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Hrs interacts with SNAP-25 and regulates Ca^{2+}-dependent exocytosis

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SUMMARY

Synaptosome-associated protein of 25 kDa (SNAP-25) is a neuronal membrane protein essential for synaptic vesicle exocytosis. To investigate the mechanisms by which SNAP-25 mediates neurosecretion, we performed a search for proteins that interact with SNAP-25 using a yeast two-hybrid screen. Here, we report the isolation and characterization of a SNAP-25-interacting protein that is the rat homologue of mouse hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs). Hrs specifically interacts with SNAP-25, but not SNAP-23/syndet. The association of Hrs and SNAP-25 is mediated via coiled-coil interactions. Using an Hrs-specific antibody, we have shown that Hrs is highly enriched in brain, where it codistributes with SNAP-25 in most brain regions. Subcellular fractionation studies demonstrate that in brain, Hrs exists in both cytosolic and membrane-associated pools. Studies using indirect immunofluorescence and confocal microscopy reveal that, in addition to early endosomes, Hrs is also localized to large dense-core secretory granules and synaptic-like microvesicles in nerve growth factor-differentiated PC12 cells. Moreover, overexpression of Hrs in PC12 cells inhibits Ca^{2+}-dependent exocytosis. These results suggest that Hrs is involved in regulation of neurosecretion through interaction with SNAP-25.

Key words: SNAP-25, Hrs, SNARE, Vesicular transport, Growth factor, Yeast two-hybrid screen

INTRODUCTION

Recent convergence from studies on constitutive and regulated vesicular transport indicates that neurotransmitter release, like other membrane fusion events, is mediated by specific interactions of proteins on transport vesicle (v-SNAREs) with proteins on the target membrane (t-SNAREs) (Rothman, 1994; Jahn and Sudhof, 1999). Synaptosome-associated protein of 25 kDa (SNAP-25) is a neuronal t-SNARE localized on the plasma membrane of presynaptic nerve terminals (Oyler et al., 1989; Söllner et al., 1993). Specific proteolysis of SNAP-25 by botulinum neurotoxins A and E inhibits neurotransmitter release, demonstrating that SNAP-25 is essential for neuronal exocytosis (Schiavo et al., 1993; Binz et al., 1994); however, the molecular mechanism by which SNAP-25 mediates neurosecretion is not fully understood. SNAP-25 forms a ternary complex with syntaxin and synaptobrevin/VAMP and this interaction is thought to be the minimal machinery for membrane fusion (Weber et al., 1998; Nickel et al., 1999). It is unclear at present how the formation of this complex is regulated to achieve the temporal and spatial specificity as well as the plasticity of synaptic vesicle exocytosis.

To understand the molecular mechanisms that regulate synaptic vesicle docking and fusion, we performed a search for proteins that interact with SNAP-25 using a yeast two-hybrid screen. We report here the isolation and characterization of a SNAP-25-interacting protein that is the rat counterpart of mouse hepatocyte growth factor (HGF)-regulated tyrosine kinase substrate (Hrs) (Komada and Kitamura, 1995). Hrs is a phosphoprotein whose tyrosine phosphorylation is induced upon stimulation by a large number of growth factors and cytokines (Komada and Kitamura, 1995; Asao et al., 1997). Moreover, Hrs interacts with signal-transducing adaptor molecule (STAM), and has been implicated in cell growth signaling (Asao et al., 1997). Very recently, Hrs has been shown to interact with Hrs binding protein (Hbp), an SH3 domain-containing protein involved in the degradation of growth factor/receptor complexes (Takata et al., 2000). Hrs contains a phosphatidylinositiol-3-phosphate-binding FYVE finger, and has been localized to early endosomes (Stenmark et al., 1996; Komada et al., 1997; Gaullier et al., 1998; Misra and Hurley, 1999). Targeted disruption of the *Hrs* gene in mice leads to abnormally enlarged early endosomes and defective ventral folding morphogenesis, implicating a role for Hrs in the vesicular trafficking via endosomes (Komada and Soriano, 1999). It has been reported that Hrs-2, a splice variant that is 80% identical to Hrs and has an additional 150 amino acids at its C terminus, binds SNAP-25 and modulates neurosecretion (Bean et al., 1997); however, it is unclear whether Hrs itself also interacts with SNAP-25 and has a similar role in regulation of exocytosis (Asao et al., 1997).

In the present study, we demonstrate that Hrs interacts with SNAP-25 both in vitro and in vivo. By using deletion analysis, we identified the regions of Hrs and SNAP-25 that mediate

their association. We generated an Hrs-specific antibody, and demonstrated that the expression pattern and subcellular localization as well as the biochemical properties of Hrs differ significantly from Hrs-2. Furthermore, we provide functional evidence for a role for Hrs in regulating Ca^{2+}-dependent exocytosis.

MATERIALS AND METHODS

Yeast two-hybrid screens

Yeast two-hybrid screens of a rat brain library were performed using a full-length mouse SNAP-25b bait (Chin et al., 2000). Prey plasmids from positive clones were rescued, and retransformed into fresh yeast cells with the SNAP-25 bait or various control baits to confirm the specificity of the interaction. The cDNA inserts of positive prey plasmids were sequenced multiple times on both strands, using an Applied Biosystems 373A DNA sequencer.

Analysis of Hrs-SNAP-25 interaction

Deletion constructs of SNAP-25 and Hrs cDNAs were made by PCR using specific primers containing the appropriate restriction sites for subcloning (Li et al., 1993). These constructs were then subcloned into the yeast two-hybrid vectors pPC97 and pPC86, and tested for interaction using the yeast two-hybrid assays with the *HIS3* and *β-galactosidase* as the reporter genes (Chin et al., 2000). Quantitative β-galactosidase assay was performed on the yeast extracts by using the substrate chlorophenol red β-D-galactopyranoside (Li et al., 1993).

Antibody production and western blot analysis

A chicken anti-Hrs antibody was generated against the C-terminal peptide of rat Hrs, CPPAQGNETQLISFD. The N-terminal cysteine residue was added for coupling purposes. The antibody was affinity-purified using the immunogen peptide coupled to a SulfoLink column (Pierce). For western blot analysis, rat tissues were homogenized in 1% SDS and subjected to SDS-PAGE. The proteins were transferred onto nitrocellulose membranes, and probed with the anti-Hrs antibody at 1:5000 dilution. Antibody binding was detected by using enhanced chemiluminescence (ECL, Amersham). The specificity of the anti-Hrs antibody was confirmed by the absence of the immunoreactive band in western blots with the antibody preabsorbed with the peptide immunogen at a ratio of 5 μg peptide per 1 μl antibody.

Production of recombinant proteins

The full-length rat Hrs was subcloned into the prokaryotic expression vectors pET32c (Novagen) to obtain the construct pET32-Hrs. This plasmid encodes a fusion protein, S-Tag-Hrs, which (from amino to carboxy terminus) consists of the 109-amino-acid thioredoxin protein, a hexahistidine tag, and a 15-amino-acid S-tag peptide fused in-frame with the Hrs protein. For the production of glutathione-S-transferase (GST)-fusion proteins, full-length Hrs, SNAP-25b and syntaxin 1B (residues 5-270) were subcloned into the vector pGEX-5X-2 (Pharmacia). Fusion proteins were expressed in bacteria, and purified as described (Chin et al., 2000).

In vitro binding assays

Bacterial lysates prepared from *E. coli* expressing S-Tag-Hrs fusion protein were incubated overnight at 4°C under gentle rocking with the GST-SNAP-25 immobilized on glutathione-agarose beads in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.1% Triton X-100 in the presence of $CaCl_2$ (1 mM), or in the absence of $CaCl_2$ but with 1 mM EGTA. For the Ca^{2+} dose-response analysis, recombinant SNAP-25 was incubated with GST-Hrs immobilized on glutathione-agarose beads in the presence of various Ca^{2+} concentrations. After extensive washes with the same solution, the bound proteins were eluted by boiling in the Laemmli sample buffer, subjected to SDS-PAGE, and analyzed by immunoblotting using horseradish peroxidase (HRP)-conjugated S-protein (Novagen) or anti-SNAP-25 antibody.

Affinity chromatography and detection of endogenous Hrs-SNAP-25 complexes

Rat brain homogenates were prepared as described (Chin et al., 2000). A mini-column of S-Tag-Hrs fusion protein immobilized on His-Bind resin matrix was loaded with the brain homogenates at 4°C, followed by extensive washes with 1× solubilization buffer. Elution of proteins bound to S-Tag-Hrs were performed with the elution buffer (1 M imidazole, 0.5 M NaCl and 20 mM Tris-HCl, pH 7.9). The eluted proteins were subjected to SDS-PAGE and immunoblotting using an anti-SNAP-25 antibody (SMI81, Sternberger Monoclonal). For detection of endogenous Hrs-SNAP-25 complexes, PC12 cells were lysed in the lysis buffer (0.1 M NaCl, 10 mM Hepes-NaOH, pH 7.4, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1% Triton X-100). After removing insoluble material by centrifugation at 120,000 ***g***, the cell lysates were incubated overnight at 4°C under gentle rocking with the GST-syntaxin 1 immobilized on glutathione-agarose beads. After extensive washes, bound proteins were analyzed by immunoblotting using antibodies against Hrs and SNAP-25.

Subcellular fractionations

Subcellular fractionations of rat brain into cytosol fraction (100,000 ***g*** supernatant) and membrane particulate fraction (100,000 ***g*** pellet) were performed as described (Chin et al., 2000). For sucrose gradient flotation analysis, brain membrane particulate fraction (100,000 ***g*** pellet) was resuspended in 55% sucrose in gradient buffer (20 mM Hepes, pH 7.4, 150 mM NaCl and 1 mM DTT) in a volume of 0.4 ml. The resuspended membranes were placed at the bottom of an ultracentrifuge tube and overlaid with 4.8 ml of a linear 25%-52.5% sucrose gradient. Flotation was carried out for 16 hours at 165,000 ***g*** in an SW50.1 rotor. Following centrifugation, the gradient was divided into 18 fractions and analyzed by western blot analysis.

Immunofluorescence microscopy

PC12 cells were grown on poly-L-lysine-coated glass coverslips and differentiated for 2 days with nerve growth factor (NGF; 50 ng/ml). The cells were fixed with 4% paraformaldehyde and processed for indirect immunofluorescence microscopy as described previously (Cameron et al., 1991; Chilcote et al., 1995). The following antibodies were used: the affinity-purified chicken anti-Hrs antibody, a mouse anti-SNAP-25 antibody (SMI 81, Sternberger Monoclonals, Inc.), a mouse anti-synaptophysin antibody (clone SVP-38, Sigma), a rabbit anti-secretogranin II antibody (Biodesign, Inc) and secondary antibodies coupled with fluorescein (FITC) or Texas Red (Jackson Immuno-Research Labs, Inc.). Stained cells were analyzed by using a Leica TCS-NT confocal microscope and the images were processed using Adobe Photoshop 5.0 (Adobe Systems, Inc.).

Expression constructs and transfections

A mammalian expression vector, pCHA, was constructed by subcloning into the multiple cloning site of pcDNA3.1(+) (Invitrogen) the following DNA fragments: a synthetic copy of the translational enhancer from the alfalfa mosaic virus RNA 4 (Jobling and Gehrke, 1987; Browning et al., 1988), a synthetic DNA sequence encoding the influenza virus hemagglutinin (HA) epitope (MGYPYDVPDYAG), and a *Not*I-*Sac*II fragment containing stop codons in all three frames from the vector pPC86 (Li et al., 1995). The expression construct pCHA-Hrs, which directs the expression of an N-terminal HA-tagged Hrs protein, was made by subcloning full-length Hrs cDNA into pCHA. Transfection of HEK293 or PC12 cells was performed using lipofectAMINE (Life Technologies) according to the manufacturer's instructions. Expression of HA-tagged Hrs in transfected cells was confirmed by immunoblot analysis using a mouse anti-HA antibody (Berkeley Antibody Co.).

A

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  1   MGRGSGTFERLLDKATSQLLLETDWESILQICDLIRQGDTQAKYAVNSIK
 51   KKVNDKNPHVALYALEVMESVVKNCGQTVHDEVANKQTMEELKELLKRQV
101   EVNVRNKILYLIQAWAHAFRNEPKYKVVQDTYQIMKVEGHVFPEFKESDA
151   MFAAERAPDWVDAEECHRCRVQFGVVTRKHHCRACGQIFCGKCSSKYSTI
201   PKFGIEKEVRVCEPCYEQLNKKAEGKAASTTELPPEYLTSPLSQQSQLPP
251   KRDETALQEEEELQLALALSQSEAEEKERMRQKSTYTAHPKSEPAPLASS
301   APPAGSLYSSPVNSSAPLAEDIDPELARYLNRNYWEKKQEEARKSPTPSA
351   PVPLTEPAAQPGEGHTAPNSMVEAPLPETDSQPITSCSGPFSEQYQNGES
401   EESHEQFLKALQNAVSTFVNRMKSNHMRGRSITNDSAVLSLFQSINSTHP
451   QLLELLNRLDERRLYYEGLQDKLAQIRDARGALSALREEHREKLRRAAEE
501   AERQRQIQLAQKLEIMRQKKQEYLEVQRQLAIQRLQEQEKERQMRLEQQK
551   QTVQMRAQMPAFPLPYAQLQAMPTAGGVLYQPSGPTSFPGTFSPAGSVEG
601   SPMHGVYMSQPAPATGPYPSMPGTTADPSMVSAYMYPAGAPGAQAAPQAQ
651   AGPTTNPAYSSYQPTPTPGYQNVASQAPQSLPAISQPPQTSNIGYMGSQP
701   MSMGYQPYNMQNLMTTLPGQDASLPAQQPYITGQQPMYQQMAPSTGPPQQ
751   QPPVAQPPPTQGPPAQGNETQLISFD
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B

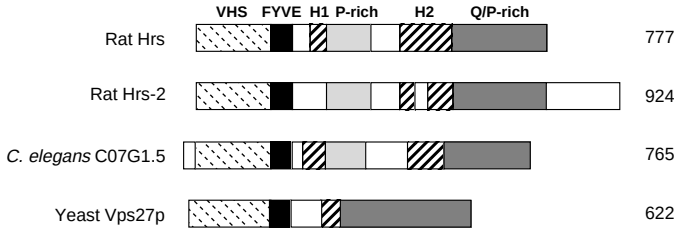

Fig. 1. Structure of rat Hrs. (A) Sequence of rat Hrs. The nucleotide sequence of rat Hrs (not shown) has been deposited in GenBank with accession number AF036344. The deduced amino acid sequence of rat Hrs is shown in single-letter code and numbered on the left. Indicated are the Zn^{2+}-binding FYVE finger (broken underline), the predicated coiled-coil domains (underline), the proline-rich domains potential WW domain interaction motif (double underline), and SH3 domain interaction motifs (black boxes). (B) Domain structures of rat Hrs and related proteins. The names and the lengths of proteins are shown on the left and right, respectively. The following domains and motif are indicated: VHS, VHS domain; FYVE, Zn^{2+}-binding FYVE finger; H1 and H2, predicted coiled-coil domains 1 and 2; P-rich, proline-rich sequence; Q/P-rich, glutamine/proline-rich sequence.

Cotransfections of PC12 cells and assays of growth hormone secretion

PC12 cells were plated in collagen-coated 6-well dishes at a density of 5×10^5 cells per well and were cultured for 24 hours. The cells were then cotransfected with 1 μg of pXGH5 encoding human growth hormone (GH) plus 2 μg pCHA or pCHA-Hrs using lipofectAMINE (Selden et al., 1986). Release experiments were performed 48 hours after transient transfection. After being washed with physiological salt solution (PSS, containing in mM: 145 NaCl, 5.6 KCl, 2.2 $CaCl_2$, 0.5 $MgCl_2$, 5.6 glucose, 0.5 ascorbate, 15 Hepes, pH 7.4), transfected PC12 cells were stimulated to secrete the expressed GH by a 15-minute incubation with PSS containing 56 mM KCl and 95 mM NaCl. The amount of growth hormone released into the medium and retained in the cells was determined by radioimmunoassay (Nichols Institute). Statistical analyses were performed by the unpaired Student's *t*-test.

RESULTS

Identification of rat Hrs as a SNAP-25-interacting protein

To identify regulatory components of neuronal exocytosis, we performed a search in rat brain for proteins that interact with SNAP-25 using a yeast two-hybrid screen. This search led to the isolation of several classes of SNAP-25-interactor clones, including proteins known to bind SNAP-25 such as syntaxin 1B (Table 1), clones corresponding to the rat counterpart of mouse and human Hrs (Table 1), as well as several novel proteins (Chin et al., 2000). Three independent overlapping clones, C2, C25 and C29, encoding rat Hrs were repeatedly isolated as positive interactors. Clone C29 is a full-length rat Hrs cDNA clone encoding a 776-amino-acid protein (Fig. 1, accession number AF036344) that is 97% and 94% identical to mouse and human Hrs, respectively (Komada and Kitamura, 1995; Asao et al., 1997; Lu et al., 1998). Clone C2 encodes amino acids 112-720 of rat Hrs. The cDNA sequence of C25 is identical to nucleotides 547-1674 (corresponding to amino acids 167-541) of rat Hrs except that it has a deletion of three nucleotides (nt), AGC, at position 1228-1230. This 3-nt deletion results in an in-

Table 1. Specific interaction of Hrs with SNAP-25

Clone number	Prey protein	β-galactosidase activity (nmol/min/mg protein)*			
		pPC97-SNAP25	pPC97-Syndet	pPC97-SyP	No bait
C29	Hrs(1-776)	30305±1351	14±1	7±2	5±3
C2	Hrs(112-720)	12301±281	ND	5±2	10±1
C25	Hrs(167-541)	10504±755	ND	7±3	6±2
C23	Syntaxin 1B(5-270)	8092±523	22983±727	11±2	8±2
C24	Syntaxin 1B(94-235)	27648±2955	ND	8±2	15±3

*Positive SNAP-25-interactor clones from a yeast two-hybrid screen were rescued from the yeast and their cDNA inserts were analyzed by DNA sequencing. Numbers in parentheses indicate amino acid positions. The specificity of interaction was tested by retransformation of the prey plasmids into the yeast cells by themselves (No bait) or with the indicated bait construct containing full-length SNAP-25 (pPC97-SNAP25), SNAP23/syndet (pPC97-Syndet) or C-terminal cytoplasmic domain of synaptophysin (pPC97-SyP). The β-galactosidase activity of cell extracts from the yeast transformants was determined using the substrate chlorophenol red β-D-galactopyranoside and normalized to the protein content.

Values are means ± s.d. of the results from triplicate determinations. ND, not determined.

frame deletion of a Gln residue at position 394 of the rat Hrs amino acid sequence, indicating that C25 encodes an Hrs splice variant. Search of the expressed sequence tag (EST) database revealed that the same 3-nt splice variation is also conserved in mouse Hrs EST sequences (accession numbers D21750 and D50050).

Sequence analysis demonstrates that rat Hrs is composed of multiple domains (Fig. 1). At the N terminus, it contains a VHS domain, a recently described domain of unknown function (Lohi and Lehto, 1998). Rat Hrs also has a Zn^{2+}-binding FYVE finger, a putative membrane-targeting motif that binds specifically to phosphatidylinositol 3-phosphate (Stenmark et al., 1996; Misra and Hurley, 1999). By using the algorithm of Lupas et al. (1991), we identified two regions with high probability (P=0.98 and P=0.99, respectively) of forming a coiled-coil structure. In addition, rat Hrs contains a proline-rich sequence (19% proline over 101 amino acids) in the linker region connecting the two coiled-coil domains, and a glutamine/proline-rich sequence (35% glutamine and proline over 212 amino acids) at the C terminus. Within these proline-rich regions, there are four PXXP motifs that can potentially interact with the SH3 domain-containing proteins (Cohen et al., 1995). Furthermore, rat Hrs contains a PPXY motif, a putative binding site for the WW-domain (Einbond and Sudol, 1996).

Sequence comparison analysis demonstrates that the domain structure of rat Hrs is conserved in mouse and human Hrs. Moreover, search of the Non-redundant Protein Database maintained at the National Center for Biotechnology Information reveals that C07G1.5, a putative protein identified by a *C. elegans* genome project, is an Hrs homologue in *C. elegans*. The amino acid sequence of *C. elegans* C07G1.5 is 35% identical to that of rat Hrs, with a conserved domain structure (Fig. 1B). Conspicuous homology is found in the VHS domain (45% identity and 56% similarity), the Zn^{2+}-binding FYVE finger (66% identity and 73% similarity), and in the predicted coiled-coil domain H1 (50% identity and 65% similarity) and H2 (48% identity and 58% similarity), indicating that these domains may contain functional sites. The amino acid sequence of rat Hrs is 82% identical to that of rat Hrs-2, a recently identified SNAP-25-interacting protein that is thought to be involved in regulated exocytosis (Bean et al., 1997). Hrs and Hrs-2 share the same VHS and the FYVE domains and the proline-rich region, with divergent coiled-coil domains H1 (61% identity and 61% similarity) and H2 (79% identity and 79% similarity) as well as the C-terminal glutamine/proline-rich region (53% identity and 53% similarity) (Fig. 1B). In addition, rat Hrs-2 contains an additional 149 amino acids at its C terminus compared to rat Hrs. The amino acid sequence of rat Hrs is also 20% identical to that of Vps27p, a yeast protein that has a role in controlling vacuolar and endocytic traffic through the prevacuolar/endosomal compartment in *Saccharomyces cerevisiae* (Piper et al., 1995). Significant homology is found in the VHS domain (24% identity and 38% similarity), the FYVE finger (42% identity and 53% similarity) and the coiled-coil domain H1 (31% identity and 47% similarity) (Fig. 1B). The sequence homology and similar domain structures suggest that Hrs, Hrs-2 and Vps27p are members of a new protein family with a putative role in vesicular trafficking.

Hrs does not interact with SNAP-23/syndet

SNAP-23/syndet is a ubiquitously expressed SNAP-25 homolog that mediates the exocytosis in non-neuronal cells (Ravichandran et al., 1996; Wang et al., 1997; Araki et al., 1997; Sadoul et al., 1997; Guo et al., 1998). SNAP-23/syndet and SNAP-25 have similar domain structure, and have been shown to interact with the same or similar proteins (Ravichandran et al., 1996; Araki et al., 1997; Foster et al., 1998; Okamoto et al., 1999; Tsujimoto et al., 1999; Chin et al., 2000). To determine whether Hrs also interacts with SNAP-23/syndet, we made an expression construct encoding mouse

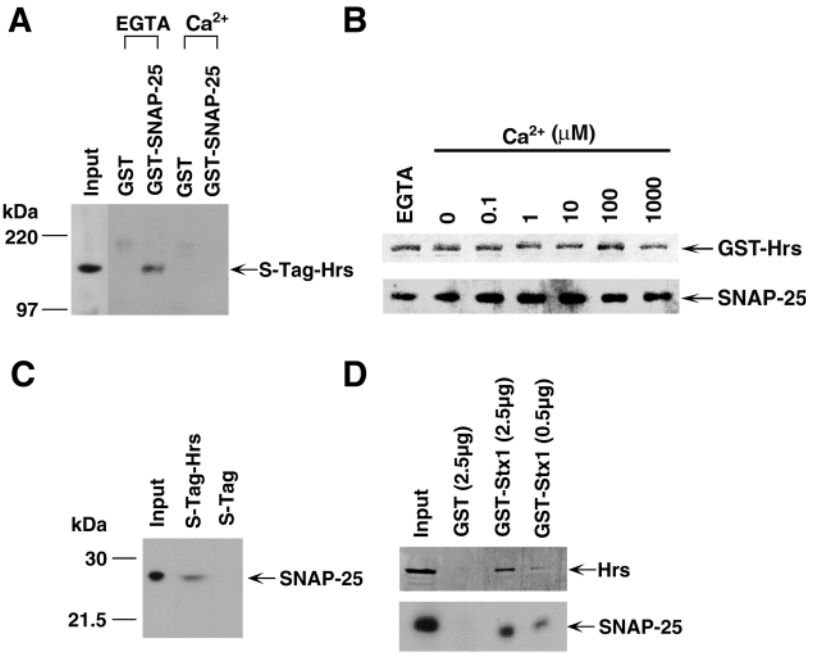

Fig. 2. Biochemical characterization of the association of Hrs with SNAP-25. (A) Binding of recombinant Hrs to immobilized GST-SNAP-25. Lysates of *E. coli* cells expressing S-Tag-Hrs fusion protein were incubated with GST or GST-SNAP-25 immobilized on glutathione-agarose beads in the presence of either 1 mM Ca^{2+} or 1 mM EGTA. After extensive washes, bound proteins were analyzed by SDS-PAGE and blotting with the HRP-conjugated S-protein that specifically binds the S-Tag. Input, 30% of the cell lysates used in the incubation. (B) Binding of recombinant SNAP-25 to immobilized Hrs. Purified recombinant SNAP-25 was incubated with immobilized GST-Hrs in the absence or presence of increasing concentrations of Ca^{2+}. Bound SNAP-25 was detected by immunoblotting, and GST-Hrs was shown as Ponceau S staining. (C) Binding of endogenous SNAP-25 to immobilized S-Tag-Hrs. Rat brain homogenates were applied to a mini-column of S-Tag-Hrs or S-Tag alone. Bound proteins were eluted and analyzed by immunoblotting for SNAP-25. Input, 30% of the brain homogenates loaded onto the columns. (D) Detection of endogenous Hrs-SNAP-25 complexes formed in vivo. PC12 cell lysates were incubated with the indicated amount of GST or GST-syntaxin 1 (GST-Stx 1) immobilized on glutathione-agarose beads. Bound proteins were analyzed by immunoblotting with antibodies against Hrs and SNAP-25. Input, 20% of the cell lysates used in the incubation.

SNAP-23/syndet (Wang et al., 1997) fused to the GAL4 DNA binding domain, and transformed it into yeast CG-1945 cells. The SNAP-23/syndet-transformed yeast cells were subsequently transformed with a plasmid encoding Hrs (clone C29) or syntaxin 1B (clone C23) fused to the GAL4 activation domain and tested for interactions using the yeast two-hybrid assays (Table 1). Unlike many of the known SNAP-25-interacting proteins such as syntaxins, Hrs-2, SNIP and EHSH1/intersectin, Hrs does not interact with SNAP-23/syndet (Table 1). Furthermore, like EHSH1/intersectin (Okamoto et al., 1999), Hrs is unable to bind a more distantly related t-SNARE, syntaxin 1 (data not shown). The inability of Hrs to interact with syntaxin 1 and SNAP-23/syndet further confirms the specificity of the observed Hrs-SNAP-25 interaction.

Biochemical characterization of the association of Hrs with SNAP-25

To examine the interaction of Hrs and SNAP-25 in vitro, the bacterial lysates prepared from the cells expressing S-Tag-Hrs fusion protein were incubated with GST-SNAP-25 fusion protein or GST control immobilized on glutathione-agarose beads in the presence or absence of Ca^{2+}. Analysis of the bound proteins with an HRP-conjugated S protein against the S-tag demonstrated that, in the absence of Ca^{2+}, S-Tag-Hrs was bound to GST-SNAP-25, but not to the control GST protein (Fig. 2A). This specific binding was inhibited by the addition of 1 mM Ca^{2+} (Fig. 2A), suggesting that the Hrs-SNAP-25 interaction may be subjected to regulation by Ca^{2+}. To further examine the Ca^{2+} dependence of the Hrs-SNAP-25 interaction, recombinant SNAP-25 was incubated with immobilized GST-Hrs under varying Ca^{2+} concentrations and the bound proteins were analyzed by immunoblotting using an anti-SNAP-25 antibody (Fig. 2B). Surprisingly, the binding of SNAP-25 to immobilized GST-Hrs was insensitive to Ca^{2+}. The same Ca^{2+}-insensitivity of SNAP-25 binding was also observed when an immobilized N-terminally truncated Hrs (residues 225-776) was used in the binding assays (data not shown). Thus, it seems that the Ca^{2+} effect shown in Fig. 2A is not a real Ca^{2+} modulation because it depends on which binding partner is immobilized.

To determine whether recombinant Hrs is able to bind endogenous SNAP-25, immobilized S-Tag-Hrs fusion protein was used to affinity-purify ('pull-down') endogenous SNAP-25 from brain homogenates. As shown in Fig. 2C, the S-Tag-Hrs fusion protein was able to pull down endogenous SNAP-25. In contrast, control S-Tag protein was unable to pull down SNAP-25, confirming the specificity of the interaction. To further test whether Hrs interacts with SNAP-25 in vivo, immobilized GST-syntaxin 1 fusion proteins were incubated with PC12 cell lysates and the bound proteins were analyzed by immunoblotting using antibodies against Hrs and SNAP-25. The results demonstrate that GST-syntaxin 1 fusion protein, but not the control GST protein, was able to pull down both Hrs and SNAP-25 (Fig. 2D). Since syntaxin 1 could not bind directly to Hrs, these data indicate that the syntaxin 1 beads were able to precipitate the endogenous Hrs-SNAP-25 complex from PC12 cell lysates.

Identification of the Hrs-binding domain of SNAP-25

To identify the specific region of SNAP-25 responsible for interaction with Hrs, we made a series of SNAP-25 deletion mutants that were expressed in yeast as fusion proteins with the GAL4 DNA binding domain (Fig. 3). The interaction of these SNAP-25 deletion mutants with the full-length Hrs was analyzed by the ability to grow on histidine-deficient medium and a β-galactosidase filter assay (data not shown) as well as a quantitative β-galactosidase assay (Fig. 3). The results demonstrated that deletion of either the N-terminal or C-terminal coiled-coil domain of SNAP-25 virtually abolished its ability to bind Hrs, suggesting that multiple domains and/or a complex folded structure of SNAP-25 are required for interaction with Hrs.

Botulinum neurotoxin E (BoNT/E), a potent inhibitor of neurotransmitter release, specifically cleaves SNAP-25 between Arg180-Ile181, resulting in the loss of the C-terminal 17 residues (Schiavo et al., 1993; Binz et al., 1994). Previous studies suggested that the C-terminal 17 residues of SNAP-25 are specifically required for a novel interaction that is functionally involved in exocytotic fusion (Banerjee et al., 1996; Rossi et al., 1997). To test if the Hrs-SNAP-25 interaction represents the novel interaction implicated by these studies, we made a SNAP-25 deletion construct (SNAP-25Δ1) that mimics the cleavage by BoNT/E and lacks the C-terminal 17 residues. As shown in Fig. 3, removal of the C-terminal 17 residues of SNAP-25 reduced its Hrs-binding ability by 97.5%, indicating the last 17 residues of SNAP-25 are required for high affinity Hrs-SNAP-25 interaction.

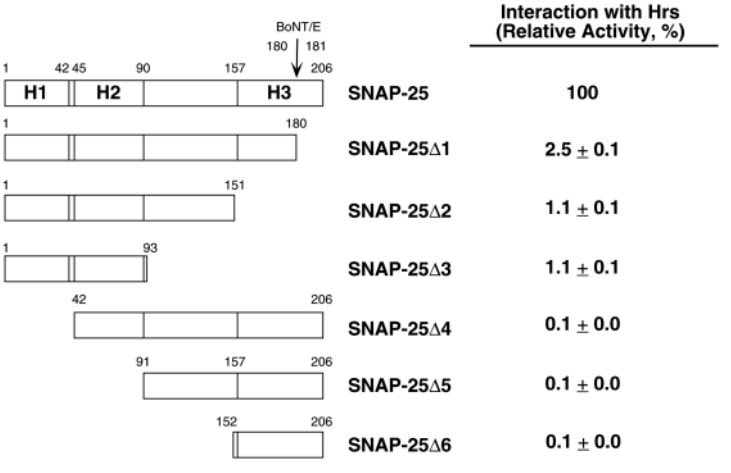

Fig. 3. Identification of the Hrs-interacting domain of SNAP-25. A domain structure of full-length SNAP-25 (residues 1-206) is illustrated, including three predicted coiled-coil domains, H1 to H3. The site of specific cleavage by botulinum neurotoxin BoNT/E is marked by an arrow. Expression plasmids encoding wild-type SNAP-25 and the indicated SNAP-25 deletion mutants fused to the GAL4 DNA binding domain were cotransformed into yeast CG-1945 cells with pPC86-Hrs, a plasmid encoding wild-type Hrs fused to the GAL4 activation domain. The interactions of SNAP-25 deletion mutants with Hrs were tested using yeast two-hybrid assays. The β-galactosidase activity of each sample was normalized to its protein content, and expressed as a percentage of the activity of wild-type SNAP-25. Values are means ± s.d. of the results from triplicate determinations.

Identification of the SNAP-25-binding domain of Hrs

To further understand the structural requirements that underlie the interaction between Hrs and SNAP-25, we carried out similar deletion analysis to map the specific region of Hrs responsible for interaction with SNAP-25 (Fig. 4). The results demonstrated that only the fusion proteins that contain the predicted coiled-coil domain H2 (residues 443-541) of Hrs were able to interact with SNAP-25. Deletions of the VHS domain, FYVE finger, H1 domain, and the two proline-rich domains had little effect on the interaction between Hrs and SNAP-25, indicating that these domains are dispensable to the Hrs-SNAP-25 interaction. Further truncations of the H2 domain abolished its ability to bind SNAP-25, indicating that the H2 coiled-coil domain represents the minimal SNAP-25-binding domain of Hrs.

Distribution of Hrs expression in rat tissues and brain regions

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed that rat Hrs mRNA is present in all the tissues examined, including brain, heart, kidney, liver, lung, and spleen (data not shown). This result is consistent with the widespread pattern of mouse and human Hrs mRNA expression reported previously (Komada and Kitamura, 1995; Asao et al., 1997; Lu et al., 1998), but the tissue distribution of Hrs protein has not yet been reported. To analyze the expression of Hrs at protein level, an Hrs isoform-specific antibody was generated against the rat Hrs C-terminal 14 amino acids, a peptide sequence that is present only in Hrs but not in Hrs-2. To characterize this antibody, HEK293 cells were transfected with an expression construct pCHA-Hrs, which directed the expression of an HA epitope-tagged Hrs recombinant protein (HA-Hrs) (Fig. 5A, lane 2). Immunoprecipitation of HA-Hrs with an anti-HA antibody and immunoblotting with the anti-Hrs antibody demonstrate that the HA-Hrs recombinant protein is specifically recognized by the chicken anti-Hrs antibody (Fig. 5A, lanes 4 and 6), but not by the preimmune chicken IgY (data not shown). The anti-Hrs antibody also recognized the 115-kDa human Hrs endogenously expressed in HEK293 cells (Fig. 5A, lanes 3 and 4). Since HA-Hrs contains an extra 14 amino acids, including the HA epitope, at the N terminus of rat Hrs, it migrated slightly slower than the endogenous human Hrs (Fig. 5A, lane 4). The specificity of the anti-Hrs antibody was further confirmed by the following experiments. (1) The preimmune chicken IgY did not react with the recombinant or endogenous Hrs protein (data not shown). (2) Pre-absorption of the anti-Hrs antibody with the peptide immunogen completely eliminated its immunoreactivity to the recombinant as well as endogenous Hrs protein (data not shown).

Using this Hrs-specific antibody, we demonstrated that rat Hrs is a 115-kDa protein that is abundantly expressed in brain, testis and pancreas, and moderately expressed in spleen (Fig. 5B), which differs significantly from the expression pattern of rat Hrs-2 (Bean et al., 1997). Longer exposure of the same blot demonstrated that Hrs is also present at a low level in all other tissues tested, namely, heart, kidney, liver, lung and skeletal muscle (data not shown). The ubiquitous expression of Hrs protein in various rat tissues, although the expression level varies from tissue to tissue, is consistent with the tissue distribution of Hrs mRNA expression (Komada and Kitamura, 1995; Asao et al., 1997; Lu et al., 1998). To further characterize the expression of Hrs in brain, various brain regions were dissected from adult rats and subjected to western blot analysis using the anti-Hrs antibody (Fig. 5C). The results show that Hrs is relatively abundant in amygdaloid area, parietal and retrosplenial areas of cerebral cortex, hippocampus, striatum, olfactory bulb, cerebellum, hypothalamus, medulla oblongata, spinal cord and pituitary gland, and expressed moderately in hindlimb area of cerebral cortex, thalamus, superior and inferior colliculi. For comparison, we also analyzed the same tissue samples with antibodies to SNAP-25 and synaptophysin (Fig. 5C). Although the abundance of SNAP-25 in pituitary gland was low relative to brain tissues and we did not detect SNAP-25 immunoreactivity in the sample of pituitary gland, it is well established that SNAP-25 is also expressed in pituitary gland (Aguado et al., 1996; Jacobsson and Meister, 1996). Thus, Hrs is coexpressed with SNAP-25 throughout the brain and in pituitary gland.

Subcellular distribution of Hrs in brain

To examine the intracellular distribution of Hrs, rat brain postnuclear supernatant was separated into cytosol (100,000 ***g*** supernatant) and membrane particulate (100,000 ***g*** pellet) fractions, and then subjected to western blot analysis with the anti-Hrs antibody (Fig. 6A). Hrs immunoreactivity was detected in both cytosol and membrane particulate fractions, although the relative amount of Hrs in the cytosol fraction was several fold more than that in the particulate fraction.

To determine whether the presence of Hrs in the particulate fractions is due to its association with membranes or with large proteinaceous complexes, the brain particulate fraction (100,000 ***g*** pellet) was further fractionated by flotation in a

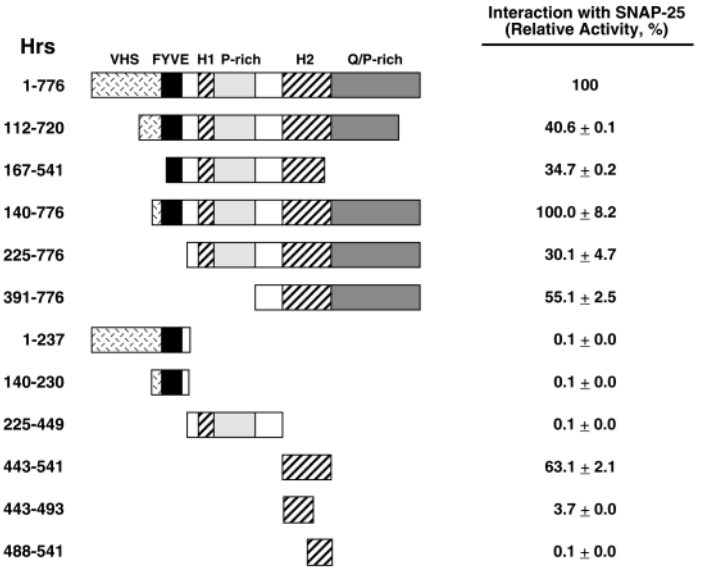

Fig. 4. Identification of the SNAP-25-interacting domain of Hrs. Expression plasmids encoding wild-type Hrs and the indicated Hrs deletion mutants fused to the GAL4 activation domain were cotransformed into yeast CG-1945 cells with pPC97-SNAP25. Yeast two-hybrid assays were performed as in Fig. 3. The β-galactosidase activity of each sample was normalized to its protein content, and expressed as a percentage of the activity of wild-type Hrs. Values are means ± s.d. of the results from triplicate determinations.

sucrose density gradient (Fig. 6B). SNAP-25, a protein that is predominantly associated with the plasma membrane, was enriched in fractions 9-13. In contrast, synaptophysin, a synaptic vesicle membrane protein, had a broader distribution pattern that not only overlapped with the SNAP-25 peak fractions but also included the dense portion of the gradient. Although a portion of Hrs stayed at the bottom of the gradient as insoluble protein aggregates, the majority of Hrs floated up to the same region of the sucrose gradient as SNAP-25, indicating an association of Hrs with brain membranes.

Immunolocalization of Hrs in NGF-differentiated PC12 cells

In non-neuronal cells with only constitutive secretory pathways, Hrs has been shown to localize on early endosomes (Komada et al., 1997). Since Hrs was isolated here as a protein that interacts with SNAP-25, a t-SNARE that is involved in regulated exocytosis, it would be of particular interest to determine the intracellular distribution of Hrs in cells with regulated secretory pathways, such as neurons or neuroendocrine cells. PC12 is a well-characterized neuroendocrine cell line that shares many characteristics of sympathetic neurons, such as secretion of neurotransmitters and the response to nerve growth factor (NGF) (Greene and Rein, 1977). The regulated secretion in PC12 cells involves two classes of secretory organelles: large dense-core vesicles, and synaptic-like microvesicles that are biochemically very similar to neuronal synaptic vesicles (Cameron et al., 1991; Bauerfeind et al., 1993). For immunofluorescence studies, PC12 cells were treated to induce the formation of neurites, which are enriched in synaptic-like microvesicles and large dense-core vesicles but not endosomes (Chilcote et al., 1995). Immunofluorescence analysis of NGF-differentiated PC12 cells using the affinity-purified anti-Hrs antibody revealed a punctate staining pattern, which demonstrates that Hrs is localized on vesicular structures concentrated at the cell periphery and in the neurite extensions (Fig. 7A,D,G,J,M,P). Control experiments using the preimmune chicken IgY or pre-absorption with Hrs antigen confirmed that the Hrs staining is specific (data not shown). Double immunofluorescence studies using antibodies against Hrs and SNAP-25 demonstrate that the overall staining pattern of Hrs is distinct from that of SNAP-25, which is primarily localized to the plasma membrane (Fig. 7A-F).

To investigate the identity of Hrs-positive vesicular structures, we performed double immunofluorescence experiments to compare the distribution of Hrs with synaptophysin (Fig. 7G-L). Synaptophysin is an integral membrane protein of synaptic vesicles that has been widely used as a marker for synaptic-like microvesicles and endosomes in PC12 cells (Cameron et al., 1991; Linstedt et al., 1992). In the cell bodies of NGF-differentiated PC12 cells, Hrs immunoreactivity partially overlaps with synaptophysin distribution, indicating that Hrs is localized on synaptic-like microvesicles and endosomes. In the neurites, a substantial overlap between Hrs and synaptophysin immunoreactivity was observed (Fig. 7G-L). Previous studies have shown that endosomes, as detected by transferrin receptor

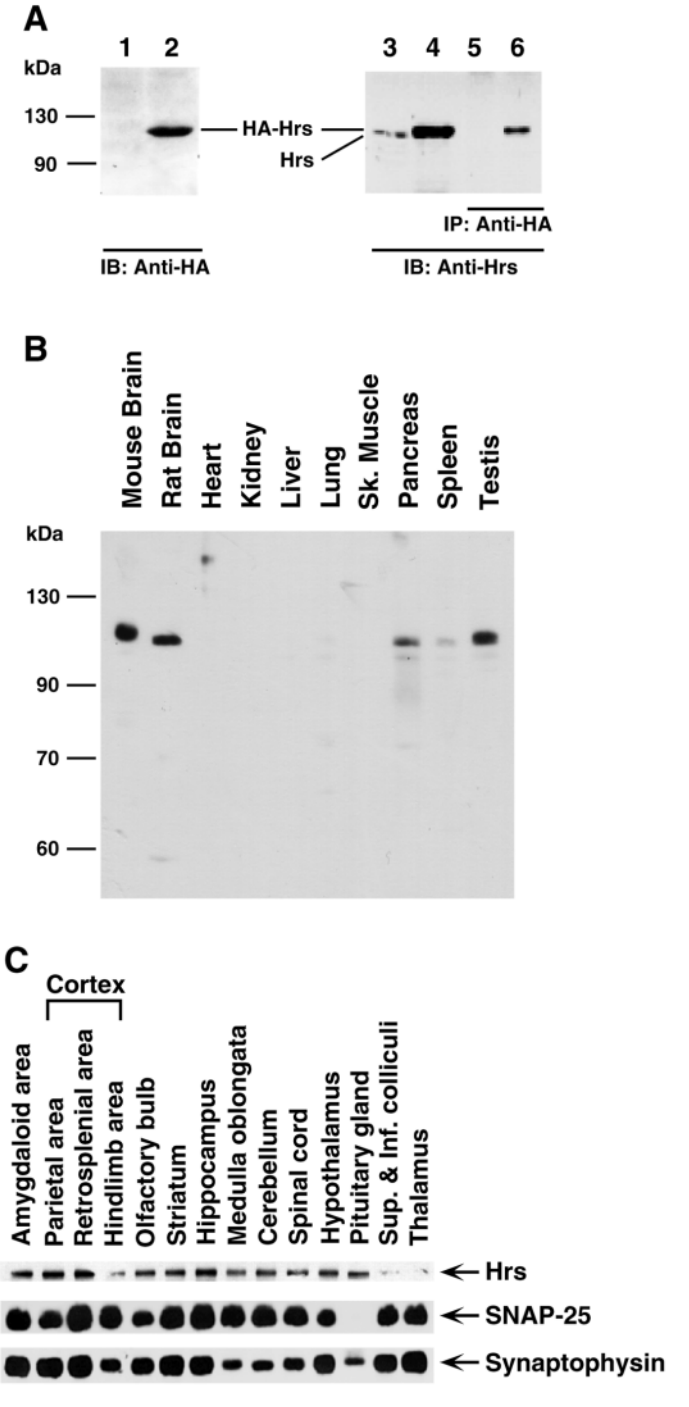

Fig. 5. Expression and distribution of Hrs protein. (A) Specificity of the anti-Hrs antibody. HEK293 cells were transfected with pCHA vector (lanes 1, 3 and 5) or pCHA-Hrs (lanes 2, 4 and 6). Cell lysates were prepared from the transfected cells and analyzed by immunoblotting using an anti-HA antibody (lanes 1 and 2) or anti-Hrs antibody (lanes 3 and 4). In lanes 5 and 6, the cell lysates were immunoprecipitated with an anti-HA antibody, and the precipitates were then analyzed by immunoblotting for Hrs. (B) Western blot analysis of Hrs expression in rat tissues. Homogenates (30 μg of protein per lane) from the indicated rat tissues (lanes 2-10) and mouse brain (lane 1) were analyzed by immunoblotting using the anti-Hrs antibody. Sk., Skeletal. (C) Regional distribution of Hrs in rat brain, compared with SNAP-25 and synaptophysin. Equal amounts of homogenate (30 μg protein per lane) from the indicated brain region were analyzed by SDS-PAGE and immunoblotting for Hrs, SNAP-25 and synaptophysin.

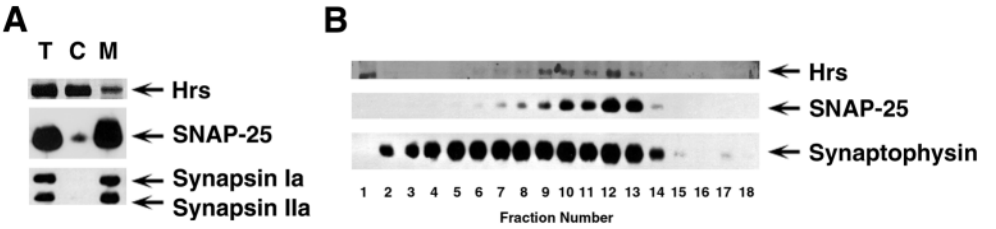

Fig. 6. Subcellular localization of Hrs in rat brain. (A) Hrs exists in both cytosolic and membrane-bound pools. Postnuclear supernatants (T) from rat brains were separated into cytosol fraction (C) and membrane particulate fraction (M). Portions representing an equal percentage of each fraction were analyzed by SDS-PAGE and immunoblotting for Hrs, SNAP-25 and synapsins. (B) Cofractionation of Hrs with SNAP-25 on a sucrose gradient. Brain membranes were placed at the bottom of a sucrose gradient and subjected to a flotation analysis. The gradient was divided into 18 fractions, with fraction 1 corresponding to the bottom of the gradient. Equal volumes of each fractions were analyzed by SDS-PAGE and immunoblotting for Hrs, SNAP-25 and synaptophysin.

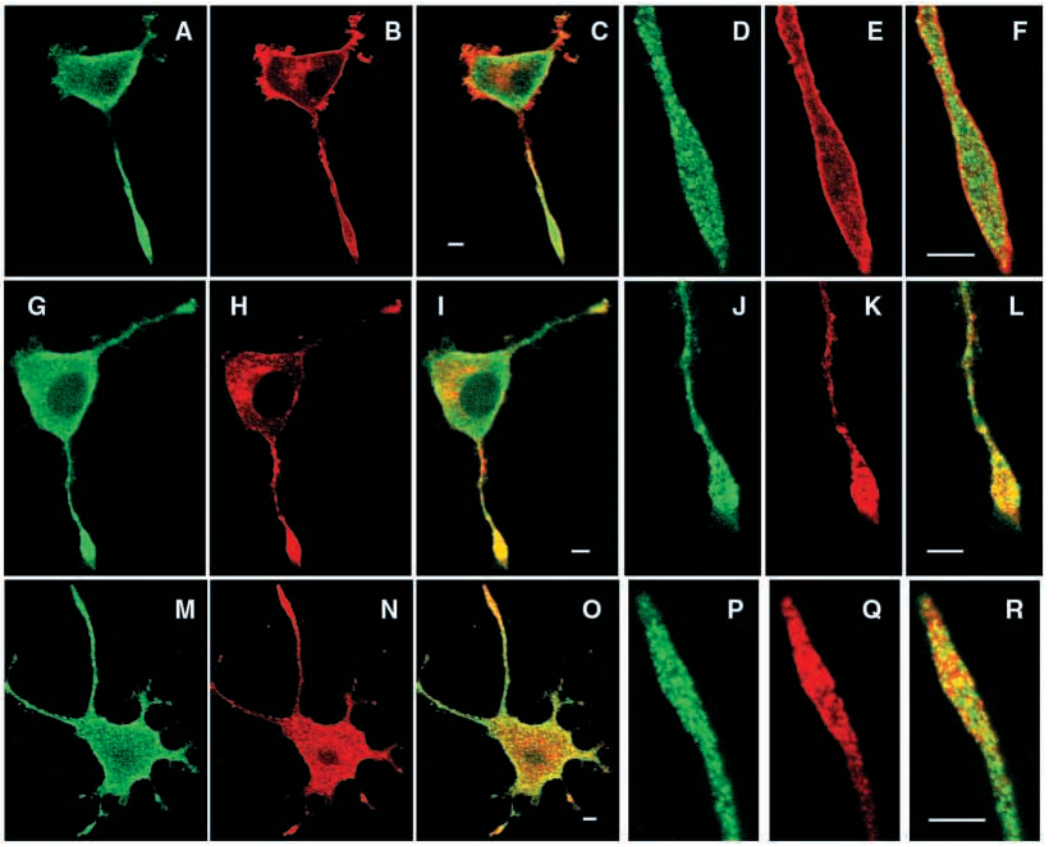

Fig. 7. Comparison of the distribution of Hrs with SNAP-25, synaptophysin and secretogranin II in NGF-differentiated PC12 cells. NGF-differentiated PC12 cells were stained with a chicken anti-Hrs antibody, which was detected with a secondary antibody conjugated to fluorescein (A,D,G,J,M,P). The same cells were simultaneously stained with primary antibodies against SNAP-25 (B,E), synaptophysin (H,K) or secretogranin II (N,Q), which were detected with secondary antibodies conjugated to Texas Red. Superimposed images (C,F,I,L,O,R) demonstrate the overlapping distribution of these proteins. The three panels on the left are higher magnification images of the same neurites shown in the three panels on the right. Bars, 5 μm.

immunoreactivity or internalized transferrin staining, are restricted to cell bodies and are virtually absent from neurites (Cameron et al., 1991; Chilcote et al., 1995). Thus, the overlapping distribution of Hrs and synaptophysin in neurites suggests that a significant portion of Hrs is localized on synaptic-like microvesicles.

To determine whether Hrs is also localized on large dense-core vesicles, double immunofluorescence experiments were performed using antibodies against Hrs and secretogranin II (Fig. 7M-R). Secretogranin II is a secretory protein stored in the dense-core secretory granules of neuronal and endocrine cells and has been widely used as a marker for large dense-core vesicles in PC12 cells (Huttner et al., 1991). A significant overlap between Hrs and secretogranin II immunoreactivity was observed, particularly at the cell periphery and in the neurite extensions (Fig. 7M-R), suggesting that a fraction of Hrs is associated with large dense-core vesicles.

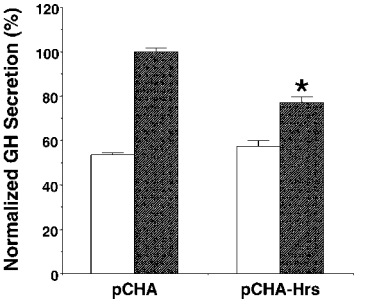

Fig. 8. Overexpression of Hrs inhibits Ca^{2+}-dependent release of growth hormone from PC12 cells. PC12 cells were cotransfected with pCMV-GH and pCHA or pCHA-Hrs. GH secretion was stimulated by a 15-minute incubation with low K^+ solution (5.6 mM, open bar) or high K^+ solution (56 mM, shaded bar). The amount of GH secreted into the medium and retained in the cells was determined by radioimmunoassay and the percentage of GH secreted was calculated. For comparison between experiments, GH secretion was normalized to the high K^+-induced GH secretion observed in the control transfection. Values are means ± s.e.m. (n=4). *P<0.001 versus GH secretion from cells transfected with control plasmid pCHA.

To verify the localization of Hrs on synaptic vesicles and secretory granules, subcellular fractionations of rat brain and PC12 cells were performed using the standard procedures (Huttner et al., 1983; Liu and Edward, 1997; Chin et al., 2000). The vast majority of Hrs was found in the cytosol fractions and very little Hrs immunoreactivity could be detected in the fractions enriched in synaptic vesicles and secretory granules (data not shown). These results suggest that Hrs is loosely associated with cellular membranes or that an essential factor necessary for Hrs membrane association was lost during biochemical procedures. Consistent with these observations, Hrs was first reported as a cytosolic protein based on fractionation studies whereas subsequent immunolocalization studies showed that it is on endosomes in HeLa cells (Komada and Kitamura, 1995; Komada et al., 1997). Similar discrepancy has previously been observed for other proteins such as α-synuclein, which was found to be localized to synaptic vesicles by immunostaining but was found in the cytosol after biochemical separation (Iwai et al., 1995).

Role of Hrs in neurosecretion

The subcellular localization of Hrs and its association with SNAP-25 suggested a possible involvement of Hrs in Ca^{2+}-dependent exocytosis. To examine this possibility, we investigated the effect of overexpression of Hrs on the regulated secretion of growth hormone (GH) from transfected PC12 cells using a GH cotransfection secretion assay (Wick et al., 1993). This assay uses human GH expressed from the cotransfected plasmid as a reporter for regulated exocytosis, and has been widely used for functional studies of presynaptic proteins (Orita et al., 1996; Fujita et al., 1998). The expressed GH is known to be stored in dense core vesicles of the transfected cells, and undergoes Ca^{2+}-dependent exocytosis in response to depolarization by high K^+ (Schweitzer and Kelly, 1985; Wick et al., 1993). As shown in Fig. 8, overexpression of Hrs resulted in a significant decrease in the high K^+-induced GH release, while it had no effect on basal GH release. These results suggest that Hrs is involved in regulated secretion from PC12 cells.

DISCUSSION

Evidence accumulated over the last few years clearly indicates that SNAP-25 plays an essential role in mediating synaptic vesicle exocytosis, but its mechanisms of action are not understood. In this study, we have isolated and characterized a SNAP-25-interacting protein that is the rat homologue of mouse and human Hrs and is likely to be a splice variant of rat Hrs-2. Through a yeast two-hybrid screen, we discovered that Hrs can physically associate with SNAP-25. In vitro binding studies have demonstrated that Hrs, like its isoform Hrs-2, can directly interact with SNAP-25. Furthermore, endogenous Hrs-SNAP-25 complexes have been detected in mammalian cells, providing direct evidence for the association of Hrs and SNAP-25 in vivo. Unlike Hrs-2 (Tsujimoto et al., 1999), Hrs is unable to bind SNAP-23/syndet, a ubiquitously expressed SNAP-25 homologue that shares 65% identity and 73% similarity with SNAP-25 (Wang et al., 1997). In addition, Hrs does not interact with syntaxin 1, a distantly related protein with a coiled-coil t-SNARE motif homologous to SNAP-25 (Weimbs et al., 1997). These results indicate that the interaction between Hrs and SNAP-25 is highly specific. Since it has been reported that Hrs-2 interacts with SNAP-25 in a Ca^{2+}-sensitive manner (Bean et al., 1997), we sought to examine whether the Hrs-SNAP-25 interaction is also modulated by Ca^{2+}. Our data demonstrated that, although the binding of soluble Hrs to immobilized SNAP-25 was sensitive to Ca^{2+}, the binding of soluble SNAP-25 to immobilized Hrs was insensitive to Ca^{2+}. Thus, Ca^{2+} is unlikely to be involved in modulating the Hrs-SNAP-25 interaction because the Ca^{2+} effect is dependent on which binding partner is immobilized. These observations also raise the question of whether the Hrs-2-SNAP-25 interaction is truly modulated by Ca^{2+}, because the Ca^{2+} effect was studied using only immobilized SNAP-25, and not the other way around, using immobilized Hrs-2 (Bean et al., 1997).

Hrs contains a VHS domain and FYVE finger, two putative coiled-coil domains, and two proline-rich regions containing multiple PPXY and PXXP motifs. Thus, Hrs could potentially interact with multiple proteins or be involved in the formation of multi-protein complexes via coiled-coil interactions, associations of its proline-rich motifs with the SH3 domain- or WW domain-containing proteins, and/or other types of protein-protein interactions involving the VHS domain or FYVE finger. By using deletion analysis, we have mapped the minimal SNAP-25-binding region to the second coiled-coil (H2) domain of Hrs. Our data indicate that the interaction between Hrs and SNAP-25 is likely to be mediated through a coiled-coil mechanism. Very recently, it has been reported that a similar region of Hrs-2 is involved in binding SNAP-25 (Tsujimoto and Bean, 2000). Since the H2 domain of Hrs is 79% homologous to the corresponding region of Hrs-2 (Fig. 1B), it is possible that the differences in amino acid sequence between this region of Hrs and Hrs-2 contribute to the observed differences in the specificity of the protein-protein interactions. For example, Hrs-2 interacts with both SNAP-25 and SNAP-23 (Tsujimoto et al., 1999), whereas Hrs only interacts with SNAP-25 but not SNAP-23.

To further elucidate the structural requirement underlying the specific association of Hrs with SNAP-25, we performed deletion analysis to delineate the minimal region of SNAP-25 responsible for its interaction with Hrs. SNAP-25 contains two coiled-coil t-SNARE motifs, both of which are involved in the formation of the four-stranded helical bundle of the SNARE complex (Weimbs et al., 1997; Sutton et al., 1998). We have shown that partial or complete deletion of either one of these t-SNARE motifs abolishes the ability of SNAP-25 to bind Hrs, indicating that both of these motifs or a complex folded structure of SNAP-25 are required for interaction with Hrs. This stringent structure requirement is consistent with the specific interaction of Hrs with SNAP-25, but not with SNAP-23/syndet. In contrast, the Hrs-2-binding domain has been localized to the N-terminal t-SNARE motif of SNAP-25 (Tsujimoto and Bean, 2000).

Hrs-2 has been shown to be an ATP-preferring nucleotidase (Bean et al., 1997). However, we were unable to demonstrate that Hrs has similar enzymatic activity. In rat Hrs-2, the putative nucleotide-binding site is thought to consist of four sequence motifs: G1, GXXXXGK; G2, RDET; G3, DXXG; and G4, TQKXD (Bean et al., 1997). The G4 motif is absent in Hrs sequences from all species, including rat Hrs. The G1 motif, which is also referred to as the phosphate-binding loop

(P-loop), is a common motif found in many ATP- and GTP-binding proteins (Saraste et al., 1990; Kjeldgaard et al., 1996). X-ray structure determination of GTP-binding proteins revealed that the G1 motif is directly involved in binding β-phosphate groups (Kjeldgaard et al., 1996). According to the structure analysis, the sixth residue in the G1 motif is always a glycine since a side chain in this position would interfere sterically with the guanine part of the nucleotide (Kjeldgaard et al., 1996). However, this glycine residue is substituted by aspartate in *C. elegans* Hrs and by glutamine in the yeast Hrs homologue, Vps27p. In addition, the critical lysine residue in the G1 motif, which is thought to stabilize the transition state of the phosphoryl transfer reaction, is substituted by glutamate in Vps27p (Saraste et al., 1990; Reinstein et al., 1990). The lack of conservation of the nucleotide-binding site in the Hrs sequences from different species raises the possibility that the nucleotidase activity may be a unique feature of the Hrs-2 isoform. It is interesting to note that the G1 motif of Hrs-2 is localized within the FYVE finger and overlaps with the conserved RRHHCRQCGNIF motif that binds phosphatidylinositol 3-phosphate and contacts soluble and micelle-embedded lipid (Misra and Hurley, 1999; Kutateladze et al., 1999).

The subcellular localization of Hrs is different from Hrs-2, which has been reported to be present on membranes of multivesicular bodies in undifferentiated PC12 cells (Tsujimoto et al., 1999). Previous studies in nonneuronal cells have demonstrated that Hrs is localized to early endosomes (Komada et al., 1997; Komada and Soriano, 1999). In this study, we have shown that Hrs is also enriched in brain, where it exists in both cytosolic and membrane-associated pools and codistributes with SNAP-25 in most regions. In NGF-differentiated PC12 cells, immunofluorescence studies revealed that Hrs is localized on vesicular structures concentrated at the cell periphery and in the neurite extensions. The staining pattern of Hrs partially overlaps with the distribution of secretogranin II, a marker for large dense-core secretory granules, and synaptophysin, a marker for synaptic-like microvesicles and endosomes. Although we could not detect Hrs in the purified synaptic vesicle fractions, perhaps as a result of loose association of Hrs with vesicle membranes, sucrose gradient flotation analysis did show that about 5% of total Hrs cofractionates with SNAP-25 on sucrose gradient, which also partially overlaps with synaptophysin distribution. Together, these data suggest that, in addition to endosomes, Hrs is also localized on dense-core secretory granules and synaptic-like microvesicles.

In addition to the previously proposed role for Hrs in the endocytic pathway, our data indicate that Hrs also functions in Ca^{2+}-dependent exocytosis. Recent studies have shown that neuronal exocytosis is modulated, on a rapid time scale, by a variety of growth factors including EGF (Barrie et al., 1996; Berninger and Poo, 1996). Our results, together with previous phosphorylation data (Komada and Kitamura, 1995), suggest that Hrs may be a downstream effector molecule that mediates the modulation of neuronal exocytosis by growth factors. As to the mechanism of action of Hrs in exocytosis, at least three possibilities exist. The first possibility is that the cytosolic form of Hrs specifically binds SNAP-25 at the plasma membrane and prevents it from interacting with other components of the exocytotic machinery. The second possibility is that the secretory vesicle/endosome-associated form of Hrs interacts with plasma membrane-associated SNAP-25 and this interaction mediates and/or regulates docking and/or fusion of secretory vesicle/endosome with the plasma membrane. The third possibility is that, rather than having a specific role in exocytosis, Hrs has a more general chaperone-like function in regulating the activity of SNAP-25 and other proteins. Molecular chaperones, such as N-ethylmaleimide-sensitive fusion protein and cysteine string protein, have been shown to function in exocytosis by coordinating sequential protein-protein interactions involved in the docking and fusion reactions, although these proteins also chaperone proteins other than those involved in exocytosis (Chamberlain and Burgoyne, 1997; Haas, 1998). A general chaperone-like function would be consistent with the ubiquitous expression of Hrs and with the interactions of Hrs with other proteins such as STAM and Hbp (Asao et al., 1997; Takata et al., 2000). Further studies are required to distinguish these possibilities and clarify the mechanism of action of Hrs in vesicular trafficking.

The nucleotide sequence reported in this article has been submitted to the GenBank/EMBL Data Bank with accession number AF036344.
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