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The IGF-1/Akt Pathway Is Neuroprotective in Huntington's Disease and Involves Huntingtin Phosphorylation by Akt

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Summary

In the search for neuroprotective factors in Huntington's disease, we found that insulin growth factor 1 via activation of the serine/threonine kinase Akt/PKB is able to inhibit neuronal death specifically induced by mutant huntingtin containing an expanded polyglutamine stretch. The IGF-1/Akt pathway has a dual effect on huntingtin-induced toxicity, since activation of this pathway also results in a decrease in the formation of intranuclear inclusions of mutant huntingtin. We demonstrate that huntingtin is a substrate of Akt and that phosphorylation of huntingtin by Akt is crucial to mediate the neuroprotective effects of IGF-1. Finally, we show that Akt is altered in Huntington's disease patients. Taken together, these results support a potential role of the Akt pathway in Huntington's disease.

Introduction

Huntington's disease (HD) is a neurodegenerative disorder characterized by involuntary movements, personality changes, and dementia. The neuropathology of HD involves a selective death of medium spiny neurons of the striatum. The mutation that causes disease is an abnormal expansion of a polyglutamine (polyQ) stretch in the N terminus of the 350 kDa protein huntingtin, and a strong inverse correlation exists between the age at onset in patients and the number of glutamines (Gusella and MacDonald, 2000).

The mechanism by which mutant huntingtin induces selective death of striatal neurons is not well understood. Several studies have revealed a series of events that could ultimately lead to the death of neurons in the striatum (for a review, see Humbert and Saudou, 2001). When huntingtin contains an expanded polyQ stretch, it localizes and aggregates in the nucleus as intranuclear inclusions that are formed of N-terminal fragments of huntingtin and ubiquitin (Davies et al., 1997; DiFiglia et al., 1997). Nuclear localization of mutant huntingtin is critical to inducing apoptosis (Saudou et al., 1998; Peters et al., 1999) by possibly altering transcription (for a review, see Cha, 2000). Intranuclear inclusions may not be causative of neuronal death (Klement et al., 1998; Saudou et al., 1998; Cummings et al., 1999; Simeoni et al., 2000), but could still have deleterious effects for the neurons by inducing dysfunction (Davies et al., 1997). In addition, huntingtin could mediate some toxicity in the cytoplasm through interaction with proteins such as HAP-1 or HIP-1 (Li et al., 1995; Hackam et al., 2000) and/or the formation of neuropil aggregates (Li et al., 2000a).

Currently, there is no treatment to prevent or delay the appearance and progression of symptoms or the death of HD patients. Several studies have identified compounds that might be of therapeutic interest in HD: creatine, histone deacetylase inhibitors, minocycline, and antiapoptotic proteins or compounds (Saudou et al., 1998; Chen et al., 2000; Andreassen et al., 2001; Steffan et al., 2001). Because of their ability to promote survival, neurotrophic factors have also been considered as potential therapeutic agents in neurodegenerative disorders. For example, we have shown using an in vitro neuronal model of HD that CNTF and BDNF block polyQ-huntingtin-induced cell death (Saudou et al., 1998). In vivo, CNTF has also been shown to be neuroprotective in rats and monkeys following excitotoxic lesions that reproduce HD (for a review, see Brouillet et al., 1999). One other interesting growth factor is insulin growth factor 1 (IGF-1), which was shown to have neuroprotective effect in several models (for a review, see Dore et al., 2000) and could be of interest in HD (Alexi et al., 1999). The ability of IGF-1 to promote cell survival has been attributed in part to the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) pathway. Akt, a serine/threonine kinase also known as protein kinase B (PKB) is a potent survival kinase that exerts its effects by acting on key substrates such as components of cell death machinery or transcription factors (for a review, see Brunet et al., 2001).

In this report, we show that IGF-1 and Akt inhibit mutant huntingtin-induced cell death and formation of intranuclear inclusions of polyQ-huntingtin. In addition, we demonstrate that huntingtin phosphorylation by Akt at serine 421 regulates mutant huntingtin-induced toxicity in primary cultures of striatal neurons. These findings reveal that the specific neuroprotective effect of the IGF-1/Akt pathway in HD is mediated by direct phosphorylation of huntingtin. Finally, we present evidence that Akt is altered in HD patients, which further supports a role of the IGF-1/Akt pathway in the context of HD.

Results

IGF-1 Inhibits Mutant Huntingtin-Induced Cell Death and Intranuclear Inclusions

Previously, we have shown that BDNF and CNTF inhibit mutant huntingtin-induced cell death, but lead to an

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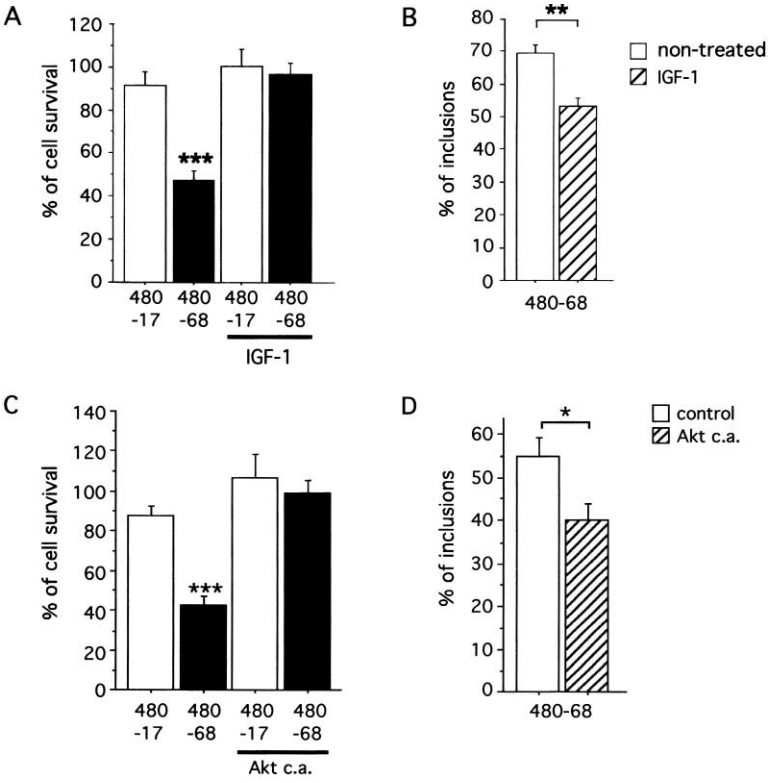

Figure 1. IGF-1 Treatment and Akt Inhibit Mutant Huntingtin-Induced Cell Death and Intranuclear Inclusion Formation

(A) Striatal neurons transfected with 480-17/68 constructs are treated with IGF-1. Data from three independent experiments (ANOVA; $F_{[1,23]} = 17.54$; $P < 0.0001$) reveal a statistically significant decrease in survival induced by 480-68 compared to 480-17 without treatment ($t_{[12]} = 5.97$; $P < 0.0001$) but not with IGF-1 treatment ($t_{[11]} = 0.38$; NS). Death induced by 480-68 without treatment is significantly different from all the other cases (Fisher's analysis: ***$P < 0.0001$).

(B) Data from three independent transfections reveal a significant decrease in the percentage of cells with intranuclear inclusions induced by the 480-68 construct when treated with IGF-1 ($t_{[11]} = 4.49$; **$P < 0.001$).

(C) 480-17/68 constructs cotransfected with Akt c.a. or with the corresponding empty vector. Data from two independent transfections (ANOVA; $F_{[1,26]} = 16.79$; $P < 0.0001$) reveal a statistically significant decrease in survival induced by 480-68 compared to 480-17 when transfected with empty vector ($t_{[14]} = 6.53$; $P < 0.0001$) but not when transfected with Akt c.a. ($t_{[12]} = 0.56$; NS). Death induced by 480-68 with the empty vector is significantly different from all the other cases (Fisher's analysis: ***$P < 0.0001$).

(D) Data from four independent transfections reveal that the percentage of striatal neurons exhibiting intranuclear inclusions is significantly reduced by Akt c.a. ($t_{[18]} = 2.56$; *$P < 0.05$).

increase in the formation of intranuclear inclusions (Saudou et al., 1998). Here, we tested the effect of another neurotrophic factor, IGF-1, on striatal neurons expressing mutant huntingtin. As expected, the 480 amino acid fragment that contains a mutant stretch of 68 glutamines (480-68) induces a statistically significant decrease in cell survival compared to the wild-type 480-17 construct. Interestingly, treatment with IGF-1 completely blocks mutant huntingtin-induced death of striatal neurons (Figure 1A) but, in contrast to BDNF and CNTF treatments, leads to a reduction in the formation of intranuclear inclusions (Figure 1B). Although IGF-1 is effective on the toxicity induced by an N-terminal fragment of mutant huntingtin, similar results were obtained with the physiological form of huntingtin (see Supplemental Figure S1 at http://www.developmentalcell.com/cgi/content/full/2/6/831/DC1). Taken together, these results show that IGF-1, in addition to inhibiting mutant huntingtin-induced cell death, leads to a decrease in the formation of intranuclear inclusions.

Akt Mediates the Neuroprotective Effect Elicited by IGF-1

We next examined signaling pathways by which IGF-1 is known to suppress apoptosis and found that in striatal neurons, treatment with IGF-1 induces specific activation of Akt through the PI3K pathway (see Supplemental Figure S2). We tested whether the neuroprotective effect of IGF-1 observed in our HD model could be attributed, at least in part, to Akt activation. By cotransfection experiments, we found that Akt completely inhibits neuronal death induced by the 480-68 construct (Figure 1C) and leads to a decrease in the formation of intranuclear inclusions (Figure 1D). Although we could not evaluate the effect of PI3K inhibition on mutant huntingtin-induced cell death due to the high toxicity of the PI3K inhibitor LY in striatal neurons, we found an increase in the percentage of striatal neurons containing intranuclear inclusions when treated with subtoxic doses of LY (100 nM; data not shown). Taken together, these findings indicate that in striatal neurons, IGF-1, through the activation of the PI3K/Akt pathway, is able to block mutant huntingtin-induced cell death and reduces the formation of intranuclear inclusions.

Akt Phosphorylates Huntingtin In Vitro

Akt could either block mutant huntingtin-induced cell death and intranuclear inclusion formation by activating survival signals (Brunet et al., 2001) or by directly acting on huntingtin protein. We analyzed the sequences of huntingtin proteins in various species for the presence of an Akt consensus phosphorylation site (Alessi et al., 1996) and found a putative site conserved between species that we refer to as S421 (serine at position 421 in the human huntingtin with 23 glutamines).

We first asked whether Akt is able to phosphorylate huntingtin within the 480 amino acid N-terminal fragments that were used to show the neuroprotective effect of IGF-1/Akt. We found that an active form of Akt (Akt c.a.), but not an inactive form of Akt (Akt k.n.; Datta et al., 1997), is able to phosphorylate the 480-17/68 fragments of huntingtin (Figure 2A). To demonstrate that S421 in the human huntingtin is phosphorylated by Akt,

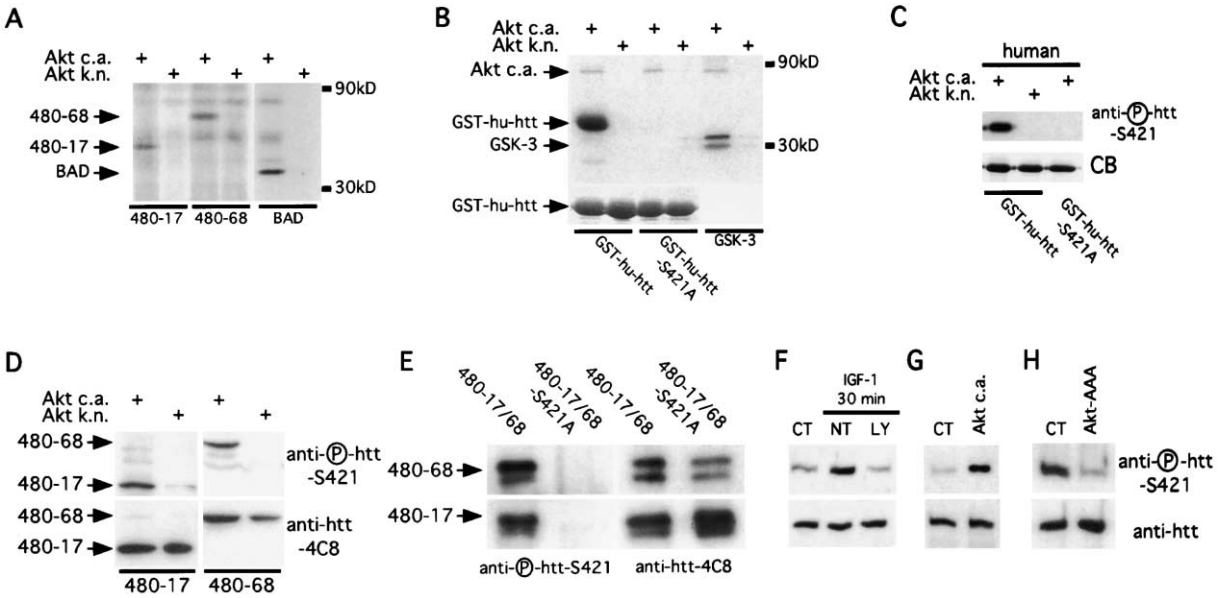

Figure 2. Akt Phosphorylates Huntingtin at S421 In Vitro and In Vivo

(A) Akt phosphorylates the 480-17/68 forms of huntingtin. Huntingtin constructs were produced in 293T cells, immunoprecipitated using 4C8 antibody, and incubated with Akt c.a. or k.n. and [γ-^{32}P]ATP.

(B) In vitro kinase assays were performed using Akt c.a. or k.n. and 4 μg of GST-fused proteins as substrates. GST-hu-htt corresponds to GST-fused human huntingtin (amino acids 384–467). The reaction products were resolved by SDS-PAGE, the gel was stained with Coomassie blue (CB, lower panel), and the ^{32}P-labeled proteins were visualized by autoradiography (upper panel). Recombinant BAD (1 μg) or GSK-3 fusion protein (1 μg; Cell Signaling Technology) were used as positive controls of Akt phosphorylation.

(C) Samples obtained as described in (B) were analyzed with anti-P-htt-S421. For immunoblotting, 1/100 of the kinase samples was loaded, whereas for CB, 1/4 of the kinase reaction was loaded.

(D and E) 293T cells were transfected with various 480-17/68 constructs and Akt c.a. or k.n. Whole-cell extracts were analyzed with the anti-P-htt-S421 antibody or an anti-htt antibody.

(F) IGF-1 leads to phosphorylation of endogenous huntingtin in a PI3K-dependent manner. SH-SY5Y cells were serum starved (48 hr), incubated with 10 μM LY 294002 (1 hr), and stimulated with 50 ng/ml IGF-1 (30 min). Immunoblotting experiments were performed using the anti-P-htt-S421 antibody and an anti-htt antibody (2E8). CT, no IGF-1 treatment; NT, no LY treatment prior to IGF-1 stimulation.

(G and H) Whole-cell extracts were analyzed as in (F).

(G) Akt phosphorylates endogenous huntingtin. 293T cells were transfected with an empty vector (CT) or Akt c.a. and serum starved for 18 hr prior to lysis.

(H) Endogenous huntingtin phosphorylation is reduced by a dominant interfering form of Akt. 293T cells were transfected with an empty vector (CT) or Akt-AAA. Transfected cells were selected by FACS.

we generated huntingtin GST-fused proteins containing either an intact S421 or a serine to alanine mutation at this site. As shown in Figure 2B, GST-hu-htt is phosphorylated, while GST-hu-htt-S421A is no longer detectably phosphorylated by Akt. This shows that S421 is a site of phosphorylation by Akt in huntingtin.

To unequivocally identify S421 as an Akt site, we raised a polyclonal antibody that specifically recognizes huntingtin phosphorylated at S421. As shown in Figure 2C, the antibody to phosphorylated huntingtin S421 (anti-P-htt-S421) recognizes GST-hu-htt that was incubated with Akt c.a. but fails to recognize nonphosphorylated GST-hu-htt or GST-hu-htt-S421A. These results demonstrate that anti-P-htt-S421 specifically recognizes huntingtin when phosphorylated at S421 and that Akt phosphorylates huntingtin at S421 in vitro. Similar results were obtained with the mouse huntingtin sequences (see Supplemental Figure S3).

Akt Phosphorylates Huntingtin In Vivo

To determine whether Akt phosphorylation of huntingtin at S421 occurs in cells, we cotransfected 293T cells with 480-17 or 480-68 with Akt c.a. or k.n. We found that Akt c.a. induces a phosphorylation of huntingtin at S421 (Figure 2D), indicating that active Akt alone is sufficient to induce the phosphorylation of huntingtin at S421. We then generated versions of 480-17/68 constructs with an S421A mutation and expressed them in 293T cells with Akt c.a. (Figure 2E). Levels of huntingtin were monitored using the 4C8 antibody, revealing no differences in expression due to the S421A mutation. The anti-P-htt-S421 recognizes 480-17/68 fragments of huntingtin but fails to recognize the proteins with the S421A mutation. Thus, we demonstrate that Akt phosphorylates the 480-17/68 forms of huntingtin in 293T cells at S421.

To test whether endogenous huntingtin becomes phosphorylated in response to IGF-1, we exposed human neuroblastoma SH-SY5Y cells to IGF-1. This treatment induces phosphorylation of huntingtin at S421 as revealed by anti-P-htt-S421 while the total level of huntingtin protein remained unchanged (Figure 2F). Phosphorylation of huntingtin was reduced when cells were treated with the PI3K inhibitor LY. We found that the

expression of Akt c.a. induces phosphorylation of endogenous huntingtin at S421 (Figure 2G), demonstrating that the observed phosphorylation of S421 in endogenous huntingtin can be due to Akt. We then asked whether the phosphorylation of endogenous huntingtin at S421 can be reduced by the presence of a dominant interfering form of Akt, Akt-AAA (Wang et al., 1999). 293T cells were transfected with GFP and Akt-AAA (1:20 ratio). To increase the percentage of cells expressing the Akt-AAA construct, GFP-positive cells were selected by FACS (Figure 2H). In contrast to FACS-sorted cells transfected with an empty vector, cells expressing Akt-AAA show reduced phosphorylation of endogenous huntingtin at S421. Altogether, our findings indicate that in cells, phosphorylation of endogenous human huntingtin is induced by IGF-1 through a PI3K-dependent mechanism and that huntingtin is a physiological substrate of Akt.

To determine whether phosphorylation of huntingtin at S421 occurs in the neuronal model in which mutant huntingtin-induced cell death is blocked by IGF-1, we analyzed the status of huntingtin phosphorylation at S421 after IGF-1 treatment (Figure 3A). Whereas the anti-P-htt-S421 antibody stains neurons transfected with the 480-17/68 constructs, the S421A mutation abolished the anti-P-htt-S421 staining. The S421A mutants of huntingtin are localized in the cytoplasm and neurites of striatal neurons as for wild-type and polyQ-huntingtin. Taken together, these data demonstrate that in 293T cells, human neuroblastoma cells, and striatal neurons, IGF-1 induces phosphorylation of huntingtin at S421.

Mutant Huntingtin-Induced Toxicity Is Regulated by Phosphorylation at S421

To analyze the biological relevance of huntingtin S421 phosphorylation, we tested the effect of the S421A mutation in huntingtin on cell survival. We found that the 480-68 and 480-68-S421A constructs elicit similar loss of survival in striatal neurons (Figure 3B). We next compared the ability of IGF-1 to increase the survival of neurons expressing the 480-68 constructs with or without the S421A mutation. Interestingly, while IGF-1 is able to completely inhibit death induced by the 480-68 construct, survival was not fully recovered by IGF-1 treatment with the 480-68-S421A construct. This result demonstrates that the complete inhibition of mutant huntingtin-induced cell death by IGF-1 requires the presence of an intact S421 in huntingtin protein. It is worthwhile to note that we observed an increase in the survival of neurons expressing the 480-68-S421A after IGF-1 treatment (Figure 3B). This indicates that IGF-1, in addition to promoting survival through the phosphorylation of huntingtin at serine 421, elicits some neuroprotection against mutant huntingtin by other mechanisms.

We next asked whether constitutive phosphorylation of huntingtin at S421 is able to reduce toxicity of mutant huntingtin. To test this hypothesis, we generated a 480-68 construct in which serine 421 is changed into an aspartic acid to mimic constitutive phosphorylation. With this S421D mutation, we found that polyQ-huntingtin is no longer able to induce neuronal death (Figure 3C) and leads to a reduction in the percentage of neurons containing intranuclear inclusions (Figure 3D). The effect is not due to differences in expression levels, since the 480-17/68-S421D are expressed in 293T cells and neurons at similar levels as the 480-17/68 constructs (data not shown).

Taken together, these results indicate that phosphorylation of huntingtin at S421 is critical to regulating mutant huntingtin-induced cell death and intranuclear inclusion formation.

Full-Length Akt Appears as a Shorter Form in the Striatum of HD Patients

Since we show that the IGF-1/Akt pathway blocks mutant huntingtin-induced toxicity, we examined the level of Akt in brain samples from HD patients. It is well established that in HD there is a selective atrophy of the brain, with the striatum being affected the most severely. Shrinkage and loss of neurons in the cerebral cortex are also observed but to a lesser extent, while the cerebellum is usually unaffected. We analyzed by anti-Akt immunoblotting human samples from control (CT) and HD grade 4 (HD4) and grade 3 (HD3) patients from three regions of the brain: cerebellum, cortex, and striatum (Figure 4). Anti-β-actin antibody is used as a control for protein levels. Interestingly, in several human brain samples, Akt appears as two species with relative molecular masses (M_r) of 60 and 49 kDa. These two species have already been characterized as Akt full-length (M_r, 60 kDa) and a caspase-3-generated Akt product (M_r, 49 kDa; Bachelder et al., 1999; Francois and Grimes, 1999; Rokudai et al., 2000). In the CT samples, from the cerebellum, cortex, and striatum, the lower Akt species is absent, except for sample 3 where a weak signal is observed in the cortex. In contrast, in HD patients the shorter Akt product is observed in the cerebellum for HD samples 5–9 and in the cortex for HD samples 4 and 6–8. More strikingly, in the striatum, the two HD samples show a high accumulation of the lower species in comparison to the CT samples, where the 49 kDa species is totally absent. Furthermore, in the striatum of HD patients, full-length Akt is either low (sample 5) or almost undetectable (sample 9; a weak band appears after overexposure; data not shown). We could not detect phosphorylated forms of huntingtin and Akt in brain samples, probably due to postmortem intervals (data not shown).

In conclusion, we observed a pronounced alteration of Akt in the most affected brain region of HD patients, the striatum. These observations further support a potential role of Akt in HD.

Discussion

We show here that phosphorylation of S421 in polyQ-huntingtin abrogates its proapoptotic activity. The absence of phosphorylation at S421 or its constitutive phosphorylation do not appear to affect the level of expression or the subcellular localization of huntingtin. Further, phosphorylation of huntingtin at S421 does not modify its ability to be ubiquitinated or degraded (data not shown). In addition, we do not observe any effects of the phosphorylation at S421 on the cleavage of huntingtin by calpain or caspases -1 and -3 (data not shown). Huntingtin interacts with several proteins (Humbert and Saudou, 2001). It is possible that phosphorylation alters

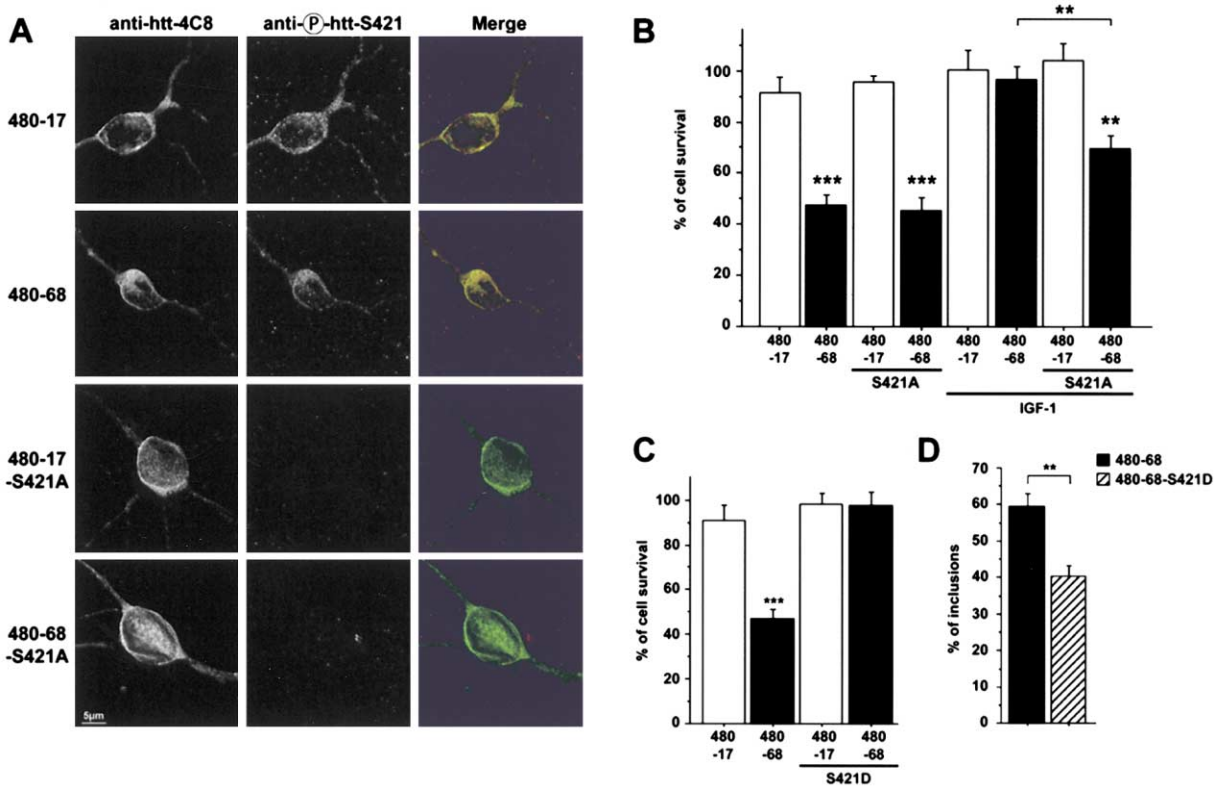

Figure 3. Phosphorylation of S421 Regulates Mutant Huntingtin-Induced Toxicity

(A) Huntingtin is phosphorylated at S421 in striatal neurons. Striatal neurons were transfected, treated with IGF-1 (50 ng/ml; 1 hr), fixed, immunostained with the 4C8 and anti-P-htt-S421 antibodies, and analyzed by deconvolution microscopy.

(B) IGF-1 suppresses mutant huntingtin-mediated cell death in an S421 phosphorylation-dependent manner. Data from three independent experiments (ANOVA; $F_{[3, 45]} = 18.44$; $P < 0.0001$) reveal that survival induced by IGF-1 on the 480-68-S421A construct is significantly lower than the survival induced by IGF-1 on the 480-68 construct ($t_{[12]} = 3.53$; $^{**}P < 0.01$) and, significantly different from all other cases (Fisher's analysis: $^{**}P < 0.01$). Death induced by the 480-68 and 480-68-S421A constructs without treatment is significantly different from all the other cases (Fisher's analysis: $^{***}P < 0.0001$).

(C) S421D mutation of huntingtin abolishes polyQ-dependent cell death. Data from three independent experiments (ANOVA; $F_{[3, 26]} = 23.97$; $P < 0.0001$) reveal that survival induced by the 480-68-S421D construct is significantly higher than the survival induced by the 480-68 construct ($t_{[13]} = 7.48$; $P < 0.0001$) but not from the survival elicited by the 480-17 construct ($t_{[13]} = 0.86$; NS) or the 480-17-S421D construct ($t_{[14]} = 0.057$; NS). Death induced by the 480-68 construct is significantly different from all the other cases (Fisher's analysis: $^{***}P < 0.0001$).

(D) Data from two independent transfections revealed that the percentage of intranuclear inclusions formed by the 480-68-S421D is lower than for the 480-68 construct ($t_{[8]} = 4.55$; $^{**}P = 0.002$).

the ability of huntingtin to interact with a yet to be identified protein.

Although we find that the complete neuroprotective effect mediated by IGF-1 requires phosphorylation of mutant huntingtin at S421, in the absence of phosphorylation of huntingtin, IGF-1 is still able to induce some neuroprotection. This suggests that in the context of mutant huntingtin-induced cell death, IGF-1 mediates its neuroprotective effect not only via a direct action of Akt on huntingtin protein but also via the phosphorylation of other substrates that increase neuronal survival. Several substrates of Akt such as Bad, FOXOs, and caspase-9 that promote neuronal survival when phosphorylated by Akt have been described (Brunet et al., 2001). Those substrates, as well as others that remain to be identified, could also participate in mediating the neuroprotective effect elicited by IGF-1 and Akt on mutant huntingtin-induced cell death.

Expansion of CAG trinucleotide repeats in otherwise unrelated proteins is known to be the cause of at least nine neurodegenerative disorders, including HD. Our experiments demonstrate that toxicity of mutant huntingtin can be inhibited by direct phosphorylation and raise the possibility that phosphorylation by Akt could also abrogate the toxicity of other proteins containing an expanded polyQ tract. One interesting candidate is the androgen receptor whose CAG expansion results in spinal and bulbar muscular atrophy (SBMA) or Kennedy's disease (Fischbeck et al., 1999). Indeed, the androgen receptor has recently been shown to be a substrate of Akt (Lin et al., 2001). However, it is not known whether this phosphorylation plays a role in the toxicity induced by the mutant polyQ androgen receptor. By scanning proteins that contain an expanded polyQ stretch and that are responsible for this class of neurodegenerative disorders, we found, for example, that ataxin-1, the protein responsible for spinal and cerebellar ataxia type 1, contains a putative phosphorylation site for Akt (data not shown). If phosphorylation by Akt inhibits death induced by mutant ataxin-1, together with this study, we

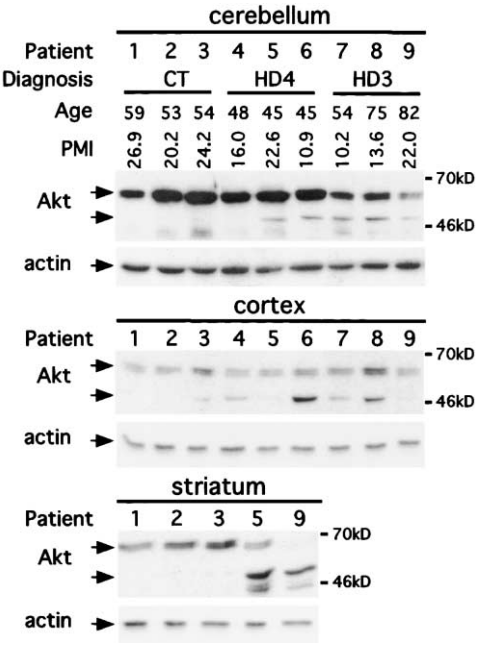

Figure 4. Akt Appears as a Shorter Form in Huntington's Disease Patients

Protein extracts were prepared from cerebellar, cortical (Brodmann area 9), or striatal postmortem samples, resolved by SDS-PAGE, and immunoblotted with anti-Akt or anti-β-actin antibodies. Brain tissues are from control (CT; samples 1–3) and HD grade 4 (HD4; samples 4–6) and grade 3 (HD3; samples 7–9) patients. PMI (hr), postmortem interval.

would have evidence for a common mechanism of neuroprotection in polyQ tract neurodegenerative disorders.

Features of apoptosis within the degenerating neurons are observed in postmortem brains from HD patients (Portera-Cailliau et al., 1995). Activation of apoptosis has also been reported in mouse and cellular models of HD and could be associated with an activation of caspases (Reddy et al., 1998; Saudou et al., 1998; Hackam et al., 2000; Li et al., 2000b). Caspases cleave specific target proteins during apoptotic death including signaling molecules that promote cell survival. In particular, Akt has been shown to be negatively regulated by apoptotic signaling pathways in vivo (Widmann et al., 1998; Bachelder et al., 1999). In vitro, Akt is cleaved by caspase-3, resulting in the generation of a shorter fragment of Akt (M_r, 49 kDa; Bachelder et al., 1999; Francois and Grimes, 1999; Rokudai et al., 2000). Interestingly, we observe a high accumulation of a 49 kDa species of Akt in the striatum of HD patients. One could hypothesize that in HD patients, this short product is the result of caspase cleavage. This cleavage may subsequently lead to inactivation of Akt as previously reported in other systems (Bachelder et al., 1999; Francois and Grimes, 1999; Rokudai et al., 2000).

By studying the mechanism of neuroprotection elicited by the IGF-1/Akt pathway in the context of HD, we have revealed a new mechanism by which Akt is able to counteract the proapoptotic properties of mutant huntingtin. Understanding the molecular basis of the inhibition of mutant huntingtin-induced toxicity by Akt as well as developing drugs that activate and/or prevent alteration of the Akt pathway could possibly lead to new therapeutic interventions in HD.

Experimental Procedures

Descriptions of constructs, cell culture, transfection, and protein extracts may be found as supplemental data at http://www.developmentalcell.com/cgi/content/full/2/6/831/DC1.

Huntingtin Phospho-Antibody S421

A phosphopeptide of the following sequence was synthesized: CSGGRSRSGpSIVELI, and coupled to KLH (Neosystem, Illkirch, France). A polyclonal antibody to the KLH-coupled peptide was obtained and affinity purified as previously described (Neosystem; Datta et al., 1997).

Brain Tissues

Tissues were obtained from the Harvard Brain Tissue Resource Center as follows: three controls (samples 1–3), three HD grade 4 (samples 4–6), and three grade 3 (samples 7–9) patients. Samples 1–9 correspond, respectively, to brain numbers 4741, 4744, 4751, 4680, 4740, 4798, 4719, 4754, and 4797 as numbered by the Harvard Brain Tissue Resource Center.

Immunocytochemistry and Immunoblotting

Antibodies used include anti-huntingtin 4C8 and 2E8 (ICC, 1:500; WB, 1:5000; Trottier et al., 1995), anti-phospho-huntingtin-S421 (ICC, 1:100; WB, 1:200), anti-ubiquitin (1:100; DAKO), anti-Akt (1:1000; New England Biolabs), anti-phospho-Akt Ser473 (1:1000; New England Biolabs), and monoclonal anti-β-actin AC15 (1:5000; Sigma).

Measurement of Neuronal Survival and Intranuclear Inclusions

For survival assays, striatal neurons were transfected with the plasmids of interest and the CMV-GFP plasmid (6:1 ratio). Twelve hours and 2 days after transfection, GFP-positive neurons were scored under fluorescence microscopy in a blinded manner. For intranuclear inclusion scoring, transfected and immunostained neurons 6 days after transfection were analyzed for the presence of ubiquitin-positive intranuclear inclusions in a blinded manner. Each graph represents two to three independent experiments performed in triplicates. Data are expressed as the mean value ± SEM. Each bar in a given graph corresponds to the scoring of about 2000 neurons in cell death experiments and to 500 neurons for inclusions scoring.

Kinase Assays

293T cells were transfected with HA-Akt vectors. Twenty-four hours after transfection, extracts were obtained and kinase assays were performed as described (Datta et al., 1997).

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