**ARTICLE**

**Dentatorubral-pallidoluysian atrophy protein interacts through a proline-rich region near polyglutamine with the SH3 domain of an insulin receptor tyrosine kinase substrate**

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Dentatorubral-pallidoluysian atrophy (DRPLA) is an autosomal dominant neurodegenerative disorder associated with CAG/glutamine repeat expansion. While the DRPLA gene is ubiquitously expressed, neuron death occurs in specific anatomical areas of the brain. This predicts that the DRPLA protein interacts with other proteins and that these interactions may play a role in pathogenesis. Here, we describe a protein that binds to the DRPLA product. One of the clones isolated with a yeast two-hybrid system was identified as a human homolog of the insulin receptor tyrosine kinase substrate protein of 53 kDa (IRSp53). The gene produced two mRNA forms by differential splicing and encoded 552 and 521 amino acids, respectively. The longer form was mainly expressed in the brain and the shorter one in other tissues. The products were phosphorylated upon stimulation of cultured cells with insulin or insulin-like growth factor 1. Binding of the DRPLA protein to IRSp53 was ascertained by co-immunoprecipitation with antibodies and also by co-localization in perinuclear oval dots in cells expressing engineered constructs. A proline-rich region near the polyglutamine tract of the DRPLA protein and the SH3 domain of IRSp53 were involved in the binding. An extended polyglutamine tract significantly reduced binding ability in yeast cells, but not in in vitro binding assays. The identification of IRSp53 and other proteins detected by the yeast hybrid system predicts that DRPLA functions in a signal transduction pathway coupled with insulin/IGF-1.

**INTRODUCTION**

Dentatorubral-pallidoluysian atrophy (DRPLA) is an autosomal dominant, progressive neurodegenerative disorder characterized by selective neuron death in the dentatofugal and pallidofugal pathways (1). It has been shown that DRPLA is caused by CAG repeat expansion (2,3). The number of CAG repeats in the DRPLA gene is highly polymorphic in the normal population with a range of 7–23, increasing to 49–88 in patients (2,4). The gene is localized to chromosome band 12p13.31 and encodes a protein of an apparent molecular size of 160 kDa (the DRPLA protein) in which the CAG repeat is translated into a polyglutamine tract (5–8). The gene is ubiquitously expressed and the levels of mRNA and protein expression are not affected by the repeat expansion (5,6,9).

In addition to DRPLA, a CAG/glutamine repeat expansion has been detected in seven other inherited neurodegenerative disorders including spinal and bulbar muscular atrophy (SBMA), Huntington’s disease (HD) and spinocerebellar ataxia (SCA) types 1–3, 6 and 7 (10–16). These disorders exhibit characteristic phenotypes resulting from dysfunction and neuron death in respective regions in the central nervous system, but each responsible gene is fundamentally expressed in ubiquitous tissues. A recent study has demonstrated that overexpression of a relatively short peptide containing an extended polyglutamine tract induces apoptosis in cultured cells, while overexpression of an intact gene product carrying the same extended polyglutamine tract does not (17). As predicted based on the anti-parallel β-sheet structure of synthetic polyglutamine tracts (18), short peptides with an extended polyglutamine tract form large, poorly soluble aggregates after expression in cultured cells. Nuclear inclusion bodies found in affected brains of patients and mice transgenic for HD, SCA1, SCA3 and DRPLA seem to be consistent with aggregate formation in such experimental systems (19–21). Most of the products for CAG repeat diseases have been shown to be cleaved by caspases during various apoptotic processes (8,22,23) and cleaved products tend to form aggregates if they...
carry an extended polyglutamine tract. These results may account for neuron death by an extended polyglutamine tract; however, little is known about the regional specificity of neuron death. Studies on associated proteins are useful to elucidate the pathogenesis of selective neuron death as well as the normal functions of the responsible genes since none has been identified except for the androgen receptor for SBMA (10) and a calcium channel subunit for SCA6 (15).

Here we report that the DRPLA protein interacts with a substrate of insulin receptor tyrosine kinase. We obtained several clones after screening libraries with the DRPLA protein as bait in a yeast two-hybrid system and one of the clones was identified to be a human homolog of a 53 kDa substrate of the insulin receptor tyrosine kinase (IRSp53). Its hamster homolog has been detected by screening for substrates of the insulin and insulin-like growth factor 1 (IGF-1) receptors with a monoclonal antibody (24). We characterized human IRSp53 and studied its interaction with DRPLA protein with respect to binding regions and effects of an extended polyglutamine tract. Since IGF-1 is known to be a trophic or anti-apoptotic factor in the brain (25–26), our finding is also relevant to the elucidation of IGF-1 signaling pathways in the brain.

**RESULTS**

**Isolation of DRPLA-interacting proteins using the yeast two-hybrid system**

Almost the entire coding region, but without its N-terminal portion, of the normal DRPLA protein was used in screening with the yeast two-hybrid system. A cDNA sequence carrying 10 CAG repeats was fused in-frame with the GAL4 DNA-binding domain to generate pBD35–1185, which carried amino acid residues 335–1185 of the DRPLA protein including a polyglutamine tract (Fig. 1A). We screened a human fetal brain Matchmaker cDNA library, which consisted of clones carrying cDNA fused in-frame with the GAL4 activation domain (AD). We finally isolated 30 His⁺/LacZ⁺ clones out of $1.2 \times 10^7$ Trp⁺/Leu⁺ auxotrophic transformants. The insert size of these isolated clones ranged from 0.5 to 3.5 kb. When the 30 clones were sequenced, seven were found to overlap and turned out to be a human homolog of a 53 kDa substrate of insulin receptor tyrosine kinase (IRSp53) by sequence homology to the previously reported IRSp53 of Chinese hamster (24). Others included proteins functioning in the signal trans-
duction pathway from insulin and Wnt, namely hDVL1, δ-catenin and the 14-3-3 protein homolog (27–30), and also nuclear proteins containing a zinc finger motif. Here we further characterize human IRSp53.

Characterization of the human IRSp53 gene

The longest insert of the isolated IRSp53 clones (clone 274) consisted of 2877 nt, with the exception of the AD portion and a poly(A) tail. By single path sequencing, two other isolated clones seemed to have the identical sequence and one carried nucleotides 1–2831. In contrast, the remaining three clones carried 2033 nt, besides the AD and poly(A) tail, in which the nucleotide sequence of nt 1–1628 was completely identical to that of clone 274. The open reading frame in the longer form started with ATG at nt 94, ended at nt 1749 and encoded 552 amino acid residues, while that in the shorter form started at the same position, ended at nt 1656 and encoded 521 amino acids (Fig. 2A). When the amino acid sequences were aligned with the reported hamster IRSp53 sequence, the shorter form resembled it in size and sequence; 94% of residues were identical after two spaces were inserted. Three putative tyrosine phosphorylation sites appeared in the N-terminal portion and an SH3 domain similar to those of human proteins VAV2 and yeast BOB1 (24) localized to positions 407–453 of the amino acid sequence (Fig. 2B).

Northern blotting using a probe common to the longer and shorter forms of human IRSp53 cDNA visualized a 3.0 kb transcript expressed in most tissues examined as well as another 3.8 kb transcript mainly expressed in the brain (Fig. 2C). Another probe, which was unique to the longer form of cDNA, predominantly hybridized with the 3.8 kb transcript (Fig. 2D). The size of the two transcripts differed by 0.8 kb, which accords with the size difference of the 3'-portion of the two cDNA forms. Thus, the two forms seem to be produced by alternative splicing. This was confirmed by RT–PCR with a primer set of a forward primer situated in the common region and a reverse primer situated in the respective unique 3'-regions (data not shown).
Characterization of the human IRSp53 protein

When in vitro translation products from each of the shorter and longer forms of human IRSp53 cDNA were analyzed by SDS–PAGE, multiple protein bands were detected (data not shown). To further examine the products, we raised a rabbit polyclonal antibody against a C-terminal portion of IRSp53 by the use of a glutathione S-transferase (GST) fusion construct (GST–IRSp53c; Fig. 1D). The antibody also detected multiple products in western blotting of the in vitro translation products (Fig. 3A). The largest products generated with the shorter and longer forms migrated at 58 and 63 kDa, respectively, which corresponds with the expected size calculated from the first methionine residue (Fig. 3A, lanes S and L). The second largest products in the respective lanes were 53 and 58 kDa, apparently consistent with the size calculated from the fifth methionine residue at position 59. The antibody also recognized endogenous products in PC12 cells (Fig. 3A, lane U). Since the 53 kDa species was the major product of in vitro translation with the shorter form of human IRSp53, Met59 seemed preferable as an initiation site for translation. Alternatively, multiple species may be generated in a post-translational process. Although IRSp53 is phosphorylated, the multiple species detected here cannot solely be explained by phosphorylation, as described below.

Phosphorylation of IRSp53

The total amount of IRSp53 in PC12 cells was not much changed upon stimulation with insulin (Fig. 3A, lanes 0 and 120 min). Phosphorylated IRSp53 was detected by immunoprecipitation with the anti-IRSp53 antibody followed by western blotting with an anti-phosphotyrosine antibody (Fig. 3B). Endogenous IRSp53 in PC12 cells was phosphorylated with as low as 10 nM insulin or IGF-1 (Fig. 3C). The phosphorylated form increased to a detectable level 5 min after insulin stimulation and continued to accumulate up to 120 min in culture with sodium vanadate, a phosphatase inhibitor. These results are consistent with previous observations in Chinese hamster ovary cells with overexpressed insulin receptor (24). The phosphorylated form migrated at the same distance as the largest form of endogenous IRSp53 in PC12 cells. Since the amount of the 58 kDa species detectable on western blotting with the anti-IRSp53 antibody was not changed by insulin stimulation, only a fraction of that migrating at the 58 kDa position was phosphorylated. Thus, phosphorylation alone does not explain the multiple forms of IRSp53. A GFP–IRSp53 fusion expressed with cloned cDNA was also phosphorylated under insulin stimulation (data not shown). Through these studies, we confirmed the isolated clone in this study to be phosphorylated upon insulin stimulation.

Interaction of IRSp53 with the DRPLA protein in yeast

We verified the interaction of IRSp53 with the DRPLA protein in yeast. As in the screening process, when yeast was co-transformed with a plasmid carrying full-length IRSp53 to produce a fusion protein with the AD (ADIRSp53) and pBDDR335–1185, both the HIS3 and LacZ reporter genes were activated (Fig. 4). The reporter genes were not activated when transformed with pADIRSp53 alone or together with either a plasmid carrying only the GAL4 DNA-binding domain (BD) portion or a control plasmid like BD–murine p53 (data not shown). Similarly, transformation with pBDDDR335–1185 alone or together with a plasmid carrying only the AD portion caused no activation (data not shown). Thus, activation of the reporter genes depended on interaction of the two proteins IRSp53 and DRPLA and did not result from self-activation nor from a single protein product. We then examined which portion of the DRPLA protein was involved in binding by the use of a series of deletion plasmids. pBDDDR335–786, which expressed a fusion protein of the BD with DRPLA amino acids 335–786, activated the reporter genes after co-transformation of yeast with ADIRSp53 and produced a higher level of β-galactosidase activity (Fig. 4). In contrast, BDDR676–793 and BDDR964–1185 showed almost no β-galactosidase activity in co-transformed yeast. Thus, the interacting portion of the DRPLA protein seemed to be localized in the amino acid 335–675 region. A construct with an extended polyglutamine tract (BDDR335–786Q+) still maintained a positive interaction with IRSp53, but β-galactosidase activities were reduced to one third of those for a similar construct with a normal range of the CAG repeat (Fig. 4C).

Co-immunoprecipitation of the DRPLA protein and IRSp53

Interaction between IRSp53 and the DRPLA protein was confirmed by immunoprecipitation. GFP-tagged IRSp53 produced by transfection in HeLa cells was detectable at the expected position by western blotting with an anti-GFP antibody. The GFP-tagged IRSp53 protein, but not GFP itself, was recovered in precipitates with the anti-DRPLA antibody (Fig. 5A). Conversely, endogenous DRPLA protein in the cells was immuno-
precipitated with the anti-GFP antibody (data not shown). Moreover, the endogenous IRSp53 and DRPLA proteins in PC12 cells were co-immunoprecipitated with the anti-IRSp53 antibody (Fig. 5B). These results clearly indicate that IRSp53 binds to the DRPLA protein. When PC12 cells were stimulated with insulin, the amount of precipitated DRPLA protein was not significantly changed. Upon insulin stimulation, phosphorylated IRSp53 levels increased markedly but the total amount of IRSp53 was not changed, as described above. Thus, phosphorylation of IRSp53 seemed not to enhance binding to the DRLPA protein.

Co-localization of the DRPLA protein and IRSp53

Subcellular localization of the DRPLA protein and IRSp53 in HeLa cells was studied under a confocal microscope. Endogenous IRSp53 detected with the anti-IPSp53 antibody was diffusely distributed in the cytoplasm (Fig. 6, panel 2). Endogenous DRPLA protein detected with the anti-DRPLA antibody was mainly localized in nuclei (Fig. 6, panel 1), although another anti-DRPLA antibody revealed cytoplasmic localization in brain tissues (6; see Discussion). Most of the GFP-tagged full-length DRPLA protein (GFP–DRQ14) was also localized in the nuclei when it carried a normal range of polyglutamines (Fig. 6, panel 3). To enrich the cytoplasmic fraction of the DRPLA protein, we made GFP-tagged constructs without the nuclear localization signal (NLS) at the N-terminal end of the DRPLA protein (GFP–DRQ14ΔN and GFP–DRQ71ΔN; Fig. 1B). Such an engineered DRPLA protein with a normal range of polyglutamines was localized in the cytoplasm as small oval dots, although a fraction of the protein still entered the nuclei (Fig. 6, panel 9). Although most endogenous IRSp53 maintained its diffuse cytoplasmic distribution in transfected cells (Fig. 6, panel 10), co-localization of GFP–DRQ14ΔN with IRSp53 was noted as yellow dots in the perinuclear region (Fig. 6, panel 11). The GFP-tagged DRPLA protein with an extended polyglutamine tract (GFP–DRQ71) formed nuclear aggregates as previously described (Fig. 6, panel 6) (31). In this condition, endogenous IRSp53 seemed not to participate in aggregate formation (Fig. 6, panels 6–8). However, the GFP-tagged DRPLA protein without NLS and carrying an extended polyglutamine tract (GFP–DRQ71ΔN) composed perinuclear and intranuclear oval dots with a diffuse distribution in the nuclei and IRSp53 was involved in aggregates in the perinuclear dots.

An SH3 domain interacts with a proline-rich region near the polyglutamine tract

To identify the region of the DRPLA protein involved in binding to IRSp53, in vitro translation products from the DRPLA constructs were subjected to an in vitro binding assay using a GST column. GST fusion proteins applied to the column were produced in Escherichia coli with constructs carrying full-length IRSp53 (GST–IRSp53f), its N-terminal portion (GST–IRSp53n) and its C-terminal portion (GST–IRSp53c), respectively, in addition to GST alone and an unrelated fusion protein (GST–Bcl2) as a control (Figs 1D and 7A). A full-sized DRPLA protein made by in vitro translation bound to GST–IRSp53f and GST–IRSp53c, but not to GST–IRSp53n and control columns (Fig. 7B–D). Although not studied extensively, an SH3 domain in the C-terminal portion of IRSp53...
was suggested to be involved in the binding. We then made a series of DRPLA products by in vitro translation of DRPLA constructs which had various sized deletions from the 3'-end (Fig. 1C) and applied them to the GST–IRSp53c column. Truncated DRPLA proteins produced from DR567, DR596 and DR749 constructs bound to the GST column but DR516 did not (Fig. 8A). These results suggest that the amino acid 517–567 region in the DRPLA protein is involved in binding. Another truncated construct with an internal deletion, DR749del, which had lost amino acid residues 428–601, did not bind to GST–IRSp53c. These results and the binding assay in yeast cells support the proposed binding region. The RPYP-PGP sequence is a consensus proline-rich motif for binding to the SH3 domain (32,33). There is one completely matched sequence in the proposed binding region, which appears 38 amino acids downstream of the polyglutamine tract, while the proposed binding region contained a few other similar sequences (Fig. 8C).

An extended polyglutamine tract modulates binding

Contrary to the binding assay in yeast, an extended polyglutamine tract in DRPLA protein seemed not to significantly affect binding in the GST column assay. Regardless of the size of the polyglutamine tract, DRPLA proteins of full length bound to GST–IRSp53c (Fig. 7). More careful quantitative assays with truncated proteins showed that products having an extended polyglutamine tract (DR749Q+ and DR696Q+) bound to GST–IRSp53c to a similar extent as their counterparts having a normal sized polyglutamine tract (Fig. 8B). However, DR598Q+, the smallest construct still retaining the proposed SH3 domain, displayed a significantly reduced affinity for IRSp53c compared with that of DR596 (Fig. 8). Thus, an extended polyglutamine tract in a certain form seemed to obstruct binding to the proposed target region. However, the proline-rich region flanking the consensus binding sequence may serve as an auxiliary binding site, on which an extended polyglutamine tract has little influence.

DISCUSSION

A growing number of neurodegenerative disorders have been shown to be caused by CAG/glutamine repeat expansion. Despite ubiquitous expression of each CAG repeat disease gene, neuronal death occurs in distinctive anatomical areas of the brain, indicating that additional regional factors are involved in
the site-specific pattern of neurodegeneration. Several candidates having a binding capacity for Huntingtonin, the gene product of the HD gene, have been detected to date and they provide a clue to understanding the normal and pathological functions of the HD gene (34). Some of the Huntingtonin-binding proteins show a cytoskeletal localization, indicating that Huntingtonin may play a role in vesicle trafficking within cells (35). A Grb2-like protein and members of the signaling complex for the epidermal growth factor receptor have been shown to bind through their SH3 domains with Huntingtonin. These studies imply that Huntingtonin is involved in signal transduction (36,37). A few proteins interacting with DRPLA protein have been identified, including WW domain-containing proteins which seem to function in the cytoskeleton (38).

In this report, we have identified an insulin receptor substrate of 53 kDa (IRSp53) as one of the DRPLA-interacting proteins. Hamster IRSp53 was previously detected during screening for substrates of the tyrosine kinase of the insulin or IGF-1 receptor by use of a monoclonal antibody and its gene was cloned (24). Our identification of human IRSp53 is grounded not only in sequence homology to the previously characterized hamster IRSp53 but also in our experiments on phosphorylation. Several insulin receptor substrates (IRSs) have been identified, including IRS-1, IRS-2, IRS-3 and IRS-4, of which IRS-1 has been most extensively characterized in the signal transduction pathway from the insulin and IGF-1 receptors (39). Several IRSs form pre-assembled complexes with other members involved in the signal transduction pathway and the complexes may be associated with the actin cytoskeleton (40). In contrast, although hamster IRSp53 is clearly demonstrated to be phosphorylated upon insulin stimulation in experimental systems, IRSp53 has not been fully characterized molecularly and in relation to the signal pathway (41). Data presented in this report on the human homolog characterize more fully the IRSp53 gene and product.

The human IRSp53 gene generated two transcripts by differential splicing and produced multiple forms of protein distinguishable by SDS–PAGE not only in cells but also by in vitro translation from each cDNA construct. Although a fraction of IRSp53 is phosphorylated, the multiple forms are not accounted for by phosphorylation alone. We assume that some of the species are generated by utilization of some other methionine as an initiation site, although any methionine in the first 70 amino acid residues is not necessarily situated in a suitable context for initiation of translation (42). The previous study on hamster IRSp53 also reported two forms, 53 and 58 kDa, detectable with the specific antibody and concluded that the 53 kDa form was present in the brain (24). Since they isolated only one form of cDNA, corresponding to the shorter form of human cDNA, it is unknown whether differential splicing takes places in rodents. Our study shows that the longer transcript is mostly expressed in the brain and potentially encodes a larger protein. Nevertheless, our preliminary study detected a smaller form comparable with the 53 kDa product in rat brain. Thus, more studies will be required to define protein species, especially the brain form, of IRSp53.

Using an antibody raised against the C-terminal portion of DRPLA, we previously detected DRPLA protein mostly in the cytoplasm in brain tissues (6). In contrast, another antiserum raised against the GST–DRPLA fusion protein used in this study as well as in a previous study revealed a primarily nuclear localization (31). This seems to be accounted for by preferential recognition by the former antibody of phosphorylated species (unpublished data). Other laboratories have also reported both cytoplasmic and nuclear localizations of DRPLA protein (20). We propose that the DRPLA protein is a shuttle plying across the nuclear membrane. Since IRSp53 is localized

Figure 7. GST column assay showing interaction of the DRPLA protein and IRSp53. (A) GST–IRSp53 fusion proteins were expressed in E.coli, analyzed by SDS–PAGE and stained with Coomassie Brilliant Blue to verify the molecular size and amount. The apparent size of each product accorded well with the calculated size indicated on the left. (B) SDS–PAGE verifying the full-length DRPLA products with normal and extended polyglutamine tracts. DRPLA constructs DR1185 and DR1185Q+, carrying normal range and expanded CAG repeats, respectively, were in vitro translated with a radioisotope. Products were analyzed by SDS–PAGE to measure the radioactivity. (C and D) GST column assay showing the interaction between the DRPLA protein and IRSp53. Radiolabeled products with a defined radioactivity were applied to the GST columns and retained protein was analyzed by SDS–PAGE. Each column was pretreated with a GST fusion protein as indicated at the bottom. Constructs for the GST fusion protein and in vitro translation products are seen in Figure 1C and D.
in the cytoplasm, we engineered a DRPLA protein without an NLS in order to detect \textit{in vivo} interaction of the two molecules. The DRPLA protein without an NLS was distributed more abundantly in the cytoplasm and co-localization with IRSp53 was ascertained. An extended polyglutamine tract in the DRPLA protein considerably reduced binding to IRSp53 in the yeast assay system, but did not significantly affect subcellular co-localization or \textit{in vitro} binding. The consensus structure for binding to the SH3 domain has been identified to be a PP II helix composed of PXXPXΦ or ΦXXPXP, where Φ is a basic amino acid residue, preferably arginine (32,33). The RPYPPGP sequence at amino acids 535–541 of the DRPLA protein exactly matches the consensus sequence and this is within the essential region for binding in the \textit{in vitro} binding assay. The binding ability of the smallest construct having the consensus sequence was much influenced by an extended polyglutamine tract, but that for larger constructs having the additional proline-rich sequence was not significantly affected. Thus, the effects of an extended polyglutamine tract seem to depend on the position, the conformational structure or the flanking sequence of the target. Since proteins are correctly folded in the \textit{in vivo} situation, overall affinity may be maintained, as observed under the microscope, but partially reduced in yeast.

**IGF-1** is known to exhibit metabolic and trophic actions in the brain. IGF-1 is transiently expressed in rat brain during maturation and expression is evident in a specific group of functionally related cerebellar projection neurons, including the deep cerebellar and red nuclei systems (43), which is comparable with the affected areas in DRPLA. Knockout mice for the IGF-1 gene show defects in the brain with a reduced size, hypomyelination and loss of neurons in particular areas (44). Treatment with exogenous IGF-1 is reported to enhance the survival of neurons \textit{in vitro} and \textit{in vivo} by activating several substrates of the receptor (24,25,45). Since our yeast two-hybrid screening reveals several other proteins potentially involved in insulin/IGF-1 signal transduction, the DRPLA protein may play a role in the neuronal signaling pathway from insulin/IGF-1, specifically presenting a docking site for formation of a multiprotein complex. An extended polyglutamine tract may influence the whole conformation of the complex, resulting in an impairment of the IGF-1 signaling pathway.

**MATERIALS AND METHODS**

**DNA techniques**

Plasmid isolation, DNA manipulation and northern blotting were carried out following standard methods described previ-
ously (5,46). DNA sequences were determined using an automated sequencer with primers situated in the vector portion and also with M13 universal primers after subcloning. In RT-PCR to confirm alternative splicing, human poly(A)^+ RNA from fetal brain, adult brain, liver, kidney and lung (Clontech, Palo Alto, CA) was reverse transcribed with Superscript II (Gibco BRL, Rockville, MD) and amplified with a sense primer, 5'-AAGGACGACGACGACGGG, and either of the antisense primers, 5'-ACCAACCAAGACAAACCA for the shorter form, or 5'-TTCTGGATGGGAGGTTGG for the longer form.

Plasmid construction

All constructs used in this study are illustrated in Figure 1. The original cDNA clones of the DRPLA gene used in this experiment have been described previously (2,5). All the constructs representing a normal range of repeats carry 10 CAG repeats but encode 14 glutamine residues because of the (CAGCAA)_2 sequence ahead of the CAG repeat. To construct a series of plasmids used as bait in the yeast two-hybrid system (pBDDDR335–1185, pBDDDR335–786, pBDDDR676–973 and pBDDDR964–1185), an NcoI–NcoI fragment (nt 1239–3814) in the DRPLA cDNA sequence; GenBank accession no. D31840), an NcoI–BglII (blunt-ended) fragment (1239–2598), a PstI–PstI fragment (2259–3162) and a blunt-ended BamHI–NcoI fragment (3125–3814) isolated from DRPLA cDNA clones were fused in-frame at the NcoI, NcoI/SmaI, PstI and SmaI sites, respectively, in the GAL4 BD of a yeast two-hybrid vector, pAS2-1 (Clontech). A series of plasmids having a truncated C-terminus (pDR749, pDR696, pDR567 and pDR516) were reported previously (8), in which the cDNA portion including the first methionine residue was located downstream of the T3 promoter in a pBluescript SK– vector (Stratagene, La Jolla, CA). To construct a plasmid without the polyaniline tract and flanking regions, pDR749 plasmid DNA was digested with AccI and BstEII, blunt-ended and then self-ligated (pDR749del). To construct plasmids carrying extended CAG repeats such as pBDDDR335–786Q+, pDR749Q+ and pDR696Q+, the AccI–AgeI fragment was replaced with the corresponding fragment of pMY1247, which is a cDNA construct carrying 71 CAG repeats. pDR598Q+ was constructed by digestion of pDR749Q+ with AgeI and NruI, followed by filling-in with the Klenow fragment and self-ligation. Plasmids for GFP–DRPLA fusion protein (pGFP–Q14), extended DRPLA protein (pGFP–Q71) and DRPLA proteins without an NLS (pGFP–Q14AN and pGFP–Q71AN) have been described previously (31). To generate a plasmid producing a fusion protein of IRSp53 with GFP (pGFP–IRSp53), a SalI–ApaI fragment (1–1846) of the longer form of IRSp53 cDNA was ligated in-frame with the Xhol–ApaI fragment of the expression vector pEGFP-C3 (Clontech). To generate plasmids producing fusion proteins of IRSp53 with GST (pGST–IRSp53f, pGST–IRSp53m and pGST–IRSp53c), a BspEII–EcoRV fragment (177–2887), an NcoI fragment (192–1194) and an NcoI–EcoRV fragment (1190–2887) of the longer form of IRSp53 cDNA were blunt-ended and then inserted in-frame into an Smal site of the pGEX-5X vector (Pharmacia, Uppsala, Sweden). For control plasmids used in the yeast two-hybrid system and expression experiments, BD/murine p53 (pVA3-1) and AD/SV40 large T antigen were purchased from Clontech and GST–Becl2 was a generous gift from Dr John C. Reed (Burnham Institute, La Jolla, CA).

Yeast two-hybrid system

Yeast of the Y190 strain (MATa, ura3-52, his3-200, lys2-801, ade2-101, trp1-901, leu2-3,112, gal1Δ, gal10Δ, cyh2, lys2::GAL1::HIS3::TATA, his3::GAL1::GAL1::TATA::LacZ) was used as host in the yeast two-hybrid system. Yeast cells were transformed with DNA following a modified lithium acetate transformation protocol and grown in YPD or a selection medium (46,47). Yeast host cells were first transformed with pBDDDR335–1185 and subsequently with plasmid DNA prepared from a human fetal brain Matchmaker cDNA library fused with the AD (Clontech). The transformants were grown on selection plates without Trp, Leu and His supplemented with 25 mM 3-amino-1,2,4-triazole (Sigma, St Louis, MO) (selection plates). Apparent His^+ clones were picked and their phenotype confirmed by restreaking onto the selection plates and by β-galactosidase filter assay as described below. Candidates were then plated on medium without Leu supplemented with 10 µg/ml cycloheximide (Cyh) to isolate Leu^- Trp^-/Cym^- segregants, which had lost the bait plasmid. Finally, plasmid DNA was propagated in E.coli cells (strain HB101) and used in subsequent experiments.

Measurement of β-galactosidase activity

Filter assays for β-galactosidase were performed in z-buffer (100 mM sodium phosphate, pH 7.0, 10 mM KCl, 1 mM MgSO_4)_2 supplemented with 50 mM 2-mercaptoethanol and 0.07 mg/ml 5-bromo-4-chloro-3-indolyl-β-d-galactoside, after transferring yeast colonies onto Hybond-N nylon membranes (Amersham, Little Chalfont, UK). Filters were then incubated at 37°C for 2–4 h until the color developed. β-Galactosidase activities were also measured after disruption of cells as follows. Log phase yeast cells were resuspended in z-buffer and disrupted by freeze–thawing with liquid nitrogen. An aliquot was incubated at room temperature with 0.67 mg/ml o-nitrophenyl-β-d-galactopyranoside in z-buffer supplemented with 2-mercaptoethanol. Subsequently, OD_420 was measured to calculate the enzyme activity by the equation of Miller (48).

Cell culture

A rat pheochromocytoma cell line (PC12) was maintained in Dulbecco’s modified Eagle’s medium (DMEM) with heat-inactivated 10% horse serum and 5% fetal bovine serum, 50 µg/ml penicillin and 0.1 mg/ml streptomycin at 37°C under a humidified atmosphere of 5% CO_2. For stimulation with growth factor, cells were preincubated in serum-free medium for 3 h and then treated in culture medium supplemented with insulin or IGF-1 at a final concentration of 10^-5–10^-6 M along with 500 µM sodium vanadate. Culture was continued at 37°C for 2 h unless otherwise indicated. The treated cells were lysed in lysis buffer (150 mM NaCl, 1% Triton X-100, 10 mM Tris–HCl, pH 7.4, 5 mM EGTA, 500 µM sodium vanadate, 1.0 mM phenylmethylsulfonyl fluoride, 18 µg/ml aprotinin, 50 µg/ml leupeptin, 1 mM benzamidine and 0.7 µg/ml pepstatin) and the supernatant fraction was obtained by centrifugation. HeLa cells were maintained in the same conditions as for PC12 cells except for using DMEM with 10% heat-inactivated fetal
bovine serum and transfected with plasmid DNA by the lipofection method (49).

Detection with antibody
A rabbit polyclonal antibody specific to human IRSp53 (anti-IRSp53) was raised with the purified product of pGST–IRSp53c and absorbed on GST–Sepharose to diminish the reactivity to GST. Western blotting was performed following a standard method (8,46). An aliquot containing 30 μg protein was fractionated by SDS–PAGE and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by electroblocting. To detect IRSp53, membranes were treated with a 1:2000 dilution of the anti-IRSp53 antibody, followed by a 1:4000 dilution of horseradish peroxide-conjugated goat anti-rabbit IgG (Sigma). To detect GFP fusion proteins, membranes were treated with a 1:500 dilution of the monoclonal anti-GFP antibody (Clontech) followed by a 1:1000 dilution of horseradish peroxide-conjugated rabbit anti-mouse IgG (Dako, Carpenteria, CA). The DRPLA protein was visualized with the anti-DRPLA antibody as previously described (8). For immunoprecipitation, an aliquot containing 500 μg protein was first incubated at 4°C with 10 μl of an indicated antibody for 3 h, then overnight with protein G/protein A–agarose (Calbiochem, San Diego, CA). Precipitates were washed three times and disassociated by boiling with the SDS–PAGE buffer and subjected to SDS–PAGE. Co-immunoprecipitated proteins were visualized using the anti-GFP antibody, anti-DRPLA antibody or a 1:2000 dilution of an anti-phosphotyrosine antibody, RC20H (Transduction Laboratories, Lexington, KY).

Confocal microscopy
Cells were treated as described previously (31). For IRSp53 detection, cells were treated with a 1:100 dilution of anti-IRSp53 antibody, followed by a 1:20 dilution of TRITC–conjugated swine anti-rabbit immunoglobulin (Dako). A fluorescent image was obtained using a confocal microscope (Fluoview; Olympus, Tokyo, Japan) equipped with an Ar laser with excitation at 488 nm and detection at 510–530 nm bandpass for GFP or with an He/Ne laser with excitation at 543 nm and detection at 565 to 590 nm bandpass for TRITC.

In vitro translation and binding assay
In vitro translation was performed using the TNT coupled reticulocyte lysate system (Promega, Madison, WI) with plasmid DNA. Translated products (2 μl) were fractionated by SDS–PAGE and visualized with an antibody. In several experiments, products were radiolabeled with [35S]methionine and detected by autoradiography or quantitatively measured with a phosphorimager (BAS2000; Fuji Film, Tokyo, Japan). For binding assays, radiolabeled in vitro translation products were incubated with 10 μg of GST fusion proteins prebound to glutathione–Sepharose 4B beads (Pharmacia) in HKM solution (10 mM HEPES, pH 7.2, 142.5 mM KCl, 5 mM MgCl2, 1 mM EGTA, 0.2% NP-40) at 4°C for 2 h and then washed with the HKM solution four times. Proteins bound to the beads were dissociated and analyzed by SDS–PAGE followed by autoradiography.

ABBREVIATIONS
AD, GAL4 activation domain; BD, GAL4 DNA-binding domain; Cyh, cycloheximide; DME, Dulbecco’s modified Eagle’s medium; DRPLA, dentatorubral-pallidoluysian atrophy; GFP, green fluorescent protein; GST, glutathione S-transferase; HD, Huntington’s disease; IGF-1, insulin-like growth factor-1; IRS, insulin receptor substrate; IRSp53, insulin receptor substrate protein of 53 kDa; NLS, nuclear localization signal; SBMA, spinal and bulbar muscular atrophy; SCA, spinocerebellar ataxia.

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