Imaging the assembly and disassembly kinetics of cis-SNARE complexes on native plasma membranes

Dana Bar-On¹,¹, Ulrike Winter²,¹, Esther Nachliel³, Menachem Gutman³, Dirk Fasshauer², Thorsten Lang²,³,⁴, Uri Ashery¹

¹ Department of Neurobiochemistry, Tel Aviv University, Tel Aviv 69978, Israel
² Department of Neurobiology, Max-Planck-Institute for Biophysical Chemistry, 37077 Göttingen, Germany
³ Laser Laboratory for Fast Reactions in Biology, Department of Biochemistry, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel
⁴ LIMES-Institute, Laboratory for Membrane Biochemistry, University of Bonn, Bonn 53115, Germany

Received 28 July 2008; revised 29 August 2008; accepted 29 August 2008

Available online 24 September 2008

Abstract Mild sonication of eukaryotic cells produces native plasma membrane sheets that retain their docked organelles, cytoskeleton structures and cytoplasmic complexes. While the delicate organization of membranous protein complexes remains undisturbed, their inner plasmalemmel leaflet can be rapidly exposed to bathing solutions, enabling specific biochemical manipulations. Here, we apply this system to track membrane-biochemistry kinetics. We monitor soluble NSF-attachment protein receptor (SNARE) complex assembly and disassembly on the plasma membrane at high time resolution. The results suggest two-phase kinetics for the assembly process and dependence of the disassembly kinetics on both N-ethyl maleimide-sensitive factor (NSF) and soluble NSF-attachment protein (z-SNAP) concentrations.

© 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Ex vivo; Plasma membrane sheet; cis-SNARE complex; Time-resolved kinetics; Assembly; Disassembly

1. Introduction

The plasma membrane (PM) is populated by many types of membrane proteins that control diverse cellular activities, such as transport, signaling and membrane fusion. Studies on membrane protein functions and in particular the roles of their intracellular domains provide a tremendous challenge for researchers. In vitro systems are generally easy to control and manipulate, but protein purification and membrane reconstitution are often tedious and the reaction conditions are artificial. In contrast, in vivo methods maintain membrane integrity and hence provide physiological conditions. However, under those conditions, access to the interior PM is limited, the bottleneck being the ability to perform rapid manipulations and visualization. PM sheet preparation is a cellular “ex vivo” method that combines the advantages of classical in vivo and in vitro approaches. Adherent cells are subjected to an ultrasound pulse which “unroofs” them, leaving behind an intact native membrane bilayer attached to a cover slip (Fig. 1A and for review see [1]). This easy preparation of “inside–out” membranes provides the full set of membrane lipids and proteins in their native environment [2]. This approach has been used to characterize the reactivity status of soluble NSF-attachment protein receptor (SNARE) proteins involved in regulated membrane fusion [3] and the effects of accessory proteins on this process [4].

In the present study, we use the ex vivo PM sheet method for a new application [5]: analysis of the kinetics of membrane protein reactions, concentrating on the assembly–disassembly dynamics of cis-SNARE complexes (Fig. 1B). SNARE complex formation between syntaxin 1A and SNAP-25 (synaptosomal associated protein of 25kDa) on the PM and synaptobrevin 2 on the vesicles (trans-SNAREs) is crucial for vesicle priming and fusion [6]. This process is impeded by the formation of the non-productive (non-fusogenic) cis-SNARE complexes formed when all three SNARE proteins reside on the PM [3,7]. These ternary SNARE complexes are extremely stable and therefore, the rate of their spontaneous dissociation is low [6]. In vivo, the specific enzymatic system consisting of the ATPase N-ethyl maleimide-sensitive factor (NSF) and its cofactor soluble NSF-attachment protein (z-SNAP) catalyzes SNARE complex disassembly to SNARE monomers [8] in order to recycle them for further rounds of membrane fusion [8]. Despite the importance of these processes for exocytosis, the kinetics of the assembly–disassembly processes in vivo has not been sufficiently described. Here, we present the kinetics of cis-SNARE complex assembly on the PM using an improved protocol that provides high temporal resolution. Our data show that the assembly process occurs in two-phases, suggesting the existence of two pools of cis-SNARE complexes that might differ in their reactivity. In addition, we show for the first time the disassembly kinetics on the PM. The results indicate that the disassembly kinetics of SNARE complexes in the native membrane environment is dependent on the concentrations of NSF and z-SNAP.

0014-5793/$34.00 © 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.febslet.2008.08.040
2. Materials and methods

2.1. Cell culture and PM sheet preparation

Pheochromocytoma 12 (PC12) cells (clone 251, [9]) were maintained and propagated as described previously [10]. For the preparation of PM sheets, cells were grown on poly-L-lysine-coated glass cover slips and disrupted by a mild, 100-ms [5] ultrasound pulse that left pure, two-dimensional, native PM sheets adsorbed to the glass (Fig. 1A).

2.2. Antibodies

For the detection of syntaxin 1, we used the mouse monoclonal antibody HPC-1 [11]. Cy3-coupled goat-antimouse was used as a secondary antibody (Dianova, Hamburg, Germany).

2.3. Purification of recombinant proteins

cDNA encoding the light chain of Botulinum neurotoxin C1 (BoNT/C1) in a pQE3 expression vector (Qiagen, Hilden, Germany) was a gift from Thomas Binz and Heiner Niemann (Medizinische Hochschule Hannover, Hannover, Germany). BoNT/C1, synaptobrevin 2 (1-96) S28C; [12]), recombinant NSF [13] and α-SNAP [13] were expressed as His6-tagged fusion proteins in Escherichia coli, purified by Ni²⁺-nitrilotriacetic acid (Ni-NTA) agarose chromatography and dialyzed against a buffer containing 150 mM NaCl, 20 mM Tris–HCl pH 7.4 and 1 mM dithiothreitol (DTT). His6-tags were removed by thrombin cleavage during overnight dialysis following elution. Dialysis buffers were based on Tris–HCl (α-SNAP) or HEPES (NSF and synaptobrevin) pH 7.4 and contained NaCl (50 mM for α-SNAP, 70 mM for synaptobrevin and 175 mM for NSF), 1 mM EDTA, 1 mM DTT, and in the case of NSF, an additional 10% glycerol and 0.5 mM ATP. To achieve a high grade of purity, all proteins were subsequently subjected to a second chromatographic step on an Äkta system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). In the case of α-SNAP and synaptobrevin, this step consisted of ion exchange using a MonoQ or a MonoS column, respectively, with a 0.1–1 M NaCl gradient for

---

**Fig. 1.** Illustration of the method and SNARE biochemistry. (A) The membrane sheet assay. Membrane sheets are generated from cells grown on glass cover slips (shown in blue) by application of a brief ultrasound pulse. The glass cover slip with the adherent membranes is incubated upside down in a drop of reaction buffer at 37 °C (example shows the formation of fluorescently labeled cis-SNARE complexes by reacting syntaxin 1A and SNAP-25 with recombinant fluorescent synaptobrevin lacking its TMR (trans membrane region). Labeled cis-SNARE complexes are quantified using fluorescence microscopy. (B) SNARE dynamics. Neuronal exocytosis is driven by complex formation between syntaxin 1A and SNAP-25 on the PM and synaptobrevin 2 on synaptic vesicles, forming trans-SNARE complexes. Alternatively, SNARE complexes can be formed when the three components reside on the same membrane (cis-SNAREs). Cis-SNAREs do not drive membrane fusion, Cis-SNAREs are disassembled by the enzymatic reaction of NSF assisted by α-SNAP. (C) BoNT/C1 action on syntaxin 1. BoNT/C1 cleaves syntaxin 1 close to its TMR, thereby removing most of its cytoplasmatic part. Syntaxin becomes cleavage-resistant upon incorporation into cis-SNARE complexes.
elution. Pure and highly active NSF was obtained by collecting the hexameric fraction after passage through a size-exclusion column (Superdex 200, Sigma–Aldrich). Covalent attachment of Alexa594 C5 maleimide (Invitrogen, Karlsruhe, Germany) to synaptobrevin was performed according to the manufacturer’s instructions.

2.4. Assembly reaction
For preparation of membrane sheets, cells were grown and disrupted as previously described [5] in ice-cold K-Glu buffer [20 mM HEPES, pH 7.2, 120 mM potassium glutamate, 20 mM potassium acetate, 10 mM ethylene glycol tetraacetic acid (EGTA)]. Membrane sheets were either fixed immediately or incubated for the indicated times at 37 °C in bovine serum albumin (BSA)-K-Glu (K-Glu buffer containing 3% BSA), then incubated for 15 min in BSA-K-Glu containing 8 μM BoNT/C1, then washed briefly and fixed for 1 h at RT in 4% paraformaldehyde (PFA) diluted in phosphate buffered saline (PBS). Membrane sheets were washed and immunostained with the primary antibody HPC-1 and with the secondary antibody Cy3-coupled goat-antimouse as described previously [14]. For each experiment, 20–60 membrane sheets were analyzed. For each condition, three to five independent experiments were averaged and values are given as mean ± S.E.M. Averaged data for the assembly reactions were fitted with one exponential fitting for each of the phases using IgorPro (WaveMetrics, Lake Oswego, OR). The time constant t indicates the time taken to increase fluorescence percentage to 1–1/e of the maximum value.

2.5. The disassembly studies
Freshly prepared membrane sheets were reacted with 2 μM recombinant synaptobrevin (of which 1 μM was labeled with Alexa594) in BSA-K-Glu at 37 °C in a humid chamber for 45 min. Then membrane sheets were washed at 37 °C for 15 min in K-Glu buffer, and incubated for the indicated times with NSF and α-SNAP in EGTA-free K-Glu buffer containing 5 mM MgCl2 and 2 mM ATP. In some cases, reaction mixtures were replaced with fresh solutions after 10 min. However, no obvious differences were observed relative to experiments in which the same reaction mixture was used for 20-min incubations. Membrane sheets were washed briefly in K-Glu buffer and then fixed at RT for 60 min, washed twice in PBS for 10 min each, incubated with 50 mM NH4Cl in PBS for 20 min, washed once with PBS for 10 min and then imaged in the presence of 1-(4-trimethylammonium)-6-phe- nyl-1,3,5-hexatriene (TMA–DPH). For each experiment, 20–60 membrane sheets were analyzed. For each condition, three to nine independent experiments were averaged and values are given as mean ± S.E.M. Averaged data for the disassembly reactions were fitted with one exponential fitting using IgorPro.

2.6. Fluorescence microscopy
Membrane sheets were analyzed using a Zeiss inverted fluorescence microscope in principle as described previously [3]. The images were analyzed using Metamorph (Universal Imaging Corporation, West Chester, PA) [3].

3. Results
The PM sheet preparation provides an ideal system for studying the kinetics of PM protein interactions. The PM sheets were created by mild sonication of cultured PC12 cells that left two-dimensional, native PM sheets adsorbed to the glass cover slip (Fig. 1A). The kinetics of endogenous SNARE complex formation was previously studied indirectly using this system [3]. In the current study however, the time resolution and measurement accuracy were improved. The PM sheets were incubated for different times after sonication to allow cis-SNARE complex formation and then treated with BoNT/C1, which specifically cleaves syntaxin molecules that are not part of the cis-SNARE complexes i.e. syntaxin monomers and syntaxin in binary complexes [3] (Fig. 1C). The sheets were then fixed and labeled with syntaxin antibody, which bound to the intact syntaxin molecules residing in the SNARE complexes (Fig. 2C).

To determine the concentration of BoNT/C1 that should be used, a calibration experiment with different concentrations of BoNT/C1 was performed. Fig. 2B depicts the level of the residual intact syntaxin, remaining after 2 min of incubation with BoNT/C1, as a function of BoNT/C1 concentration. After 2 min with 8 μM BoNT/C1 the level of residual intact syntaxin was 30% (Fig. 2B). To assure a maximal cleavage of the free syntaxin molecules, we carried out the BoNT/C1 treatment with the highest level (8 μM) and extended the incubation period to 15 min reaching a level of 10% (Fig. 2B, dashed line). Under these conditions, the baseline fluorescence immediately after PM sheet formation was low (10%), suggesting that in freshly prepared membranes, about 90% of the syntaxin is present in its free form (Fig. 2C and D, t = 0 min) consistent with earlier reports [3]. With time, the membrane fluorescence increased, representing the kinetics of cis-SNARE complex assembly in the absence of disassembly proteins (Fig. 2C and D). Surprisingly, the assembly dynamics exhibited two-phase kinetics; in the first, rapid phase (on the order of minutes), ~15% of the total cis-SNAREs are formed (Fig 2D, left), then the process plateaus transiently and continues at a slower rate, reaching a steady-state level of ~85% of the total syntaxin in cis-SNAREs after ~60 min (Fig. 2D, right). These two, previ- ously non-differentiated [3] kinetic phases of SNARE complex formation were revealed by the higher time resolution provided by these experiments.

The disassembly dynamics was monitored by adding known quantities of the disassembly factors NSF and α-SNAP in the presence of Mg-ATP to PM sheets. Prior to the disassembly process, the sheets were incubated with fluorescently labeled recombinant synaptobrevin 2 which reacted with the endoge- nous SNARE proteins to form labeled cis-SNARE complexes (Fig. 3A and B (t = 0)). Upon disassembly, the soluble fluorescently labeled synaptobrevin was released from the SNARE complexes and diluted in the bathing solution. Therefore, the decrease in PM fluorescence directly reflected the cis-SNAREs’ disassembly kinetics.

The reaction dynamics exhibited clear dependence on NSF/ α-SNAP concentrations. There was an initial rapid phase of disassembly that lasted for up to 2 min (Fig. 3C), followed by a slower phase. The amplitude of the fast phase was a function of NSF/α-SNAP concentration. The remaining fraction of the cis-SNARE complexes after the disassembly process (Fig. 3B, all curves at t = 20 min) showed dependence on NSF/α-SNAP concentration as well.

4. Discussion
4.1. Differentiation of two kinetic phases in cis-SNARE complex assembly
The assembly of cis-SNARE complexes was previously moni- tored indirectly by incubating the PM sheets for predetermined times followed by an additional, long (45 min) incubation with fluorescently labelled synaptobrevin. The de- crease in the incorporation of exogenous synaptobrevin over time indicated endogenous cis-SNARE complex formation. However, the relatively long half time (~5 min) of synaptobre- vin binding [3] and the very few time points examined led to a systematic underestimation of the determined half time of
SNARE complex formation. In the present study, we sampled cis-SNARE complex assembly directly and more frequently and took advantage of the specific and rapid cleavage of free syntaxin by BoNT/C1 to gain higher time resolution and accuracy. The increased temporal resolution using the new readout revealed the existence of two kinetic phases of assembly: a rapid one that used up 15% of the total syntaxin followed by a second step that used up about 60% of the total syntaxin.

The two-phase dynamics might be explained by assuming that at \( t = 0 \) min, the PM already contains a fraction of pre-existing binary complexes (syntaxin-SNAP-25). Formation of the binary complex is rather slow [15] and therefore, its existence on the PM at the beginning of the experiment might accelerate ternary complex formation [15]. An alternative explanation is that the two-phase kinetics represents two populations of syntaxin that differ in their reactivity. The syntaxin on the PM has been shown to form clusters via homophilic protein–protein interactions [16]: Sieber et al. detailed the anatomy of syntaxin nano-sized clusters [17] that included 84% of the syntaxin molecules. The rest (~16%) of the syntaxin molecules freely diffused outside the clusters. Thus, fast-forming complexes might be attributed to the free syntaxin population that is more accessible to SNAP-25 and synaptobrevin for the formation of cis-SNARE complexes. Under this scenario, the slow phase might correspond to cis-SNARE complexes that are formed from the clustered syntaxin population [17], since clustered syntaxin is less accessible or has to dissociate from the clusters. In either case, further information is needed to understand the mechanistic basis of this phenomenon.

### 4.2. Dynamics of cis-SNARE disassembly

The disassembly reaction was measured under conditions in which cis-SNARE complexes were, as under physiological conditions, embedded in the native membrane. The experimental results indicated that at various concentrations and stoichiometric ratios of NSF and \( \alpha \)-SNAP, the disassembly components work efficiently enough to cope with the speed of SNARE complex assembly on the PM sheets (as shown in Fig. 2). As the level of cis-SNAREs in the cells is expected to be very low [3], it is reasonable to assume that efficient cis-SNARE disassembly also occurs under physiological conditions in intact cells. The disassembly dynamics clearly exhibits
dependence of the fast-amplitude fraction (Fig. 3C, t = up to 2 min) and the remaining fraction of cis-SNAREs on NSF/α-SNAP concentration. The remaining fraction of cis-SNAREs could be a result of additional processes that interfere with cis-SNARE complex disassembly, such as the formation of syntaxin-α-SNAP complexes and their disassembly by NSF [13], or lower accessibility of the disassembly machinery to a certain population of SNARE complexes.

5. Conclusions

Assays using native PM sheets are highly flexible and enable the study of diverse aspects of membrane proteins. In the current study, we expanded the use of this method by analyzing the kinetics of cis-SNARE complex assembly/disassembly. The availability of free SNARE proteins for fusion-producing trans-SNARE complex formation depends on maintaining low levels of cis-SNARE complexes. An interfering process such as cis-SNARE complex formation could be of crucial importance to the regulation of trans-SNARE formation, thereby having an influence on exocytosis in general. We distinguished two-phase kinetics in the assembly process. Moreover, the dependence of cis-SNARE complex disassembly kinetics on NSF and α-SNAP was characterized for the first time on PM sheets.

Acknowledgements: The authors would like to thank Professor R. Jahn for his constant support, Dr. F. E. Zilly for providing recombinant protein, and D. Diezmann for excellent technical assistance and for providing recombinant proteins. D.B.O. and U.W. were supported by the Max Planck Society and a grant from the Deutsche Forschungsgemeinschaft (SFB 523), respectively.

References


