Evidence for the putative docking/fusion complex of exocytosis in parotid acinar cells

Taishin Takuma[a,*], Mitsuo Tagaya[b], Tokuro Ichida[a]

[a]*Department of Oral Biochemistry, School of Dentistry, Health Sciences University of Hokkaido, Tobetsu, Hokkaido 061-02, Japan*
[b]*School of Life Science, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan*

Received 20 December 1996; revised version received 21 January 1997

Abstract In lysates of the rat brain, the SNARE complex, a putative membrane fusion machinery of synaptic exocytosis, is extremely stable and is detected after SDS-PAGE. Applying this technique to parotid acinar cells, however, we could only detect the monomeric VAMP-2, but not the high molecular forms associated with other components of the SNARE complex. Parotid acini did not contain brain-type t-SNAREs, but contained NSF and αSNAP. When VAMP-2 was immunoprecipitated from parotid acinar cell lysates, NSF and αSNAP were coprecipitated with it. Since NSF and αSNAP are unable to bind directly to VAMP-2 but indirectly bind via t-SNAREs, the immunoprecipitate very likely contained unidentified t-SNAREs.

Key words: Exocytosis; Membrane fusion; SNARE hypothesis; VAMP-2; NSF; αSNAP

1. Introduction

Exocytosis is a fusion process of secretory granule membranes with the plasma membrane. According to the most prevailing theory (SNARE hypothesis) [1–3], the *N*-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF attachment proteins (α,β,γ-SNAPs) in the cytosolic fraction make a complex with the specific receptor proteins (SNAP receptors; SNAREs) on the vesicle membrane (v-SNAREs) and those on the target membrane (t-SNAREs). The specific protein-protein interaction of the SNARE complex is believed to lead to docking and fusion of secretory vesicles with their proper destination. In neuronal cells, vesicle-associated membrane protein-2 (VAMP-2) is recognized as a v-SNARE on synaptic vesicles, and synaptosome-associated protein of 25 kDa (SNAP-25) and syntaxin 1A/B are known as t-SNAREs on presynaptic membranes. In vitro reconstitution system, VAMP-2, SNAP-25, and syntaxins are tightly bound to form a 7S complex, and then make a 20S complex in the presence of NSF and SNAPs. These protein-protein interactions have been established to be mediated via the predicted coiled-coil domain near the C-terminus of syntaxin; this domain binds SNAP-25, VAMP-2, and αSNAP that binds NSF. NSF has two ATP binding sites in each molecule and dissociates the 20S SNARE complex by hydrolyzing ATP. The ATPase activity of NSF plays an essential role in membrane fusion, since mutation of the ATP binding sites eliminates both ATPase activity and fusion [6–9].

Several lines of evidence strongly support the SNARE hypothesis: (1) v-SNAREs and t-SNAREs make a stable complex in cell lysates prepared with non-ionic detergents; (2) fatal neurotoxins such as tetanus and botulinum toxins specifically cleave these SNARE proteins and inhibit exocytosis[11–13]; and (3) the depletion of homologs of these SNARE proteins inhibits various steps of vesicle transport in yeast [14].

Although proteins of the SNARE complex are widely distributed among eukaryotic cells, in mammals only neuronal and endocrine cells have so far been fully established to have both v-SNAREs and t-SNAREs for exocytosis [15–18]. In exocrine cells, including parotid acinar cells, VAMP-2 of v-SNAREs has been identified, but brain-type t-SNAREs have not been detected [19,20]. Recently, however, tetanus toxin and botulinum toxin B, both of which specifically hydrolyze VAMP-2, inhibited amylase release from streptolysin-O permeabilized pancreatic [21] and parotid acinar cells [22], suggesting that the SNARE hypothesis is also applicable to exocrine glands. Therefore, in this study we have examined whether or not the SNARE complex is formed in the lysate of parotid acinar cells.

2. Materials and methods

2.1. Materials

Anti-rat VAMP-2 antibody (rabbit IgG, polyclonal), anti-rat syntaxin (monoclonal) and anti-rat SNAP-25 (monoclonal) antibodies were purchased from Wako (Osaka, Japan). Anti-NSF (monoclonal) was prepared as described previously [6] and anti-αSNAP (rabbit IgG, polyclonal) antibody was raised against His-tag αSNAP. Pefabloc SC was from Merck (Darmstadt, Germany). Enhanced chemiluminescence (ECL) kit was from Amersham (Little Chalfont, England). Block Ace was from Dainippon Seiyaku (Osaka, Japan). All other chemicals utilized were the highest grade commercially available.

2.2. Immunoprecipitation and immunoblotting

Rat parotid acinar cells (small acini) were prepared as described earlier [23,24]. Parotid acini were pelleted and lysed with 0.5 ml PBS containing 1% Triton X-100, 2 mM DTT, and 0.2 mM Pefabloc SC on ice for 30 min. The lysate was centrifuged at 15000 rpm for 5 min at 4°C, and aliquots of the supernatant (100 μl) were incubated with either 0.5 mM ATP+2 mM $MgSO_4$ or 0.5 mM ATP+2 mM EDTA at 4°C for 30 min. After incubation, the mixture was diluted 10-fold with the incubation medium without Triton X-100; and then anti-VAMP-2 antibody or control rabbit IgG was added to it. After the mixture had been rotated at 4°C for 1 h, the antigen-antibody complex was recovered with protein A Sepharose beads and washed 5 timed with the incubation medium without Triton X-100. The beads were boiled for 5 min in the Laemmli cocktail.

Proteins in the immunoprecipitate were resolved by SDS-PAGE on a 5–20% gradient gel (Atto, Tokyo). Proteins in the gel were transferred to a PVDF membrane at 100 mA per mini-gel (90×73×1 mm) for 60 min with a semi-dry blotter using 0.1 M Tris-0.192 M glycine

*Corresponding author. Fax: (81) (1332) 3-1391.
E-mail: takuma@hucc.hokudai.ac.jp

Abbreviations: NSF, *N*-ethylmaleimide-sensitive fusion protein; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; VAMP, vesicle-associated membrane protein; SNAP-25, synaptosome-associated protein of 25 kDa; MEM, minimum essential medium

PII S0014-5793(97)00081-1

buffer containing 5% methanol. The membrane was washed twice with water, blocked with Block Ace at room temperature for 1 h, and incubated with properly diluted primary antibody in PBS containing 0.05% Tween 20 and 20% Block Ace for 1 h at room temperature or overnight at 4°C. SNARE proteins were then visualized by the ECL system.

3. Results and discussion

Hayashi et al. reported that the binding between v-SNAREs and t-SNAREs in rat brain lysates is maintained during SDS-PAGE unless the sample in the Laemmli cocktail is boiled [4,10]. This point was confirmed in this study (Fig. 1). When the sample was not boiled, monomers of syntaxin and SNAP-25 were markedly decreased, and the high molecular mass complexes of these t-SNAREs were clearly seen. On the other hand, the monomer of VAMP-2 was not markedly decreased in the same sample, although the high molecular complexes were clearly detected. These results suggest that either (1) the binding between VAMP-2 and t-SNAREs is less stable in Laemmli cocktail than that between syntaxin and SNAP-25 or (2) VAMP-2 is in relative excess for making SNARE complexes compared with t-SNAREs or both. Furthermore, among bands of the high molecular mass complexes, the relative intensity of syntaxin, SNAP-25, and VAMP-2 varied greatly, indicating that these SNARE complexes are heterogeneous in their composition.

Although brain-type t-SNAREs do not exist in parotid acini, high molecular mass complexes of VAMP-2 should be detected in the non-heated sample, if similar SNARE complexes are formed in parotid acinar cell lysates. However, we could only detect the monomeric VAMP-2, but not the high molecular mass complex of VAMP-2 even when a large amount of proteins was subjected to SDS-PAGE and the immunoblots were exposed for a long time to detect weak

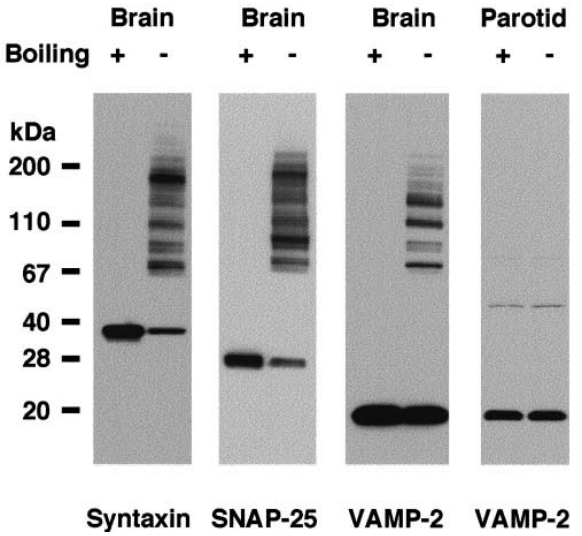

Fig. 1. SNARE complexes in rat brain and parotid acinar cell lysates. Rat brain and parotid acini were homogenized with PBS containing 1% Triton X-100, 2 mM DTT, 2 mM EDTA and 0.2 mM Pefabloc SC in a Teflon-glass homogenizer. The lysate was kept on ice for 30 min and then centrifuged at 15000 rpm for 5 min at 4°C. Aliquots of the supernatant were mixed with the Laemmli cocktail and kept at room temperature or boiled for 5 min. The boiled sample was re-boiled just before SDS-PAGE. After SDS-PAGE, SNARE proteins were visualized by immunoblotting with the ECL system.

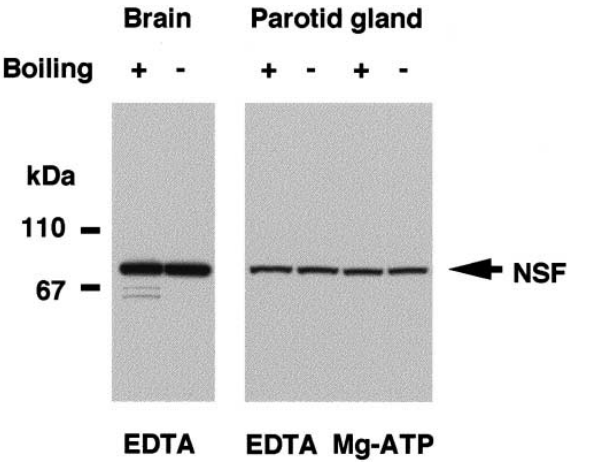

Fig. 2. Detection of NSF in brain and parotid acinar cell lysates. The lysates were prepared as described in the legend to Fig. 1, except that parotid acini were homogenized with the buffer without EDTA. After centrifugation, lysates of parotid acini were incubated with either 0.5 mM ATP+2 mM $MgSO_4$ or 0.5 mM ATP+2 mM EDTA at 4°C for 30 min. Aliquots of the lysates were mixed with the Laemmli cocktail and kept at room temperature or boiled for 5 min. After SDS-PAGE, NSF was visualized by immunoblotting.

signal of VAMP-2 (Fig. 1). A faint band seen at a molecular mass of 50 kDa was a non-specific one that was observed even if blots of parotid lysates were incubated with the secondary antibody for rabbit IgG without primary antibodies (data not shown). These results suggest that the binding between VAMP-2 and t-SNAREs in parotid acini is less stable than that in brain.

Parotid acinar cells was found to contain NSF and αSNAP (Figs. 2 and 3). NSF and SNAPs are believed to be ubiquitous components of SNARE complexes in all vesicle transport, including exocytosis. In this study, even when the Laemmli cocktail was not boiled, NSF in both brain and parotid acini was only detected as a monomer irrespective of the presence or absence of Mg-ATP.

Next we tried to isolate SNARE complexes by immunoprecipitation with anti-VAMP-2 antibody. Since the antigen-antibody complex of VAMP-2 was fairly unstable in the presence of 1% Triton X-100, we incubated anti-VAMP-2 with a 10-fold diluted lysate of parotid acini (0.1% Triton X-100). As shown in Fig. 3, VAMP-2 was precipitated only with anti-VAMP-2 antibody, but not with control rabbit IgG or protein A Sepharose beads alone, indicating that the immunoprecipitation is specific and that the washing process completely removed unbound VAMP-2. The recovery of VAMP-2 with anti-VAMP-2 antibody was almost complete, since we could not detect VAMP-2 in 10-fold concentrated supernatant.

Then we examined the existence of NSF and αSNAP in the immunoprecipitate obtained with anti-VAMP-2. As shown in Fig. 3, NSF was coprecipitated with VAMP-2, and was completely removed from the immunoprecipitate in the presence of Mg-ATP. The Mg-ATP-dependent release of NSF from the SNARE complex is the most characteristic behavior of this protein [1,2]. αSNAP was also coprecipitated with VAMP-2 and was released from the immunoprecipitate in the presence of Mg-ATP. αSNAP is recognized to be released from the SNARE complex only in the presence of both NSF and Mg-ATP [4,5]. In the latter experiment the background reaction was very high, since antibodies for VAMP-2 and αSNAP

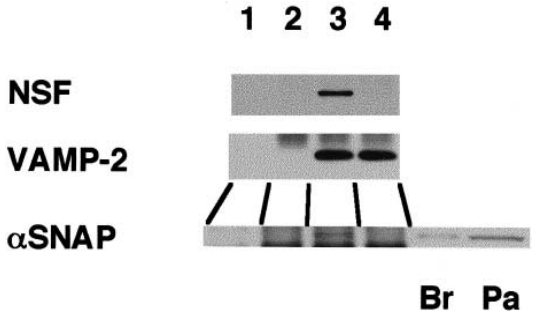

Fig. 3. Coprecipitation of NSF and αSNAP by immunoprecipitation with anti-VAMP-2 antibody. The immunoprecipitation was performed as described in Section 2. Lysates of parotid acini and brain were prepared as described in the legend to Fig. 1. After SDS-PAGE, NSF, VAMP-2, and αSNAP were visualized by immunoblotting. Lane 1, protein A Sepharose beads alone; lane 2, control rabbit IgG; lane 3, anti-VAMP-2 antibody incubated with 0.5 mM ATP+2 mM EDTA; lane 4, anti-VAMP-2 antibody incubated with 0.5 mM ATP+2 mM $MgSO_4$. Br, brain; Pa, parotid acinar cells.

were both polyclonal rabbit IgG and the molecular mass of αSNAP (35 kDa) was just between the masses of heavy and light chains of anti-VAMP-2 that were used for immunoprecipitation.

The present study clearly shows that the antibody for VAMP-2 coprecipitates NSF and αSNAP, essential components of membrane fusion in various steps of vesicle transports. In vitro study reveals that NSF and αSNAP are unable to bind directly to VAMP-2, but bind indirectly via t-SNAREs that bind to VAMP-2 [4,5]. Recently the cDNA of SNAP-23, a new homolog of SNAP-25, was cloned from a human B lymphocyte cDNA library by use of the yeast two-hybrid system, in which syntaxin 4 was used as the 'bait' [25]. Northern blot analysis revealed that SNAP-23 is ubiquitously expressed in many human tissues, including pancreas [25]. Thus, it is very likely that rat parotid glands also contain the rat counterpart of SNAP-23. In addition, syntaxin 4 is also expressed widely in non-neuronal cells [26] and is specifically located on the plasma membrane [27]. These results and our present findings collectively suggest that the immunoprecipitate obtained with anti-VAMP-2 contains unidentified t-SNAREs of the rat parotid acinar cells in addition to NSF and αSNAP. Further study is necessary to identify t-SNAREs in parotid acinar cells.

Acknowledgements: This study was supported in part by a grant-in-aid for special research projects from the Health Sciences University of Hokkaido.

References

[1] Sollner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R.H. and Rothman, J.E. (1993) Cell 75, 409–418.
[2] Sollner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P. and Rothman, J.E. (1993) Nature 362, 318–324.
[3] Rothman, J.E. (1994) Adv. Second Messenger Phosphoprotein Res. 29, 81–96.
[4] Hayashi, T., Yamasaki, S., Nauenburg, S., Binz, T. and Niemann, H. (1995) EMBO J. 14, 2317–2325.
[5] Hanson, P.I., Otto, H., Barton, N. and Jahn, R. (1995) J. Biol. Chem. 270, 16955–16961.
[6] Tagaya, M., Wilson, D.W., Brunner, M., Arango, N. and Rothman, J.E. (1993) J. Biol. Chem. 268, 2662–2666.
[7] Sumida, M., Hong, R.M. and Tagaya, M. (1994) J. Biol. Chem. 269, 20636–20641.
[8] Whiteheart, S.W., Rossnagel, K., Buhrow, S.A., Brunner, M., Jaenicke, R. and Rothman, J.E. (1994) J. Cell Biol. 126, 945–954.
[9] Morgan, A., Dimaline, R. and Burgoyne, R.D. (1994) J. Biol. Chem. 269, 29347–29350.
[10] Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Sudhof, T.C. and Niemann, H. (1994) EMBO J. 13, 5051–5061.
[11] Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B.R. and Montecucco, C. (1992) Nature 359, 832–835.
[12] Schiavo, G., Poulain, B., Rossetto, O., Benfenati, F., Tauc, L. and Montecucco, C. (1992) EMBO J. 11, 3577–3583.
[13] Link, E. et al. (1992) Biochem. Biophys. Res. Commun. 189, 1017–1023.
[14] Ferro-Novick, S. and Jahn, R. (1994) Nature 370, 191–193.
[15] Jacobsson, G., Bean, A.J., Scheller, R.H., Juntti-Berggren, L., Deeney, J.T., Berggren, P.O. and Meister, B. (1994) Proc. Natl. Acad. Sci. USA 91, 12487–12491.
[16] Regazzi, R. et al. (1995) EMBO J. 14, 2723–2730.
[17] Roth, D. and Burgoyne, R.D. (1994) FEBS Lett. 351, 207–210.
[18] Morgan, A. and Burgoyne, R.D. (1995) EMBO J. 14, 232–239.
[19] Stecher, B., Ahnert-Hilger, G., Weller, U., Kemmer, T.P. and Gratzl, M. (1992) Biochem. J. 283, 899–904.
[20] Braun, J.E., Fritz, B.A., Wong, S.M. and Lowe, A.W. (1994) J. Biol. Chem. 269, 5328–5335.
[21] Gaisano, H.Y., Sheu, L., Foskett, J.K. and Trimble, W.S. (1994) J. Biol. Chem. 269, 17062–17066.
[22] Fujita-Yoshigaki, J., Dohke, Y., Hara-Yokoyama, M., Kamata, Y., Kozaki, S., Furuyama, S. and Sugiya, H. (1996) J. Biol. Chem. 271, 13130–13134.
[23] Takuma, T. and Ichida, T. (1994) FEBS Lett. 3401, 29–33.
[24] Takuma, T. and Ichida, T. (1994) J. Biol. Chem. 269, 22124–22128.
[25] Ravichandran, V., Chawla, A. and Roche, P.A. (1996) J. Biol. Chem. 271, 13300–13303.
[26] Bennett, M.K., Garcia-Arraras, J.E., Elferink, L.A., Peterson, K., Fleming, A.M., Hazuka, C.D. and Scheller, R.H. (1993) Cell 74, 863–873.
[27] Brumell, J.H., Volchuk, A., Sengelov, H., Borregaard, N., Cieutat, A.M., Bainton, D.F., Grinstein, S. and Klip, A. (1995) J. Immunol. 155, 5750–5759.