Tetanus Toxin-mediated Cleavage of Cellubrevin Impairs Exocytosis of Transferrin Receptor-Containing Vesicles in CHO Cells

Thierry Galli,* Tamie Chilcote,* Olaf Mundigl,* Thomas Binz,‡ Heiner Niemann,‡ and Pietro De Camilli*

*Department of Cell Biology and Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut 06510; and ‡Bundesforschungsanstalt für Viruskrankheiten der Tiere, D-72076 Tübingen, Federal Republic of Germany

Abstract. Cellubrevin is a member of the synaptobrevin/VAMP family of SNAREs, which has a broad tissue distribution. In fibroblastic cells it is concentrated in the vesicles which recycle transferrin receptors but its role in membrane trafficking and fusion remains to be demonstrated. Cellubrevin, like the synaptic vesicle proteins synaptobrevins I and II, can be cleaved by tetanus toxin, a metallo-endoprotease which blocks neurotransmitter release. However, non-neuronal cells are unaffected by the toxin due to lack of cell surface receptors for its heavy chain. To determine whether cellubrevin cleavage impairs exocytosis of recycling vesicles, we tested the effect of tetanus toxin light chain on the release of preinternalized transferrin from streptolysin-O–perforated CHO cells. The release was found to be temperature and ATP dependent as well as NEM sensitive. Addition of tetanus toxin light chain, but not of a proteolytically inactive form of the toxin, resulted in a partial inhibition of transferrin release which correlated with the toxin-mediated cleavage of cellubrevin. The residual release of transferrin occurring after complete cellubrevin degradation was still ATP dependent. Our results indicate that cellubrevin plays an important role in the constitutive exocytosis of vesicles which recycle plasmalemma receptors. The incomplete inhibition of transferrin release produced by the toxin suggests the existence of a cellubrevin-independent exocytotic mechanism, which may involve tetanus toxin-insensitive proteins of the synaptobrevin/VAMP family.

SYNAPTIC vesicles are highly specialized secretory organelles which undergo regulated exocytosis at synapses. They store and secrete nonpeptide neurotransmitters and are continuously regenerated in nerve endings by cycles of exo-endocytosis (Ceccarelli et al., 1973; Heuser and Reese, 1973). They differ in many biochemical and functional properties from the secretory granules of the classical regulated secretory pathway which are assembled and loaded with content in the region of the *trans*-Golgi network (De Camilli and Jahn, 1990). Instead, they appear to be closely related to the vesicular carriers which functionally connect early endosomes to the plasmalemma since many similarities between the recycling pathways of plasmalemma receptors (for example the transferrin [Tf][1] receptor) and the recycling of synaptic vesicles have become apparent (Cameron et al., 1991; Linstedt and Kelly, 1991; McPherson and De Camilli, 1994). One important difference between the properties of synaptic vesicles and those of vesicles which carry recycling receptors to the cell surface, is the regulation of the exocytotic event. In the case of synaptic vesicles (Del Castillo and Katz, 1954), but not in the case of Tf receptor-containing vesicles (Mellman et al., 1987), exocytosis is highly regulated in a phasic fashion by cytosolic Ca^{2+}. However, even in the case of synaptic vesicles, a low rate of constitutive exocytosis can be observed in the absence of stimuli, suggesting that Ca^{2+} is involved in some regulatory control of the fusion event, rather being an integral participant in the fusion reaction (Del Castillo and Katz, 1954; Katz and Miledi, 1968; Matteoli et al., 1992). In agreement with these considerations, a convergence of biochemical work carried out primarily in neurons and of genetic studies in yeast, have suggested that the same basic fusion machinery may be used both for regulated and constitutive exocytosis in all cells (Aalto et al., 1992; Bennett and Scheller, 1993; Söllner et al., 1993*b*; Sudhof et al., 1993).

Putative key components of the fusion complex are small polypeptides anchored either to the vesicle (v-SNAREs) or to the plasmalemma (t-SNAREs) and cytosolic factors which are recruited to the site of fusion. In the case of synap-

Address correspondence to Pietro De Camilli, Department of Cell Biology, Howard Hughes Medical Institute, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06510.

1. *Abbreviations used in this paper:* NEM, *N*-ethylmaleimide; NSF, *N*-ethylmaleimide-sensitive fusion protein; SCAMP, secretory carrier membrane protein; SNAP, soluble NSF attachment protein; SL-O, streptolysin-O; SNAP-25, synaptosome-associated protein of 25 kD; v-SNARE, vesicle SNAP receptors; t-SNARE, target-membrane SNAP receptors; TeTx, tetanus toxin; TeTx-LC, tetanus toxin light chain; Tf, transferrin; VAMP, vesicle-associated membrane protein.

tic vesicle exocytosis, the v-SNAREs are the synaptobrevins (or vesicle-associated membrane proteins [VAMPs]) and their plasmalemma counterparts are syntaxin and the synaptosome-associated protein of 25 kD (SNAP-25) (Söllner et al., 1993*a*,*b*). Synaptobrevin was found to interact with syntaxin and SNAP-25, and this complex in turn interacts in an ATP-dependent fashion with NSF and α/β and γ soluble NSF attachment protein (SNAP) (Söllner et al., 1993*a*,*b*).

The hypothesis that synaptobrevin, syntaxin, and SNAP-25 are key membrane components of the exocytotic complex for synaptic vesicles is strongly supported by the finding that each of these three proteins is the target for the proteolytic action of clostridial neurotoxins (Link et al., 1992; Schiavo et al., 1992*a*,*b*, 1993*a*,*b*; Blasi et al., 1993*a*,*b*; Binz et al., 1994; Yamasaki et al., 1994*a*,*b*), which act as potent inhibitors of synaptic vesicle exocytosis (Niemann, 1991; Montecucco and Schiavo, 1993). Since homologues of synaptobrevin (Protopopov et al., 1993), syntaxin (Aalto et al., 1993), SNAP-25 (V. Bankaitis, personal communication), *N*-ethylmaleimide-sensitive fusion protein (NSF) (Wilson et al., 1989), and α/β- and γ-SNAPs (Clary et al., 1990; Griff et al., 1992) are known to be involved in exocytosis from yeast, members of these protein families must participate in exocytosis from all cells. Accordingly, NSF and SNAPs (Whiteheart et al., 1993) are ubiquituously expressed and isoforms of synaptobrevin and syntaxins have been recently identified outside the nervous system (Cain et al., 1992; Bennett et al., 1993; Chin et al., 1993; McMahon et al., 1993).

Cellubrevin, which shares a very high degree of homology with synaptobrevin I and II (Trimble et al., 1988; Baumert et al., 1989; Elferink et al., 1989) is expressed in all nonneuronal tissues examined (McMahon et al., 1993). Proteins of the synaptobrevin/VAMP family are also expressed in all tissues of *Drosophila* (Chin et al., 1993; DiAntonio et al., 1993). In fibroblastic cells, cellubrevin is present on Tf receptor-containing vesicles (McMahon et al., 1993), emphasizing the close similarity of Tf receptor-containing vesicles to synaptic vesicles (Cameron et al., 1991). The great homology between cellubrevin and synaptobrevin at the site where synaptobrevin is cleaved by tetanus toxin (TeTx) (McMahon et al., 1993; Yamasaki et al., 1994*a*) offers an opportunity to test the role of cellubrevin in vesicle recycling. Indeed, cellubrevin was found to be cleaved by tetanus toxin in both cell extracts and living cells (Link et al., 1993; McMahon et al., 1993; Yamasaki et al., 1994*a*). Since nonneuronal cells do not have the receptor for the toxin at their surface, experiments on living cells were performed by introducing the proteolytically active portion of the toxin (the light chain [TeTx-LC]) via a transient cDNA transfection procedure (McMahon et al., 1993). In these experiments, however, an effect of the toxin on exocytosis was not investigated and a role of cellubrevin in vesicle exocytosis remains to be demonstrated.

In the present study we have established an assay to monitor exocytosis of Tf-containing vesicles from streptolysin-O (SL-O)-perforated cells. We have then used this assay to determine whether cleavage of cellubrevin produced by TeTx-LC leads to an impairment of the constitutive exocytosis of these vesicles. Our results demonstrate that TeTx-LC produces a reduction, although not a block, of exocytosis which correlates with cleavage of cellubrevin. They provide a first direct functional demonstration that a protein of the synaptobrevin family is involved in the constitutive exocytosis of recycling vesicles in nonneuronal cells, and suggest the existence of a tetanus toxin-insensitive fusion mechanism in CHO cells.

Materials and Methods

Materials

[^{125}I]diferric Tf (90 μCi/ml) and [^{125}I]protein A (126 μCi/ml) were purchased from NEN (Wilmington, DE), brefeldin A from Epicentre Technologies (Madison, WI), reduced SL-O from Murex Diagnostics (Norcross, GA), fluorescein-labeled phalloidin from Molecular Probes, Inc. (Eugene, OR), ATP (magnesium salt) and hexokinase from SIGMA (St. Louis, MO), and human diferric Tf from GIBCO BRL (Gaithersburg, MD). Tissue culture reagents were from GIBCO BRL. Stock solutions of the recombinant light chain of TeTx-LC and of the proteolytically inactive mutant ([Q234]-TeTx-LC) were prepared as described (McMahon et al., 1993) and were 13 μM in buffer T (10 mM PIPES, 150 mM K-glutamate, pH 7.2).

Antibodies

Antibodies specific for cellubrevin (MC16) and for synaptobrevin I (MC9), respectively, were generated in rabbits by injecting synthetic peptides corresponding to the NH_2-terminal amino acids of the two proteins followed by a cysteine (sequences: MSTGVPSGSSAATGC and MSAPAQPPAEGTEGAC, respectively). For immunocytochemistry, the MC16 serum was affinity purified using the cellubrevin peptide coupled to EAH Sepharose 4B (Pharmacia, Uppsala, Sweden). The following antibodies were obtained through generous gifts: anti-human Tf receptor monoclonal antibodies (H68.4) (Dr. I. Trowbridge, Salk Institute, La Jolla, CA); anti-SCAMPs monoclonal antibodies (SG7C12) (Dr. D. Castle, University of Virginia School of Medicine, Charlottesville, VA); anti-synaptobrevin II serum and monoclonal antibody (Cl10.1) which recognizes all synaptobrevin isoforms (Dr. R. Jahn, Yale University School of Medicine, New Haven, CT); polyclonal serum (anti-VAMP2/trpE) raised against the core region of synaptobrevin II (residues 32 to 97) (Trimble et al., 1988) which is highly conserved (McMahon et al., 1993) in synaptobrevin/VAMP isoforms from invertebrates to mammals (Dr. R. Scheller, Stanford University, Stanford, CA). Monoclonal antibodies directed against α- and β-tubulins and human Tf were purchased from Amersham Corp. (Arlington Heights, IL) and Accurate Chemical and Science Corp. (Westbury, NY), respectively.

Cell Culture

CHO cells were grown as monolayer cultures in Dulbecco's modified Eagle's media supplemented with 10% (vol/vol) fetal calf serum, 34 μg/ml proline, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Semiconfluent monolayers containing 4.5–6.0 $\times$ 10^4 cells/cm^2 were used for all experiments.

Immunocytochemistry

CHO cells were grown for 48 h on poly-[L]ornithine-coated glass coverslips and fixed with 4% formaldehyde in phosphate buffer containing 4% sucrose. Immunofluorescence was performed as described (Cameron et al., 1991). In some cases cells were allowed to internalize Tf (human diferric Tf, 20 μg/ml final concentration) for 1 h at 37°C in culture medium, briefly rinsed in the same buffer, and then fixed. Internalized Tf was then stained with secondary antibodies as described (Cameron et al., 1991). Brefeldin A (10 μg/ml final concentration) was added during the last 15 min of the 1-h Tf internalization period in some experiments (Mundigl et al., 1993). SL-O perforation of the cells was performed as described by Miller and Moore (1991, 1992) (see also below). Cells were then incubated at 37°C for 65 min in the presence of 2 mM ATP (with and without TeTx-LC) and, subsequently, fixed.

Tf release from SL-O–perforated Cells

CHO cells were perforated with SL-O essentially as described by Miller and Moore (1991, 1992). Approximately 24 $\times$ 10^6 cells were resuspended in phosphate-buffered saline containing 5 mM EDTA, pelleted at 350 g for 5 min at 4°C, resuspended in 10 ml of buffer K (20 mM Hepes, 128 mM NaCl, 3 mM KCl, 1 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 2.7 mM $CaCl_2$, and

11 mM glucose, pH 7.4, with NaOH), and then repelleted. The pellet was then resuspended in buffer K at a concentration of 5×10^4 cells/μl and incubated in the presence of [^{125}I]Tf (10 μg/ml) for 1 h at 37°C (Podbilewicz and Mellman, 1990; Lippincott-Schwartz et al., 1991). The cell suspension was washed with 1 ml of ice-cold buffer K by sedimentation and resuspension using an Eppendorf 5154C microcentrifuge. An aliquot of this material was used to study [^{125}I]Tf release from intact cells. The remaining cell suspension was washed two times with 1 ml of ice-cold buffer A (20 mM Hepes, 110 mM NaCl, 5.4 mM KCl, 0.9 mM Na_2HPO_4, 10 mM $MgCl_2$, 2 mM $CaCl_2$, and 11 mM glucose, pH 7.4, with NaOH), resuspended in buffer A at the final concentration of 10^4 cells/μl, and then mixed with an equal volume of buffer A containing SL-O (0.2 U/ml final concentration). After incubation on ice for 10 min, excess SL-O was removed by centrifugation and one wash with 1 ml of buffer B (20 mM Hepes, 100 mM K-glutamate, 40 mM KCl, and 5 mM EGTA, pH 7.2, with KOH) containing 2 mM Mg-ATP and 5 mM free Mg^{2+} (added as $MgCl_2$). The concentration of free Mg^{2+} was calculated using the software by Foehr and Warchol (Ulm, Federal Republic of Germany). The pellet was then resuspended in buffer B at a final density of 10^4 cells/μl and split into aliquots to which the concentrated toxins in buffer T, buffer T alone, or hexokinase and glucose for the ATP depletion experiments (final concentration of 30 U/ml and 3 mM, respectively), were added (Miller and Moore, 1991, 1992). 100-μl aliquots of treated and control cell suspensions were then incubated at 37°C for 5, 20, 35, or 65 min and the release of [^{125}I]Tf in the medium was determined. As previously reported, the SL-O–induced formation of cell pores occurs as soon as the cells are shifted to 37°C (Ahnert-Hilger et al., 1989*b*). The 4°C control samples were obtained by returning the cells to an ice bath after 5 min at 37°C to allow pores to form. To study the effect of *N*-ethylmaleimide (NEM), cells were first brought to 37°C for 2 min to allow SL-O–induced pore formation, then incubated on ice with 1 mM NEM for 15 min and subsequently with 2 mM dithiothreitol (DTT) to quench NEM (Beckers et al., 1989; Miller and Moore, 1991). Subsequently, cells were incubated at 37°C to assay release of [^{125}I]Tf. Control incubations were performed by adding DTT together with NEM at the beginning of the incubation on ice (Beckers et al., 1989).

At each time point studied, triplicate samples were centrifuged and the supernatants and pellets were collected separately. The latter were resuspended in 20 μl of SDS gel sample buffer or 1% Triton X-100. The amounts of [^{125}I]Tf in the supernatants and corresponding pellets were measured in a γ counter (LKB, Bromma, Sweden). In each experiment, an aliquot of the SL-O–treated cell suspension was pelleted at the beginning of the 37°C incubation (defined as 0 min). The amount of [^{125}I]Tf recovered in the supernatant of this sample was defined as background and therefore substracted from each individual release value obtained at later incubation times. The amount of [^{125}I]Tf released was expressed as the percent of total amount of [^{125}I]Tf present in each sample (supernatant plus pellet). Each experiment was repeated at least three times, and a single representative experiment is shown in each figure. Values are reported as the mean ± SD of three determinations.

In pilot experiments, the effectiveness of the permeabilization procedure for cells in suspension was assessed by incubating SL-O–treated cells with propidium iodide (1 μg/ml) or fluorescein-labeled phalloidin (50 nM) followed by flow cytometry analysis with a FACSTARplus cell sorter (Becton Dickinson, San Jose, CA) (excitation at 488 nm and emission at 630 nm and 530 nm, respectively).

Western Blotting

Proteins from pellets used for the release experiments described above were separated by SDS-PAGE (Schagger and von Jagow, 1987) and analyzed by Western blotting for cellubrevin, secretory carrier membrane protein (SCAMPs), α- and β-tubulin, and Tf receptor. Immunoreactive bands were visualized with [^{125}I]protein A.

Results

Localization of Cellubrevin in CHO Cells

As a premise to our studies we raised a rabbit anti-serum directed against the NH_2-terminal region of rat cellubrevin which is divergent from the corresponding regions of synaptobrevin I and II. The affinity-purified antibodies, which are highly specific for cellubrevin and do not recognize synaptobrevin I and II, reacted very strongly with cellubrevin in formaldehyde-fixed CHO cells and allowed localization of the endogenous protein by immunofluorescence. Cellubrevin immunoreactivity appeared as fine puncta sparse throughout the cytoplasm (Fig. 1, *B*, *D*, and *F*). These puncta were particularly concentrated in the region of the microtubule-organizing center as demonstrated by double immunofluorescence with anti-β-tubulin antibodies (Fig. 1, *C* and *D*). This localization was very similar to the distribution of Tf internalized by receptor-mediated endocytosis during 1-h incubation (compare Fig. 1, *A* and *B*), a distribution which is known to closely reflect the intracellular localization of Tf receptors (Cameron et al., 1991). More puncta appeared to be positive for cellubrevin than for internalized Tf; however, virtually all spots positive for Tf were also positive for cellubrevin. After brefeldin A treatment (Lippincott-Schwartz et al., 1991) tubules positive for internalized Tf were also positive for cellubrevin (Fig. 1, *A* and *B*, insets), further emphasizing the colocalization of the two proteins. These immunofluorescence results are in agreement with those previously obtained by the staining of exogenous cellubrevin in cellubrevin-overexpressing CV1 cells (McMahon et al., 1993).

Release of Preinternalized Tf from SL-O–perforated CHO Cells

The assay utilized in this study to monitor exocytosis of recycling vesicles is based on the well-characterized pH and iron dependency of the affinity of Tf for its receptor: the affinity of free Tf (apo-Tf) is higher at acid pH, while the affinity of diferric Tf is higher at neutral pH (Maxfield and Yamashiro, 1991; Dautry-Varsat et al., 1983; Hopkins and Trowbridge, 1983). Diferric Tf binds to receptors at the cell surface and is internalized by receptor-mediated endocytosis. At the acid pH of endosomes iron dissociates from Tf, while apo-Tf remains bound to the receptor and is incorporated together with the receptor in endosome-derived recycling vesicles (McGraw and Maxfield, 1990; Maxfield and Yamashiro, 1991; Dautry-Varsat et al., 1983; Hopkins and Trowbridge, 1983). Following vesicle exocytosis, apo-Tf is released into the medium. Thus, the release of [^{125}I]Tf after a period of [^{125}I]Tf internalization closely reflects exocytosis of constitutively recycling endosome-derived vesicles. This hypothesis has been validated in previous studies (Podbilewicz and Mellman, 1990; Lippincott-Schwartz et al., 1991).

We used the protocol of permeabilization of CHO cells with SL-O described by Miller and Moore (1991, 1992). This procedure was found to allow the study of constitutive secretion via the biosynthetic pathway from CHO cells up to at least 90 min after perforation (Miller and Moore, 1991). Cells grown on coverslips were used for immunofluorescence experiments, and cells in suspension for quantitative studies on the release of preinternalized [^{125}]Tf. To determine the efficiency of the perforation procedure under our experimental conditions, perforated cells were stained with two membrane-impermeable compounds: fluorescein-labeled phalloidin, which stains actin filaments, and propidium iodide, which stains the nucleus. They were then analyzed by immunofluorescence (cells on coverslips) or by flow cytometry (cells in suspension). Virtually all cells were found to be perforated. The efficiency of the perforation procedure

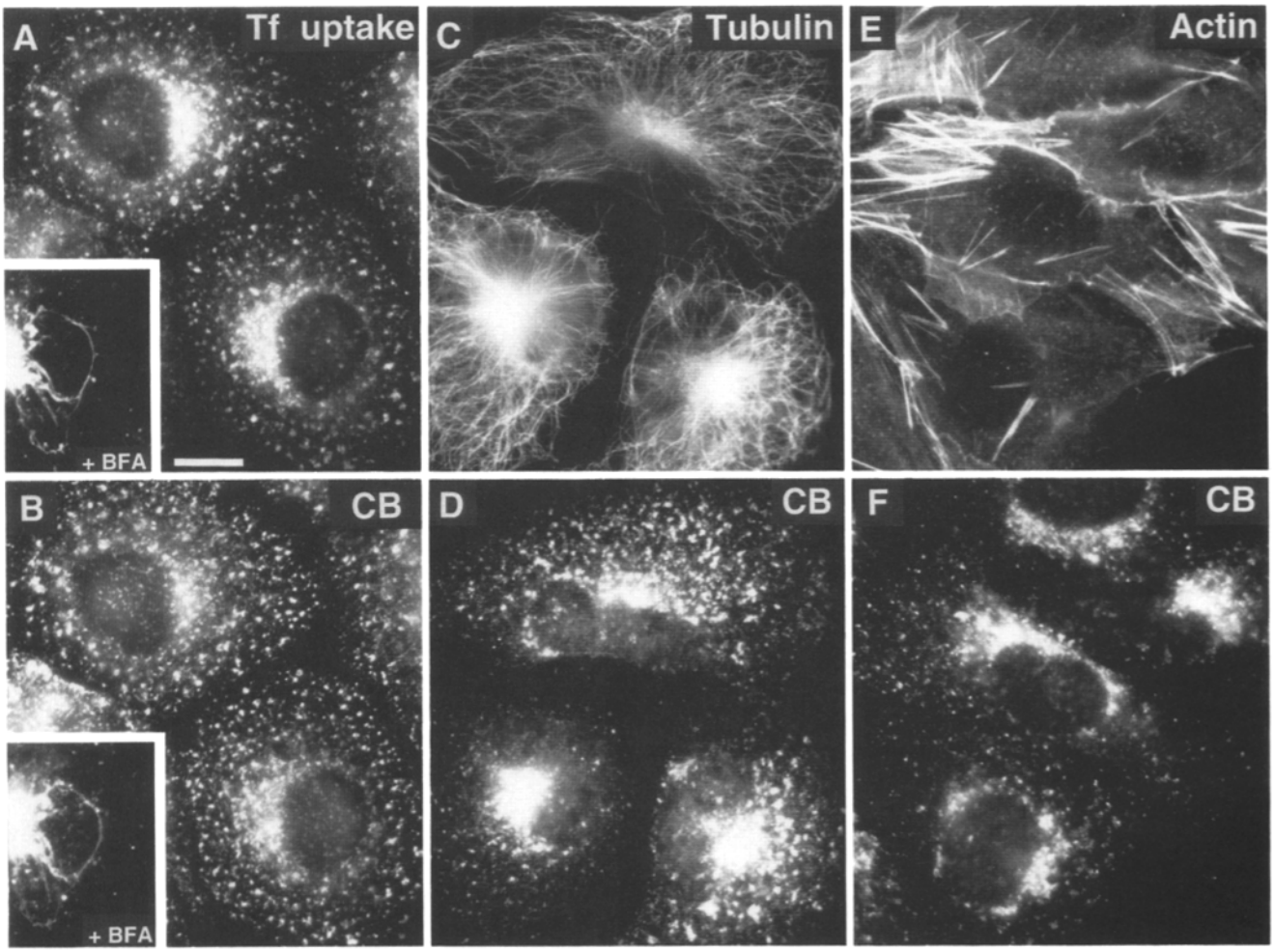

Figure 1. Localization of endogenous cellubrevin in CHO cells. Double immunofluorescence micrographs. (*A* and *B*) Double labeling for cellubrevin (*CB*) and for human Tf (*Tf uptake*) after exposure of the cells to human Tf (20 μg/ml) for 1 h. The insets in the two fields show a detail of a cell which had been treated with brefeldin A (+BFA) during the last 15 min of Tf uptake. (*C* and *D*) Double labeling for β-tubulin and for cellubrevin (*CB*). (*E* and *F*) Double labeling for actin (fluorescein-conjugated phalloidin) and for cellubrevin (*CB*). Bar, 14 μm (17 μm for the two insets).

was further validated by the virtual complete cleavage of cellubrevin produced by TeTx-LC added to the incubation medium (see below).

Light microscopy observation of perforated cells incubated for 65 min in the presence of 2 mM ATP demonstrated a slight retraction of the cell edges. However, the intracellular morphology was still strikingly preserved. As shown in Fig. 2, the microtubular and actin arrays were still present and cellubrevin immunoreactivity was not substantially affected. The only noticeable difference from intact cells was a partial disassembly of microtubules (compare Fig. 2 *A* [perforated cells] with Fig. 1 *C* [intact cells]) and a finer appearance of cellubrevin immunoreactivity in the perforated cells (compare Fig. 2, *B* and *D* [perforated cells] with Fig. 1, *B*, *D*, and *F* [intact cells]). The distribution of actin (Fig. 2 *C*) was similar to that seen in control cells (Fig. 1 *E*). A well preserved intracellular organization was confirmed by electron microscopy, although the cytosolic matrix appeared to have been partially extracted in perforated cells (not shown).

The release of preinternalized [^{125}I]Tf from perforated CHO cells in suspension cultures is shown in Fig. 3. Cells were incubated with [^{125}I]Tf for 1 h at 37°C and then washed and incubated for 65 min with or without exposure to SL-O perforation. In 65 min, SL-O–treated cells incubated with 2 mM ATP released about half of the amount of [^{125}I]Tf released by intact cells (Fig. 3). The kinetics of release were similar in intact and perforated cells but with values ~50% lower at any time point in perforated cells (Fig. 3). Both intact and SL-O–perforated cells released within the first 5 min ~40% of the total Tf released in 65 min. In the case of perforated cells some variability (24–32%) in the total amount of [^{125}I]Tf released over 65 min was observed. Most of this variability depended upon variability occurring during the first 5 min, when the rate of release is the fastest, while the fraction of release occurring between 5 and 65 min was always remarkably constant. The release of internalized Tf from SL-O–perforated cells was confirmed by immunofluorescence experiments. A significant fraction of Tf immunoreactivity visible at the end of a 1-h internalization

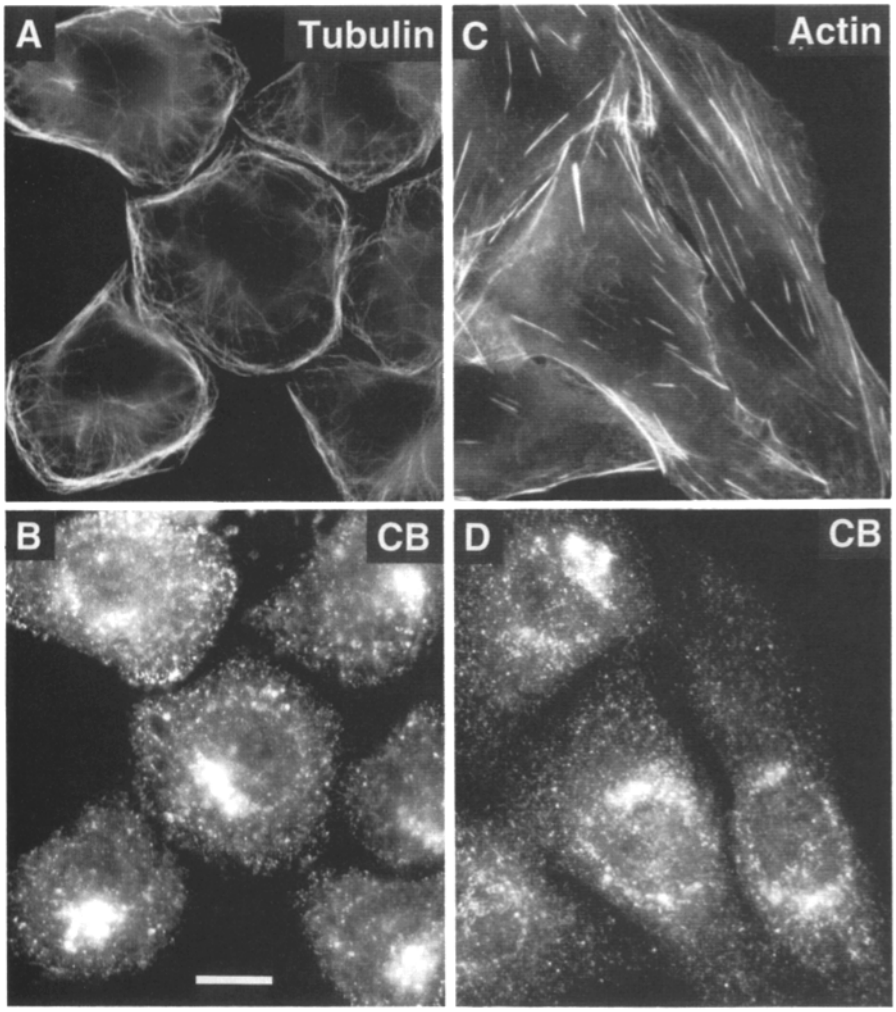

Figure 2. Structural preservation of SL-O-perforated CHO cells as demonstrated by immunofluorescence. Perforated cells were incubated for 65 min in the presence of 2 mM ATP before fixation. (*A* and *B*) Double labeling for β-tubulin and for cellubrevin (*CB*). A comparison with Fig. 1 *C* suggests that some, but not all, the microtubules have depolimerized. (*C* and *D*) Double labeling for actin (fluorescein-conjugated phalloidin) and for cellubrevin (*CB*). Bar, 14 μm.

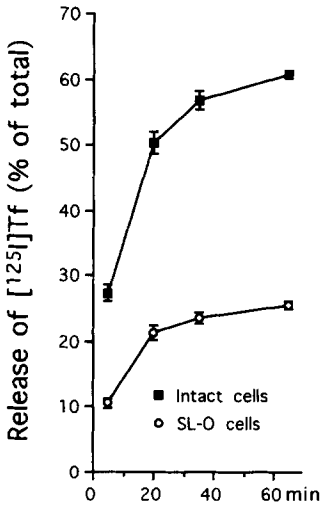

Figure 3. Release of preinternalized [^{125}I]Tf from intact and SL-O-perforated CHO cells. Intact cells (*full squares*) and SL-O perforated cells (in 2 mM ATP) (*open circles*) were incubated for 5, 20, 35, and 65 min at 37°C. The amount of [^{125}I]Tf released in each sample is expressed as the percentage of the total [^{125}I]Tf present in that sample (cell-associated plus released Tf). For each time point and treatment, values represents the mean ± SD of three determinations from a representative experiment. The amount of [^{125}I]Tf present in the supernatant of the cell suspension at 0 min was substracted from the amount of [^{125}I]Tf present in each sample supernatant.

(Fig. 4 *A*) was lost after cell perforation and a 65-min incubation in the presence of ATP (Fig. 4 *C*). Tf immunoreactivity was generally drastically decreased from all cell regions, consistent with a good preservation of the cell cytoskeleton, and therefore, most likely, of mechanisms of vesicle translocation.

We next investigated whether [^{125}I]Tf release from SL-O-perforated CHO cell exhibits the properties expected for a release occurring by exocytosis. The release was found to be dependent on the presence of ATP, because exposure of the perforated cells to an ATP-depleting system inhibited release (Fig. 5). This inhibition was complete after a lag phase, possibly needed to achieve a maximal ATP depletion. A similar lag phase in the effect of the ATP-depletion system on constitutive exocytosis of glycosaminoglycans from perforated CHO cells was reported by Miller and Moore (1991). Release of [^{125}I]Tf from perforated CHO cells was also dependent on temperature; when cells previously shifted for 5 min to 37°C to allow SL-O to act were returned to ice, [^{125}I]Tf release was completely blocked (Fig. 5). Additionally, release was also completely inhibited by treatment with NEM, as expected for a release requiring NSF, and there-

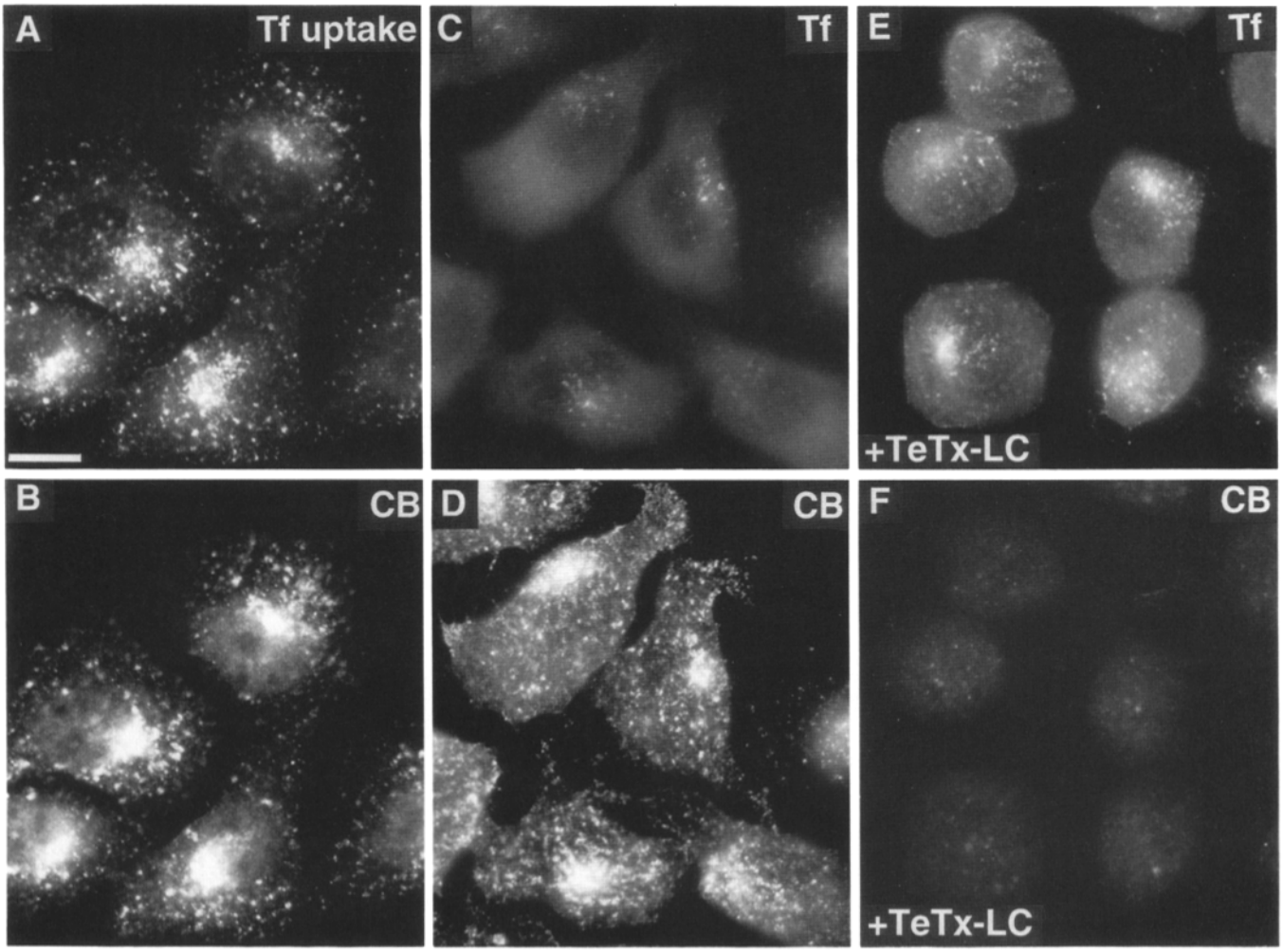

Figure 4. Release of preinternalized human Tf from SL-O–perforated CHO cells in the absence or presence of TeTx-LC. Cells were first incubated with human Tf (20 μg/ml) for 1 h then exposed to SL-O and incubated for 65 min with 2 mM ATP and in the absence or presence of 200 nM TeTx-LC before fixation. The figure shows three pairs of micrographs double labeled for human Tf (*Tf*) and for cellubrevin (*CB*) at the end of the internalization period (*A* and *B*), or at the end of the 65 min incubation without (*C* and *D*) or with toxin (*E* and *F*). Comparison of *A* with *C* demonstrates the significant, but incomplete, release of Tf from perforated cells treated in control conditions. Residual cell-associated Tf immunoreactivity is more intense in toxin-treated cells (*E*) than in control cells (*C*). In toxin-treated cells (*F*) cellubrevin immunoreactivity has virtually disappeared. Bar, 14 μm.

fore, bona fide membrane fusion (Söllner et al., 1993*a*,*b*). These findings rule out the possibility that some of the release of [^{125}I]Tf from perforated cells may be due to a major cell lysis or to lysis of Tf-containing vesicles independent from exocytosis.

Effect of TeTx–mediated Cleavage of Cellubrevin on Tf Release from Perforated Cells

Cells in suspension were perforated and incubated for 20 min at 37°C in the presence of increasing concentrations (0, 10, 30, 100, and 300 nM) of TeTx-LC. As illustrated in Fig. 6, TeTx-LC treatment resulted in a dose-dependent inhibition of [^{125}I]Tf release which correlated with a dose-dependent cleavage of cellubrevin with a nearly maximal effect at 300 nM. At this concentration no cellubrevin was left in the cell at the end of the 20-min incubation. The half-maximal inhibition of [^{125}I]Tf release occurred at ~30 nM of TeTx-LC.

Further experiments were then carried out with the maximal concentration of the toxin (300 nM) to achieve a complete cleavage of cellubrevin in the first few minutes of incubation. At this high toxin concentration, cellubrevin was completely degraded within 5 min (Fig. 7) and could not be detected even after overexposure of the autoradiogram (data not shown). SCAMPs (Brand and Castle, 1993) and Tf receptor (McMahon et al., 1993), two other proteins present in the same vesicular carrier as cellubrevin, and tubulin, remained unaffected by this treatment even after 65 min (Fig. 7), demonstrating that the proteolytic activity of the toxin was specific for cellubrevin. Furthermore, no difference in the overall protein composition was observed between control and toxin-treated cells in Coomassie blue–stained gels (data not shown). The time-course of [^{125}I]Tf release in the presence or absence of 300 nM TeTx-LC is shown in Fig. 8 *A*. The inhibition of release produced by TeTx was already visible at 5 min and continued thereafter. At 65 min the inhi-

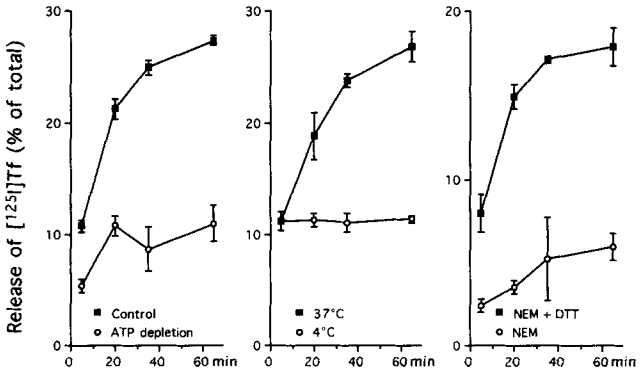

Figure 5. Sensitivity of [^{125}I]Tf release to ATP depletion, temperature and NEM. (*Left*) [^{125}I]Tf release from SL-O-perforated cells at 37°C in the presence of 2 mM ATP (*squares*) or of an ATP-depleting system consisting of 30 U/ml hexokinase and 3 mM glucose (*open circles*). (*Center*) [^{125}I]Tf release from SL-O-perforated cells at 37°C (*squares*) and 4°C (*open circles*) in the presence of 2 mM ATP. All cells were first incubated for 5 min at 37°C after SL-O addition to allow pore formation. The 4°C samples were then transferred to an ice-water bath. (*Right*) Release from SL-O-perforated cells at 37°C in the presence of 2 mM ATP. Cells were first incubated at 37°C for 2 min after SL-O addition to allow pore formation; they were subsequently incubated for 15 min on ice with 1 mM NEM plus 2 mM DTT (*squares*) or 1 mM NEM alone (*open circles*). In the latter case, NEM was quenched with 2 mM DTT for 5 min at the end of the 15-min incubation. Cells were then allowed to release [^{125}I]Tf at 37°C for 5, 20, 35, and 65 min. Results are expressed as described in Fig. 3.

bition ranged from 20–33 % of the control release. Nevertheless, in spite of the complete cleavage of cellubrevin, release of [^{125}I]Tf still occurred although at a lower rate.

Immunofluorescence staining of perforated cells incubated for 65 min in the presence of 200 nM TeTx-LC confirmed these biochemical findings. Loss of the immuno-

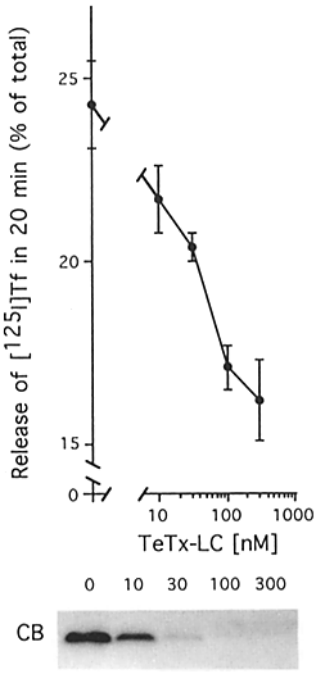

Figure 6. Dose-dependent effect of TeTx-LC on [^{125}I]Tf release and cellubrevin cleavage in SL-O-perforated CHO cells. (*Top*) Release of [^{125}I]Tf during a 20-min incubation in the presence of 2 mM ATP and 0, 10, 30, 100, or 300 nM TeTx-LC. Results are expressed as described in Fig. 3. (*Bottom*) The pellets from the same cell suspensions used to measure [^{125}I]Tf release were subjected to SDS-PAGE and levels of cellubrevin were demonstrated by Western blotting.

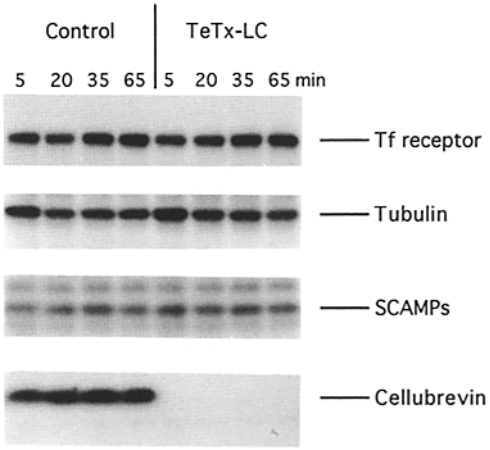

Figure 7. Specificity of the proteolytic effect of TeTx-LC on cellubrevin. SL-O-perforated cells were incubated with 2 mM ATP in the absence or presence of 300 nM TeTx-LC. Levels of Tf receptor, tubulin, SCAMPs, and cellubrevin remaining in the cell at the end of 5-, 20-, 35-, and 65-min incubations were demonstrated by Western blotting.

reactivity for Tf preinternalized during a 1-h incubation (Fig. 4 *A*) from TeTx-LC-treated cells was less prominent (Fig. 4 *E*) than in perforated cells incubated without toxin (Fig. 4 *C*). Furthermore, the typical cellubrevin immunoreactive pattern (Fig. 4, *B* and *D*) had practically disappeared in TeTx-LC-treated cells (Fig. 4 *F*).

Control experiments were performed with a mutant TeTx-LC which harbors a point mutation at position 234 (E→Q) and which does not have proteolytic activity (McMahon et al., 1993; Yamasaki et al., 1994*a*). This mutation is thought to abolish the coordination of a water molecule in one of the four positions of the tetrahedron around the catalytic zinc ion and thus disrupts protease activity (Yamasaki et al., 1994*a*). As shown in Fig. 8 *B*, the mutant toxin referred to as [Q234]TeTx-LC, did not produce any cellubrevin cleavage. Accordingly, the difference between the release of [^{125}I]Tf occurring in the presence of wild-type toxin or of the mutant toxin was similar to the difference observed between TeTx-LC-treated sample and control samples (Fig. 8 *A*). When measured in the same experiment, the release of [^{125}I]Tf observed in the presence of [Q234]TeTx-LC was not statistically different from the release observed in control preparations (Fig. 8 *C*).

The TeTx-LC-insensitive release of [^{125}I]Tf was ATP dependent. Fig. 9 shows that in the same experiment, an ATP-depleting system produced a greater inhibition of [^{125}I]Tf release than 300 nM TeTx-LC. Furthermore, the inhibitory effect of TeTx-LC on [^{125}I]Tf release was not additive to that of the ATP-depleting system alone, demonstrating that TeTx-LC inhibits a release which is ATP dependent and therefore, most likely, an exocytotic release.

The persistence of a fraction of an ATP-dependent [^{125}I]Tf release after complete cellubrevin cleavage raises the possibility that CHO cells may contain another v-SNARE of the synaptobrevin/VAMP family resistant to TeTx-LC. To test for this possibility, we analyzed CHO cells by Western blotting with anti-synaptobrevin antibodies (see Materials and

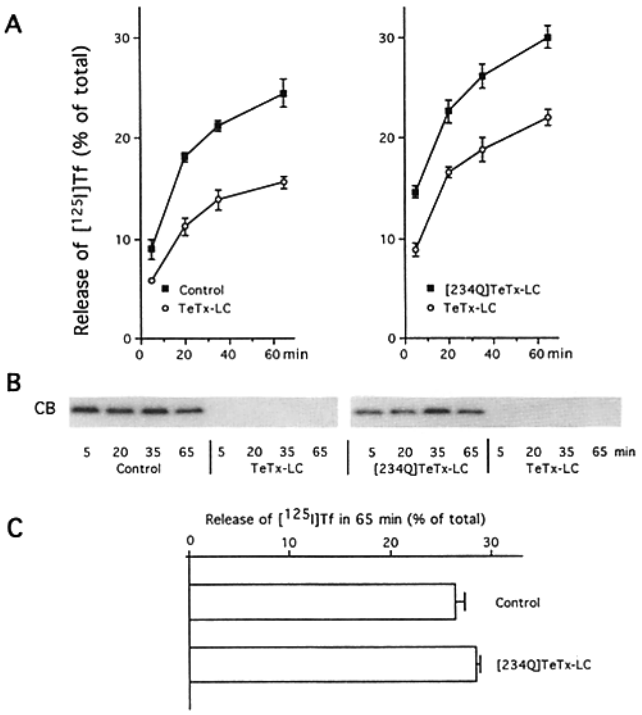

Figure 8. Time-course of the action of TeTx-LC and comparison with the action of the mutant, proteolytically inactive, toxin [234Q]TeTx-LC. All incubations were performed in the presence of 2 mM ATP and in the absence or presence of 300 nM toxin as indicated. Results are expressed as described in Fig. 3. (*A, Left*) [^{125}I]Tf release in the presence (*open circles*) or absence (*squares*) of TeTx-LC. The effect of the toxin is significant compared to controls at all time points with $P < 0.01$ using student's T-test. (*Right*) [^{125}I]Tf release in the presence of TeTx-LC (*open circles*) or [234Q]TeTx-LC (*squares*). The effect of the toxin is significant compared to the mutant at all time points with $P < 0.01$ using student's T-test. (*B*) Western-blot analysis of cellubrevin content in cell pellets corresponding to the samples used for the [^{125}I]Tf release assays shown in *A*. Already at 5 min cellubrevin has been completely cleaved. (*C*) [^{125}I]Tf released in 65 min in the absence or presence of [234Q]TeTx-LC. The difference between the amount of [^{125}I]Tf released in these two conditions is statistically not significantly different.

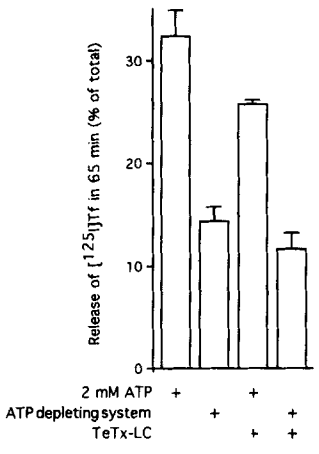

Figure 9. ATP dependence of the release of [^{125}I]Tf which is resistent to the action of TeTx-LC (300 nM). SL-O-perforated cells were incubated for 65 min with the additions indicated in the figure. Results are expressed as the mean ± SD of six values. Using the student's T test, the release observed in the presence of 2 mM ATP alone was found to be statistically different from the other three conditions ($P < 0.01$). The effect of the toxin in the presence of ATP was also significantly different ($P < 0.01$) from the effect of the ATP-depleting system alone. The effect of the toxin in the presence of the ATP depleting system was not significantly different when compared to the effect of the ATP-depleting system alone.

Methods) known to cross-react with the three mammalian synaptobrevin/VAMP homologues. None of these antibodies detected a TeTx-LC–resistant protein in the molecular weight range of synaptobrevins. These data suggest either that a cellubrevin isoform which is not detected by available antibodies is present in CHO cells or, alternatively, that the TeTx-LC–resistant release relies on a synaptobrevin/VAMP-independent secretion mechanism.

Discussion

Our findings provide evidence that cellubrevin plays a role in exocytosis and more generally that proteins of the synaptobrevin/VAMP family participate in exocytosis of recycling vesicles in nonneuronal cells. Exocytosis of vesicles which recycle Tf receptor takes place constitutively and is not subject to phasic regulation. Thus, these results support an important and general role of proteins of the VAMP/synaptobrevin family both in constitutive and regulated exocytosis.

Key tools which have made this study possible were two toxins: TeTx-LC and SL-O. TeTx is a Zn^{2+} protease which cleaves proteins of the synaptobrevin/VAMP family including cellubrevin with remarkable specificity (Link et al., 1992; Schiavo et al., 1992*a*,*b*; McMahon et al., 1993). Since nonneuronal cells do not have a plasmalemma receptor for the tetanus holotoxin (heavy and light chain), they do not internalize the toxin and do not translocate the light chain into the cytoplasm as is the case for neuronal cells. We have therefore used the toxin in combination with a cell perforation procedure based on SL-O.

SL-O is a pore-forming toxin which we have used here to selectively perforate the plasmalemma (Ahnert-Hilger et al., 1989*a*, 1993). The pores make possible the introduction of TeTx-LC and other proteins into cells, while preserving sufficient cell integrity to allow the study of vesicular traffic over limited time scales (Miller and Moore, 1991, 1992). The well known property of apotransferrin to be released into the medium following exocytosis of the vesicles which recycle it to the cell surface, offered the possibility to monitor exocytosis of these vesicles by detecting release of preinternalized Tf (Podbilewicz and Mellman, 1990). Temperature and ATP dependence, as well as NEM sensitivity of [^{125}I]Tf release supported the hypothesis that such release takes place by an exocytotic mechanism. Furthermore its NEM sensitivity provides a first piece of evidence suggesting that the underlying exocytotic event is mediated by the fusion machine involving NSF (Wilson et al., 1989) and SNAREs (Söllner et al., 1993*b*).

Purified recombinant TeTx-LC, which had been produced in bacteria, was applied to SL-O–perforated CHO cells. Its effect was extremely rapid and at high toxin concentrations, cellubrevin cleavage was complete by 5 min. Cellubrevin cleavage correlated with a significant reduction of Tf release, which could be demonstrated both biochemically and by immunofluorescence. Since the toxin inhibits, rather than stimulates, release, one cannot attribute release to a nonspecific action of the toxin leading to vesicle lysis. Specificity of the effect was further suggested by the lack of action of the mutant toxin ([Q234]TeTx-LC), which does not have any proteolytic activity (McMahon et al., 1993). The proteolytic effect of TeTx-LC appeared to be extremely specific for cellu-

brevin in agreement with previous studies (Link et al., 1992; Schiavo et al., 1992*a*,*b*).

In spite of the clear impairment of [^{125}I]Tf release produced by the toxin, 70% of [^{125}I]Tf release was independent of cellubrevin and TeTx-LC insensitive. This finding suggests two alternatives. One is that one or more other proteins of the synaptobrevin/VAMP family (a v-SNARE), and not sensitive to TeTx-LC, coexist(s) with cellubrevin on recycling vesicles. The other is that a fusion mechanism based on v- and t-SNARE interactions may coexist with a v-SNARE-independent exocytotic mechanism. The former possibility is more attractive. First, the release assay is completely sensitive to NEM, a compound known to block NSF–SNARE-dependent fusion mechanisms. Second, at least two genes encoding synaptobrevin/cellubrevin homologues are present in yeast (Protopopov et al., 1993) and disruption of one of the two genes is insufficient to produce a significant impairment of secretion. It is therefore likely that cellubrevin may not be the only synaptobrevin homologue expressed in non-neuronal cells and that its function may be partially compensated by other synaptobrevin related proteins. These other proteins cannot be synaptobrevin I or II because antibodies which recognize these two isoforms failed to detect significant levels of such isoforms in CHO cells (our unpublished observations). Furthermore, preliminary experiments with botulinum toxins D and F, which cleave proteins of the synaptobrevin family at sites that differ from the site cleaved by TeTx-LC (Schiavo et al., 1993*b*; Yamasaki et al., 1994*a*), did not produce a more severe inhibition of [^{125}I]Tf release than obtained with TeTx-LC.

TeTx-LC was previously expressed transiently in CV-1 cells resulting in virtually complete cleavage of cellubrevin (McMahon et al., 1993). The effect of this treatment on exocytosis was not investigated. Strikingly, however, cells in which cellubrevin had been cleaved were still able to internalize Tf by receptor-mediated endocytosis, indirectly suggesting that the Tf receptor is still brought to the cell surface by exocytosis. TeTx-LC was also expressed in the Sertoli cells of transgenic mice (Eisel et al., 1993). These mice were sterile, consistent with a functional impairment of these cells. However, even in this case the presence of viable Sertoli cells rules out an essential role of either cellubrevin or synaptobrevin I and II which are all sensitive to TeTx-LC cleavage in the mouse (Patarnello et al., 1993).

As indicated by its localization on Tf receptor-containing vesicles, cellubrevin must recycle between the cell surface and intracellular vesicles including endosomal intermediates. This recycling is similar to the recycling of synaptobrevin I and II via synaptic vesicles (McPherson and De Camilli, 1994). Potentially, cellubrevin may participate in fusion reactions in more than one step of the pathway. In a previous study Link et al. (1993) have used a cell-free endosome fusion assay to determine whether cellubrevin plays a role in endosome–endosome fusion. They found that complete cleavage of cellubrevin by TeTx-LC did not produce any effect on the efficiency of endosome fusion. Notably, the endosome–endosome fusion is an homotypic fusion event (fusion between membranes of the same compartment), while exocytosis is a heterotypic fusion event (fusion between two different membranes). Homotypic and heterotypic membrane fusion play distinct roles in the physiology of the cell and may be controlled by different mechanisms. Whether cellubrevin is involved in the fusion of endocytotic vesicles with early endosomes remains to be elucidated.

It also remains to be demonstrated whether cellubrevin is involved in pathways to the plasmalemma other than the receptor-mediated recycling pathway, such as, for example, the constitutive secretory pathway. The distribution of endogenous cellubrevin in CHO cells (this study) and of overexpressed cellubrevin in CV-1 cells (McMahon et al., 1993) appear to be more widespread than the distribution of internalized Tf. Thus, the involvement of cellubrevin in other pathways can be anticipated.

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