D53 is a novel endosomal SNARE-binding protein that enhances interaction of syntaxin 1 with the synaptobrevin 2 complex *in vitro*

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Synaptobrevin 2 (Sb2), syntaxin1 (Stx1), and synaptosomal-associated protein of 25 kDa (SNAP-25) are the main components of the soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) complex involved in fusion of synaptic vesicles with the presynaptic plasma membrane. We report the characterization of D53, a novel SNARE-binding protein preferentially expressed in neural and neuro-endocrine cells. Its two-dimensional organization, established by the hydrophobic cluster analysis, is reminiscent of SNARE proteins. D53 contains two putative helical regions, one of which includes a large coiled-coil domain involved in the interaction with Sb2 *in vitro*. Following subcellular fractionation, endogenous D53 was specifically detected in the membrane-containing fraction of PC12 cells, where it co-immunoprecipitated with Sb2. Analysis by confocal microscopy showed that, in these cells, endogenous D53 co-localized partially with the transferrin receptor in early endosomes. *In vitro* assays revealed that binding properties of D53 to Stx1 and Sb2 are comparable with those of SNAP-25. Furthermore, D53 forms Sb2/Stx1/D53 complexes *in vitro* in a manner similar to SNAP-25. We propose that D53 could be involved in the assembly or disassembly of endosomal SNARE complexes by regulating Sb2/Stx interaction.

Key words: coiled-coil, early endosomes, intracellular trafficking, PC12 cells.

INTRODUCTION

Fusion of synaptic vesicles with the plasma membrane is mediated by the formation of SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) complexes involving proteins specifically located on the synaptic vesicles (v-SNAREs), synaptobrevin 2 (Sb2), and target proteins located on the plasma membrane (t-SNAREs), syntaxin1 (Stx1) and the synaptosomal-associated protein of 25 kDa (SNAP-25). The synaptic vesicle protein, Sb2, is considered to be one of the key proteins involved in exocytotic membrane fusion. Sb2 and Stx1 are transmembrane proteins containing a coiled-coil domain. SNAP-25 contains two coiled-coil domains located in the N- and C-terminal regions and is anchored to the plasma membrane by palmitoylated cysteine residues present in the middle part of the molecule [1,2]. *In vitro*, Sb2, SNAP-25 and Stx1 form a bundle of four α helices [3,4] that bridges donor and target membranes in close proximity, thereby leading to lipid bilayer fusion [5]. Disassembling of the SNARE complex, allowing further rounds of membrane fusion, is triggered by binding of soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein and the ATP-dependent activity of *N*-ethylmaleimide-sensitive fusion protein (for review see [6]). Major evidence for the involvement of these proteins in neurosecretion results from the fact that secretion was inhibited by treatment with botulinum or tetanus neurotoxins which specifically cleave Sb2, Stx1 and SNAP-25 (for reviews see [7–9]).

Sb2, Stx1 and SNAP-25 are the prototypes of protein families whose members have been identified in cells as distantly related as yeast and mammalian neurons. This suggests that the SNARE mechanism is highly conserved throughout secretory pathways [10–12].

We have characterized R10 [13], an avian protein containing a coiled-coil domain, expression of which is developmentally regulated in chick embryo neuroretina. It was reported that the gene encoding the human orthologue of this protein is overexpressed in tumours and tumour cell lines, in particular in breast and lung adenomas and adenocarcinomas [14].

In this study we report the biochemical characterization of D53, a highly R10-related protein expressed in neural and neuroendocrine cells. We show that both D53 and R10 belong to a structurally conserved family of proteins sharing a coiled-coil domain. This domain is necessary in the location of D53 on early endosomes. We also show that D53 directly interacts with Sb2 and Stx1 and potentiates, *in vitro*, the formation of Sb2/Stx1/D53 complexes.

Analysis of its two-dimensional (2D) organization together with its *in vitro* properties, suggest that D53 could regulate SNARE complexes on early endosomes.

EXPERIMENTAL

Cell culture and transfection

PC12 cells were plated on collagen-coated plastic Petri dishes, and grown as described previously [15]. HeLa and COS-7 cells

Abbreviations used: DTT, dithiothreitol; 2D, two-dimensional; 3D, three-dimensional; GFP, green fluorescent protein; GST, glutathione S-transferase; HCA, hydrophobic cluster analysis; LB, Luria–Bertani; NSF, *N*-ethylmaleimide sensitive fusion protein; Sb2, synaptobrevin2; SNAP-25, 25 kDa synaptosomal-associated protein; SNARE, soluble *N*-ethylmaleimide sensitive fusion protein attachment protein receptor; t-SNARE, SNARE complexes involving target proteins located on the plasma membrane; v-SNARE, SNARE complexes involving proteins located on the synaptic vesicles; Stx1, syntaxin1; u*A*, uncalibrated absorbance values; wt, wild-type.

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The chicken D53 sequence has been assigned GenBank® accession number AY004870.

were maintained in Dulbecco's modified Eagle medium containing 7 % fetal calf serum. The cells were transiently transfected with expression vectors by the DEAE Dextran-chloroquin method as described previously [16].

Construction of two-hybrid cDNA library

Oligo (dT)- and random-primed two-hybrid system libraries of neuroretina from 13-day-old chick embryos were prepared in lambdaHybriZAP vector using ZAP-cDNA Gigapack II Gold Cloning Kit and HybriZAP Two-Hybrid Predigested Vector Kit (Stratagene, La Jolla, CA, U.S.A.).

Plasmid construction and mutagenesis

The pGBT9 R10 plasmid, used as a bait for the two-hybrid screening, was constructed by cloning amino acids 52–251 of R10 [13] in frame with the GAL4 DNA-binding domain of the vector.

To obtain constructs synthesizing glutathione S-transferase (GST)-D53 and GST-Sb2 fusion proteins, the *Eco*RI/*Sal*I fragments of pAD-GAL4D53 and pAD-GAL4Sb2 plasmids, isolated by the two-hybrid screening, were inserted in frame with the GST gene in the pGEX-4T1 vector (Amersham Biosciences Europe, Saclay, France) resulting in pGEX/D53 and pGEX/Sb2. The *Eco*RI/*Sal*I fragment of pAD-GAL4D53 was also inserted in frame with the green fluorescent protein (GFP) gene in the pEGFP-C2 vector (Clontech Laboratories, Palo Alto, CA, U.S.A.) to produce pEGFP/D53.

The pGEX/NterD53 construct was prepared from the pGEX/D53 plasmid by deleting the *Blp*I and *Xho*I restriction fragment which encoded the C-terminal part of the protein. This plasmid encodes the first 40 amino acids of D53 coupled to GST.

To obtain the construct synthesizing a haemagglutinin-tagged D53 protein, the coding region of D53 was fused in frame at its 3′ end with the DNA fragment encoding the nine-residue epitope HA1 of the influenza virus haemagglutinin, by PCR. The primers used were: 3005 (5′), 5′-GACGCGGTGCTGCGACC-3′; and 3006HA (3′), 5′-TCAGAGGCTAGCATAATCAGGAACATCATACGGACACTGAAGCTCTTCTT-3′, encoding the HA epitope. The PCR product was cloned into the pMOSBlue vector (Amersham Bioscience), sequenced, digested with *Xba*I and *Bam*HI and inserted into the pSVL vector between *Xba*I and *Bam*HI sites (pSVLD53-HA).

Plasmid expressing the $D53P_{57–64}$ mutant protein was generated by PCR using oligonucleotide-mediated site-directed mutagenesis. Briefly, the fragment of pAD-GAL4D53 encoding amino acids 1–147 was amplified with the appropriate primers (forward primer, 5′-GATGTATATAACTATCTATTCG-3′, and reverse primer, 5′-AGTGGGAGAATTCCTCAT-3′), digested with *Eco*RI and used to replace the corresponding fragment of pGEX/D53 and pEGFP/D53. These mutagenic oligonucleotides were 5′-GTACCAGCAGCCAAAGAAAAGCACCCGATT-3′ and its complementary strand.

pBKSD53 and $pBKSD53P_{57–64}$ plasmids used for the *in vitro* transcription and translation experiments were obtained by inserting the *Hin*dIII/*Sal*I fragments of pEGFP/D53 and $pEGFP/D53P_{57–64}$ constructs into the pBluescript vector (Stratagene).

To synthesize a histidine-tagged D53, the *Eco*RI/*Xba*I fragment of pAD-GAL4D53 was inserted into the pET28a vector (Novagen, Madison, WI, U.S.A.).

$GST\text{-}Syntaxin1A_{1–261}$ plasmid encoding the cytoplasmic region of Syntaxin1A (amino acids 1–261) fused to GST and referred as GST-Stx1, was kindly provided by Dr S. Quetglas [17].

Sequence analysis

Searches for similarities were performed at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) using the BLAST2 and PSI-BLAST programs [18]. Coiled-coil regions were predicted by using the COILS and PAIRCOILS programs [19,20]. The bidimensional hydrophobic cluster analysis (HCA) was also used in order to highlight the organization of the protein sequence into domains and their associated secondary structure features [21]. This method adds secondary-structure prediction to sequence comparison and is therefore particularly efficient for comparing sequences sharing low levels of identity (below the so-called 'twilight' zone, i.e. 20–25 % identity), as structure is often much more conserved than sequence [22,23]. Secondary structure predictions were performed using the JPred program [24].

Cell fractionation

PC12 cells (10^7) were homogenized in 2 ml of PBS containing protease inhibitors (protease inhibitor cocktail tablets, Roche) using a cell cracker. After 5 min centrifugation at 1000 ***g***, two-thirds of the resulting supernatant [post-nuclear supernatant (PNS)] were centrifuged at 200000 ***g*** for 30 min at 4 °C. The supernatant fraction was the cytosol fraction. The pellet corresponding to the membrane fraction was further re-suspended in 600 μl of PBS and homogenized.

Immunoprecipitation and Western blotting

Cell lysates from COS-7 cells transfected with D53-expressing vectors, from PC12 cells or from embryonic chicken neuroretinas were prepared as described previously [25]. They were analysed by immunoprecipitation followed by SDS/PAGE and Western blotting. Blots were probed with either the anti-D53 and the anti-HA (12CA5, Roche) or the anti-Sb2 antibodies at dilutions of 1:1500, 1:1000 and 1:2000 respectively. Proteins were visualized by chemiluminescence as recommended by the suppliers (Amersham Bioscience; Pierce, Tattenhall, Cheshire, U.K.).

Purification of bacterial recombinant proteins

GST fusion proteins were prepared according to Frangioni and Neel [26]. A single colony of DH5α, transformed with the plasmid of interest, was grown overnight in Luria–Bertani (LB) broth containing 100 μg/ml ampicillin. A 1:50 (v:v) dilution of the overnight culture was grown in LB, up to A_{600} 0.4, then induced with 0.1 mM IPTG for 4 h at 37 °C. Bacteria were pelleted by centrifugation at 7000 ***g*** for 7 min at 4 °C and washed with STE (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA). Pellets were lysed by sonication with 100 mg/ml lysozyme, 1.5 % laurylsarcosine in STE. Lysates were clarified by centrifugation at 10000 ***g*** for 5 min, at 4 °C. The supernatant was adjusted to 2 % Triton X-100, and incubated with glutathione–Sepharose beads at 4 °C for 4 h. Beads were washed several times with ice-cold PBS and resuspended in storage buffer [50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM dithiothreitol (DTT), 10 % (v/v) glycerol].

Recombinant Stx1 was purified from the GST fusion protein by treatment with 11 units of human thrombin (Sigma)/ml in 150 mM NaCl, 25 mM $CaCl_2$, 10 mM Hepes, pH 7.4, and 0.05 mM Tween 20, for 3 h at 25 °C. Thrombin was removed by adding *p*-aminobenzamidine coupled to agarose beads (Sigma) and centrifugation at 2000 rev./min for 1 min at 4 °C. His-tagged proteins were purified according to Weber et al. [5]. Briefly, *Escherichia coli* strain Bli5 (Novagen), transformed with the plasmid of interest, was grown under conditions described above,

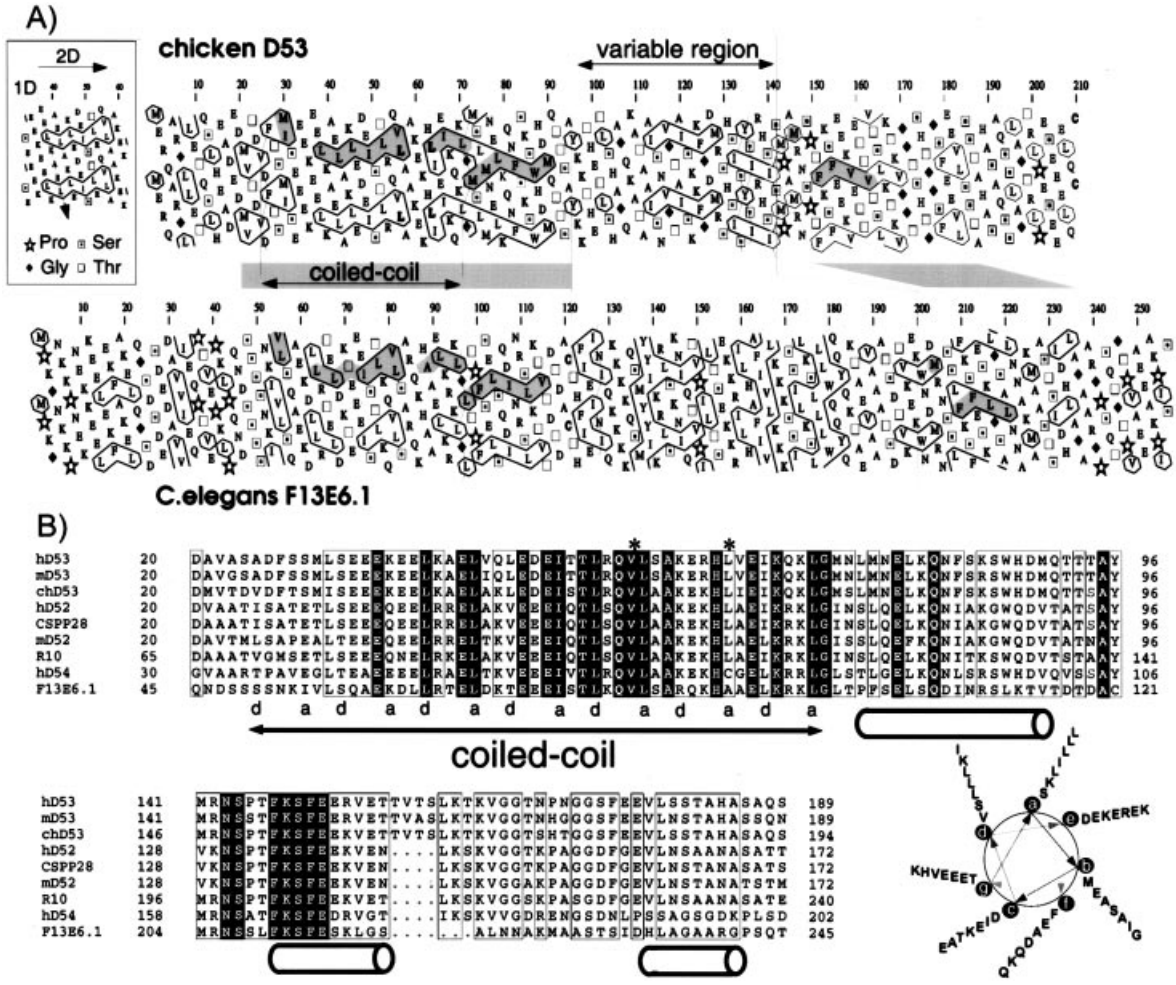

Figure 1 Structure of D53 protein

(**A**) Comparison of the HCA plots of the chicken D53 and *C. elegans* F13E6.1 protein sequences. The sequence is shown as a duplicated α-helical net, where hydrophobic amino acids (V, I, L, F, Y, W) are enclosed. The sequence is presented in duplicate in order to restore the full environment of each amino acid. Hydrophobic amino acids form clusters which mainly correspond to the internal faces of regular secondary structures (α-helices and β-strands) [21]. The way to read the sequence in one-dimension (1D) and with secondary-structure distribution (2D) is indicated in the inset. Symbols used for amino acids with a particular structural behaviour are also indicated in the inset. The most conserved regions are indicated with shading below the sequence (also denoted 'coiled-coil'). Within these regions, the conserved hydrophobic amino acids are enclosed and those which should participate in the core of the structure are shaded. They form the main part of the hydrophobic clusters whose shape strongly indicate α-helical structures. (**B**) Linear alignment of the conserved regions of the R10/D53 protein family. Identical amino acids are white on a black background, similar amino acids are boxed. The 'a' and 'd' positions of the coiled-coil's heptad repeat, as well as the position of the predicted α-helices are indicated below the sequences. The amino acids of the coiled-coil are also shown on a helical wheel (right). Leucine residues 57 and 67 are indicated by asterisks in (**B**) and in **bold** in (**A**).

in LB medium containing 50 μg/ml kanamycin and 30 mg/ml chloramphenicol. Bacteria were lysed by lysozyme treatment (100 μg/ml) and sonication for 1 min. Protein was purified on nickel-nitriloacetic acid (Ni-NTA) agarose (Qiagen, Courtaboeuf, France) in 25 mM Hepes, KOH, pH 7.4, 100 mM KCl, 10 % (v/v) glycerol, 2 mM β-mercaptoethanol, 1 % octyl β-D-glucopyranoside in the presence of 250 mM imidazole.

In vitro binding experiments

pBKSD53 and pBKSD53P$_{57-64}$ constructs were used for *in vitro* T7-driven transcription and translation using the TNT-coupled transcription/translation kit (Promega) with [^{35}S]methionine as the radioactive tracer amino acid in a final volume of 50 μl. For binding experiments, 10 μl of the reactions were incubated overnight at 4 °C with 10 μg of GST alone, or GST-Sb2 and GST-D53 immobilized on glutathione–Sepharose beads in the presence of 50 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM DTT, 10 % (v/v) glycerol and 1 % Triton X-100 in a final volume of 400 μl. After centrifugation, the supernatant containing the unbound fraction was removed and the beads were washed four times with 1 ml of binding buffer, resolved by SDS/PAGE and revealed by fluorography.

In binding experiments involving bacterial recombinant GST-Sb2, GST-Stx1, HisD53 and the cytoplasmic domain of Stx1, 0.3 or 1 μM GST-Sb2 or GST-Stx was incubated overnight at 4 °C with increasing concentrations of recombinant His-tagged D53 in 21 mM Hepes, pH 7.5, 20 mM KCl, 120 mM potassium acetate, 1.6 mM EDTA, 2 % (v/v) glycerol, 0.4 mM β-mercaptoethanol, 0.2 % octyl β-D-glucopyranosyde, 0.01 % Tween 20 and 10 mM imidazole. In the GST-Sb2/HisD53 complex, 0.3 μM Stx1 was added and incubation was continued for 1 h. After washing three times with 400 μl of 20 mM Hepes, pH 7.5, 150 mM potassium acetate, 2 mM EDTA, 0.05 % Tween 20 and

5% (v/v) glycerol, proteins were analysed by SDS/PAGE and immunoblotting. EC_{50} was defined as the half-maximal binding of each soluble protein, based on pixel intensity quantified on the Gel Documentation System 2000 (Bio-Rad). For HisD53 (Figure 6A), uncalibrated absorbance (u*A*) values/mm² were: 0, 1.75; 7.75, 21.2, 29.6, 33. For HisD53 (Figure 6B), u*A* values/mm² were: 0, 2.32; 4.17, 11, 20.4, 21.4, 29. For Stx1 (Figure 6C), u*A* values/mm² were: 0, 0.08, 3.19, 3.97, 21.7, 29.4, 41.7. For HisD53 (Figure 6D), u*A* values/mm² were: 0, 6.53, 11.7, 18, 26.3, 37.7. For Stx1 (Figure 6D), u*A* values/mm² were: 4.22, 10.6, 8.7, 20.6, 24.3, 30.7.

Preparation of D53-specific antiserum

The pGex/NterD53 construct was used to produce a bacterial protein containing the first 40 amino acids of D53 fused to GST, as described previously. After purification, the protein was concentrated in a Centricon 10 (Amicon, Danvers, MA, U.S.A.), following the manufacturer's instructions, and prepared for immunization of rabbits (Agrobio, la Chavannerie, La Ferté St Aubin, France).

Antibodies

Monoclonal antibodies were gifts from: Dr R. Jahn (Max-Planck Institute, Göttingen, Germany) for rat-Sb2 cl69.1; Dr C. Barnstable (Yale University, New Haven, CT, U.S.A.) for rat-Syntaxin1 HPC-1; Dr I. Trowbridge (Salk Institute, San Diego, CA, U.S.A.) for Transferrin Receptor, H68.4. N-terminal His6 antibody was purchased from Clontech and 12CA5 antibody from Roche.

The secondary antibodies used were FITC-conjugated anti-rabbit IgG (Sigma), and Texas Red dye-conjugated AffiniPure Goat Anti-Mouse IgG (H and L; Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.).

Immunofluorescence

PC12 cells were seeded in collagen-coated eight-well chambers (Laptek chamber; Nalge Nunc International, Naperville, IL, U.S.A.) at a density of 12000 cells per well. After 3 days, cells were fixed with 3% (w/v) paraformaldehyde in PBS for 20 min and permeabilized by treatment with 0.25% Triton X-100 in PBS for 10 min at 22 °C. After treatment with 0.2% (w/v) gelatin in PBS, for 20 min, cells were incubated for 1 h with the first antibodies at 22 °C. After washing three times with PBS, they were incubated with the secondary antibodies for 1 h, washed several times with PBS, and mounted in Vectashield mounting medium (Vector Laboratories, Peterborough, U.K.). HeLa cells were permeabilized before fixing, by digitonin treatment (50 μg/ml in PBS) for 90 s on ice.

RESULTS

D53 belongs to a structurally conserved family of proteins

R10 was isolated in proliferating quail neuroretina cell cultures infected with the avian lymphomatosis virus RAV-1, as a hybrid mRNA containing R10 sequence associated with retroviral regulatory elements [13]. R10 encodes a protein containing a coiled-coil domain involved in homo- and hetero-oligomeric protein interactions [13,27].

To investigate the function of the R10 gene product, the yeast two-hybrid system was used to identify interacting proteins. A neuroretina cDNA library, prepared from 13-day-old chick embryos, was screened by using R10 as bait. Among approximately 169000 transformants screened, three classes of clones showed specific interaction with the bait. They encoded the R10 protein, confirming its capacity to form homo-oligomers [13], the chicken orthologue of the mammalian D53, a highly R10-related protein shown to be overexpressed in several human breast carcinomas [14] and the chicken orthologue of the synaptic-vesicle-associated protein Sb2.

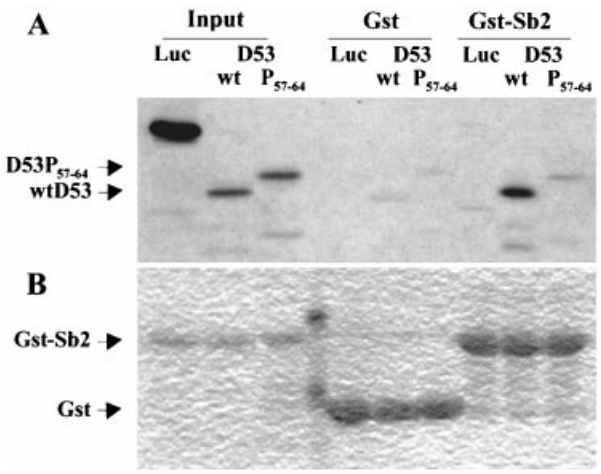

Figure 2 D53 interacts with Sb2 *in vitro*

(**A**) D53wt and $D53P_{57–64}$ were radiolabelled by *in vitro* transcription and translation and incubated with GST-D53, GST and GST-Sb2. One-tenth of each reaction mixture (Input), and bound proteins of the remaining nine-tenths were analysed by SDS/PAGE and fluorography. An internal negative control (background) was measured in each lane by quantifying the same surface area just above the specific signal. The obtained values were subtracted from the values obtained for the specific signal. The values obtained in u*A* were: 47.92 for the wtD53 input; 48.17 for the $D53P_{57–64}$ input; 5.15 for the retention of the wtD53 on the GST alone; 4.3 for the retention of the $D53P_{57–64}$ on the GST alone; 65.34 for the retention of the wtD53 on the GST-Sb2; and 9.91 for the retention of the $D53P_{57–64}$ on the GST-Sb2. Luciferase (Luc) was used as a negative control for interaction with GST-Sb2. (**B**) Equal amounts of GST and GST-Sb2 loading were checked by Coomassie Blue staining.

A BLAST search for similarities revealed that several R10/D53-related proteins existed in species from *Caenorhabditis elegans* to human [14,28–32]. The overall similarities, ranging from 28.2–81.5%, suggested the existence of an R10/D53 protein family. Comparison of D53 amino acid sequence with that of the most distantly related sequence of the family, i.e. that of the *C. elegans* F13E6.1 putative protein, allowed organization of the protein into domains and delineation of the most conserved regions within these domains. These should be essential to the structure and function of such proteins. By using the bidimensional HCA [21], we identified two conserved regions (shaded blocks between the two compared plots) separated by a variable region, which cannot be aligned between chicken D53 and the *C. elegans* protein, and should, therefore, correspond to different structures (Figure 1A).

The first conserved region in all members of the R10/D53 protein family includes a typical coiled-coil domain, as defined by the COILS [19] and PAIRCOIL [20] programs (Figure 1B) and easily detectable in the HCA plot (Figure 1A). Indeed, the hydrophobic positions of the heptad repeat, constituting the core of the coiled-coil structure (positions 'a' and 'd'), form a typical long horizontal cluster. In fact it corresponds to the 2D projection of the three-dimensional (3D) helical structure, as the support used in this sequence representation is the α-helix. Within this first conserved region, the coiled-coil is immediately followed by a region predicted, by HCA and JPred, to adopt a helical

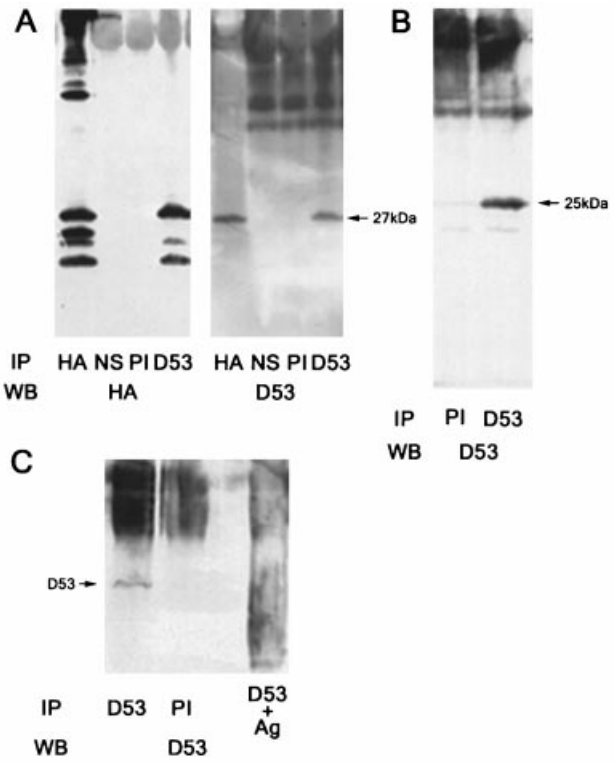

Figure 3 Characterizations of D53 protein and antibody

(**A**) Expression of HA-tagged D53 protein. Lysates of COS-7 cells transfected with pSVLD53-HA construct were immunoprecipitated with anti-HA antibody (HA), non-specific rabbit serum (NS), pre-immune serum (PI), or D53-specific antiserum (D53), resolved by SDS/PAGE (15% gel) and immunoblotted with either the anti-HA-specific antibody or the D53 antiserum. (**B**) Specific recognition of endogenous D53 protein. A lysate of neuroretina, extracted from 13-day-old chicks, was immunoprecipitated either with preimmune serum, or with D53-specific antiserum, and revealed with the D53 antiserum. (**C**) Competition experiment. D53-specific antiserum was incubated for 3 h at 4 °C in the presence of GST-D53 beads, the supernatant was used for the immunoprecipitation (D53 + Ag) of neuroretina lysates and compared with precipitation obtained in the same conditions with the preimmune (PI) or the specific D53 antiserum.

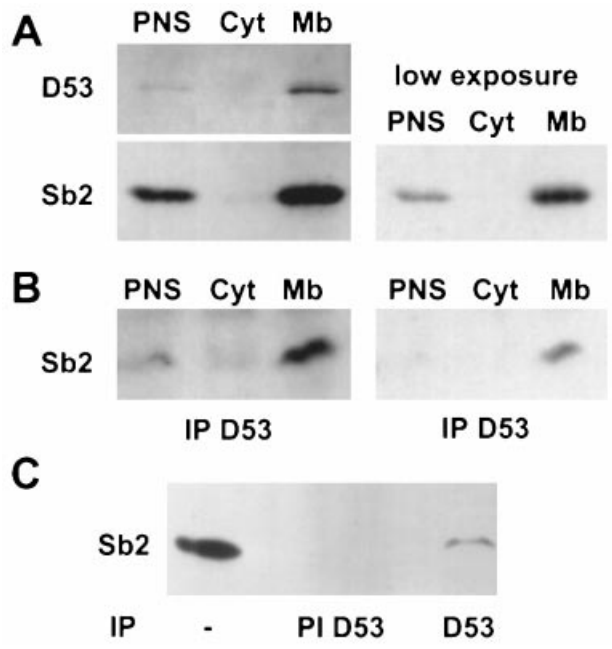

Figure 4 Subcellular localization of D53 and Sb2 in PC12 cells

(**A**) Proteins (100 μg) contained in the post-nuclear supernatant (PNS), cytosolic (Cyt) and membrane-containing fractions (Mb) of PC12 cells were resolved by SDS/PAGE (12% gel) and probed with D53- and Sb2-specific antibodies. (**B**) Sb2 co-immunoprecipitates with D53 in the membrane-containing fraction of PC12 cells. Proteins (200 μg) from each fraction were immunoprecipitated with D53 antiserum, analysed by SDS/PAGE (12% gel) in parallel with proteins indicated in (**A**) and simultaneously revealed with the Sb2-specific antibody. The values from the quantification (uncalibrated optical density), done on the low exposure, were 81.24 for the direct Western blot, 25.30 for the immunoprecipitation and 15.81 for the unspecific signal. (**C**) Specificity of the D53-Sb2 interaction. Lysates of PC12 cells were immunoprecipitated with either preimmune (PI) or specific D53 antiserum.

structure (Figure 1B). The second conserved region is predicted to contain two other α-helices with a small domain which has a moderate probability of forming coiled-coils (Figure 1B). These results strongly suggested that members of this family could have similar functions involving their helical domains.

D53 is a Sb2-binding protein

Because of the strong similarity between R10 and D53, and the specific expression of D53 in avian neuroretina (V. Proux-Gillardeaux, unpublished work), we investigated whether D53 was also able to interact with Sb2. We found that interaction of D53 with Sb2 was as efficient as that of R10, in the two-hybrid system (results not shown). A direct interaction between the two proteins was assessed by an *in vitro* binding assay. Thus, a purified recombinant GST-Sb2 fusion protein was immobilized on glutathione–Sepharose beads and incubated with [^{35}S]methionine-labelled D53 protein obtained by *in vitro* translation. D53 was able to bind to GST-Sb2, but not to GST-coupled beads (Figure 2A). Indeed, a quantification of the fluorography revealed that 15.15% of radiolabelled D53 was retained on GST-Sb2-coupled beads versus 1.19% on GST alone, leading to about 14% of specific retention.

As mentioned above, D53 contains a coiled-coil domain conserved in all members of the R10/D53 protein family. Moreover, the coiled-coil domain of Sb2 is involved in heterotypic protein–protein interactions. Thus we investigated whether the binding of D53 to Sb2 was mediated by the N-terminal coiled-coil domain of D53. We constructed a plasmid expressing a mutant form of D53 (pBKSD53P$_{57-64}$), in which we disrupted the coiled-coil structure by substituting leucine residues 57 and 64 for proline residues, as predicted by the PAIRCOIL program. Only a very weak interaction of the mutant protein with GST-Sb2 was detected (Figure 2A). Only 2.3% of the mutant protein was retained on GST-Sb2 beads versus 1% on GST alone, resulting in a 1.3% specific retention. Thus, D53 was retained about 11 times more than the mutant protein. This clearly showed that the binding of D53 to Sb2 requires the integrity of its N-terminal coiled-coil domain. Taken together, these results provided evidence for a direct *in vitro* interaction between D53 and Sb2 mediated by the coiled-coil domain of D53.

To confirm *in vivo* the D53/Sb2 interaction, we immunized rabbits with a GST fusion protein containing the first 40 amino acids of D53. Antiserum specificity was validated by immunoprecipitation and immunoblotting analysis, using lysates from COS-7 cells transfected with the pSVLD53-HA plasmid. We detected a 27 kDa protein, also identified by the HA-Tag antibody. This protein was not recognized by preimmune or non-specific rabbit serum (Figure 3A). Moreover, this antiserum specifically recognized an endogenous D53 protein of 25 kDa in

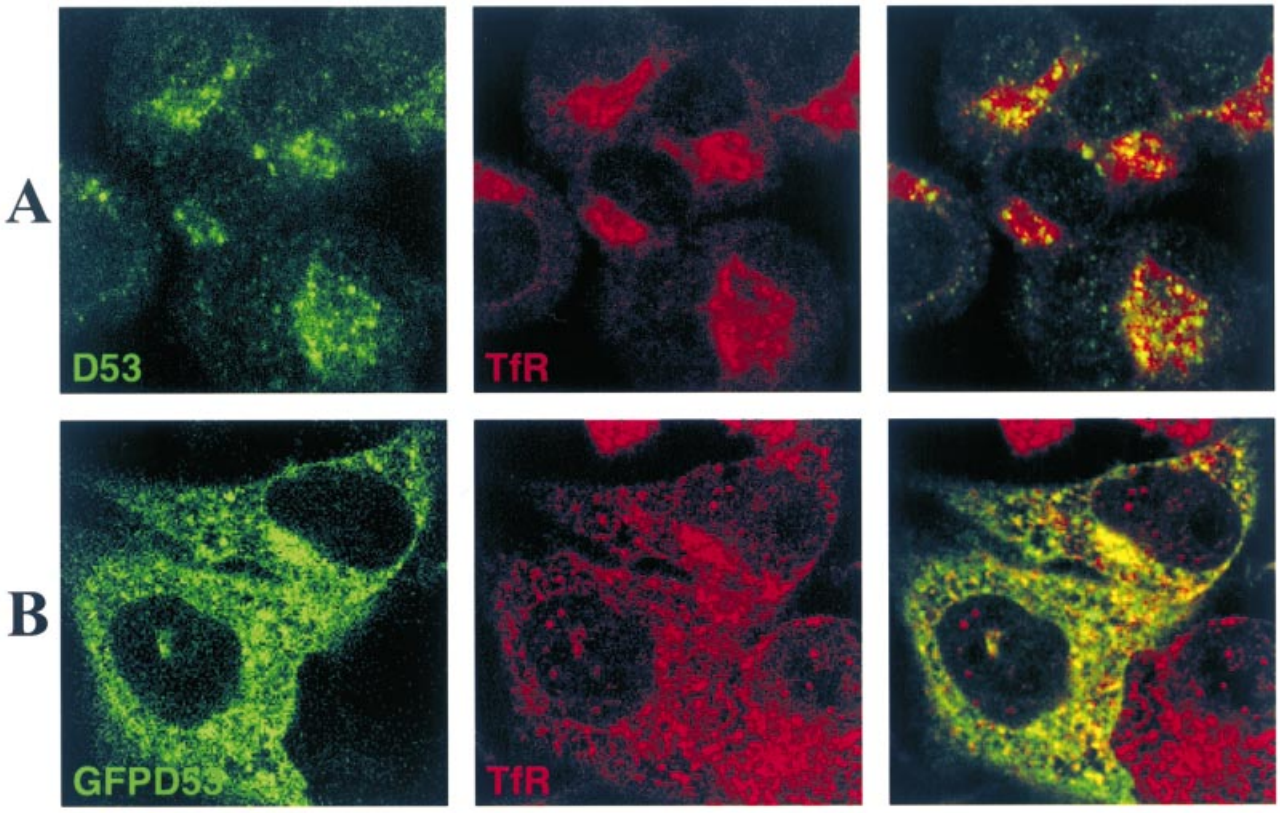

Figure 5 Localization, by confocal microscopy, of D53 in PC12 cells (A) and of GFPD53 in HeLa cells (B) in comparison with the endosomal recycling compartment (TfR)

For double labelling, D53 was revealed with a FITC-conjugated anti-rabbit secondary antibody, whereas TfR was revealed with a Texas Red dye-conjugated secondary antibody. GFPD53 was revealed by direct fluorescence. The panels on the right-hand side are colour overlays.

13-day-old chicken neuroretina (Figure 3B). A competition experiment done by incubating the D53 antiserum previously with a D53 purified protein abolished this detection (Figure 3C).

Sb2 is known to be a transmembrane protein. Given that D53 does not contain any membrane targeting motif, we asked whether the subcellular localization of D53 overlaps with that of Sb2. Therefore we performed subcellular fractionation of PC12 cells in which both D53 and Sb2 were expressed. We found that both proteins are enriched in the membrane-containing fraction (Figure 4A), as compared with the post-nuclear supernatant fraction. They were not detected in the cytosolic fraction.

To assess the biological significance of the *in vitro* binding of D53 to Sb2, we investigated whether we could detect the presence of D53/Sb2 complexes in the membrane-containing fraction. We detected Sb2 in complexes immunoprecipitated by D53-specific antibody and about 7.25% of the total Sb2 was pulled down with D53-specific antibody (Figure 4B). This co-immunoprecipitation is specific, because only the D53 antibody, and not the pre-immune serum co-immunoprecipitated Sb2 (Figure 4C). Given that D53 is predicted to be soluble, this suggests that its association with membranes could be mediated through its interaction with Sb2 in PC12 cells.

D53 partially localizes on early endosomes

Next, we investigated the subcellular localization of endogenous D53 in PC12 cells by confocal microscopy. Using affinity-purified D53 polyclonal antibody, we observed the labelling of punctate structures throughout the cytoplasm that were enriched in a perinuclear region (Figure 5A).

We investigated whether D53 co-localized with the transferrin receptor, an established marker of early endosomes [33]. We found that detection of D53 immunoreactivity overlapped with that of the transferrin receptor (Figure 5A). Given that Sb2 was previously found not only on the small synaptic-like vesicles, but also on early endosomes of PC12 cells [34], these results suggest that D53 and Sb2 may co-localize on early endosomes.

The discrepancy between the absence of a membrane-targeting motif on the D53 amino acid sequence, and its enrichment in the membrane-containing fraction, suggested that the association of D53 with early endosomes could be mediated by protein–protein interactions involving the coiled-coil domain of the protein. To assess the role of this domain, we examined whether its disruption affected localization of the protein on early endosomes. Therefore, we transfected Hela cells with plasmids expressing either GFPD53wt (wild-type) or GFPD53P_{57-64} mutant protein and we studied their subcellular localization by confocal microscopy, in correlation with the distribution of the transferrin receptor. Both wt and mutant D53 proteins were revealed by the direct fluorescence of GFP. We observed that GFPD53wt and mutant proteins were predominantly detected as cytosolic proteins, masking the detection of transferrin-receptor-containing organelles (results not shown). Therefore, we permeabilized the transfected cells with digitonin, before their fixation, in order to

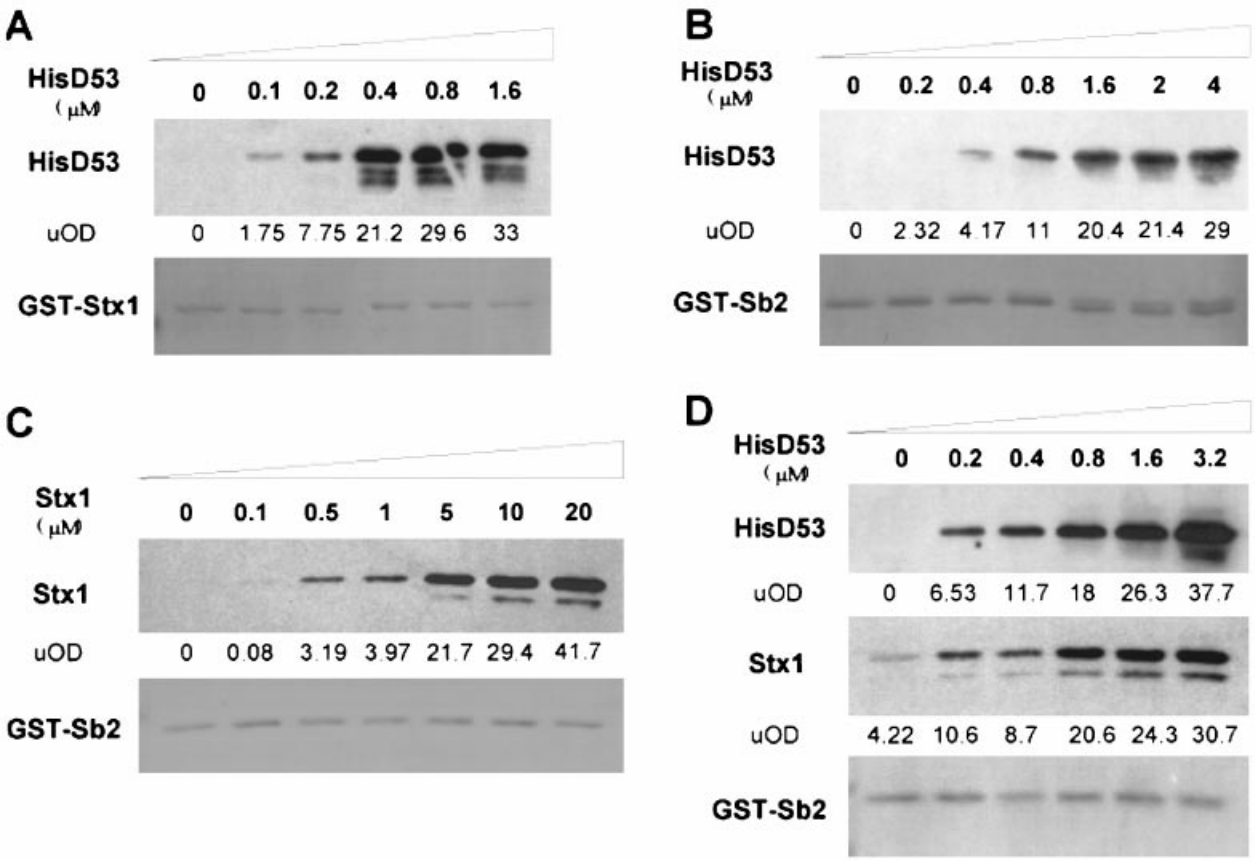

Figure 6 ***In vitro*** **interactions of Sb2, Stx1 and D53**

(**A**) D53 binding to Stx1 and (**B**) to Sb2 *in vitro*. GST-Stx1 (1 μM) or GST-Sb2 (0.3 μM) immobilized on glutathione–Sepharose beads were incubated with increasing amounts of purified HisD53. (**C**) Stx1 binding to Sb2. GST-Sb2 (0.3 μM) was incubated with increasing amounts of purified Stx1. (**D**) D53 potentiates interaction of Stx1 with Sb2 *in vitro*. GST-Sb2 beads (1 μM) were incubated with increasing amounts of purified HisD53; each sample was then incubated with 0.3 μM of purified Stx1. After washing and boiling, bound HisD53 and Stx1 were identified by SDS/PAGE and Western blotting using His6- or Stx1-specific antibodies. Values obtained by quantification of the bands are written under each corresponding band as uncalibrated optical density (uOD). The presence of equal amounts of GST-Stx1 and GST-Sb2 was confirmed by Ponceau S staining.

wash out excess of exogenous cytosolic proteins. We observed a perinuclear and punctate vesicular distribution of GFPD53wt similar to that observed in PC12 cells. D53 partialy overlapped with the transferrin receptor, but a non-endosomal localization of the protein was also observed (Figure 5B). However, the mutant GFPD53P$_{57-64}$ protein was no longer detectable under these conditions, indicating its inability to associate with membranes (results not shown). These experiments confirm that D53 is predominantly present on early endosomes and that its localization is dependent on the coiled-coil domain integrity.

D53 enhances interaction of Stx1 with the Sb2 complex

The results presented above showed that D53 resided on vesicular structures that presumably contained a fraction of Sb2 and that it interacted with Sb2 through its coiled-coil domain. Sb2 was shown previously to be the v-SNARE interacting with Stx1, the membrane-associated t-SNARE [35]. However, in a binary *in vitro* system, Sb2 and Stx1 exhibited a relatively weak interaction, whereas the addition of SNAP-25 significantly increased the binding of Sb2 to Stx1 [36]. We therefore investigated whether the presence of D53 could also modulate Sb2/Stx1 interaction *in vitro*.

Given that SNAP-25 binds directly to Sb2 and Stx1 in a dose-dependent manner [37], we examined whether D53 exhibited similar binding properties. We incubated increasing amounts of His-tagged D53 with constant amounts of either GST-Stx1A$_{1-261}$ (Figure 6A) or GST-Sb2 (Figure 6B) fusion proteins immobilized on glutathione–Sepharose beads. Binding of D53 to Stx1 and Sb2 was saturable, with a 50 %-effective concentration (EC$_{50}$) of about 0.42 μM and 2 μM respectively. These values were comparable with those obtained under the same conditions for SNAP-25 binding to the same proteins [37]. These results indicated that, like SNAP-25, D53 bound directly to v- and t-SNAREs.

We then studied whether D53 also affects binding of Stx1 to Sb2. We found that in a similar binding assay, association of Stx1 with GST-Sb2 was not saturable at concentrations up to 20 μM of Stx1 (Figure 6C). By using the Microcal™ Origin program (see the Experimental section), the EC$_{50}$ of this interaction was estimated at about 10.2 μM of Stx1. This was consistent with previous data indicating that EC$_{50}$ of Sb2 binding to GST-Stx1 was lower than 12 μM [37]. Finally we incubated increasing amounts of recombinant His-tagged D53, with 1 μM of the GST-Sb2 fusion protein bound to glutathione–Sepharose beads and added 0.3 μM of purified recombinant Stx1 was added to each sample (Figure 6D). We observed that under these

conditions, D53 bound to GST-Sb2 with an EC_{50} of about 2.2 μM, a value similar to that obtained in pairwise association. As expected, only barely detectable amounts of Stx1 interacted with Sb2 in the absence of D53. However, by increasing the concentration of D53, we found that there was a dose-dependent increase in the retained amount of Stx1. Interestingly, Stx1 binding to GST-Sb2 was enhanced 7-fold in the presence of 3.2 μM D53. The EC_{50} of Stx1 to GST-Sb2 was obtained at 0.61 μM D53. This concentration is comparable with the amount of SNAP-25 necessary to obtain a EC_{50} of Sb2 to GST-Stx1, as observed by Pevsner et al. (0.7 μM) [37].

DISCUSSION

In the present study we analysed the biochemical properties of D53, a novel v- and t-SNARE binding protein expressed in avian neuroretina and in PC12 cells. We showed that D53 contains a large N-terminal coiled-coil domain required for its localization on early, transferrin-receptor-containing endosomes and for its interaction with the v-SNARE, Sb2.

The main finding of this study was that D53 enhances interaction of Stx1 with the Sb2 complex *in vitro*. Using recombinant Sb2, Stx1 and D53, we showed that D53 binds to both Sb2 and Stx1, with EC_{50} values similar to those obtained for SNAP-25. Furthermore, in the presence of increasing amount of D53, binding of Stx1 to Sb2 was enhanced in a dose-dependent manner. These biochemical characteristics are similar to those assigned to SNAP-25 [36,37]. Analysis of the 3D structure of the synaptic SNARE complex has shown that it is a heterotrimer containing Stx1, Sb2 and SNAP-25 in a ratio of 1:1:1. In fact, four coiled-coil domains are involved in the formation of this complex, one provided by Stx1, one by Sb2 and two originating from SNAP-25. These domains are arranged in parallel to form a cylinder [3,4]. The 2D organization of D53 consists of two helical regions, conserved in all members of the R10/D53 family, one of which contains a highly conserved coiled-coil domain. This structure is reminiscent of the proteins included in SNARE complexes. Based on our results, it is likely that the canonical N-terminal coiled-coil of D53 could participate in a four-helix bundle similar to those formed in SNARE complexes. This hypothesis is supported by the propensity of the D53 coiled-coil to form tetrameric structures, as predicted by the PAIRCOIL program [20] and based on the high proportion of non-branched amino acids, such as Leu, at a and d positions of the heptad register (Figure 1B) [38]. Moreover, the length of its N-terminal helical domain, which contains nine a and ten d positions (Figure 1B), is consistent with previous reports showing that in the different SNARE proteins, eight a and eight d positions are necessary to form the four-helix bundle structures [39,40]. However, D53 contains at its C-terminal portion two α-helices, instead of a long coiled-coil domain in the case of SNAP-25. This suggests that two molecules of D53 could take part in the formation of a tetrameric complex involving Sb2 and Stx. It was reported that in yeast, an intracellular t-SNARE is built from a 'heavy chain', homologous to syntaxin, and two separate non-syntaxin 'light chains' [41]. According to this model, the C-terminal helices of D53 could either interact together and stabilize the complex, or bind to other proteins and regulate the complex assembly.

Alternatively, D53 could behave like SNAP-25. In this case, the C-terminal α-helices, together with the N-terminal coiled-coil domain, would participate in the formation of the core structure of a trimeric complex. This hypothesis is strengthened by the fact that a discontinuous SNAP-25 C-terminal coil supports exocytosis [42]. Accordingly, the two C-terminal α-helices of D53 could behave as a truncated coiled-coil domain. It is also supported by the fact that the concentration of D53 necessary to obtain a half-maximal saturation of Stx1 to Sb2 is comparable with that of SNAP-25.

Results of immunolocalization experiments showed that D53 is located on endosomes characterized by the presence of the transferrin receptor, in PC12 and in the non-neuronal HeLa cells. This suggests that D53 could regulate the formation of SNARE complexes involved in recycling synaptic vesicles, possibly by activating association of Sb2 with endosomal t-SNAREs. Syntaxin13 could be a good candidate, since it has been identified as an endosomal t-SNARE implicated in homotypic endosome fusion and found in a complex with Sb2 in rat brain Triton X-100 extracts [43]. According to the model proposed above, D53 could have a SNAP-25-like function on early endosomes. In mammalian cells only four proteins of the SNAP-25 family have been characterized so far: SNAP-25 at the presynaptic plasma membrane, SNAP-23 and Syndet at plasma membranes of non-neuronal cells [44–47], and SNAP-29 with a broad distribution on multiple membranes [48]. Interestingly, D53, like SNAP-29, but unlike SNAP-25, Syndet and SNAP-23, does not contain a membrane-association domain. Nevertheless, D53 is located in the membrane fraction of PC12 cells, where it binds to Sb2. We propose that D53 could be a novel actor in the biochemical pathway that underlies homotypic or heterotypic membrane fusion. It could act as a SNAP-25-like protein involved in the formation of endosomal SNARE complexes. However, we cannot exclude a function on early endosomes similar to that described for SNAP-29, which was shown to inhibit SNARE disassembly in synapses [49].

Both helical regions identified in D53 protein are highly conserved in all members of the R10/D53 family. Immunolocalization experiments showed that R10 was located on the endoplasmic reticulum, where it co-localized with β-COP (V. Proux-Gillardeaux and T. Galli, unpublished work), suggesting that proteins of the R10/D53 family have similar functions in different cell compartments involving vesicular trafficking.

Human orthologs of R10 and D53 are overexpressed in tumours and tumour cell lines, in particular in breast and lung adenomas and adenocarcinomas [14,29]. Interestingly, it was also reported that SNAP-25 is overexpressed in prolactin-secreting human pituitary adenomas [50]. Although, at present, our data do not shed light on whether proteins involved in vesicular trafficking may have a direct link with oncogenesis, it seems crucial to further explore their potential role in human cancer.

We thank Ms Marie Cambot and Ms Naïma Chamil for technical assistance. This work was supported by the Centre National de la Recherche Scientifique and by the Institut Curie. V.P.-G. was supported by fellowships from the Ligue Nationale Contre le Cancer and from the Association pour la Recherche sur le Cancer.

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Received 20 August 2002/10 October 2002; accepted 11 October 2002
Published as BJ Immediate Publication 11 October 2002, DOI 10.1042/BJ20021309