Syntaxin 12, a Member of the Syntaxin Family Localized to the Endosome*

(Received for publication, October 30, 1997, and in revised form, January 13, 1998)

Bor Luen Tang‡, Andrew E. H. Tan‡, Lay Kheng Lim, San San Lee, Delphine Y. H. Low, and Wanjin Hong§

From the Membrane Biology Laboratory, Institute of Molecular and Cell Biology, Singapore 117609, Republic of Singapore

We have cloned a new member of the syntaxin family of proteins. The open reading frame encodes a polypeptide of 272 amino acids with potential coiled-coil domains and a C-terminal hydrophobic tail. Northern blot analysis showed that the transcript is fairly ubiquitous. A soluble recombinant form of the polypeptide without the hydrophobic region binds to α-SNAP (soluble N-ethylmaleimide-sensitive factor attachment protein) and synes/SNAP-23 in vitro. Polyclonal antibody against the recombinant protein recognized a 39-kDa protein in the membrane fraction of cell lysates. Indirect immunofluorescence studies using the polyclonal antibody showed that the protein is localized to intracellular membrane structures. Selective permeabilization studies with digitonin and saponin indicate that the epitope(s) recognized by the antibody is exposed to the cytoplasm, consistent with the predicted orientation characteristic of SNAP receptor molecules. Morphological alterations of the staining pattern of the protein with brefeldin A and wortmannin treatment indicate that the protein is localized to the endosome. The cDNA we have cloned apparently corresponded to three previously described expressed sequence tags named as syntaxins 12, 13, and 14, respectively. We therefore propose to retain the name syntaxin 12 for this protein.

A biochemical and biophysical understanding of vesicular transport at the molecular level has been facilitated by in vitro assays, which reconstitute transport processes in cell-free systems (1). Thus, molecular components required for both vesicle budding (2) and vesicle docking/fusion processes (3–4) have been isolated. The N-ethylmaleimide-sensitive factor (NSF),1 an ATPase whose activity regulates the formation and dissociation of fusion complexes, is the first cytosolic factor characterized of SNAP receptor molecules. Morphological alterations of the staining pattern of the protein with brefeldin A and Wortmannin treatment indicate that the protein is localized to the endosome. The cDNA we have cloned apparently corresponded to three previously described expressed sequence tags named as syntaxins 12, 13, and 14, respectively. We therefore propose to retain the name syntaxin 12 for this protein.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF035632.

‡ The first two authors contributed equally to this work.

§ Supported by a grant from the Institute of Molecular and Cell Biology. To whom correspondence should be addressed: Membrane Biology Laboratory, Institute of Molecular and Cell Biology, 30 Medical Dr., Singapore 117609, Republic of Singapore. Tel.: 65-874-3762; Fax: 65-779-1117; E-mail: mchbw@imcbnus.sg.

1 The abbreviations used are: NSF, N-ethylmaleimide-sensitive factor; RPMI medium, Rosewell Park Memorial Institute medium; SNAP, soluble NSF attachment proteins; SNAPs, SNAP receptor; NRK, normal rat kidney; EST, expressed sequence tags; GST, glutathione S-transferase; TGN, trans-Golgi network; BFA, brefeldin A.

SYNTHESIS/ASSEMBLY OF MEMBRANE PROTEINS IN Eukaryotic CELLS*

The Journal of Biological Chemistry 1998, 273(12), 6944–6950
© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
A

ATGTCCTACGGTCCCTTAGACATGTATCGGAAATCCGGGGCCTCGAGAGCTTGAGCAGCTCATCCAGAGCTG

M S Y G P L D M Y R N P G R R S L R D F S S I T Q T C

CAGCCGCAACATCAGCCGATCAGCCAGAGCAGCTCAGATTAAGGAATTTGAGGCCAACCTGGAGCAGCT

180 S G N I Q R I S Q A T A Q I K N L M S Q L G T K O D

CAGGCAAAACTCGAGAATTGCAACAGTATGCCAGACACTCCACAAATCACCAGCTTGCGAGAGAGACT

240 S S K L Q O E N L Q Q F Q H S T N Q L A K E T N E L L K

GAATTAGGTCTTGTGGGCTATCCCTTGTCATGCAGAGCAGGCGACCAAGAACAGCTTCTCAGAGGAGGCGCT

320 C E G S L P L P S A S E Q R Q Q K L G K R L M N D

CTTCCTGCTGGGCTGAGACAAATTTGGAGAGGTAAGTGCAAGAAGAAGTATCAGGAAGAAGAAGAAAGAAC

400 T C S S A L N N F Q V V Q V R Q E S K E K E S I A R A

GGGCTGAGATCGATGTCTCTTCTTGAGAGAAGAGAGAAGAGAGCAGTCGTCTCATTGGAGCGATAGAGAAG

480 R A G S R L A E D R O R Q E E Q O L V S F D S H E W N

CAGATGCAAGAGGAAGAAGAGGCGACATCAGTGCAGAAGACCTTGAGAATTAAGAGAGAAGAAGGCGAC

560 Q M Q S G E E E A J T E Q D L E L I E K E R T A I G

GCAGCTGAGGCTGAGATTTGGATGCTAATCAGATTTAAGACCTTAGCTATGAGATGATGCAGAGAAGTAC

640 C O L T A D I L V U N O B E V E S Y V R A S D L O R A A Y

TACTGGAAAAATCGCCGAAGAAGATCTGATATCCGCTGCTGCTCTCAGTGATTTGATCAGAGCTTCT

720 I D S I E A N V E S V H E R A S D O L R A A Y

Y Q K K S R C K M C I L V L V L S V I V V V V

CIGGTTCCTTCAGTGA

W V A S K

B

C

FIG. 1. Molecular cloning of a novel rat syntaxin. A, the DNA sequence and derived amino acid sequence of the coding region of rat syntaxin 12. The putative transmembrane is boxed. The coiled-coil region, which is most homologous to other known syntaxins, is underlined. B, Kyte-Doolittle hydrophobicity plot of the primary sequence of syntaxin 12 performed by the DNA Strider 1.1 program. C, coils output for syntaxin 12 coils 2.1 analysis of potential coiled-coil domains of syntaxin 12. The window of analysis is 21 amino acids wide.
EXPERIMENTAL PROCEDURES

Materials—Cell lines were primarily from the American Type Culture Collection. Monoclonal antibody against trans-Golgi network 38 (TGN38) was kindly provided by Dr George Banting (University of Bristol, United Kingdom). Expressed sequence tag clones were generated by the Washington University MERCK EST project and were obtained from the IMAGE consortium. Syndet cDNA (15) was kindly provided by Dr. G. Baldini (Columbia University, New York). Syntaxin 1A cDNA (6) was kindly provided by Dr. R. Scheller (Stanford University, CA). mSEC13 cDNA (16) was kindly provided by Dr. Anand Swaroop (University of Michigan).

Methods—Data base searches were performed with the various BLAST algorithms available at the National Center for Biotechnology (NCBI) World Wide Web server. Library screening, cloning, and DNA sequencing were performed using standard methods as described (17). Northern blot analysis was performed using a rat multiple tissue Northern blot from CLONTECH.

In vitro translation of various constructs was performed using in vitro translation kits from Promega according to the manufacturer’s protocol. For in vitro binding assays (18), the cytoplasmic domain of syntaxin 12 (amino acids 1–248) was generated by the polymerase chain reaction and cloned into the plasmid pBSK (Stratagene).

[35S]Methionine-labeled translation product was incubated with glutathione-Sepharose beads coated with either glutathione S-transferase (GST), GST-aSNAP, or GST-syndet in binding buffer (20 mM Hepes, pH 7.5, 25 mM NaCl, 3% glycerol, 7 mM MgCl2, 1 mM CaCl2, and 1 mM EDTA) with 0.1% bovine serum albumin and 0.5% Nonidet P-40 at 4 °C for 3 h. The beads were washed twice with the complete incubation buffer, twice in buffer without bovine serum albumin, and twice in buffer without bovine serum albumin and Nonidet P-40. SDS sample buffer was then added, and the SDS eluates were analyzed by SDS-polyacrylamide gel electrophoresis.

The cytoplasmic domain of the protein is expressed either as hexahistidine-tagged or GST fusion proteins in bacteria. The fusion proteins were also used to immunize rabbits. Polyclonal antibodies were affinity-purified from serum harvested after several booster injections by the fusion proteins immobilized on nitrocellulose strips.

Cells were maintained in RPMI medium supplemented with 10% fetal bovine serum. Immunofluorescence microscopy was performed as described previously (19–20). Cells plated on coverslips and subjected to various treatments were fixed with 3% paraformaldehyde followed by sequential incubation with the primary antibodies and fluorescein isothiocyanate or rhodamine-conjugated secondary antibodies. Fluorescence labeling was visualized using an Axioskop microscope (Carl Zeiss,)
RESULTS

Molecular Cloning and Sequencing of a Novel Member of the Syntaxin Family—Data base searches have allowed us to identify human ESTs (accession numbers R21569 and N99549) potentially coding for a syntaxin-like molecule. A complete cDNA was isolated from a rat brain cDNA library, and sequencing revealed a 272-amino acid open reading frame as shown in Fig. 1A. The predicted amino acid sequence has a stretch of 22 hydrophobic residues at the C terminus, as illustrated by a Kyte-Doolittle hydrophobicity plot (Fig. 1B). This primary structure is characteristic of a hydrophobic tail anchor. The polypeptide has several potential regions that may form coiled-coil structures, as revealed by the Coils version 2.1 program (Fig. 1C).

A data base search using the NCBI BLAST program revealed that the coding sequence has the highest homology with members of the syntaxin family, particularly in the coiled-coil region preceding the C-terminal transmembrane domain (Fig. 2A). A multiple tissue Northern blot with the full-length cDNA showed that the transcript has a fairly ubiquitous expression, being more abundant in brain, lung, and kidney (Fig. 2B).

Based on ESTs identified by data base searches, a series of 10 novel syntaxins has been previously described (21). A search of the GenBank™ dbest data base revealed several human ESTs that, in view of their sequence homology, represent the human homologs of our rat cDNA. Among these, three human ESTs (R29508, AA167677, and T08774) have been listed as syntaxin 12, syntaxin 13, and syntaxin 14, respectively (21). Fig. 2C is a schematic diagram of how the translated sequence of these ESTs match with the coding region of the rat cDNA. As shown, the limited and inaccurate sequence information of these ESTs only allowed each of them to be matched with a portion of the coding region. There is a short overlap between AA167677 and R29508. T08774 matches to the C-terminal portion of the rat protein. Apparently, these assumed syntaxins 12, 13, and 14 represent the human homolog of the rat protein. Based on the limited sequence data alone, these ESTs were assumed to represent individual syntaxin-like molecules. Our results therefore caution against the assignment of individual identity to an EST before full-length sequence information of each is available. To avoid any further confusion in the nomenclature of new members of the syntaxin family, we have retained the name syntaxin 12 for this protein.

Syntaxin 12 Is a SNAP Receptor Molecule Localized to the Endosome—The predicted primary structure of syntaxin 12 and its homology to other syntaxins suggest that it is a SNARE molecule. We sought to confirm this by investigating if syntaxin 12 binds to α-SNAP in vitro. [35S]Methionine-labeled translation product of the soluble cytoplasmic domain of both syntaxin 1A and syntaxin 12 was incubated with glutathione-Sepharose beads coated with either GST or GST-α-SNAP. As shown in Fig. 3, the binding of the syntaxin 1A cytodomain to GST-α-SNAP is significantly higher than to GST itself. Such is also the case for syntaxin 12. On the other hand, mSEC13 (20), a protein with multiple β-transducin or WD-40 repeats known to participate in protein-protein interactions, did not exhibit significant binding to GST-α-SNAP. The ability of syntaxin 12 to bind α-SNAP is in good agreement with its putative function as a SNARE.

Syntaxin 1 is known to exist in complex with another neuronal-specific SNARE molecule, SNAP-25 (22–23). A novel nonneuronal molecule that binds syntaxin and synaptobrevin and is homologous to SNAP-25 has since been cloned from human B lymphocyte (18), and based on its molecular size, is called SNAP-23. Recently, syntet, a ubiquitous mouse protein homologous to SNAP-25 has also been cloned (15). Based on the sequence similarity, syntet appear to be the mouse homolog of human SNAP-23. We sought to determine if syntaxin 12 could also interact in vitro with syntet/SNAP-23. As shown in Fig. 3, both syntaxin 1A and syntaxin 12 binds to syntet/SNAP-23 with high specificity, whereas mSEC13 again exhibits no binding. The ability of syntaxin 12 to bind SNAP-23 is highly suggestive of its functional similarity with other members of the syntaxin family.

That syntaxin 12 is indeed a membrane-anchored protein was confirmed by the fact that its full-length translated product could not be stripped off membranes by high salt (1 M KCl) or high pH (sodium bicarbonate, pH 11) treatment but could be solubilized with a detergent such as Triton X-100 (Fig. 4A). To further characterize the molecule, rabbit polyclonal antibodies were raised using bacterially expressed fusion protein. The affinity-purified antibody detected a ~39-kDa band by immunoblot analysis of the in vitro translated product of the full-length cDNA and NRK cell lysates (Fig. 4B). Detection can be abolished by co- or preincubation of the antibody with excess amount of the fusion protein (not shown).

As a first step toward functional characterization of syntaxin 12, we performed indirect immunofluorescence microscopy to localize the protein in several cell lines. As shown in Fig. 5, the antibody-stained intracellular structures clustered at the perinuclear region of mouse (MEF), human (HeLa), and rat (NRK) cells. To confirm the membrane topology of syntaxin 12, we performed indirect immunofluorescence microscopy to localize the protein in several cell lines. As shown in Fig. 5, the antibody-stained intracellular structures clustered at the perinuclear region of mouse (MEF), human (HeLa), and rat (NRK) cells.
characteristic of all syntaxins but not all SNAREs identified to date. All syntaxins discovered to date were localized to either the Golgi region or the cell surface, indicating that they function along the exocytic pathway. As shown in Fig. 6, the antibody labeled perinuclear structures in NRK cells. The labeling pattern in NRK cells resembles that of the TGN marker, TGN38 (24), double-labeled in the same cell although not completely colocalized. The fungal metabolite brefeldin A (BFA) has varying effects on the morphology of subcellular organelles and on the distribution of various markers on these organelles (25). The distribution of Golgi markers to the endoplasmic reticulum (25) and the collapse of TGN markers TGN38 (26) and furin proprotein convertase (27) and endosomal markers (28–30) into the microtubule-organizing center upon BFA treatment had been extensively documented. The effect of BFA on the morphology of a particular protein is therefore often useful in determining its subcellular localization. Treatment of cells with 10 μg/ml BFA resulted in the collapse of the structure into a compact structure characteristic of the microtubule-organizing center, colocalizing well with that of TGN38. This result suggests that the perinuclear staining of syntaxin 12 is not that of the Golgi apparatus (which under this condition would have redistributed to the endoplasmic reticulum) but may be that of the TGN or the endosomes. BFA, however, also causes a fusion of the endosomes with the trans-Golgi network (28), and endosomal markers behave quite like TGN markers at the end point of the BFA effect.

To further determine the exact localization of the rat syntaxin and differentiate between the two possibilities, we treat cells with wortmannin, the phosphatidylinositol 3-kinase inhibitor (31). This drug has been shown to alter the morphology of endosomes but not the Golgi apparatus or the trans-Golgi network (32–33). Although wortmannin treatment did not alter the perinuclear Golgi staining marked by the TGN38 monomeric antibody, the perinuclear structure marked by the rat syntaxin antibody in the same cells were converted into swollen vacuoles, characteristic of wortmannin-induced changes to the stainings of endosomal markers such as the mannose 6-phosphate receptor (32). We observed this as well in another cell line, L2 (not shown). The above results strongly suggest that the rat syntaxin-like molecule is localized to a BFA and wortmannin-sensitive endosomal compartment.

DISCUSSION

We have therefore cloned a novel member of the syntaxin family with a unique subcellular localization. None of the syntaxins 1–6 published to date are associated with the endosome. Exogenous expression of syntaxin 1A does result in intracellular localization in Madin-Darby canine kidney cells, and exogenous expression of syntaxin 3 does have an intracellular component (10). These intracellular stainings were, however,
shown not to be endosomal in nature but rather colocalized with a lysosomal marker (10). Another novel syntaxin, known as syntaxin 7, has recently been cloned in our laboratory (34) as well as by Wang et al. (35). Based on its homology to yeast and plant vacuolar syntaxins, Wang et al. proposed that syntaxin 7 may have a role in trafficking between the Golgi apparatus and the lysosomes. Our immunolocalization data, however, suggest that syntaxin 7 is localized to the endosomes (34). In view of the localizations of syntaxin 7 and syntaxin 12, the transport machinery of the endocytic pathway, like its exocytic counterpart, also utilizes members of the syntaxin family.

What may the function of syntaxin 12 be? There are several possibilities. Judging by its compact, perinuclear staining, syntaxin 12 may well reside in a late endosomal compartment. Indeed it does not colocalize with transiently internalized transferin (not shown). However, we could not rule out that small amounts of syntaxin 12 may reside in the early endosomes. If solely localize to a late endosomal compartment, syntaxin 12 may function to receive vesicles either from the TGN or the early endosome or participate in the recycling of surface receptors. Elucidation of its exact role in transport awaits experiments involving effective functional disruption either by the introduction of negative dominant mutants, inhibitory antibodies, or targeted disruption of the gene.

Acknowledgments—We thank Dr. George Banting for monoclonal antibody against TGN38, Dr. G. Baldini for syned cDNA, Dr. R. Scheller for syntaxin 1A cDNA, Dr. Anand Swaroop for mSEC13 cDNA, Dr. S. H. Wong for GST-aSNAP, and Mr. Robin Philips for protein sequencing.

REFERENCES

1. Rothman, J. E., and Orci, L. (1992) Nature 355, 409–415
2. Waters, M. G., Serafini, T., and Rothman, J. E. (1991) Nature 349, 248–251
3. Rothman, J. E., and Warren, G. (1994) Curr. Biol. 4, 220–233
4. Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) Nature 362, 318–324
5. Bennett, M. K., and Scheller, R. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2559–2563
6. Bennett, M. K., Garcia-Arraras, J. E., Elferink, L. A., Peterson, K., Fleming, A. M., Hanuka, C. D., and Scheller, R. H. (1995) Cell 74, 863–873
7. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) J. Mol. Biol. 215, 403–410
8. Bennett, M. K., Calakos, N., and Scheller, R. H. (1992) Science 257, 255–259
9. Bock, J. B., Lin, R. C., and Scheller, R. H. (1996) J. Biol. Chem. 271, 17961–17965
10. Low, S. H., Chapin, S. J., Weimbs, T., Komives, L. G., Bennett, M. K., and Mostov, K. (1996) Mol. Biol. Cell 7, 2067–2078
11. Gaisano, H. Y., Gai, M., Malkus, P. N., Sheu, L., Bouquillon, A., Bennett, M. K., and Trimble, W. S. (1996) Mol. Biol. Cell 7, 2019–2027
12. Whitney, J. A., Gomez, M., Shreff, D., Kreis, T. E., and Mellman, I. (1995) Cell 83, 763–766
13. Robinson, L. J., Aniento, F., and Gruenberg, J. (1997) J. Cell Sci. 110, 2079–2087
14. Link, E., McMahon, H., Fischer von Mollard, G., Yamashita, S., Niemann, H., Sudhof, T. C., and Jahn, R. (1993) J. Biol. Chem. 268, 18423–18426
15. Wang, G., Witkin, J. W., Hao, G., Bankaitis, V. A., Scherer, P. E., and Baldini G. (1997) J. Cell Sci. 110, 565–573
16. Swaroop, A., Yang-Feng, T. L., Liu, W., Gieser, L., Barrow, L. L., Chen, K.-C., Agarwal, N., Meisler, M. H., and Smith, D. I. (1994) Hum. Mol. Genet. 3, 1281–1286
17. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1993) Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, New York
18. Ravichandran, V., Chawla, A., and Roche, P. A. (1996) J. Biol. Chem. 271, 13300–13303
19. Tang, B. L., Low, S. H., and Hong, W. (1995) Eur. J. Cell Biol. 68, 199–205
20. Tang, B. L., Peter, F., Krijnse-Locker, J., Low, S. H., Griffiths, G., and Hong, W. (1995) Mol. Biol. Cell 7, 255–256
21. Bock J. B., and Scheller, R. H. (1997) Nature 387, 133–135
22. Otto, H., Hansom, P. I., and Jahn, R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6197–6201
23. Sollner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H., and Rothman,
A Syntaxin Molecule in the Endosome

24. Luzio, J. P., Burke, B., Banting, G., Howell, K. E., Braghetta, P., and Stanley, K. K. (1990) *Biochem. J.* **270**, 97–102

25. Klausner, R. D., Donaldson, J. G., and Lippincott-Schwartz, J. (1992) *J. Cell Biol.* **116**, 1071–1080

26. Reaves, B., and Banting, G. (1992) *J. Cell Biol.* **116**, 85–94

27. Molloy, S. S., Thomas, L., VanSlyke, J. K., Stenberg, P. E., and Thomas, G. (1994) *EMBO J.* **13**, 18–33

28. Lippincott-Schwartz, J., Yuan, L. C., Tipper, C., Amherdt, M., Ori, L., and Klausner, R. (1991) *Cell* **67**, 601–616

29. Wood, S. A., and Brown, W. J. (1991) *J. Cell Biol.* **119**, 273–285

30. Wood, S. A., Park, J. E., and Brown, W. J. (1991) *Cell* **67**, 591–600

31. Arcaro, A., and Wymann, M. P. (1990) *Biochem. J.* **296**, 297–301

32. Brown W. J., DeWald, D. B., Emr, S. D., Plutner, H., and Balch, W. E. (1995) *J. Cell Biol.* **130**, 781–796

33. Reaves, B. J., Bright, N. A., Mullock, B. M., and Luzio, J. P. (1996) *J. Cell Sci.* **109**, 749–762

34. Wong, S. H., Xu, Y., Zhang, T., and Hong, W. (1998) *J. Biol. Chem.* **273**, 375–403

35. Wang, H., Frelin, L., and Pevsner, J. (1997) *Gene* **199**, 39–48
Syntaxin 12, a Member of the Syntaxin Family Localized to the Endosome
Bor Luen Tang, Andrew E. H. Tan, Lay Kheng Lim, San San Lee, Delphine Y. H. Low and Wanjin Hong

J. Biol. Chem. 1998, 273:6944-6950. doi: 10.1074/jbc.273.12.6944

Access the most updated version of this article at http://www.jbc.org/content/273/12/6944

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 19 of which can be accessed free at http://www.jbc.org/content/273/12/6944.full.html#ref-list-1