SH3 Domain-dependent Association of Huntingtin with Epidermal Growth Factor Receptor Signaling Complexes*

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Ya Fang Liu‡§, Richard C. Deth¶, and Didier Devys

From the ‡Center for Neurological Disease, Brigham and Women's Hospital and Department of Neurology, Harvard Medical School, Boston, Massachusetts 02114, the ¶Department of Pharmaceutical Sciences, Northeastern University, Boston, Massachusetts 02115, and the §Institut de Genetique et de Biologie Moleculaire et Cellulaire, Université Louis Pasteur, 1 rue Lautent Fries, BP 163 Illkirch, Cédex C.U. de Strasbourg 67404, France

Huntington’s disease (HD) is an autosomal dominant inherited neurodegenerative disorder characterized by massive loss of striatal neurons in caudate and putamen (1, 2). The defect in the HD gene locus involves a moderate expansion of the polymeric CAG trinucleotide repeat near the N terminus of huntingtin (3). The age of onset of HD is roughly correlated to the number of the CAG repeats (4, 5). Although two proteins have been recently identified to interact with huntingtin (3), the normal function of huntingtin remains a mystery.

Identification of associated proteins is an initial step to characterize the biological function of any novel protein. Huntingtin does not contain any kinase, SH2, SH3, PH, or PTB domains, but does possess multiple proline-rich motifs in its sequence which resemble SH3 domain binding motifs (8, 9). Most SH3 domain-containing proteins are adapter proteins for tyrosine kinase receptor-mediated signaling (8). We, therefore, examined the possible association of huntingtin with several SH3 domain-containing signaling molecules in EGF receptor signaling complexes. We found that huntingtin is directly associated with both Grb2 and RasGAP and indirectly associated with Shc and the EGF receptor. Huntingtin is not associated with Nck and does not co-exist with Sos in the same signaling complex. Our results suggest that huntingtin may be a unique signaling intermediate for EGF receptors and may be involved in the regulation of Ras-dependent signaling pathways.

EXPERIMENTAL PROCEDURES

Immunoblotting and Co-immunoprecipitation—Human epithelial carcinoma A431 or embryonic kidney 293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. These cells, starved in serum-free medium for 24–48 h, were stimulated with 100 ng/ml EGF for 5 min at 37 °C. Cells were then washed once with ice-cold phosphate-buffered saline (PBS) and lysed with Nonidet P-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1% Nonidet P-40, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 10% glycerol, 10 μM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A). The anti-huntingtin monoclonal antibody 4C8 has been characterized previously (10). The anti-huntingtin polyclonal antibody 437, raised against a conjugated peptide derived from the first 17 amino acids of huntingtin, was prepared and characterized as described in Ref. 11. The rabbit immune serum was subsequently purified using a peptide-antigen-affinity column. To prepare peptide preabsorbed antibody 437, the peptide antigen was incubated with 437 in 10 volumes of PBS for 4 h at 4 °C. 1 ml of A431 or 293 cell lysates was incubated with different antibodies as indicated in the figure legends and protein A- or G-Sepharose 4B beads (Pharmacia Biotech Inc.) for 2–3 h. Beads were washed three times with lysis buffer and once with PBS. The cell lysates or precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with primary antibody as indicated in the figure legend, followed by a peroxidase-linked secondary antibody. Immunoblotting was visualized by an enhanced chemiluminescence reaction and autoradiography.

Preparation of GST-fusion Proteins and in Vitro Binding Studies—Construction, site-directed mutagenesis, and characterization of the full-length Grb2 W36/193K SH3 domain-inactivating mutant have been described previously (12). Different fragments of Grb2 cDNA were cloned into pGEX-2T (Pharmacia) by polymerase chain reaction. Induction of expression and purification of various Grb2 GST-fusion proteins were performed as described previously (13). All GST-fusion proteins were freshly prepared on glutathione-Sepharose 4B beads (Pharmacia). These beads were incubated with EGF-stimulated 293 cell lysates for 1 h at 4 °C and washed three times with ice-cold lysis buffer and one time with PBS. Bound proteins were analyzed with huntingtin immunoblotting. About 0.5–1 μg of Grb2, 2–4 μg of RasGAP or Nck GST-fusion proteins, or GST alone was used for in vitro binding experiments.

RESULTS

To test the hypothesis that huntingtin is involved in tyrosine kinase receptor-mediated signal transduction, we examined its tyrosine phosphorylation status in cultured A431 cells. Although we did not detect any significant tyrosine phosphorylation of huntingtin (Fig. 1A), we found that EGF receptor stimulation increased the level of tyrosine phosphorylation in a protein of molecular mass ~170–185 kDa which was associated with huntingtin (Fig. 1A). Because A431 cells overexpress the EGF receptor (14), and the association with huntingtin...
appears to be regulated by activation of the receptor, we hypothesized that the EGF receptor might be one of these tyrosine-phosphorylated proteins associated with huntingtin.

We then explored the possible association between huntingtin and the EGF receptor in vivo by co-immunoprecipitation. A431 cells express large amounts of the EGF receptor and have a low level of huntingtin, while 293 embryonic kidney cells are rich in huntingtin and have a low level of the EGF receptor. We therefore decided to examine the association of huntingtin with the EGF receptor in both cell lines. Cell lysates from quiescent and EGF-stimulated A431 cells were incubated with an anti-huntingtin polyclonal antibody 437 (Fig. 1B, lanes 1 and 2) or peptide antigen preabsorbed 437 (Fig. 1B, lanes 3 and 4) or mouse IgG (lane 5), or 437 preimmune serum (lane 6). The precipitated proteins were analyzed by Western blotting using an anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Inc.). B, cell lysates were immunoprecipitated with 437 or peptide preabsorbed 437. The precipitated proteins were analyzed by Western blotting using an anti-EGF receptor (EGFR) antibody (gift from Dr. T. Roberts). Lanes 1 and 2, cell lysates from quiescent A431 cells; lanes 3 and 4, 293 cells; lanes 5 and 6, controls: A431 (lane 5) or 293 (lane 6), cell lysates were immunoprecipitated with the peptide antigen preabsorbed 437; lane 7, EGF-stimulated A431 cell lysates were precipitated with mouse IgG; lane 8, A431 cell lysates. C, cell lysates from quiescent and EGF-stimulated A431 (lanes 1 and 2) or 293 (lanes 3 and 4) cells were precipitated with an anti-EGF receptor antibody and immunoblotted with 437. HDP, Huntington’s disease protein.

Next, we explored the mechanism of the association of huntingtin with the EGF receptor. Because huntingtin contains multiple proline-rich motifs, which may mediate binding to SH3 domains (8, 9), we tested for an association between huntingtin and Grb2, an adapter protein containing one SH2 domain flanked by two SH3 domains (15, 16). Grb2 binds to the activated EGF receptor through its SH2 domain and with Sos through its two SH3 domains (15, 16). Cell lysates were incubated with anti-huntingtin monoclonal antibody 4C8 or mouse IgG (control), and the precipitated proteins were analyzed by Western blotting. The results show that only a small fraction of Grb2 is associated with huntingtin in quiescent A431 or 293 cells (Fig. 2A). Upon stimulation of the EGF receptor, huntingtin-associated Grb2 is increased (Fig. 2B). To further examine the association of huntingtin with Grb2 in vivo, cell lysates were immunoprecipitated with an anti-Grb2 antibody and analyzed by Western blotting with 4C8. Similarly, stimulation of the EGF receptor increased the association between huntingtin and Grb2 in vivo in both 293 (lanes 1 and

**Fig. 1.** Association between huntingtin and the EGF receptor in vivo. A, cell lysates from quiescent and EGF-stimulated A431 cells were immunoprecipitated with 4C8 (lanes 1 and 2), 437 (lanes 4 and 5), mouse IgG (lane 3), or 437 preimmune serum (lane 6). The precipitated proteins were analyzed by Western blotting using an anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology, Inc.). B, cell lysates were immunoprecipitated with 437 or peptide preabsorbed 437. The precipitated proteins were analyzed by Western blotting using an anti-EGF receptor (EGFR) antibody (gift from Dr. T. Roberts). Lanes 1 and 2, A431 cells; lanes 3 and 4, 293 cells; lanes 5 and 6, controls: A431 (lane 5) or 293 (lane 6), cell lysates were immunoprecipitated with the peptide antigen preabsorbed 437; lane 7, EGF-stimulated A431 cell lysates were precipitated with mouse IgG; lane 8, A431 cell lysates. C, cell lysates from quiescent and EGF-stimulated A431 (lanes 1 and 2) or 293 (lanes 3 and 4) cells were precipitated with an anti-EGF receptor antibody and immunoblotted with 437. HDP, Huntington’s disease protein.

**Fig. 2.** Association of huntingtin with Grb2 in vivo and in vitro. A, cell lysates from A431 or 293 cells were precipitated with 4C8 (lanes 1–4) or mouse IgG (lanes 5–8). The precipitated proteins were analyzed by Western blotting with an anti-Grb2 antibody. Lanes 1 and 2 and 5 and 6, A431 cells; lanes 3 and 4 and 7 and 8, 293 cells; lanes 9 and 10, A431 and 293 cell lysates. About 10% of the volume of cell lysates used for immunoprecipitation was loaded on lanes 9 or 10. Except for the Grb2 band, the rest result from detection of mouse IgG, which is also seen in the controls. B, cell lysates from quiescent or EGF-stimulated A431 or 293 cells were precipitated with an anti-Grb2 antibody, and the precipitated proteins were analyzed by immunoblotting with 437. Lanes 1 and 2, A431 cells; lanes 3 and 4, 293 cells; lanes 5, 293 cell lysate; lane 6, A431 cell lysate. About 10% of the volume of cell lysates used for immunoprecipitation was loaded on lanes 5 and 6. C, in vitro binding studies with Grb2 GST-fusion proteins. EGF-stimulated 293 cell lysates were incubated with GST alone or with various Grb2 GST-fusion proteins as indicated in the figure: GST, GST alone; Grb2SH2, GST-Grb2 SH2 domain; Grb2Mu, the full length of GST-Grb2 W36/193K SH3 domain-inactivating mutant; Grb2F, the full-length GST-Grb2; Grb2CSH3, GST-Grb2 C-terminal SH3 domain; Grb2NSH3, GST-Grb2 N-SH3; HDP, huntingtin immunoprecipitation with 4C8 as the control.
tween huntingtin and Shc by co-immunoprecipitation. Unlike the Grb2 SH2 domain alone or with the full-length Grb2 SH3 domain-inactivating mutant (Fig. 2B). No huntingtin binding was observed with either Grb2 SH2 domain alone or with the full-length Grb2 SH3 domain-inactivating mutant (Fig. 2C). These results indicate that the two flanking SH3 domains of Grb2 are required for binding to huntingtin.

Stimulation of the EGF receptor increases tyrosine phosphorylation of Shc and results in assembly of the Shc-Grb2-Sos complex (16, 17). Therefore we tested for an association between huntingtin and Shc by co-immunoprecipitation. Unlike the two flanking SH3 domains of Grb2 are required for binding to huntingtin.

FIG. 3. Analysis of the huntingtin-associated signaling complex. A, cell lysates from quiescent or EGF-stimulated A431 or 293 cells (as indicated in the figure) were immunoprecipitated with anti-huntingtin polyclonal antibody 437, the precipitated proteins were analyzed with an anti-Shc antibody (Transduction Laboratories). B, cell lysates from quiescent or EGF-stimulated A431 or 293 cells (as indicated in the figure) were immunoprecipitated with anti-huntingtin monoclonal antibody 4C8; the precipitated proteins were analyzed by Western blotting using an anti-Sos1 polyclonal antibody (Upstate Biotechnology, Inc.). In the last lane, cell lysates from EGF-stimulated A431 cells were immunoprecipitated with anti-Grb2 antibody as a control.

2) and A431 cells (lanes 3 and 4) (Fig. 2B). The 330-kDa protein band detected from 293 cell lysates is not huntingtin, because it is not recognized by anti-huntingtin monoclonal antibody 4C8 (Fig. 2B), and it does not bind to Grb2. These results indicate that huntingtin is associated with Grb2 both in vivo and in vitro, and the association is regulated by activation of the EGF receptor. Similar to most signaling molecules (8, 15, 16), only a small proportion of huntingtin participates in the assembly of signaling complexes upon activation of the EGF receptor.

To determine whether the SH3 domains of Grb2 mediate binding to huntingtin, deletion and substitution mutants of Grb2 were generated as GST-fusion proteins and purified on glutathione-Sepharose beads. GST-fusion protein beads containing full-length Grb2, Grb2 SH2 domain, N- or C-terminal SH3 domains, or the full-length Grb2 W36/I93K SH3 domain-inactivating mutant (12) were incubated with lysates from EGF-stimulated 293 cells for 1 h, and the bound proteins were analyzed by 4C8 Western blotting. Only full-length Grb2, and individual SH3 domains of Grb2, were able to bind to huntingtin (Fig. 2C). No huntingtin binding was observed with either Grb2 SH2 domain alone or with the full-length Grb2 SH3 domain-inactivating mutant (Fig. 2C). These results indicate that the two flanking SH3 domains of Grb2 are required for binding to huntingtin.

FIG. 4. Association between huntingtin and RasGAP in vivo and in vitro. A, EGF-stimulated 293 cell lysates were incubated with different antibodies or GST-fusion protein beads as indicated in the figure. NckGST, cell lysates were incubated with the full-length Nck GST fusion protein; RasGAPIP, immunoprecipitated with anti-RasGAP antibody; RasGAPSH3, incubated with GST-RasGAP SH3 domain; RasGAPFull, incubated with the RasGAP SH2-SH3-SH2 domain. HD-PIP, precipitated with 437; GST, incubated with GST alone. The bound proteins were analyzed by 4C8 Western blotting. B, cell lysates from quiescent or EGF-stimulated A431 (lanes 1 and 2) and 293 (lanes 3 and 4) were precipitated with anti-huntingtin monoclonal antibody 4C8; the precipitated proteins were analyzed with an anti-RasGAP antibody.

Grb2, Shc appears to be weakly associated with huntingtin, and only the 52-kDa form of Shc was detectable in 437 immunoprecipitates (Fig. 3A). Moreover, in Shc immunoprecipitates huntingtin could not be detected (data not shown). These data suggest that huntingtin may be indirectly associated with Shc.

Upon activation of the EGF receptor, Grb2 binds to the activated receptor through its SH2 domain, and to proline-rich motifs of Sos through its two-flanking SH3 domains (15, 16). We therefore examined whether huntingtin co-exists with Sos in Grb2-associated signaling complexes by co-immunoprecipitation. Sos1 was not detected in the anti-huntingtin immunoprecipitates in either quiescent or EGF-stimulated A431 and 293 cells (Fig. 3B). In contrast, Sos1 could be readily detected in the Grb2 immunoprecipitates in EGF-stimulated A431 cells (Fig. 3B). These results suggest that Grb2-huntingtin and Grb2-Sos are two different signaling complexes. Because huntingtin and Sos both bind to the two flanking SH3 domains of Grb2, huntingtin may serve as an inhibitor for the Ras-dependent signaling pathway by competitive inhibition of the assembly of the Grb2-Sos complex.

To examine other potential SH3 domain-mediated interactions with huntingtin in the EGF receptor signaling complex, we tested for the association of huntingtin with RasGAP and Nck in vivo and in vitro. RasGAP only contains a single SH3 domain (18). In response to activation of the EGF receptor, RasGAP is phosphorylated and associates with the receptor through its SH2 domain (18). Nck contains three N-terminal SH3 domains followed by a SH2 domain (19). It binds to the autophosphorylated EGF receptor through its SH2 domain and acts as an adapter module for EGF receptor signaling (19). We did not detect any significant association between huntingtin and Nck both in vivo (data not shown) and in vitro (Fig. 4A). However, we observed an association between RasGAP and huntingtin in vivo as well as in vitro (Fig. 4). In RasGAP...
immunoprecipitates, huntingtin can be readily detected (Fig. 4A). Conversely, RasGAP can be also detected in huntingtin immunoprecipitates, and the association is regulated by activation of the EGF receptor (Fig. 4B). To obtain further evidence for the association of huntingtin with RasGAP, in vitro binding assays were performed with the RasGAP GST-fusion proteins. The SH2-SH3-SH2 or SH3 domain alone of RasGAP was able to bind to huntingtin (Fig. 4A), whereas individual RasGAP SH2 domains failed to bind (data not shown). Taken together, these results indicate that huntingtin selectively associates with a subset of SH3 domain-containing molecules in the EGF receptor signaling complex.

**DISCUSSION**

In the present study, we demonstrate that huntingtin associates with the EGF receptor signaling complex through binding to the SH3 domains of Grb2 and RasGAP in vivo as well as in vitro. Assembly of these huntingtin complexes is highly dependent on autophosphorylation and activation of the EGF receptor, suggesting that the formation of these complexes is part of cellular signaling cascades mediated by the receptor. Although the SH3 domains of Grb2 are known to bind to several proline-rich proteins (19, 20), evidence for these associations is mainly limited to in vitro binding studies. In the present study, we demonstrate that the association of huntingtin with Grb2 or RasGAP is regulated by activation of EGF receptors in two different intact cell systems. These results suggest that assembly of Grb2-huntingtin and RasGAP-huntingtin complexes are a part of EGF receptor-mediated cellular signaling cascades. Grb2 relays signals from activated EGF receptors to the Ras/mitogen-activated protein kinase signaling pathways. Accumulating evidence indicates that elevation of GTP-bound Ras in cultured cells is correlated with an increase in apoptosis (21, 22). Mice with null mutation of either the RasGAP or huntingtin gene exhibit increased neuronal apoptosis in early embryonic development (23, 24). Moreover, neuronal apoptosis is evident in brains of the HD patients, particularly at the later stage of the disease (25, 26). It will be interesting to investigate whether mutant huntingtin containing an expansion of the CAG trinucleotide repeat alters Ras-dependent signaling in neuronal cells.

It is of interest to understand why neuronal degeneration in HD is delayed for some 20–60 years after such a CAG repeat expansion-dependent gain of function. As observed in our studies and many others (11, 17, 20), only a small proportion of signaling molecules is required for the assembly of signaling complexes and activation of cellular signaling cascades, with most of these molecules being kept in reserve. Thus a small disturbance in the assembly of signaling complexes with the CAG repeat-expanded huntingtin may not significantly affect the receptor-mediated certain cellular functions for a long period of time. The high capacity for compensation within the tyrosine kinase receptor signaling system may explain latency in the onset of HD. On the other hand, the HD huntingtin-associated signaling complex could mediate a novel signaling pathway, which has a dominant function. This might account for the fact that HD is inherited as an autosomal dominant trait and neuropathological changes occur in both homozgyous and heterozygous individuals.

In summary, our findings suggest that huntingtin is an important intermediate for cellular signaling by the EGF receptor, and possibly by other tyrosine kinase receptors. These results also provide the first clue that a tyrosine kinase receptor-mediated signaling cascade may underlie the pathogenesis of a neurodegenerative disease.

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