated by growth factor receptors would be inhibited as supported by our observation that NGF-mediated neurite
outgrowth is blocked in both 48F and 89F clones. Consequently, one would expect that the occurrence of a diversity of cancer might be reduced. Although 48F and 89F clones proliferate faster than PC12 cells and 16F clones, this change is very likely due to the fact that these cells attach much better to the plate than both PC12 and 16F cells, and cell attachment is an important factor to control the growth rate of cultured cells.

The PI-3-Akt pathway plays a critical role in controlling glucose metabolism by insulin (24). Although the current study did not directly examine effects on insulin receptors, it is generally believed that the PI 3-kinase pathway is common to many growth factor receptors (25). Our observations, therefore, may signify an effect on insulin-mediated glucose metabolism. Deficiency in glucose metabolism is widely reported (9, 10) in HD patients, and all HD patients lose a considerable amount of body weight which cannot be explained by other clinical manifestations of HD (26).

Inhibition of the PI-3-Akt pathway would explain why glucose utilization and fat storage are inhibited in HD patients; however, further studies are needed to evaluate whether the mutated Huntingtin affects cellular signaling and function mediated by the insulin receptor family. Akt is also an important survival signal in neurons (27), and it is well known that inhibition of Akt activation promotes neuronal death (14, 27). Thus, inhibition of the PI-3-Akt pathway may represent an important pathological change in HD and also requires further examination.

Huntingtin is a ubiquitously expressed protein (4, 5), and dysfunction of Huntingtin may not only cause pathological changes in the central nervous system but may also result in changes in peripheral tissues. Because most peripheral tissues are capable of regeneration, degenerative features may only be found in the central nervous system. To understand the pathogenesis of HD, one has to investigate how polyglutamine expansion affects the normal function of Huntingtin. In our current study, we demonstrate that polyglutamine-expanded Huntingtin inhibits growth factor receptor-mediated cellular signaling and biological function. Because these actions are highly related to many clinical manifestations in HD, our data may reflect an important molecular mechanism underlying the pathogenesis of HD.

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