

# Regulated secretion: SNARE density, vesicle fusion and calcium dependence

Jens R. Coorsen<sup>1,3,\*</sup>, Paul S. Blank<sup>1</sup>, Fernando Albertorio<sup>1</sup>, Ludmila Bezrukov<sup>1</sup>, Irina Kolosova<sup>1</sup>, Xiongfong Chen<sup>2</sup>, Peter S. Backlund Jr<sup>1</sup> and Joshua Zimmerberg<sup>1,\*</sup>

<sup>1</sup>Laboratory of Cellular and Molecular Biophysics, and <sup>2</sup>Unit on Biologic Computation, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892-1855, USA

<sup>3</sup>Department of Physiology and Biophysics, Neuroscience Research Group, Faculty of Medicine, University of Calgary, Calgary, Alberta T2N 4N1, Canada

\*Authors for correspondence (e-mail: jcoorsse@ucalgary.ca or joshz@helix.nih.gov)

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## Summary

SNAREs such as VAMP, SNAP-25 and syntaxin are essential for intracellular trafficking, but what are their exact molecular roles and how are their interactions with other proteins manifest? Capitalizing on the differential sensitivity of SNAREs to exogenous proteases, we quantified the selective removal of identified SNAREs from native secretory vesicles without loss of fusion competence. Using previously established fusion assays and a high sensitivity immunoblotting protocol, we analyzed the relationship between these SNARE proteins and Ca<sup>2+</sup>-triggered membrane fusion. Neither the extent of fusion nor the number of intermembrane fusion complexes per vesicle were correlated with the measured density of identified egg cortical vesicle (CV) SNAREs. Without

syntaxin, CVs remained fusion competent. Surprisingly, for one (but not another) protease the Ca<sup>2+</sup> dependence of fusion was correlated with CV SNARE density, suggesting a native protein complex that associates with SNAREs, the architecture of which ensures high Ca<sup>2+</sup> sensitivity. As SNAREs may function during CV docking in vivo, and as further proteolysis after SNARE removal eventually ablates fusion, we hypothesize that the triggered steps of regulated fusion (Ca<sup>2+</sup> sensitivity and the catalysis and execution of fusion) require additional proteins that function downstream of SNAREs.

Key words: Secretory vesicles, Exocytosis, Membrane fusion, Sea urchins, Quantitative immunoblotting

## Introduction

Research on Ca<sup>2+</sup>-triggered exocytosis, the fundamental cellular process underlying most secretion, has focused on three important proteins that form a SNARE complex (Burgoyne and Morgan, 1998; Jahn and Sudhof, 1999; Wickner and Haas, 2000; Chen and Scheller, 2001; Pelham, 2001) – proteins that are absolutely required for membrane trafficking in a variety of biological systems. The appealing ‘SNARE hypothesis’ – that SNAREs force membranes together to promote fusion (Jahn and Hanson, 1998; Sutton et al., 1998; Weber et al., 1998; Chen et al., 1999; Hua and Charlton, 1999; McNew et al., 2000) – is now a major paradigm in cell biology. However, the SNARE complex has been shown to markedly affect the Ca<sup>2+</sup> regulation of exocytosis but not fusion per se (Nonet et al., 1993; Bittner et al., 1996; Broadi, 1996; Nagamatsu et al., 1996; Coorsen et al., 1998; Deitcher et al., 1998; Reist et al., 1998; Tahara et al., 1998; Yoshihara et al., 1999; Peters et al., 2001; Schoch et al., 2001; Washbourne et al., 2002; Zimmerberg et al., 2000), and there are indications that other proteins function downstream of SNAREs in both exocytosis and in yeast vacuolar homotypic fusion (Coorsen et al., 1998; Tahara et al., 1998; Ungermann et al., 1998; Peters et al., 2001). To explain these contrary results, modified hypotheses that retain SNARE centrality to fusion include catalysis of fusion reactions by SNARE-stabilized transition states (Schoch et al., 2001) and multistep,

Ca<sup>2+</sup>-dependent assembly of SNARE complexes (Xu et al., 1999; Chen et al., 2001; Scales et al., 2001). However, the exact function of SNAREs is difficult to establish because the regulated exocytotic pathway is interconnected and cyclic: interfering with any step eventually blocks secretion. By studying the massively synchronous cortical vesicle (CV) exocytosis of fertilization, which occurs but once, the cycle is bypassed and the final Ca<sup>2+</sup>-dependent fusion steps are isolated. The exocytosis of CV in vitro occurs with physiological [Ca<sup>2+</sup>]<sub>free</sub> (Baker and Whitaker, 1978).

To differentiate between the density of CV SNAREs, changes in the Ca<sup>2+</sup> sensitivity of CV fusion, and the rate and extent of CV fusion, advantage was taken of the size, purity homogeneity and high preparative yields that make CV useful for coupled functional-biochemical analyses (Coorsen et al., 1998; Tahara et al., 1998; Zimmerberg et al., 2000). Endogenously docked CVs from eggs of *Strongylocentrotus purpuratus* are fully Ca<sup>2+</sup> sensitive and release-ready, and they fuse with the plasma membrane (PM) within milliseconds of exposure to optimal [Ca<sup>2+</sup>]<sub>free</sub> (exocytosis in vitro) (Shafi et al., 1994). Ca<sup>2+</sup>-triggered homotypic CV fusion retains the essential features of regulated exocytosis (Coorsen et al., 1998; Zimmerberg et al., 2000); only by removing CVs from the PM is there full access to all vesicle surface proteins. Our approach uses proteases with differing spectra of endogenous substrates on intact CVs, to quantitatively change the density

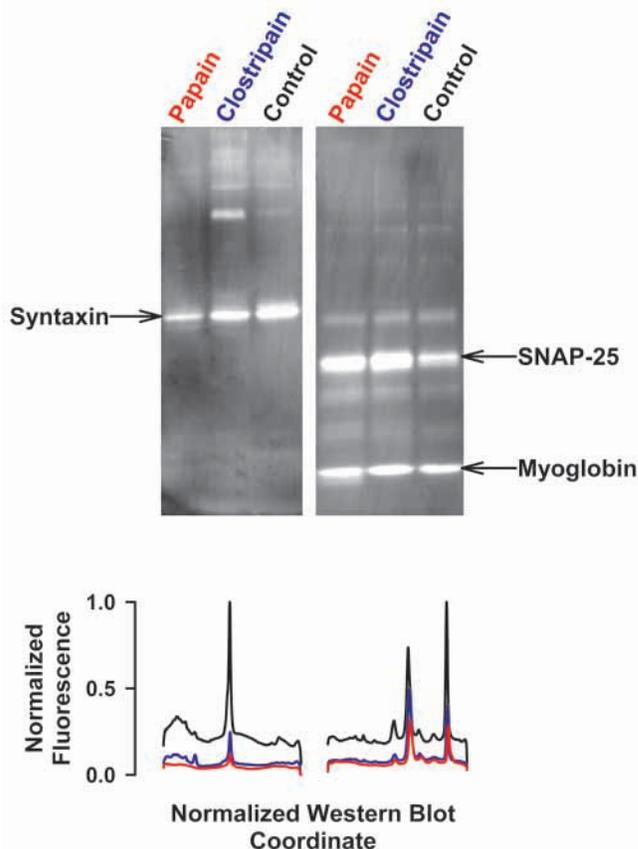
of different sets of proteins; the  $[Ca^{2+}]_{free}$ -dependent rate and extent of CV fusion is then tested, and the density of SNARE proteins measured. This approach (1) makes no assumptions as to which proteins are essential, (2) allows for the involvement of low-abundance proteins, (3) uses native vesicles of endogenous size and composition, and (4) is not subject to issues of compensatory proteins as are genetic knockout studies. This approach does require quantitative assays of absolute protein amount that are antibody dependent (Coorssen et al., 2002).

On average, isolated CV have ~5500, ~700 and ~330 copies of VAMP, SNAP-25 and syntaxin, respectively (Coorssen et al., 2002). We combined (1) a sensitive, quantitative immunoblotting assay and polyclonal antibodies (to minimize underestimation due to epitope loss) to measure SNARE proteins (Coorssen et al., 2002); (2) fusion assays to assess the effects of SNARE removal; and (3) a method for determining the average number of active fusion complexes per vesicle,  $\bar{n}$ , that combines the relationship between the extent of fusion and  $\bar{n}$ , with the exponential decrease in  $\bar{n}$  after protease treatment (Vogel et al., 1996; Coorssen et al., 1998). This analysis was important because there is redundancy in the number of fusion complexes on CV; for any one vesicle, fusion will not be inhibited until all functional fusion complexes on that vesicle are inactivated, although the rate of fusion will be progressively inhibited by reductions in the density of critical components (Vogel et al., 1996; Blank et al., 2001).

## Materials and Methods

Quantitative immunodetection of SNAREs was as described previously (Coorssen et al., 2002). Sea urchin (*S. purpuratus*) maintenance and most methods were as previously described (Vogel et al., 1996; Coorssen et al., 1998; Tahara et al., 1998). For incubations with exogenous proteases, CV suspensions ( $A_{405} \sim 1.0-1.2$ ) were mixed with equal volumes of baseline-intracellular medium (B-IM) buffer (Coorssen et al., 2002) containing trypsin (Fluka, St Louis, MO), papain (Calbiochem, La Jolla, CA) or clostripain (Sigma) and incubated for 1-4 hours at 25°C; CV integrity and clumping were assessed throughout. To optimize protease exposures, suspensions were gently triturated when minor clumping was evident. Incubations were stopped with an equal volume of ice cold B-IM containing a standard mix of protease inhibitors (8 mM benzamidine HCl, 8 mM dithiothreitol (DTT), 2.5  $\mu$ M aprotinin, 23.4  $\mu$ M pepstatin, 33.7  $\mu$ M leupeptin and 168  $\mu$ M 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF)) supplemented with 10  $\mu$ M soybean trypsin inhibitor (SBTI) and 3.4 mM *N*<sup>α</sup>-tosyl-Lys chloromethyl ketone (TLCK) (for trypsin) or 2.1 mM AEBSF and 2.3 mM *N*<sup>α</sup>-tosyl-Phe chloromethyl ketone (TPCK) (for papain) or 2 mM benzamidine, 4 mM AEBSF and 0.5 mM TLCK (for clostripain). Stopped samples were immediately centrifuged (2000 *g* for 15 minutes at 4°C) and recovered CVs were suspended in B-IM (pH 6.7) containing the protease inhibitors described above; aliquots of each were taken for counting, protein extraction and fusion assays.

The determination of proteolytic fragmentation patterns for syntaxin and SNAP-25 (Fig. 1) provided a guide for cutting the blotting membrane into three pieces that could be probed independently for SNAREs. In most experiments, using the molecular weight markers as a guide, the membrane was cut between syntaxin and SNAP-25 and just below the smallest SNAP-25 fragment. Myoglobin, recognized by the SNAP-25 antibody, was added only when blotting the entire membrane for syntaxin and SNAP-25 and was not included when processing blotting membranes for the presence of VAMP.



**Fig. 1.** Full-length western blot probed for syntaxin and SNAP-25. 4 hour control, clostripain- and papain-treated samples (5, 15 and 20  $\mu$ l load) were evaluated. The normalized fluorescence scans represent the load-adjusted intensity as a function of the normalized western blot coordinate (see Coorssen et al., 2002).

For kinetic assays, CVs were centrifuged onto coverslips and transferred to a perfusion chamber, permitting constant monitoring both by time-resolved light scattering and by direct visual inspection (Blank et al., 1998a; Blank et al., 1998b; Coorssen et al., 1998). A flow rate of ~1 ml/s was used to wash CV lawns and exchange buffers. All experiments used B-IM containing  $[Ca^{2+}]_{free}$  of ~100 nM, 150  $\mu$ M or 1 mM. Light-scattering signals were normalized so that  $[Ca^{2+}]_{free}$  of ~100 nM and 1 mM gave 0% and 100% fusion, respectively. In all cases, aliquots of the same CV suspensions were used to determine and verify  $Ca^{2+}$  activity.

The analysis of fusion is based on the number of active fusion complexes/CV, a Poisson distributed random variable in which the average number of committed fusion complexes,  $\bar{n}$ , varies between 0 and ~9 depending on  $[Ca^{2+}]_{free}$  (Vogel et al., 1996; Coorssen et al., 1998; Blank et al., 2001). Although 1 mM  $[Ca^{2+}]_{free}$  is routinely used to saturate  $\bar{n}_{Max}$  and establish the sigmoid plateau, ~100% fusion occurs at much lower  $[Ca^{2+}]_{free}$ ; cumulative log-normal sigmoid curves can be characterized by the distribution midpoint and width and/or the midpoints and corners. Thus, for comparing activity curves, both the width and the  $[Ca^{2+}]_{free}$  associated with 5% and 50% fusion are used. The relationships between extent of fusion (%F),  $\bar{n}$ , and trypsin treatment are  $\%F=100 \times (1 - \exp(-\bar{n}))$  and  $\bar{n}=\bar{n}_{Max} \times \exp(-[\text{trypsin}]/\tau)$ , respectively, where  $\tau$  is the concentration decay constant (Vogel et al., 1996). Changes in %F as a function of the trypsin concentration were fit using the equation  $\%F=100 \times (1 - \exp(-\bar{n}_{Max} \times \exp(-[\text{trypsin}]/\tau)))$ . For kinetic studies, low solution flow was used to minimize disruption of CV on coverslips. Under

these conditions, the transition from active to committed state is not resolved; the kinetic data were fit using the original two-state model and a temporal offset (Vogel et al., 1996; Blank et al., 2001). The initial rate of fusion can be approximated by  $\bar{n} \times p$  (Vogel et al., 1996; Blank et al., 2001). With  $p = \kappa \times \bar{n}$  (Blank et al., 2001), where  $\kappa$  represents a characteristic efficiency constant, the initial rate can be expressed as  $\kappa \times \bar{n}^2$ . This function suggests that  $\kappa$  is proportional to a rate constant, whereas  $\bar{n}$  is analogous to a concentration term. With  $\kappa$  proportional to a fusion rate constant, the change in energy resulting from papain treatment can be calculated using the relationship  $K_{\text{control}}/K_{\text{papain}} = \exp(-(E_{\text{control}} - E_{\text{papain}})/kT)$ .

The exponential correlation between SNARE density and the midpoint of the calcium activity curve,  $\text{Midpoint} = A \times \exp(-\text{Density}/B) + C$ , was evaluated with weighting using  $1/(s.e.)^2$  of the midpoint estimates. The error in SNARE density was ignored in the fitting after determining that the error range in the parameter estimates (95% confidence) was greater than changes in the estimates arising from fitting the midpoint vs. the density values shifted by  $\pm$  the error in the density. Overall, the error in the density divided by the density was 19%, 16% and 5% for VAMP, SNAP-25 and syntaxin, respectively. We could determine syntaxin density to better than 1 molecule/CV<sup>†</sup>.

## Results

The  $\text{Ca}^{2+}$  activity and kinetics of CV fusion are described by an analysis developed for CV-PM fusion (Vogel et al., 1996; Coorssen et al., 1998; Blank et al., 2001). CV-CV fusion is characterized by sigmoidal  $\text{Ca}^{2+}$  activity curves; midpoints and widths of the  $\text{Ca}^{2+}$  threshold distributions were determined (Fig. 2A; Table 1) (Coorssen et al., 1998; Blank et al., 1998a). The  $\text{Ca}^{2+}$  activity curves for CV-CV fusion are minimally shifted from those for CV-PM fusion (Coorssen et al., 1998), which predict  $[\text{Ca}^{2+}]_{\text{free}}$  that are comparable to those measured during fertilization (Vogel et al., 1996); 5%/50% fusion occurs at a  $[\text{Ca}^{2+}]_{\text{free}}$  of 3/6  $\mu\text{M}$ . One hour in buffer has little effect on CV fusion, with 5%/50% fusion occurring at  $[\text{Ca}^{2+}]_{\text{free}}$  of 3/7  $\mu\text{M}$  (Fig. 2A; Table 1); after 4 hours in buffer, there is a significant shift in the midpoint of the  $\text{Ca}^{2+}$  activity curve and 5%/50% fusion occurs at a  $[\text{Ca}^{2+}]_{\text{free}}$  of 36/76  $\mu\text{M}$ . Thus, results of protease treatments were compared to parallel, untreated controls.

Trypsin had a systematic and progressive effect on  $\text{Ca}^{2+}$  activity (sensitivity and extent) (Fig. 2A; Table 1). 700 units/ml trypsin (1 hour) shifted  $\text{Ca}^{2+}$  activity rightward, but with no loss in maximal extent of fusion elicited by a saturating

**Table 1. CV-CV  $\text{Ca}^{2+}$  activity curve parameters**

Protease (units/ml)	Extent of fusion	Midpoint ( $\mu\text{M}$ )	Width (pCa units)
0 hours control (0)	100%	5.8 $\pm$ 0.1	0.21 $\pm$ 0.01
1 hour control (0)	100%	7.1 $\pm$ 0.2	0.23 $\pm$ 0.02
4 hours control (0)	100%	76.3 $\pm$ 2.2	0.20 $\pm$ 0.02
1 hour clostripain (100)	100%	11.2 $\pm$ 0.7	0.44 $\pm$ 0.03
4 hours clostripain (100)	100%	68.5 $\pm$ 1.4	0.14 $\pm$ 0.01
1 hour papain (2000)	100%	45.3 $\pm$ 3.9	0.72 $\pm$ 0.05
4 hours papain (2000)	100%	196.9 $\pm$ 7.2	0.28 $\pm$ 0.02
1 hour trypsin (700)	100%	16.9 $\pm$ 0.7	0.44 $\pm$ 0.02
1 hour trypsin (3500)	71%	41.7 $\pm$ 3.0	0.51 $\pm$ 0.04

**Table 2. CV-PM  $\text{Ca}^{2+}$  activity curve parameters**

Protease (units/ml)	Extent of fusion (%)	Midpoint ( $\mu\text{M}$ )	Width (pCa units)
1 hour control (0)	100	21.4 $\pm$ 0.6	0.28 $\pm$ 0.02
Clostripain (100)	100	23.8 $\pm$ 0.7	0.38 $\pm$ 0.02
Papain (3000)	96	35.9 $\pm$ 0.5	0.22 $\pm$ 0.01
Trypsin (700)	100	31.3 $\pm$ 0.7	0.30 $\pm$ 0.01
Trypsin (14,000)	51	35.3 $\pm$ 0.5	0.20 $\pm$ 0.01

$[\text{Ca}^{2+}]_{\text{free}}$ ; the 5%/50% fusion level occurs at 3/17  $\mu\text{M}$ . 3500 units/ml trypsin (1 hour) caused a further rightward shift in  $\text{Ca}^{2+}$  activity and  $\sim$ 30% loss of fusion. Prolonged incubations or higher trypsin doses further digested certain CV proteins, correlating with losses in  $\text{Ca}^{2+}$  activity and progressively greater inhibition of fusion; 700 units/ml (3 hours) resulted in  $\sim$ 70% inhibition (data not shown). In comparison, 35,000 units/ml trypsin (1 hour) rendered CV fusion incompetent (99 $\pm$ 1% inhibition at saturating  $[\text{Ca}^{2+}]_{\text{free}}$ ). Like N-ethylmaleimide (NEM), trypsin inhibited fusion in a manner consistent with a single, crucial site of action and the number of these sites,  $\bar{n}_{\text{Max}} = 8.4 \pm 1.0$ , agrees with previous determinations (Vogel et al., 1996; Coorssen et al., 1998). By contrast, papain (2000 units/ml, 1 and 4 hours) shifted and broadened  $\text{Ca}^{2+}$  activity without altering the maximal extent of fusion (Fig. 2A,B; Table 1); 5%/50% fusion occurs at 3/45 and 68/197  $\mu\text{M}$   $[\text{Ca}^{2+}]_{\text{free}}$ , respectively, following 1 and 4 hour treatments. Clostripain (100 units/ml, 1 and 4 hours) had minimal effect on  $\text{Ca}^{2+}$  activity (Fig. 2A,B; Table 1, 5%/50% fusion occurs at 2/11 and 40/69  $\mu\text{M}$   $[\text{Ca}^{2+}]_{\text{free}}$ , respectively, following 1 and 4 hour treatments), but distinct effects on the CV protein profile (Fig. 2C). When added hourly, fresh papain (but not clostripain) gradually inhibited fusion ( $\sim$ 20-30% fusion at saturating  $[\text{Ca}^{2+}]_{\text{free}}$  after 3 hours treatment, data not shown). In all cases, comparable results were obtained after simply settling protease-treated CV into contact, confirming that the measured fusion is dependent on proteins rather than on centrifugation (Coorssen et al., 1998). Similar results were also obtained for exocytosis in vitro after 1 hour treatment of CV-PM preparations (Fig. 2D; Table 2). Trypsin does not appear to affect docking per se, as CV undocking was not observed. The effects of papain (3000 units/ml) were less dramatic, suggesting limited access to the CV-PM contact site. Thus, each of these proteases had a different effect on CV fusion, enabling us to distinguish between changes in the  $\text{Ca}^{2+}$  sensitivity or extent of fusion.

To determine whether the differential protease effects on  $\text{Ca}^{2+}$  activity and fusion extent were due to differential proteolysis of

<sup>†</sup>Confidence intervals for syntaxin copies per CV (Copy #/CV).

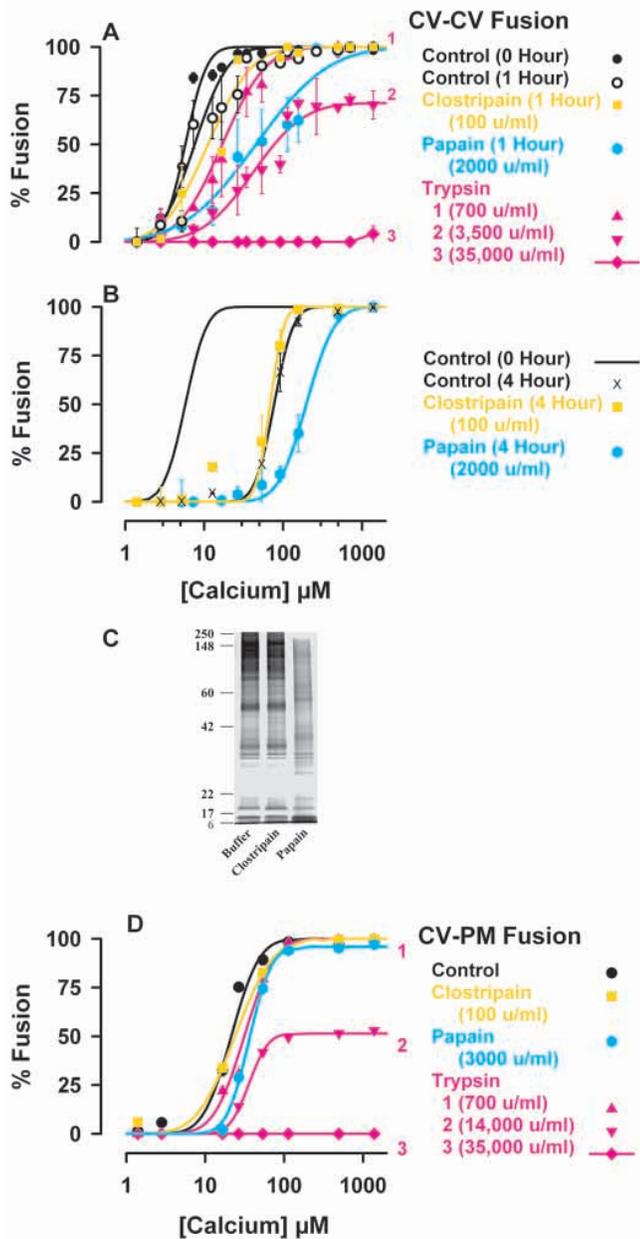
Copy #/CV = (fmol syntaxin)  $\times$  6.02  $\times$  10<sup>8</sup> / [(CV count)  $\times$  (Dilution factor)  $\times$  (Extraction efficiency)].

The error in determining Copy #/CV is:

$\sigma_{\text{Copy \#/CV}} = \text{Copy \#/CV} \times [(\sigma_{\text{fmol}}/\text{fmol})^2 + (\sigma_{\text{CV count}}/\text{CV count})^2 + (\sigma_{\text{Dilution factor/dilution factor}})^2 + (\sigma_{\text{Extraction efficiency/Extraction efficiency}})^2]^{0.5}$ .

Assuming that the relative error for each of these terms is 5% (0.05), an extremely conservative value for all terms except the extraction efficiency, then the total error can be approximated by  $\sigma_{\text{Copy \#/CV}} = \text{Copy \#/CV} \times 0.1$ .

Using a weighted average of the results obtained at the two different protein loads (30 and 15  $\mu\text{l}$  of sample), the average copy numbers are 0.107 $\pm$ 0.008 and 0.404 $\pm$ 0.032. If the calculated error is representative of the underlying error then the Copy #/CV is significantly less than one copy at the 99% confidence level ( $3 \times \sigma_{\text{Copy \#/CV}}$ ). If the calculated error represents an estimate of the unknown underlying distribution then the appropriate *t* value with one degree of freedom (two samples were averaged, *n*-1 degrees of freedom) must be used. In this case, the *t* value for one degree of freedom at the 95% confidence interval is 12.71 and the error would be 0.107 $\pm$ 0.102 and 0.404 $\pm$ 0.407; these values also indicate significantly less than one copy at the 95% confidence level. Therefore, within >95% probability, these two experiments show that our determination of less than one copy of syntaxin per CV is statistically significant.



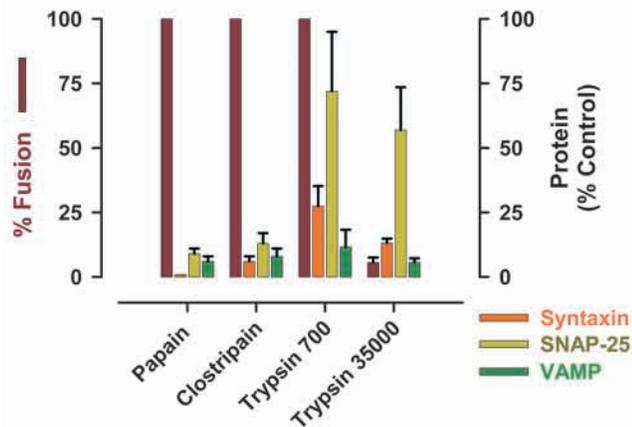
**Fig. 2.** Effects of exogenous proteases on  $\text{Ca}^{2+}$ -triggered fusion and CV membrane proteins. (A) Isolated free-floating CVs in suspension were treated with the indicated protease concentrations. Following protease neutralization and washing, recovered CVs were suspended in B-IM buffer. A fraction of each sample was taken for protein extraction and analysis, and the remainder was assayed for  $\text{Ca}^{2+}$ -triggered fusion. Control CVs (black circle) were sampled and tested immediately after preparation (0 hours;  $n=16$ ); incubated controls (open circle) were left for 1 hour at  $25^\circ\text{C}$  in the absence of protease ( $n=26$ ). CVs were incubated with 700 (purple triangle;  $n=5$ ), 3500 (inverted purple triangle;  $n=9$ ) and 35,000 (purple diamond;  $n=4$ ) units/ml trypsin, respectively, with 2000 units/ml of papain (blue hexagon;  $n=9$ ), or with 100 units/ml clostripain (yellow square;  $n=3$ ) for 1 hour at  $25^\circ\text{C}$ . Data presented are means  $\pm$  s.e.m. of the normalized data from a total of 32 separate CV preparations made over the course of two seasons. (B) Isolated CVs, treated as in (A), but with a 4 hour incubation time; control CVs (0 hours) are represented by a solid black curve as in (A). Control samples without protease ( $\times$ ;  $n=5$ ) were incubated in parallel with samples containing 2000 units/ml papain (blue hexagon;  $n=6$ ) or 100 units/ml clostripain (yellow square;  $n=3$ ). (C) Differential effects of exogenous proteases on the profile of hydrophobic proteins extracted from CVs. CVs treated as described in (B) were analyzed by SDS-PAGE and silver staining. Incubations (4 hour) were with buffer only (lane 1), 100 units/ml clostripain (lane 2) or 2000 units/ml papain, as indicated. 1 hour incubations yielded similar protein profiles, although changes were less pronounced (not shown). (D) Endogenously docked CV-PM preparations were treated (1 hour) with the indicated protease concentrations, neutralized, washed, suspended in B-IM buffer and assayed for  $\text{Ca}^{2+}$ -triggered fusion. Controls (black circle) were sampled and tested 1 hour after preparation, and other samples were incubated (1 hour,  $25^\circ\text{C}$ ) with 700 (purple triangle), 14,000 (inverted purple triangle) and 35,000 (purple diamond;  $n=4$ ) units/ml trypsin, respectively, with 3000 units/ml of papain (blue hexagon), or with 100 units/ml clostripain (yellow square). Data are representative examples of one to three determinations.

SNARE proteins, we used the fact that known SNAREs have substantial numbers of potential trypsin, papain and clostripain cleavage sites throughout their cytoplasmic domains (Table 3). The initial number of accessible proteolytic cleavage sites is

expected to be lower due to structural constraints (Hubbard et al., 1998; Hubbard, 1998). Using the program Nickpred (Hubbard et al., 1998), examination of SNAREs in the Brookhaven Protein Data Base predicted multiple limited proteolytic sites. Subsequent loss of structure is expected to expose additional sites to further proteolysis. Indeed, ladders of multiple proteolytic fragments migrating below the main VAMP and SNAP-25 protein bands were detected with lower protease concentrations or shorter treatment time (1 hour). Following 4 hour protease treatments, syntaxin appears to be either intact or fully proteolyzed, as no fragments were detected (Fig. 1). This observation is consistent with the complete loss of syntaxin complex immunoreactivity observed following trypsin proteolysis (Lawrence and Dolly, 2002). SNAP-25

**Table 3. Summary of proteolytic cleavage sites in all known SNARE proteins**

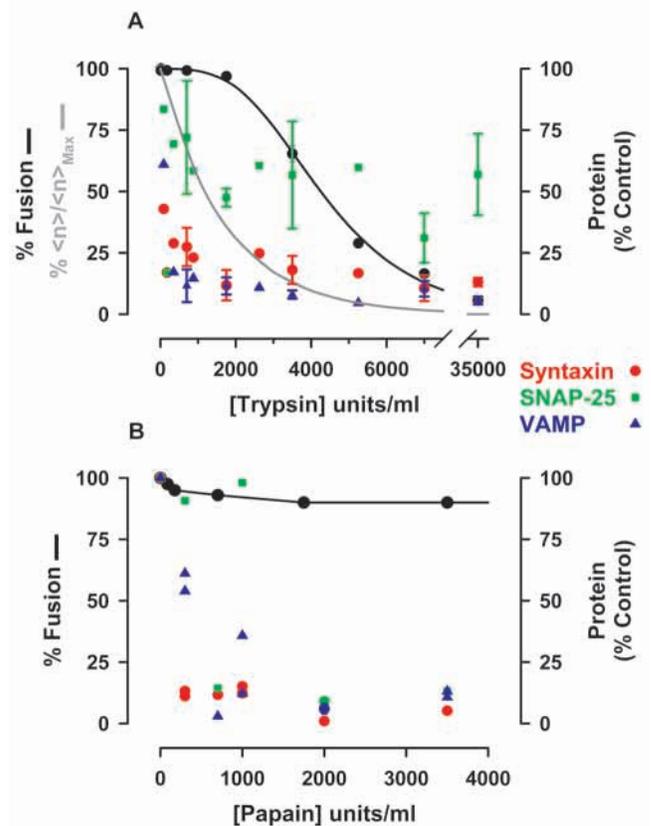
SNARE protein	Number of isoforms analyzed	Total number of protease cleavage sites						Number of amino acids from transmembrane region to first cleavage site					
		Trypsin		Papain		Clostripain		Trypsin		Papain		Clostripain	
		Ave. #	Min. #	Ave. #	Min. #	Ave. #	Min. #	Ave. #	Max. #	Ave. #	Max. #	Ave. #	Max. #
Syntaxin	90	44	31	84	54	20	12	3	4	3	4	6	14
SNAP25/23 (NH <sub>2</sub> -term)	25	12	8	25	20	6	5	4	6	3	6	21	29
SNAP25/23 (C-term)	25	17	10	37	17	8	5	3	6	3	6	9	14
VAMP	92	19	10	42	21	9	4	9	11	9	11	11	63



**Fig. 3.** Average effects of exogenous proteases on fusion and CV SNARE proteins. Isolated CVs were treated with proteases and their hydrophobic proteins extracted, separated by SDS-PAGE and the constituent SNARE proteins analyzed by quantitative immunoblotting. An aliquot of each sample was also analyzed for fusion. The SNARE complements of isolated CVs are shown in the bar graph following 1-4 hour incubations at 25°C with 2000 units/ml papain ( $n=10-14$  for the individual SNAREs), 100 units/ml clostripain ( $n=8$ ), or 700 and 3500 units/ml ( $n=5$  each) trypsin, as indicated. Papain treatment reduced VAMP, SNAP-25 and syntaxin to, on average,  $341 \pm 108$ ,  $62 \pm 21$  and  $4 \pm 1$  copies/CV, respectively. Clostripain reduced VAMP, SNAP-25 and syntaxin to  $424 \pm 143$ ,  $93 \pm 17$  and  $20 \pm 5$ , respectively. Also shown is the extent of post-treatment CV-CV fusion elicited by a maximal  $[Ca^{2+}]_{free}$  (1 mM).

immunoreactivity, which may correspond to fragments, was detected in both control and treated samples (Fig. 1); VAMP immunoreactivity often appeared as a doublet (data not shown). The amounts of these SNARE fragments decreased with increased protease concentration or treatment time and were included in the quantitative determinations of SNARE density. Thus, unlike *Clostridial* toxins, these proteases could destroy all known SNAREs (including toxin-insensitive homologs).

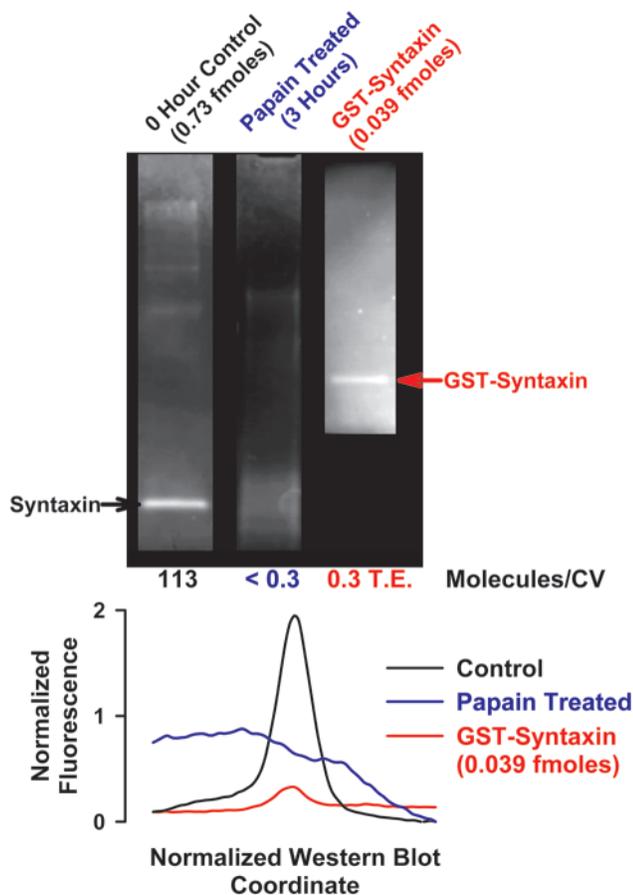
Trypsin (700 units/ml, 1 hour) digested substantial amounts of VAMP, SNAP-25 and syntaxin but had no effect on the extent of fusion (Fig. 3). Increased trypsin (3500 units/ml) removed >90% of VAMP and syntaxin; ~60% SNAP-25 remained (Fig. 4). Higher trypsin doses (Fig. 4) or longer incubation times (700 units/ml, 3 hours) did not reduce SNAREs to levels lower than detected after 1 hour with 3500 units/ml, but did block fusion. Papain (2000 units/ml) and clostripain (100 units/ml) removed most SNAREs after 1 hour; there was little added affect of 4 hour incubations. Papain reduced VAMP, SNAP-25 and syntaxin by 94%, 91% and >99%, respectively, and clostripain by 92%, 87% and 94%; CV remained fully  $Ca^{2+}$  sensitive and 100% fusion competent (Fig. 3). A post-protease wash with chaotropic buffer resulted in some further loss of SNARE fragments nonspecifically bound to CV, but there was no change in fusion at saturating  $[Ca^{2+}]_{free}$ , indicating that free SNARE fragments were not promoting fusion; although ineffective, these fragments were still included in our density assessments to ensure exhaustive SNARE quantification. Intact SNAREs are not removed by the chaotropic buffer wash (data not shown). Post-protease fusion was not due to nonphysiological  $Ca^{2+}$ -induced membrane destabilization, or fusion would remain high after extensive



**Fig. 4.** Poisson analysis. Proteolytic loss of SNAREs with increasing trypsin concentrations (1 hour) does not correlate with either the observed decrease in fusion or the derived number of active fusion complexes ( $\bar{n}$ ) at 1 mM  $[Ca^{2+}]_{free}$ . Protein proteolysis data are from five separate experiments; the fusion curve and  $\bar{n}$  data are from 26 experiments. Proteolytic loss of SNAREs with increasing papain concentrations (1 hour) does not correlate with a loss in fusion. Protein proteolysis and fusion data are from three experiments, except for 2000 units/ml papain, which are the averaged data of 10-14 experiments.

trypsinization; this was not the case (Figs 2, 3 and Fig 4A; 35,000 units/ml trypsin). Thus, despite SNARE removal, fusion activity remained. Furthermore, when added hourly, fresh papain, but not clostripain, gradually inhibited fusion despite comparable reductions in SNARE densities. Fusion appears to require proteins other than the SNAREs.

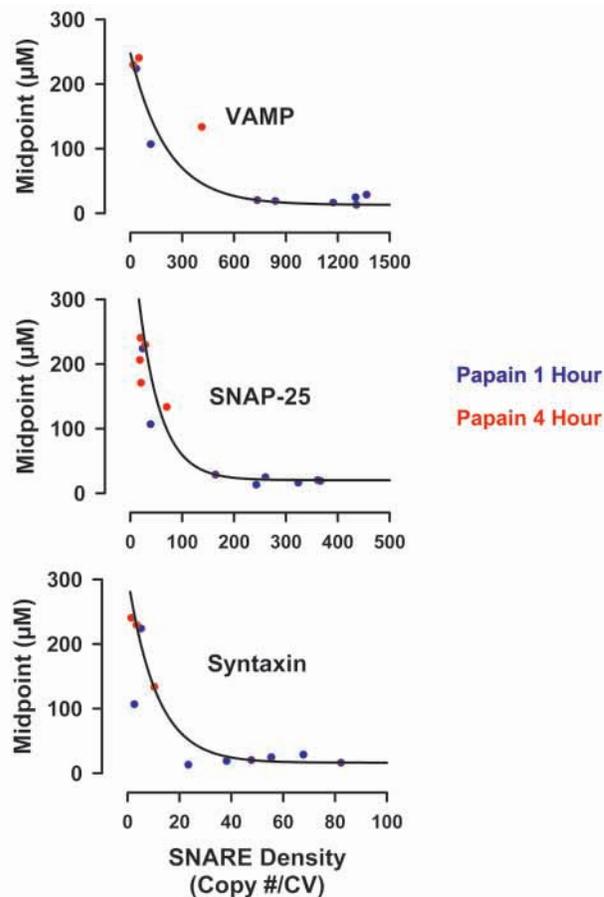
Reducing syntaxin to <1 copy/CV ('biochemical knockout'; 0.1 or 0.4 copy/CV at the 95% confidence level for each of two samples with a range from 0-0.8<sup>†</sup>) by the most potently SNARE-destructive treatment (papain; 2000 units/ml replaced every hour for 3 hours) (Fig. 5) decreased but did not abolish the extent of fusion elicited by saturating  $[Ca^{2+}]_{free}$  (two trials, final extent 20-30%). Eight more trials with 2000 units/ml papain gave  $100 \pm 1\%$  fusion with only 1-3 syntaxin copies/CV in 4 trials and >3 copies/CV in 4 trials; 50% fusion occurred at 45 and ~200  $\mu M$   $[Ca^{2+}]_{free}$  after 1 and 4 hour treatments, respectively. Overall, the reduced syntaxin density as a function of papain treatment ranged from <1-80 copies/CV depending upon the papain concentration (300-5000 units/ml) and treatment time (1-4 hours). For comparison, the reduced syntaxin density as a function of clostripain treatment ranged



**Fig. 5.** Proteolytic ablation of syntaxin: average density <math>< 1</math> copy/CV. Using a calibration curve derived from serial dilution of purified glutathione S-transferase (GST)-syntaxin (Coorssen et al., 2001) 0.73 fmoles, corresponding to 113 molecules/CV, were detected in 0 hour controls. No syntaxin was detected in papain-treated (2000 units/ml, replaced every hour for 3 hours) samples. The limit of detection for this calibration was <math>< 0.039</math> fmoles or 0.3 Treated Equivalent (T.E.) molecules/CV for the same protein load (CV number) used to visualize the papain-treated sample. The maximal syntaxin density in this sample is 0.3 molecules/CV. The plot of normalized fluorescence vs. Western Blot Coordinate shows how the signal associated with 0.039 fmoles is easily quantified. The curves are drawn with an offset for visual comparison.

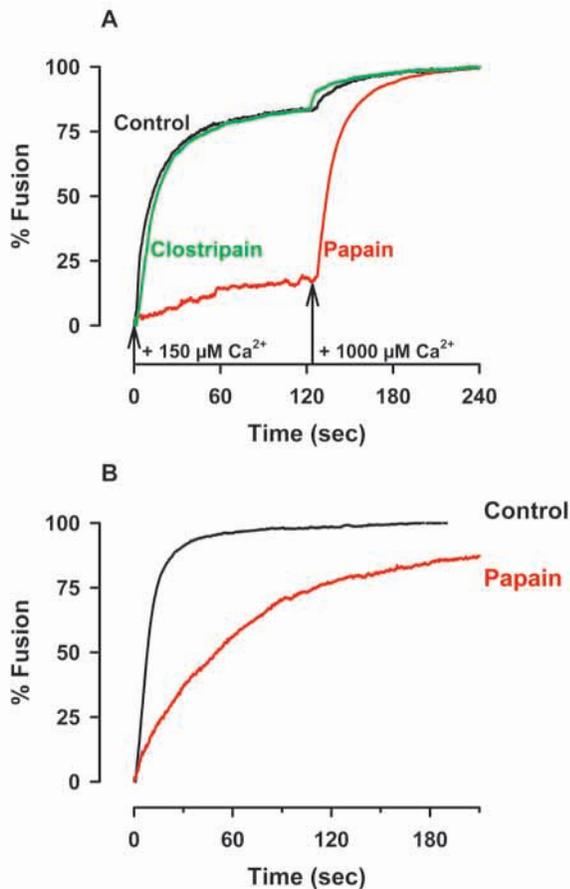
from 5 to 50 copies/CV. If an individual SNARE complex catalyzes fusion, and proteolysis is random, then Poisson analysis (Vogel et al., 1996; Coorssen et al., 1998; Blank et al., 2001) can be used to predict the extent of fusion at saturating

Shifts in



**Fig. 6.**

suggesting disruption of a



**Fig. 7.** Kinetics of  $\text{Ca}^{2+}$ -triggered CV–CV fusion. (A) Differential effects on  $\text{Ca}^{2+}$ -triggered CV–CV fusion kinetics following 4 hour protease treatments. Papain, but not clostripain, inhibits response to  $150 \mu\text{M} [\text{Ca}^{2+}]_{\text{free}}$ . However, at  $1 \text{ mM} [\text{Ca}^{2+}]_{\text{free}}$ , 100% fusion is observed in all cases. The decrease in the rate and extent of fusion at  $150 \mu\text{M} [\text{Ca}^{2+}]_{\text{free}}$  is consistent with a reduction in  $\bar{n}$ , as determined from a rightward shift in the  $\text{Ca}^{2+}$  activity curve. (B) Limiting papain treatment to 3 h alters the rate of fusion elicited with  $150 \mu\text{M} [\text{Ca}^{2+}]_{\text{free}}$ , with minimal change in the extent of fusion. Thus, the rate of fusion decreased without a substantial change in  $\bar{n}$ ; limited papain treatment decreased the fusion probability,  $p$ .

density at 1 hour and the high-density midpoint constrained to the value of the 1 hour control ( $7.1 \mu\text{M}$ ), all three SNAREs were described by a common exponential decay with a low-density midpoint of  $\sim 250 \mu\text{M} [\text{Ca}^{2+}]_{\text{free}}$ . Thus, papain treatment reveals an intrinsic  $\text{Ca}^{2+}$ -dependent process with an activity midpoint that can be modulated by proteolysis between  $\sim 6$  and  $\sim 250 \mu\text{M} [\text{Ca}^{2+}]_{\text{free}}$ . If this reflects the inherent  $\text{Ca}^{2+}$  sensitivity of the native regulated fusion complex, then the  $\text{EC}_{50}$  of this endogenous fusion complex is  $\sim 250 \mu\text{M} [\text{Ca}^{2+}]_{\text{free}}$ .

Decreases in the density of components crucial to triggered fusion are predicted to inhibit the rate of fusion (Vogel et al., 1996; Blank et al., 2001). As clostripain (100 units/ml, 4 hours) failed to affect  $\text{Ca}^{2+}$  activity (Fig. 2B) and had no effect on fusion kinetics (Fig. 7A), but did digest SNAREs, it is unlikely that the identified SNAREs in this system are crucial to fusion. Papain (2000 units/ml, 4 hours) reduced the kinetics and extent triggered by  $150 \mu\text{M} \text{Ca}^{2+}$  but not the final extent of fusion triggered by saturating  $\text{Ca}^{2+}$ , indicating that both the

probability per unit time ( $p$ ) and  $\bar{n}$  decreased (Fig. 7A). However, a limited papain treatment (2000 units/ml, 3 hours) altered the kinetics triggered by  $150 \mu\text{M} \text{Ca}^{2+}$  with minimal changes in the extent (Fig. 7B). Analysis (see Materials and Methods) ruled out a direct reduction in  $\bar{n}$ , indicating that papain reduced  $P$ , the probability that any given vesicle will fuse (Vogel et al., 1996; Blank et al., 2001). After 3 hours,  $p/\bar{n}$  (a measure of efficiency) in papain-treated CV is only  $\sim 17\%$  of control, but is unaffected by clostripain (4 hours) (Fig. 7A). The changes in fusion rate after 3 hours papain treatment are consistent with an increased energy barrier for fusion. Treating  $p/\bar{n}$  as a rate constant (see Materials and Methods), the energy contributed by papain-sensitive proteins is estimated to be  $\sim 2 \text{ kT}$  ( $0.6 \text{ kcal/mol}$ ) per fusion complex. This is about half that required to overcome the hydration layer at membrane surfaces (Leiken et al., 1993) or to mediate molecular rearrangements thought necessary for bilayer merger (Kuzmin et al., 2001). Thus, papain (but not clostripain or low doses of trypsin) removes or modifies crucial modulatory proteins that have pronounced effects on the efficiency of triggered fusion.

## Discussion

The ability to determine with precision the number of copies of syntaxin/CV allowed a quantitative evaluation of SNARE hypotheses, as the SNARE core complex requires SNARE motif helices from syntaxin that cannot be substituted by other SNAREs (Scales et al., 2000a).  $\text{Ca}^{2+}$ - and protein-dependent fusion of native secretory vesicles proceeded after substantial removal of identified SNAREs, even after syntaxin was stoichiometrically eliminated (biochemical ‘knockout’). It should be noted that the effective CV SNARE densities remaining after proteolysis may be even lower than the values we report, as we included all detectable SNARE fragments in the overall quantification of our high sensitivity immunoblots. If ineffective SNARE fragments are present (as indicated by the use of chaotropic washes), they have, for the sake of thoroughness, nevertheless been included in the quantitative evaluations of density. Thus, although the identified SNAREs do not effect fusion, the  $\text{Ca}^{2+}$  sensitivity data suggest that they may interact with modulators of  $\text{Ca}^{2+}$  efficiency that directly influence the capacity for fusion in this  $\text{Ca}^{2+}$ -triggered system.

## Lack of a direct role for SNAREs in membrane fusion: alternate interpretations

Our conclusion, that SNAREs are neither driving CV membranes together nor inextricably linked to  $\text{Ca}^{2+}$ -regulated fusion via an absolute requirement for the presence of their cytosolic domains, is dependent on a correct interpretation of SNARE proteolysis, antibody interactions and the fusion process. Two classes of alternative hypothesis that preserve a direct role for SNARE proteins can be considered. The first class is that the ‘wrong’ SNAREs were evaluated in this study: the necessary SNAREs were still present on protease-treated CV at a sufficient density to support fusion. This class can be subdivided into SNARE homologs and SNARE fragments that do not cross-react with the antibodies used. The second class is that multiple fusion pathways exist, and include SNARE-dependent and -independent mechanisms; the latter can be

subdivided into protein-mediated and protein-independent mechanisms.

#### Do we have the right SNAREs?

The interpretation of genetic studies of SNARE activity can be influenced by the presence of gene homologs (e.g. Vilinsky et al., 2002). By contrast, the biochemical knockout approach relies on the structural conservation of putative homologs. The conservation of the different potential protease cleavage sites in all known SNARE homologs is very high (Table 3), particularly for the trypsin, papain and clostripain cleavage sites closest to the PM (i.e. cleavage at this site would result in the complete release of the cytoplasmic domain) and in the potential coiled-coil domains postulated to be essential to SNARE-mediated fusion. Thus, although we do not know all the epitopes recognized by the antibodies employed, complete destruction of other SNARE homologs should also occur under the conditions of our experiments. If this interpretation is incorrect, then a putative protease resistant 'SNARE' would either have a sequence that is significantly different from all known SNAREs, such that not even one functional proteolytic site exists (a feature that would question the nature of SNARE homology), or exist in a stable conformation such that its cleavage sites were never accessible to protease action during the extended incubation times used in our experiments.

Antibodies raised specifically against nonconserved regions of the protein can certainly identify different homologs of SNAREs. In this study, polyclonal antibodies raised against the whole cytosolic region (e.g. including the extended highly conserved domains) were used. Because SNAREs maintain highly conserved regions it is more likely that our antibodies would cross-react with any protein containing these conserved regions than would monoclonal antibodies. We have used antibodies against SNAREs from other species in the past (Coorssen et al., 1998; Coorssen et al., 2002; Tahara et al., 1998), and have always identified only these SNAREs on the CV membrane. To accept the alternate hypothesis, that SNAREs drive the fusion step, occult SNARE homologs to (or proteolytic fragments of) syntaxin, VAMP and SNAP-25 need to exist in protease-resistant conformations that are not recognized by our polyclonal antibodies (i.e. lack all the epitopes recognized by the antibodies used here) at all protein concentrations and dilutions assessed with an ultrasensitive immunodetection protocol (Coorssen et al., 2002), and the putative proteolytic fragments would have to remain stably bound in the presence of chaotropic buffers. Although this is an alternative explanation, we think it simpler and thus more likely that CV–CV fusion can occur in either the absence of syntaxin or with 91–99% overall loss of SNARE proteins. This interpretation is also most consistent with the substantial differences in SNARE densities measured on native secretory vesicles compared with those required for fusion in reconstituted preparations (Weber et al., 1998; Coorssen et al., 2002). In the absence of SNARE and other modulatory influences, the fusion process has an endogenous  $\text{Ca}^{2+}$ -activity whose midpoint is  $\sim 250 \mu\text{M} [\text{Ca}^{2+}]_{\text{free}}$ .

#### Are there multiple protein-dependent or protein-independent fusion mechanisms?

Our analysis of the  $\text{Ca}^{2+}$  activity and kinetics of fusion is based

on the hypothesis that an increase in  $[\text{Ca}^{2+}]_{\text{free}}$  increases the number of participating fusion complexes and that these complexes are randomly distributed among CVs (Vogel et al., 1996; Blank et al., 2001). This analysis has consistently explained the experimental data observed in this system (Vogel et al., 1996; Blank et al., 1998a; Blank et al., 1998b; Coorssen et al., 1998; Tahara et al., 1998; Blank et al., 2001; Ikebuchi et al., 2001). However, if changes in the  $\text{Ca}^{2+}$  activity and the kinetics of fusion do indicate a shift from one fusion pathway to another, then perhaps SNARE proteins normally mediate fusion, but in their absence alternative mechanisms become evident; redundant pathways may exist. This is an intriguing and provocative alternative hypothesis. However, no changes occurred in kinetics following clostripain treatment, despite substantial decreases in SNARE density, whereas comparable decreases in SNARE density following papain treatment did alter the kinetics of fusion. This is perhaps the most direct evidence against an essential role for the SNAREs in fusion, as any change in the density of an essential component of the fusion complex would be predicted to inhibit the rate of fusion (Vogel et al., 1996; Blank et al., 2001). The differential effects of protease treatments are difficult to reconcile with a simple switch from a SNARE-mediated to a SNARE-independent, protein-mediated fusion pathway without postulating the existence of multiple classes of SNAREs such that clostripain preserves the SNARE-mediated pathway by acting only on nonfunctional SNAREs while papain and trypsin target only the functional SNAREs. Rather, the demonstrated relationship between SNARE density and  $\text{Ca}^{2+}$  activity, seen in the papain data (Fig. 6), clearly indicates the existence of a single fusion pathway (with an inherent  $\text{Ca}^{2+}$  activity). The  $\text{Ca}^{2+}$  sensitivity of this fundamental fusion pathway is modulated in the 'physiological' range of  $[\text{Ca}^{2+}]_{\text{free}}$  either by SNAREs coupled with other modulatory components such as 'Ca<sup>2+</sup> sensors,' or by other components alone that track the papain-dependent changes in SNARE density via similar proteolytic sensitivity. Redundancy in modulatory factors may be a conserved hallmark of triggered exocytosis; the data indicate that trypsin and papain more broadly affect these modulatory factors than does clostripain.

A switch from a SNARE-mediated to a protein-independent mechanism (e.g.  $\text{Ca}^{2+}$ -mediated lipid fusion) can not explain the data because extensive trypsinization ablates the fusion response, even to several millimolar  $[\text{Ca}^{2+}]_{\text{free}}$ ; if any of the fusion observed was nonphysiological or purely lipid-mediated, then it should also have been observed under these conditions. As the fusion response is also lost after treatments with other broad-spectrum proteases (e.g. chymotrypsin; data not shown), it is unlikely that purely lipid-mediated fusion occurs under our experimental conditions. In fact, even when using pure lipid vesicles (LV) as the target membrane (CV–LV fusion;  $\text{Ca}^{2+}$ -dependent fusion at  $<60 \mu\text{M} [\text{Ca}^{2+}]_{\text{free}}$ ), proteinaceous machinery is still essential to the triggered fusion steps, as determined by thiol sensitivity (Vogel et al., 1992). The shift in the midpoint of the  $\text{Ca}^{2+}$  activity curve for CV–CV fusion never enters the range of  $[\text{Ca}^{2+}]_{\text{free}}$  required to induce purely lipid fusion; fusion of lipid membranes containing mixtures of neutral and negatively charged lipids requires tens of millimolar  $[\text{Ca}^{2+}]_{\text{free}}$  (Cohen et al., 1980; Cohen et al., 1984; Duzgunes et al., 1981; Coorssen and Rand, 1995; Zimmerberg and Chernomordik, 1999). The  $\text{Ca}^{2+}$

activity curves for all conditions, except for papain where the distribution of  $\text{Ca}^{2+}$  sensitivity is believed to be significantly altered, indicate that >95% fusion occurs between  $\sim 10$  and  $160 \mu\text{M} [\text{Ca}^{2+}]_{\text{free}}$ , a range that is consistent with the physiology of  $\text{Ca}^{2+}$ -triggered exocytosis. Because it takes time for the proteases to work, we must also consider a shift in mechanism due to incubation time. However, the invariance of  $p/\bar{n}$  with incubation time (0–4 hours), indicating no change in the estimated energy of fusion, makes a time-dependent shift in mechanism unlikely.

Although extensive trypsinization ( $\geq 3500$  units/ml, 1 hour) does not simply create an environment for 'lipid-mediated' fusion, this proteolytic treatment did reduce VAMP and syntaxin to <10% of control, or  $\sim 30$  syntaxin/CV; a maximum of  $\sim 30$  syntaxin-limited complexes (1:1:1 VAMP, SNAP-25, syntaxin complexes or syntaxin with other binding partners) are possible per trypsin-treated CV. If syntaxin and fusion complexes are stoichiometrically proportional, then reducing  $\bar{n}_{\text{Max}}$  by 90% ( $\bar{n}_{\text{Max, trypsin}}=0.84$ ) would still support >50% fusion at saturating  $[\text{Ca}^{2+}]_{\text{free}}$ . If an individual syntaxin complex (trimeric SNARE complex or other) (Lawrence and Dolly, 2002) causes fusion (i.e. is both fusogen and  $\text{Ca}^{2+}$  sensor) (Sutton et al., 1998; Chen et al., 2001; Peters et al., 2001) then, assuming a uniform surface distribution, any contacting CV domains >130 nm in diameter would ensure 100% fusion at saturating  $[\text{Ca}^{2+}]_{\text{free}}$  (for CVs having 30 syntaxin/CV). But extensively trypsinized CVs do not fuse (Fig. 3, Fig. 4A). As trypsin effects on  $\text{Ca}^{2+}$  activity did not correlate with SNARE removal (Fig. 2A, Fig. 3, Fig. 4A), other trypsin-sensitive proteins must function in defining the activity curve for  $\text{Ca}^{2+}$ -sensitive fusion; notably, the trypsin sensitivity of synaptotagmin is well established (Tugal et al., 1991).

### SNAREs and the process of exocytosis

The simplest hypothesis is that there is only one endogenous mechanism of protein-mediated fusion in this system, and our dissection of this mechanism results in altered responses. According to this hypothesis, although SNAREs have key roles in the pathway of exocytosis, they are not members of any minimal protein set required for  $\text{Ca}^{2+}$ -triggered fusion in this regulated exocytotic system – certainly not in any capacity that requires their intact cytosolic domains; their rapid intermembrane binding does not appear to be essential to the minimal mechanism of native membrane merger (Coorssen et al., 1998). Of course, SNAREs are enormously important to the process of exocytosis; there is good evidence that SNAREs affect an early stage of target membrane recognition, vesicle docking and/or priming (Pelham, 2001; McNew et al., 2000; Scales et al., 2000a; Scales et al., 2000b). Indeed, our new estimates indicate that SNAREs, together with other modulatory proteins, can contribute energy to the fusion mechanism, although not enough to directly trigger fusion, except perhaps at densities in excess of those in native vesicle membranes (Weber et al., 1998; Coorssen et al., 2002). SNAREs probably play a role in trafficking and the localization of CV to PM docking sites, which occurs during the oocyte-to-egg transition (Berg and Wessel, 1997), because fusion can be disrupted by clostridial toxins injected into eggs whose CVs have been de-docked (Bi et al., 1995); these PM docking sites may be associated with fusion complexes (Ikebuchi et al., 2001).

In other systems, genetic knockouts of VAMP and SNAP-25 affect the regulation of exocytosis but not the ultimate capacity to release vesicular contents (Nonet et al., 1998; Yoshihara et al., 1999; Schoch et al., 2001; Rao et al., 2001; Washbourne et al., 2002), leading to two hypotheses – that SNAREs catalyze fusion reactions by stabilizing transition states (Schoch et al., 2001) and that multistep,  $\text{Ca}^{2+}$ -dependent assembly of SNAREs forces membranes together to promote fusion (Xu et al., 1999; Chen et al., 2001; Scales et al., 2001). Considering the findings presented here, neither of these hypotheses is probable because the rate of CV fusion (rather than of multiple late steps in the exocytotic pathway) in clostripain-treated samples was independent of SNARE density, and the removal of SNAREs and other modulatory proteins suggests an inherent  $\text{Ca}^{2+}$  activity in the triggered steps of fusion.

In regulated fusion, SNAREs may function as structural proteins that prepare or optimize local release sites (Coorssen et al., 1998), perhaps by coordinating the right vesicles (Rothman, 1994; McNew et al., 2000) to the right  $\text{Ca}^{2+}$  channels (Rettig et al., 1997; Jarvis et al., 2000), for which they then act as regulatory components. This represents another interpretation of SNARE knockout data (Schoch et al., 2001; Washbourne et al., 2002). If VAMP loss alters presynaptic architecture such that vesicles dock less effectively (outside domains of increased intracellular  $\text{Ca}^{2+}$ ), leading to diminished functional docking by  $\sim 10\%$ , then spontaneous release and measures of docked, primed vesicles (i.e. sucrose pools) would be diminished by  $\sim 10\%$  (Parsons et al., 1995) and the fast evoked response to  $\text{Ca}^{2+}$  would be lost (Xu et al., 1998).

### A calcium regulatory complex that is papain sensitive

Although we do not yet know whether the fusion complex itself is inherently  $\text{Ca}^{2+}$  sensitive, or whether this property is resident to an associated sensor, this sensitivity can be correlated to SNARE density following proteolysis by papain. This suggests a native papain-sensitive complex that includes SNAREs and associated proteins. However, this complex is not essential per se in the triggered fusion steps of exocytosis because in our work higher  $[\text{Ca}^{2+}]_{\text{free}}$  overcame the block to fusion (seen at 'physiological'  $[\text{Ca}^{2+}]_{\text{free}}$ ) caused by proteolysis. Such recovery has also been documented in earlier experiments with *Clostridial* toxins (Dreyer and Schmitt, 1983; Ahnert-Hilger and Weller, 1993; Bittner and Holz, 1993; Lawrence et al., 1994; Glenn and Burgoyne, 1996; Lawrence et al., 1996; Capogna et al., 1997; Land et al., 1997; Fassio et al., 1999), suggesting that the concept of a regulatory complex may extend beyond the present system. In general, the testing and rescue of exocytosis at only one  $[\text{Ca}^{2+}]_{\text{free}}$  after complex disruption tells us a great deal about the modulatory role of the complex. Incomplete  $\text{Ca}^{2+}$  activity curves provide only a partial analysis of the pathway in question, leading to models that explain only a very circumscribed stage of exocytosis, upstream of the fusion steps. Complexes of SNAREs and accessory proteins may promote fusion efficiency in vivo. Our finding that proteolysis by papain reduced the probability of fusion supports this interpretation. Although similar influences of SNAREs on the probability of fusion have been noted in other systems (Finley et al., 2002; Stewart et al., 2002), the mechanism of this action was not discernible; here, we

hypothesize that a complex of SNAREs and associated proteins may modulate the inherent  $\text{Ca}^{2+}$  sensitivity of the native fusion complex, and that these modulatory proteins are more sensitive to papain than to clostripain.

In summary, the isolated CV preparation is unique in its stage specificity, allowing the dissection of post-SNARE,  $\text{Ca}^{2+}$ -dependent steps of exocytosis. Proteolysis has revealed two different functional activities involving the  $\text{Ca}^{2+}$  sensor(s) and the fusogen, in accordance with other studies indicating the involvement of additional proteins acting after SNAREs (Coorsen et al., 1998; Tahara et al., 1998; Ungermann et al., 1998; Peters et al., 2001). In the current study we achieved a biochemical 'knockout' of syntaxin in stage-specific native secretory vesicles, without the loss of triggered fusion. These studies have also provided intriguing estimates of the inherent  $\text{Ca}^{2+}$  sensitivity of the endogenous fusogen-fusion complex separate from a regulatory complex that appears to include SNAREs.

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