Fluorescence Resonance Energy Transfer Reports Properties of Syntaxin1A Interaction with Munc18-1 in Vivo*

Received for publication, August 31, 2004, and in revised form, October 13, 2004
Published, JBC Papers in Press, October 15, 2004, DOI 10.1074/jbc.M410024200

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Syntaxin1A, a neural-specific N-ethylmaleimide-sensitive factor attachment protein receptor protein essential to neurotransmitter release, in isolation forms a closed conformation with an N-terminal α-helix bundle folded upon the SNARE motif (H3 domain), thereby limiting interaction of the H3 domain with cognate SNAREs. Munc18-1, a neural-specific member of the Sec1/Munc18 protein family, binds to syntaxin1A, stabilizing this closed conformation. We used fluorescence resonance energy transfer (FRET) to characterize the Munc18-1-syntaxin1A interaction in intact cells. Enhanced cyan fluorescent protein-Munc18-1 and a citrine variant of enhanced yellow fluorescent protein-syntaxin1A, or mutants of these proteins, were expressed as donor and acceptor pairs in human embryonic kidney HEK293-S3 and adrenal chromaffin cells. Apparent FRET efficiency was measured using two independent approaches with complementary results that unambiguously verified FRET and provided a spatial map of FRET efficiency. In addition, enhanced cyan fluorescent protein-Munc18-1 and a citrine variant of enhanced yellow fluorescent protein-syntaxin1A colocalized with a Golgi marker and exhibited FRET at early expression times, whereas a strong plasma membrane colocalization, with similar FRET values, was apparent at later times. Trafficking of syntaxin1A to the plasma membrane was dependent on the presence of Munc18-1. Both syntaxin1A(L165A/E166A), a constitutively open conformation mutant, and syntaxin1AI209A, an H3 domain point mutant, demonstrated apparent FRET efficiency that was reduced ~70% from control. In contrast, the H3 domain mutant syntaxin1AI209A had no effect. By using phosphomimetic mutants of Munc18-1, we also established that Ser-313, a Munc18-1 protein kinase C phosphorylation site, and Thr-574, a cyclin-dependent kinase 5 phosphorylation site, regulate Munc18-1/syntaxin1A interaction in HEK293-S3 and chromaffin cells. We conclude that FRET imaging in living cells may allow correlated regulation of Munc18-1/syntaxin1A interactions to Ca\(^{2+}\)-regulated secretory events.

Synaptic transmission is triggered by Ca\(^{2+}\)-dependent fusion of synaptic vesicles with the presynaptic membrane. Central to the vesicular targeting, docking, and fusion process that underlie this regulated exocytotic event are soluble N-ethylmaleimide-sensitive factor and its attachment protein (SNAP)\(^1\) and membrane-bound receptors (SNAREs) (1–3). Synaptic vesicles contain the v-SNARE synaptobrevin (also termed VAMP) that interacts with high specificity to the integral presynaptic plasma membrane t-SNAREs syntaxin1A and SNAP25. These SNARE proteins assemble into a ternary complex via interaction of their SNARE motifs into parallel four-helix bundles (4, 5). SNARE complex assembly is believed to facilitate close membrane apposition and is a step essential to the fusion of synaptic vesicles with the plasma membrane (6). The assembly of trans-SNARE complexes for Ca\(^{2+}\)-regulated exocytosis is subject to strict spatial and temporal regulation (7).

Members of the Sec1/Munc18 (SM) gene family have also emerged as proteins essential for membrane trafficking and fusion (8). This is evidenced by the accumulation of vesicles in loss of function SM mutants in yeast (9, 10), Drosophila (11, 12) and Caenorhabditis elegans (13). SM proteins bind with high affinity to members of the syntaxin gene family and exhibit strong specificity to a membrane transport pathway, although members of the family are highly homologous and may interact with more than one syntaxin isoform (14). Consistent with a strict specificity, the way SM proteins interact with syntaxins is fundamentally different among different SM/syntaxin pairs. For example, yeast Sec1p binds to assembled SNARE complexes at the cell membrane, yeast Sly1p binds to a conserved N-terminal domain of syntaxin for fusion of transport vesicles from the endoplasmic reticulum to Golgi, whereas interaction of mammalian Munc18-1 with syntaxin at the plasma membrane requires the complete cytosolic domain of syntaxin and is absent in assembled SNARE complexes (15–18). Multiple functions have been proposed for SM proteins, including those of a trafficking factor for syntaxin (19), a regulator of vesicle docking (20, 21), a facilitator of priming processes required for assembly of SNARE complexes (22), and a mediator of the kinetics of expansion of the fusion pore (23, 24). However, despite considerable evidence demonstrating their necessity for normal membrane trafficking and fusion, the precise function(s) of SM proteins remains poorly defined.

In the case of synaptic transmission, the SM protein Munc18-1 binds with high affinity to the plasma membrane t-SNARE syntaxin1A. Syntaxin1A consists of an N-terminal domain composed of an antiparallel three-helix bundle (termed the Habc domain) that is connected via a short flexible linker to

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\(^*\) This work was supported by Grant NS39914 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\(^1\) The abbreviations used are: SNAP, soluble N-ethylmaleimide-sensitive factor attachment protein; SNARE, SNAP receptor; SM, Sec1/Munc18; PKC, protein kinase C; Cdk, cyclin-dependent kinase; FRET, fluorescence resonance energy transfer; CFP, enhanced cyan fluorescent protein; eYFP, citrine mutant of enhanced yellow fluorescent protein; HEK, human embryonic kidney; PSS, physiological saline solution.
occurs on Ser-306 and Ser-313 associated protein kinase (34–37). Phosphorylation by PKC kinase C (PKC), cyclin-dependent kinase 5 (Cdk5), and death shown both has been best characterized. For example, Munc18-1 has beenpete with Munc18-1 for binding syntaxin1A complexes (42, 43). In addition, tomosyn has been shown to com- substitute for the VAMP SNARE in syntaxin1A SNARE com-plexes (37, 44). Munc18-1, which contains a single C-terminal V-SNARE motif that cancyclin to syntaxin1A and this interaction has been proposed to stabilize an open confor-mation of formation of Munc18-1/syntaxin1A complexes (34). Func-tionally, PKC phosphorylation of Ser-313 has been reported toalter the dynamics of vesicle release events (23, 24). By com-parison, Cdk5 phosphorylation of Munc18-1 on formation of Munc18-1/syntaxin1A complexes (18) has been shown to alter the dynamics of vesicle release events (23, 24). Thus, there is compelling evidence to sup-port regulation of the Munc18-1/syntaxin1A interaction as a critical determinant in the SNARE complex assembly that is necessary for the Ca2+-dependent exocytotic process of neuro-transmitter release and synaptic transmission (33). Therefore, there is compelling evidence to sup-port regulation of the Munc18-1/syntaxin1A interaction as a critical determinant in the SNARE complex assembly that is necessary for the Ca2+-dependent exocytotic process of neuro-transmitter release and synaptic transmission (33). Therefore, there is compelling evidence to sup-port regulation of the Munc18-1/syntaxin1A interaction as a critical determinant in the SNARE complex assembly that is necessary for the Ca2+-dependent exocytotic process of neuro-transmitter release and synaptic transmission (33).

A number of mechanisms regulating the assembly and dis-association of the Munc18-1/syntaxin1A complex have been proposed. Among these, regulation by protein phosphorylation has been best characterized. For example, Munc18-1 has been shown both in vitro and in vivo to be a substrate for protein kinase C (PKC), cyclin-dependent kinase 5 (Cdk5), and death-associated protein kinase (34–37). Phosphorylation by PKC occurs on Ser-306 and Ser-313 in vitro and results in a reduc-tion in the affinity of Munc18-1 for syntaxin1A and inhibition of formation of Munc18-1/syntaxin1A complexes (34). Function-ally, PKC phosphorylation of Ser-313 has been reported to alter the dynamics of vesicle release events (23, 24). By com-parison, Cdk5 phosphorylates Munc18-1 at a single site in vitro (Thr-576), and this phosphorylation is capable of mediating disassembly of preassembled Munc18-1/syntaxin1A complexes (38). Pharmacological inhibition of Cdk5 inhibited induced se-cretion from adrenal chromaffin cells (38) and pancreatic beta cells (39). In addition to protein phosphorylation, a number of direct protein- mediated mechanisms have also been suggested as important regulators of the Munc18-1/syntaxin1A complex. Included among these are proteins of the Munc13 and tomosyn gene families. Munc13 binds to the N terminus of syntaxin1A, and this interaction has been proposed to stabilize an open conformation of syntaxin that is necessary for pairing the syntaxin1A SNARE motif with cognate SNAREs (40, 41). Tomosyn contains a single C-terminal V-SNARE motif that can substitute for the VAMP SNARE in syntaxin1A SNARE complexes (42, 43). In addition, tomosyn has been shown to com-pete with Munc18-1 for binding syntaxin1A in vitro. Munc18-1 also interacts with other proteins, such as Mints (44, 45) and DOC2 (46), although their role in modulating the Munc18-1/ syntaxin1A interaction is unknown.

To date, characterization of specific mechanisms of formation and disassembly of a Munc18-1/syntaxin1A complex have re- lied on in vitro biochemical assays of protein-protein interac-tion and mechanistic interpretations of alterations in func-tional responses using genetic approaches in cell or animal models (2, 6, 8). However, to characterize the temporal and spatial aspects of regulation associated with activation of the secretory pathway requires an experimental approach that directly reports the state of the bimolecular Munc18-1/syntaxin1A protein interaction within living cells. In the pres-ent study, we used fluorescence resonance energy transfer (FRET) as a non-invasive imaging method to characterize the interaction of Munc18-1 with syntaxin1A in both the HEK293-S3 cell line and in primary cultures of bovine adrenal chromaffin cells. As FRET probes we used enhanced cyan flu-orescent protein (CFP) and the citrine variant of enhanced yellow fluorescent protein (eYFP) fused to the N termini of Munc18-1 and syntaxin1A, respectively. Two independent FRET methods have been used to provide additional confidence in measurement and quantification of apparent FRET effi-ciency of the bimolecular interactions. To establish the selec-tivity of the FRET signal and to establish the sensitivity of the approach to report changes in the affinity of the bimolecular interaction within cells, we have tested the effects of various expression paradigms and mutants of Munc18-1 and syntaxin1A. In addition, we evaluated the effect of PKC and of Cdk5 phosphorylation of Munc18-1 on formation of Munc18-1/ syntaxin1A complexes and on the trafficking of syntaxin1A from the Golgi complex to the plasma membrane.

MATERIALS AND METHODS

Chemicals and Expression Constructs—Mouse monoclonal antibody against syntaxin1A was from Sigma; mouse monoclonal antibody against Munc18-1 and the Golgi marker protein GS27 were from BD Transduction Lab (San Jose, CA); Alexa Fluor 488 and Alexa Fluor 548 goat anti-mouse antibodies were from Molecular Probes (Eugene, OR). All other chemicals were obtained from Sigma. HEK293 cells stably expressing the rat α11 and human β3 and α2β voltage-gated calcium channel subunits (termed HEK293-S3 cells) were a gift from D. Rock (Warner-Lambert Parke Davis, Ann Arbor, MI). The LoxP sequence from pLP-EGFP-C1 (Clontech) was subcloned into the multiple cloning site regions of pcDNA3.1, pECFP-C1, and pEcYFP-C1 (Q39M mutant of pEYFP-C1) to generate recipient vectors for subcloning using the Cre recombinase-mediated Creator System (Clontech). Rat Munc18-1 and rat syntaxin1A were merged to the C terminus of the enhanced cyan mutant of green fluorescent protein (ECFP) and EYFP. Mutants of Munc18-1 and syntaxin1A were constructed using the polymerase chain reaction-based Quick Site-Directed Mutagenesis kit from Stratagene (Cedar Creek, TX). The sequence fidelity of all expression constructs was confirmed by DNA sequencing (University of Michigan DNA Sequencing Core).

Cell Culture and Transfections—HEK293-S3 cells were plated and cultured in RPMI 1640 with l-glutamine supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (Life Technologies, Inc.), 0.4 mg/ml of hygromycin, and 0.6 mg/ml of genetin at 37 °C in 95% O2-5% CO2 for 2 days on coverslips (thickness 1 mm) affixed to the bottom of 35-mm culture dishes before transfection. Transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manu-facturer’s instructions. One h before transfection, cells were placed in Dulbecco’s modified Eagle’s medium lacking antibiotics and supple-mented with 1% l-glutamine, 1% non-essential amino acids, and 10% fetal bovine serum. After 4–6 h with DNA, the culture medium was changed to RPMI 1640. Cells were used for optical investigation 24–36 h after transfection.

Chromaffin cells were isolated and cultured from bovine adrenal glands using divalent metal ion-free rinse, collagenase digestion, and gradient centrifugation as described previously (47). Cells were cul-tured and transfected in 35-mm culture dishes and replated onto colla-gen-coated glass coverslips 4 h before imaging. Chromaffin cells were transfected using biolistic particle bombardment (gene gun, Bio-Rad) according to the manufacturer’s instructions.

Immunocytochemistry—HEK293-S3 cells cultured on coverslips were fixed with 4% paraformaldehyde in physiological saline solution (PSS) for 20 min, rinsed with PSS, and quenched with 50 mm NH4Cl-PSS for 12 min. PSS contained 145 mM NaCl, 5.6 mm KCl, 5.6 mm glucose, 0.5 mm CaCl2, 0.2 mm MgCl2, 10 mm HEPES, and 0.5 mm glucose, 0.5 mm ATP, 2 mm GTP, 10 mm sodium pyruvate, and 0.1 mm sodium orthovanadate in PSS. After fixation, cells were permeabilized using 1% Triton X-100 in PSS for 5 min and blocked with 5% normal goat serum in PSS for 1 h. Cells were incubated with rabbit anti-Munc18-1 (1:1000) and mouse anti-syntaxin1A (1:1000) antibodies in PSS containing 1% bovine serum albumin (BSA) for 1 h and washed with PSS. After incubation for 1 h, cells were washed with PSS and quenched with 50 mM NH4Cl-PSS for 10 min. Cells were incubated with either donkey anti-rabbit Alexa Fluor 594 or donkey anti-mouse Alexa Fluor 488 (1:200) in PSS containing 1% BSA for 1 h, and coverslips were mounted in Mowiol. Images were acquired using a Nikon inverted microscope equipped with a cooled charge-coupled device camera and a 100× objective. Fluorescence images were acquired at the appropriate excitation and emission wavelengths, and images were pseudocolored using Adobe Photoshop (CS2) and presented as maximum projections of Z stacks of 10 optical sections spaced 0.5 μm apart. Results were analyzed using ImageJ (National Institutes of Health).
mm MgCl₂, 2.2 mm CaCl₂, and 15 mm HEPES (pH 7.4). After rinsing with PSS, cells were permeabilized with 0.2% Triton X-100 in PSS containing 2% normal goat serum for 20 min and then incubated sequentially with Munc18-1 or syntaxin1A antibody (1:400 to 1:800 dilution) for 2 h and with Alexa Fluor-488 goat antimouse secondary antibody (1:200 dilution) for 1 h. After rinsing, coverslips were mounted on glass slides with 75% glycerol-PSS. The Golgi marker GS27 was localized in HEK293-S3 cells using a mouse monoclonal antibody (1:200 dilution) and Cy5-conjugated anti-mouse secondary antibody (1:200 dilution). Digital fluorescence images from HEK293-S3 and chromaffin cells were viewed with either an Olympus fluorescence microscope or a Zeiss LSM 510 META confocal microscope (conventional channel mode; 488-nm argon laser line), and images were processed using Photoshop 6.0 software (Adobe Systems Inc.). Controls with no primary antibody were used to confirm the specificity of the immunological reactivity.

**Measurement of FRET Stoichiometry by Sensitized Emission on a Conventional Fluorescence Microscope—FRET imaging was performed on transfected cells 24–36 h after transfection. The microscope consisted of an inverted fluorescence microscope (Olympus, IX71) interfaced to a TILL-Photonics Polychrome IV xenon lamp-based monochromator (TILL-Photonics, Grafelfing, Germany), a polychromic mirror that allowed detection of multiple fluorophores (436–500 nm, Chroma Technology Corp., Brattleboro, VT), a Planapo ×60 water immersion objective (1.2 NA), a multispectral microimager (Optical Insights, Santa Fe, NM) containing a dichroic splitter (505dcrx) and emission filters (D465/30 and HQ535/30) to allow simultaneous two-channel monitoring of emission fluorescence, and a cooled digital CCD camera (TILL-4 MAGO QE). Control of image acquisition and excitation wavelengths was carried out under software control (TILL-Vision). Pixel-by-pixel alignment of images was performed by hardware adjustments of the multispectral microimager and offline positioning of images using TILL-Vision software. All analysis of the acquired and adjusted images was performed using Metamorph image-processing software (version 6.1, Universal Imaging, Inc., Mahwah, PA).

Individual images were acquired with an exposure time of 250 ms, and a single or 10-frame averaged image was generated for each filter set combination. No differences were found between data for a single- and frame-averaged acquisition, but as a result of lowered image noise, the FRET values reported and the images shown were based on the 10-frame averaged images. Images corresponding to three excitation/emission wavelength settings were recorded. The CFP/yellow fusion protein was used as a FRET donor with the cYFP fusion protein as the FRET acceptor. These include acceptor excitation/acceptor emission (AA), donor excitation/donor emission (DD), and donor excitation/acceptor emission (DA). In addition, for each excitation/emission filter combination, an averaged background and shade correction image was collected before an experiment for offline correction of cell images for camera bias and/or uneven illumination or fluorescence signal across the field imaged. The background image was a 20-frame average of images collected with the illumination light source blocked. The shade correction image was a 20-frame averaged image of purified solutions of bacterially expressed CFP and cYFP (~50 nm) in phosphate-buffered saline (Life Technologies, Inc.) buffer sandwiched between a coverslip and glass slide precoated with bovine serum albumin (1 mg/ml in phosphate-buffered saline). Background and shade images were used to generate corrected images (C) of the cells for each excitation/emission setting according to the equation,

$$C_i = (S_{max} - S) / (B - S) $$

where $S_{max}$ is the shade image maximum value, $S$ is the shade image, and $B$ is the background.

Quantification of FRET from the corrected cell images (i.e. $I_{100}^AA$, $I_{100}^DA$, $I_{100}^DD$) was determined according to a recently described procedure termed FRET quantification by the fluorescence microscope method (48). The method determines FRET efficiencies for each pixel of a cell image. The first parameter is the apparent FRET efficiency ($E_p$), which is the product of the FRET efficiency of the specific biomolecular interaction, termed characteristic FRET efficiency ($E_c$), and the fraction of acceptor in complex with the donor. $E_p$ per se is dependent upon orientation and distance between the fluorescent probes. Because $E_p$ is proportional to the fraction of acceptor in complex, it can be used to measure changes in the fraction of the acceptor in complex. $E_p$ was determined according to the relation,

$$E_p = \gamma(I_{100}^{DA} - \beta I_{100}^{DD})/(\alpha I_{100}^{AA} - \beta I_{100}^{DD})$$

where $I_{100}^AA$, $I_{100}^DD$, and $I_{100}^AA$ represent shade and background corrected fluorescent intensity corresponding to wavelengths of donor excitation-acceptor emission (DA), donor excitation-donor emission (DD), and acceptor excitation-acceptor emission (AA). The proportionality constants $\alpha$, $\beta$, and $\gamma$ were determined as described below. The second parameter determined is $E_p$, which is the apparent donor efficiency and is the product of $E_p$ and the fraction of the donor in complex. $E_p$ was determined according to the relation,

$$E_p = 1 - I_{100}^{DD} - I_{100}^{AA} - \alpha I_{100}^{DA} - \beta I_{100}^{DD} + I_{100}^{AA}$$

The third parameter is the acceptor-donor molar ratio (RATIO). RATIO indicates the mole fraction of acceptor to donor in each pixel of the collected cell image. RATIO was calculated according to the formula

$$RATIO = \gamma(I_{100}^{AA} - \alpha I_{100}^{DA} - \beta I_{100}^{DD})/(\alpha I_{100}^{AA} + I_{100}^{DD})$$

A RATIO of 1 indicates equal mole fractions of acceptor to donor. Equations used to determine these three parameters are derived in Hoppe et al. (48) and require determination of four constants identified as $\alpha$, $\beta$, $\gamma$, and $\xi$ on the optical system used for measurements. The
proportionality constant $\alpha$ relates the intensity of acceptor emission with donor excitation to that with acceptor excitation and is determined from transfected cells expressing only cYFP. $\beta$ relates the intensity of donor emission to that of acceptor emission with donor excitation and is determined from transfected cells expressing CFP alone. The constant $\gamma$ was determined from cells expressing a CFP-cYFP fusion protein construct in which the two fluorophores are directly linked by a 16-amino acid linker (48). This constant defines the ratio of the extinction coefficients of the acceptor to the donor at the donor excitation.

The proportionality constant $\xi$ relates the intensity of acceptor emission resulting from donor energy transfer to the concomitant decrease in donor fluorescence. In our optical system, $\alpha$ and $\beta$ were 0.015 and 0.942, respectively, and $\gamma$ and $\xi$ were 0.067 and 0.014, respectively. An $E_C$ value of 0.37 determined for the linked CFP-cYFP construct was taken from the literature (48).

**Measurement of Efficiency of Syntaxin1A Trafficking to Plasma Membrane**—The relative amount of expressed cYFP-syntaxin1A associated with plasma membrane regions was quantified offline across treatments from background- and shade-corrected cYFP images. For each imaged cell, the total area of plasma membrane, and area and average intensity of the “cytoplasm region” was determined for each cell by constricting the region measured by five pixels from the outer perimeter of the plasma membrane. Averaged cYFP signal area at the plasma membrane region was then calculated according to the formula,

$$\frac{(A_{\text{cell}} - A_{\text{cyto}} - A_{\text{cyto}} - A_{\text{cyto}})(A_{\text{cyto}} - A_{\text{cyto}})}{A_{\text{cyto}}}$$

where $A_{\text{cell}}$ is the total area of cell, $I_{\text{cell}}$ is the averaged cYFP intensity of the cell, $A_{\text{cyto}}$ is the total area of the cytoplasm, and $I_{\text{cyto}}$ is the averaged cYFP intensity of the cytoplasm. Measurements of images were performed using Photoshop 6.0 software.

**Electrophysiological Measurements of Steady-state Inactivation of Voltage-gated Calcium Channels**—Recordings were made using an Axopatch 200a amplifier (Axon Instruments, Foster City, CA) through an ITC-16 AD/DA interface (Instrutech Corp., Great Neck, NY) under the control of Pulse Control integrated into IGOR PRO software version 4.0.2a (Wavemetrics Inc., Lake Oswego, OR). Patch pipettes (2–4 MΩ) were pulled on a Sutter Instruments P-87 microelectrode puller using Carlsborg, WA) and coated with elastomer (Sylgard, Dow Corning, Midland, MI) to minimize capacitance. All recordings were made using a voltage-clamp at room temperature. The patch pipette recording solution consisted of 120 mM tetraethylammonium chloride, 10 mM HEPES, 1 mM CaCl2, and 1 mM EGTA (pH 7.3). Ba2+ was added immediately before recording. The extracellular solution consisted of 140 mM tetraethylammonium chloride, 10 mM BaCl2, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES (pH 7.3). Ba2+ was used as charge carrier to minimize Ca2+-induced current inactivation or activation of Ca2+-dependent signaling pathways. Whole-cell conductance and series resistance were compensated electronically, and evoked currents were filtered at 5 kHz. All currents were leak subtracted (P4 protocol) before analysis.

To study the properties of voltage-dependent steady-state inactivation, a three-pulse voltage protocol was used. Two 20-ms test pulses to 0 mV from a −100-mV holding potential were separated by a conditioning inactivating voltage step. The voltage dependence of inactivation was measured in response to 3-s conditioning steps that ranged from −110 to 0 mV. Application of each three-pulse protocol was separated by a 25-s period at the holding potential to allow recovery from voltage-dependent inactivation. Voltage-dependent steady-state inactivation data were fitted with a Boltzmann function of the form

$$I/I_0 = A_0 + (A_\infty - A_0)/(1 + e^{(V - V_0)/k})$$

where $I/I_0$ is the current normalized to maximum current, $A_\infty$ and $A_0$ are the maximum and base of the curve, $V_0$ is the midpoint of voltage dependence of inactivation, and $k$ is the slope factor. Curve fits to data were made using an iterative, non-linear, least-squares fitting algorithm provided in the IGOR PRO software.

**RESULTS**

**Expression and Functional Analysis of Munc18-1 and Syntaxin1A FRET Probes**—HEK293-S3 cells were initially used to establish FRET measurements between CFP-Munc18-1 and cYFP-syntaxin1A. These cells allowed high transient transfection efficiency and protein overexpression, and they lack endogenous syntaxin1A and Munc18-1 proteins, which likely facilitated interactions between the expressed neural-specific recombinant proteins. Also, HEK293-S3 cells lack neuronal proteins that modify the molecular mechanism of Ca2+-regulated exocytosis in neurons and may affect Munc18-1 interactions with syntaxin1A.

Initially, we tested whether the addition of CFP and cYFP tags to Munc18-1 and syntaxin1A, respectively, altered the subcellular targeting or function of these proteins. As shown in the confocal images of Fig 1, CFP-Munc18-1 fluorescence localized predominantly to the cytosolic compartment without specific labeling of the nucleus or plasma membrane. Examination of the expression of a non-tagged Munc18-1 by immunocytochemistry using anti-Munc18 antibody revealed a subcellular distribution similar to that of CFP-Munc18-1. Expression of cYFP-syntaxin1A in the absence of Munc18-1 consistently resulted in labeling of the perinuclear membrane, numerous small punctate intensities of cytosolic fluorescence, and often a few sites of intense fluorescence likely representing protein compartmentalization within organelles such as the
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Fig. 2. Energy transfer detected from an increase in donor fluorescence after acceptor photobleaching in CFP-Munc18-1 and cYFP-syntaxin1A cotransfected and fixed HEK293-S3 cells. A, representative example of mixed emission spectra of CFP-Munc18-1 donor and cYFP-syntaxin1A acceptor fluorophores (excitation, 458-nm laser line) taken before (black line) and after (red line) photobleaching (with the 514 argon laser line). Spectra are shown for a region that was photobleached (upper plot) and for another region in the same cell that was not photobleached (lower plot). Note that CFP donor emission increased on photobleach only in the photobleached region of the cell. B, set of unmixed CFP and CFP images of cells taken before photobleach (left set) and after acceptor photobleach (right set). The region of photobleach is indicated by the white outlined box. Pseudocolored intensity images at the bottom show regions of cell membrane in the photobleached (between the arrowheads) and non-photobleached (between the arrowheads) regions taken before and after bleaching. C, averaged FRET efficiency (%) measured for coexpression of non-linked CFP and cYFP protein (N-L; n = 11), for expression of a control FRET construct resulting in direct linkage of CFP to cYFP via a short peptide linker sequence (L; n = 14), and coexpression of CFP-Munc18-1 with cYFP-syntaxin1A (M18 + S1A; n = 37). FRET was determined for the photobleached region at the cell membrane region, whereas results in non-photobleached regions show the percentage of change in CFP after the photobleach step. The averaged intensity ratio of cYFP to CFP (before photobleach) for the cells corresponding to each condition is shown (right plot). Significant differences are relative to the non-linked CFP, cYFP expression condition.
from the Zeiss Meta confocal microscope of the mixed emission spectra of the CFP-Munc18-1 donor and cYFP-syntaxin1A acceptor fluorophores (excitation, 458-nm line of argon laser) taken from cell images before and after localized photobleach for photobleached and non-photobleached regions of the cell. A selective increase in the peak emission intensity corresponding to the region of CFP emission was observed in the photobleached, and not in the non-photobleached, regions of the cell. These results are consistent with a FRET signal originating from the transfer of resonant energy from CFP to cYFP and the dequenching of this energy transfer upon photobleaching of the acceptor fluorophore. Fig 2B shows a representative set of spectrally unmixed images (CFP and cYFP) taken from HEK293-S3 cells before and after photobleach of the acceptor (514-nm line of argon laser). The CFP images show an increase in the donor emission (also pseudocolored intensity images) after photobleach that occurred only in the region of the cell exposed to the photobleach. Fig 2C provides the averaged relative FRET efficiency value for coexpression of CFP-Munc18-1 with cYFP-syntaxin1A at the plasma membrane (22.0 ± 1.0%, ± S.E., n = 37) in the bleached area of the cells. For comparison and as a further control, Fig. 2C also shows relative FRET efficiency values observed in cells coexpressing only the CFP and cYFP proteins (non-linked, N-L) and in cells expressing a construct in which the CFP and cYFP proteins were directly linked (L) by a short peptide sequence to produce a FRET configuration. Although the averaged cYFP/CFP fluorescence intensity ratios were similar (range, 0.7 to 1.4) for each condition, only coexpression of CFP-Munc18-1 and cYFP-syntaxin1A and the linked CFP-cYFP protein demonstrated substantial FRET.

In addition to interaction of CFP-Munc18-1 and cYFP-syntaxin1A at plasma membrane regions, cell images also frequently showed discrete regions of CFP and cYFP fluorescence within the cytosol. We, therefore, tested the hypothesis that these regions of CFP and cYFP fluorescence resulted from targeting of cYFP-syntaxin1A to the Golgi complex and its interaction with CFP-Munc18-1 because syntaxin1A is sorted for trafficking to the plasma membrane. Initially, we evaluated whether the CFP and cYFP fluorescence colocalized with immunofluorescence against a Golgi antigen (GS27 protein) and whether FRET occurred between CFP-Munc18-1 and cYFP-syntaxin1A within the Golgi region. To facilitate accumulation of fluorescent signal within the Golgi, imaging was performed on cells that were fixed 6 h after cotransfection. Confocal microscopy of these cells showed a predominant colocalization of immunofluorescence against GS27 protein with CFP and cYFP fluorescence (Fig. 3). At this early time point after transfection, few cells showed significant localization of CFP-Munc18-1 or cYFP-syntaxin1A at the plasma membrane. In addition, immunofluorescence against Golgi marker GS27 in control, non-transfected HEK293-S3 cells demonstrated a similar subcellular localization and immunofluorescent signal to that observed in the cells cotransfected with CFP-Munc18-1 and cYFP-syntaxin1A, albeit with slightly increased perinuclear labeling of the transfected cells. These data indicate that overexpression of Munc18-1 with synt1X1A did not lead to significant disruption or reorganization of the Golgi complex. Importantly, the colocalization of CFP-Munc18-1 and cYFP-syntaxin1A at the Golgi region represented a direct bimolecular interaction as determined by measurement of FRET using the acceptor photobleach approach. Indeed, averaged relative FRET efficiency for this interaction (27.3 ± 0.9%, ± S.E., n = 8) was not significantly different from that measured for plasma membrane regions after 24–48 h of expression. These data demonstrate that Munc18-1 interacts with syntaxin1A at the region associated with the Golgi complex and suggests that the interaction between these proteins is maintained during trafficking and after delivery of syntaxin1A to the plasma membrane.

Measurement of CFP-Munc18-1 and cYFP-Syntaxin1A FRET by Sensitized Emission—As an alternative and independent measure of FRET between CFP-Munc18-1 and cYFP-syntaxin1A, we used a filter-based approach with a conventional fluorescence microscope. This approach measured FRET as an increase in acceptor fluorescence resulting from donor excitation (i.e. sensitized emission FRET). Three FRET parameters were quantitatively determined: $E_A$, $E_D$, and $RATIO$ (Eq. 48). $E_A$ is the apparent efficiency of acceptor interaction with donor, whereas $E_D$ is the apparent efficiency of donor interaction with acceptor. $RATIO$ is the molar ratio of cYFP to CFP. Fig 4A provides representative cYFP images showing subcellular distribution in HEK293-S3 cells of syntaxin1A and corresponding images of $E_A$, $E_D$, and $RATIO$ for coexpression of CFP-Munc18-1 and cYFP-syntaxin1A and for control constructs (CFP + cYFP-syntaxin1A or peptide-linked CFP-cYFP). CFP-Munc18-1 + cYFP-syntaxin1A coexpression resulted in membrane localization of syntaxin1A and substantial $E_A$ and $E_D$ values ($E_A$, 31.6 ± 0.9%; $E_D$, 27.0 ± 1.1%, n = 27). An additional experiment evaluated FRET between Munc18-1 and syntaxin1A that were linked to nondimerizing mutant forms of CFP and cYFP fluoroproteins (A206K mutants, (56)), respectively, as green fluorescent proteins, and their variants may self-aggregate and thus FRET. Syntaxin1A distribution and FRET values were nearly identical to those observed with the non-monomeric CFP and cYFP fusion proteins. By comparison, a negative control involving coexpression of CFP + cYFP-syntaxin1A showed no specific FRET signal ($E_A$, 2.7 ± 0.5%; $E_D$, 1.2 ± 0.1%, n = 14), whereas a positive control with the linked CFP-cYFP construct demonstrated spatially uniform FRET throughout the cell cytoplasm. The averaged FRET efficiency values $E_A$ and $E_D$ for all treatments are shown in Fig. 4B. Differences in the FRET values between treatments occurred without substantial variations in averaged molar RATIO values (Fig 4C).

**Fig. 3. Distribution of CFP-Munc18 and cYFP-syntaxin1A to a Golgi region with a limited expression period in HEK293-S3 cells.** Cells were non-transfected (Control) or cotransfected with CFP-Munc18-1 and cYFP-syntaxin1A and fixed after a 6-h expression period. Cells were then immunolabeled using the monoclonal anti-GS27 antibody, followed by Alexa Fluor-647-conjugated secondary antibodies. Confocal images were collected for control and cotransfected cells of the immunoreactive signal by using linear unmixing of the corresponding CFP and cYFP signals. A representative set of images from cotransfected cells is shown, indicating overlap of GS27 immunoreactivity with CFP and cYFP fluorescence. Scale bar, 10 μm.
changes in the level of interaction of these proteins, we measured the effects of mutants of Munc18-1 and syntaxin1A. Initially, the effect on FRET was determined using CFP-Munc18-1 and a cYFP-tagged mutant of syntaxin1A (L165A, E166A) that assumes a constitutively open conformation (syntaxin1A "open" mutant) and that has been reported not to bind to Munc18-1 in vitro (28). The results demonstrated a strongly reduced apparent FRET efficiency ($E_A$ and $E_D$) as compared with the wild-type protein pair (Fig. 5). $E_A$ was reduced from 31.6 ± 0.9% to 13.1 ± 1.0%, whereas $E_D$ decreased from 27.0 ± 1.1% to 8.9 ± 0.6%. Images of cYFP-syntaxin1A for the syntaxin1A "open" mutant also showed localized densities of cYFP fluorescence with no apparent increased signal at the plasma membrane. It is important to note, however, that FRET between the syntaxin1A "open" mutant and Munc18-1 was significantly above the background value (2.7 ± 0.5%, from CFP + cYFP-syntaxin1A treatment), indicating that the syntaxin1A "open" mutant retained a capacity to interact with Munc18-1 in vivo.

As a complementary experiment, we determined the effect on apparent FRET efficiency of a site-directed mutant of Munc18-1 (R39C) that exhibits strongly reduced affinity for interaction with syntaxin1A in vitro (57). FRET images from cells coexpressing the CFP-tagged version of this mutant with cYFP-syntaxin1A (Fig. 5) demonstrated that expression of the Munc18-1 (R39C) mutant resulted in a significant reduction in FRET ($E_A$, 15.3 ± 1.3%; $E_D$, 25.2 ± 1.0; n = 27) as compared with wild-type proteins. However, the reduction in FRET is less than that observed with coexpression of CFP-syntaxin "open" mutant + CFP-Munc18-1. Comparison of CFP and the RATIO images between treatments indicates that the amount of syntaxin1A at the plasma membrane region with Munc18-1 (R39C) expression is less than that with coexpression of wild-type proteins but greater than with the syntaxin1A "open" condition. Notably, coexpression of both syntaxin1A "open" and Munc18-1 (R39C) mutants resulted in a lower apparent FRET efficiency ($E_A$ of 7.0 ± 0.5%; $E_D$ of 7.8 ± 0.2%; n = 11) than when only one of the FRET pair was mutated. These results further substantiate that the syntaxin1A "open" mutant can bind to Munc18-1 in vivo.

To evaluate the effect of mutations directly within the SNARE motif of syntaxin1A on apparent FRET efficiency in vitro, we examined two site-directed mutants of cYFP-syntaxin1A, i.e., cYFP-syntaxin1A (I233A) and cYFP-syntaxin1A (I209A). The I233A mutation of syntaxin1A rests within the N-terminal domain of the SNARE motif that normally occupies the central cavity of Munc18-1 during interaction of Munc18-1 with syntaxin1A. Previously reported in vitro binding assays demonstrated that this mutation reduces syntaxin1A/Munc18-1 binding without reduction in syntaxin1A binding to its cognate t-SNARE SNAP25 (58). As shown in Fig. 5, measurement of FRET between CFP-Munc18-1 and cYFP-syntaxin1A (I233A) showed that apparent FRET efficiency ($E_A$, 9.9 ± 0.5%; $E_D$, 15.8 ± 1.1%) was reduced from that of the control CFP-Munc18-1 and cYFP-syntaxin1A proteins. The I209A mutation of syntaxin1A is also within the SNARE motif of syntaxin1A, but as a result of its placement, this mutant has been reported to retain full capacity for Munc18-1 binding while reducing binding to SNAP25 (59). Coexpression of cYFP-Munc18-1 with a CFP-syntaxin1A (I209A) mutant resulted in apparent FRET efficiency values ($E_A$, 31.9 ± 1.5%; $E_D$, 34.2 ± 1.8%) that were not significantly different from control. Taken together, the data demonstrated that FRET is sensitive to expected differences in interaction between Munc18-1 and syntaxin1A.

Relationship of Apparent FRET Efficiency to RATIO Allows Unambiguous Comparison of FRET between Treatments—In the present study, determination of the molar ratio (cYFP/CFP)
using a FRET stoichiometry method provided a means of relating the measured apparent FRET efficiency to the relative levels of expression of donor and acceptor labeled protein. Fig 6A compares frequency histograms of molar ratio values between treatments. Molar ratio values were determined for each pixel of the cells imaged in each condition. The mean molar ratio for coexpression of CFP-Munc18-1 (R39C) and cYFP-syntaxin1A was, for example, significantly different (1.95 ± 0.2, n = 27) from expression of CFP-Munc18-1 + cYFP-syntaxin1A open (1.04 ± 0.2, n = 20) or the wild-type constructs (CFP-Munc18-1 + cYFP-syntaxin1A open, 0.93 ± 0.1, n = 25). Relationships between the apparent efficiency of the donor (E$_D$) or acceptor (E$_A$) in complex and the molar RATIO are plotted in Fig. 6B. Comparison of the relationships between the wild-type FRET protein pair and CFP-Munc18-1 + cYFP-syntaxin1A “open” showed a marked decrease in apparent FRET efficiency throughout the range of RATIO values. In contrast, the data for the wild-type FRET protein pair showed considerable overlap with that for CFP-Munc18-1 (R39C) + cYFP-syntaxin1A. Importantly, however, in each case apparent FRET efficiency was sensitive to the relative expression levels of the acceptor and donor proteins, and the apparent FRET efficiency/RATIO relationship resembled that of a single-site binding curve. Therefore, to calculate a value for E$_A$ and E$_D$ that can be unambiguously compared between treatments, determination of averaged FRET efficiency was restricted to include only those FRET efficiency values where RATIO fell within the range 0.9 to 1.1. This ratio was chosen based on an established 1:1 stoichiometry for Munc18-1 and syntaxin1A interaction. Fig. 6C shows the E$_A$ and E$_D$ values calculated over this restricted ratio range for each of the treatments. The results demonstrated the expected correspondence between the E$_A$ and E$_D$ values when the molar ratio approaches 1. In addition, the results confirmed a strong reduction in FRET signal with the syntaxin1A “open” mutant (67%) and an ~30% reduction with the Munc18-1 (R39C) mutant from control.

Effects of Mutations of Munc18-1 Phosphorylation Sites on CFP-Munc18-1/cYFP-Syntaxin1A FRET—To determine the effects of Munc18-1 phosphorylation by PKC and Cdk5 on Munc18-1/syntaxin1A interaction in intact cells, we measured FRET using phosphomimetic mutants of the PKC and Cdk5 sites of Munc18-1. Fig. 7A compares averaged E$_A$ values in the 0.9–1.1 RATIO interval between the control CFP-Munc18-1 + cYFP-syntaxin1A FRET pair and a series of phosphomimetic mutants of CFP-Munc18-1 coexpressed with cYFP-syntaxin1A. To better mimic electrostatics of a PO$_4^2-$ group at Ser-313, the effects of double dGlu mutations at Ser-312, the effects of double dGlu mutations at Ser-312 and Ser-313 were also tested. Results showed that the PKC phosphomimetic mutants demonstrated the strongest reductions in FRET when expressed as double Ser-312-Ser-306 or triple Ser-306-Ser-312-Ser-313 dGlu mutants. The Ser-306-Ser-313 double dGlu mutation showed no statistically significant change in apparent FRET efficiency values from control, demonstrating that within the cell environment this phosphomimetic mutant is capable of interacting with syntaxin1A. This suggests that the effects of the Ser-306-Ser-313 phosphomimetic mutant on secretory responsiveness result from actions independent of Munc18-1/syntaxin1A binding. Coexpression of Munc18-1 mutants carrying a single dGlu mutation at either the Ser-306 or Ser-313 site also had no significant effect on the measured FRET. The Cdk5 phosphomimetic mutant demonstrated a reduction in FRET. We next examined whether phosphorylation altered plasma membrane targeting of syntaxin1A by analysis of subcellular distribution of cYFP. As shown in Fig. 7B, coexpression of the control CFP-Munc18-1 + cYFP-syntaxin1A resulted in a pronounced targeting of cYFP to the plasma membrane regions. By comparison, expression of Munc18-1 with
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Fig. 6. Relationships of energy transfer efficiencies between CFP-Munc18-1 and cYFP-syntaxin1A detected by sensitized emission to molar ratio of cYFP acceptor to CFP donor. A, frequency histograms of molar ratio values for coexpression of CFP-Munc18-1 + cYFP-syntaxin1A (WT), CFP-Munc18-1 + cYFP-syntaxin1A L165A,E166A (S1A Open), and CFP-Munc18-1 R39C + cYFP-syntaxin1A (M18 R39C) in HEK293-S3 cells. B, plots of apparent FRET efficiency (EA, ED) for all pixels of imaged cells against their corresponding RATIO values for given treatment conditions. E_A (EA) and E_D (ED) relationships for CFP-Munc18-1 + cYFP-syntaxin1A (WT) (black) are compared with those of CFP-Munc18-1 + cYFP-syntaxin1A L165A,E166A (S1A Open) and Munc18-1 R39C + cYFP-syntaxin1A (M18 R39C) (gray). The box indicates the RATIO interval 0.9 to 1.1 over which E_A and E_D values were averaged for each treatment condition. C, averaged E_A and E_D values encompassing the RATIO interval 0.9 to 1.1 for each treatment condition. Significant differences are relative to the wild type (WT) CFP-Munc18 + cYFP-syntaxin1A condition.

either the PKC phosphorylation site mutations (Ser-306-Ser-313 or the Ser-306-Ser-312-Ser-313) or the Cdk5 phosphorylation site mutation (Thr-574) resulted in restriction of coexpression of syntaxin1A to perinuclear regions and areas of intense fluorescence within the cytoplasm that likely reflect accumulation in Golgi and endoplasmic reticulum compartments. Averaged results quantifying the effect on trafficking of cYFP-syntaxin1A to the plasma membrane for each of the mutants of Munc18-1 are shown in Fig 7C. None of the single dGlu mutations of the PKC sites differed from control in targeting cYFP-syntaxin1A. To verify that the Munc18-1 and its mutant constructs were expressed at similar levels relative to syntaxin1A, the averaged molar cYFP/CFP ratios were compared across treatments (Fig 7D). The results showed similar expression ratios, indicating that differences in FRET efficiency or syntaxin1A targeting across treatments did not result from changes in relative expression of the proteins.

Imaging of FRET between CFP-Munc18-1 and cYFP-Syntaxin1A in Adrenal Chromaffin Cells—To examine the properties and regulation of Munc18-1 and syntaxin1A in a cell environment where these proteins are endogenous and where components normally regulating their interaction in the Ca^{2+}-dependent exocytotic pathway are functionally active, we used neuroendocrine adrenal chromaffin cells. A comparison of the subcellular distribution of CFP-Munc18-1 and cYFP-syntaxin1A when expressed alone or in combination in chromaffin cells is shown by confocal microscopy in Fig 8A. Unlike expression in HEK293-S3 cells, the expression of CFP-Munc18-1 in chromaffin cells resulted in specific enrichment of fluorescence intensity at the plasma membrane regions, although the fluorescent signal was also present throughout the cytosol. The presence of endogenous syntaxin1A in the plasma membrane likely promoted the targeting and association of Munc18-1 to this domain. In contrast, overexpression of cYFP-syntaxin1A in chromaffin cells resulted in intense fluorescent labeling of the perinuclear region, an effect similar to that observed for cYFP-syntaxin1A expression in HEK293-S3 cells. Because Munc18-1 is critical for trafficking of syntaxin1A, these results suggest that Munc18-1 may be a limiting factor for syntaxin1A trafficking in chromaffin cells. Coexpression of CFP-Munc18-1 and cYFP-syntaxin1A resulted in strong fluorescent labeling at or near the plasma membrane without accumulation of cYFP-syntaxin1A at the perinuclear region. Overlap in the subcellular distribution between CFP-Munc18-1 and cYFP-syntaxin1A occurred as expected. To confirm that the overlap in the distribution reflected a true bimolecular interaction, we first evaluated FRET between the CFP-Munc18-1 and cYFP-syntaxin1A using the acceptor photobleach method. Fig 8B shows a representative set of spectrally unmixed images (CFP and cYFP) taken from transfected and fixed adrenal chromaffin cells before and after photobleach of the acceptor. With photobleaching, donor emission increased only in the region of the cell exposed to the photobleach. Fig 8C compares the mixed emission spectra of CFP-Munc18-1 and cYFP-syntaxin1A taken before and after photobleach for bleached and non-bleached regions of the cell. Consistent with the incidence of FRET, the peak of CFP emission intensity was selectively increased in the photobleached region of the cell. The averaged relative FRET efficiency was 36.8 ± 2.2% (n = 14). As a final test for FRET, we imaged transfected, living chromaffin cells and measured FRET by sensitized emission. Fig. 8D shows representative cYFP images and the FRET parameters (E_A, E_D, and RATIO). Averaged apparent FRET efficiency values determined by sensitized emission were: E_A, 30.6 ± 3.0%; E_D, 19.8 ± 2.9% molar RATIO, 0.75 ± 0.1% (n = 10); E_A, 28.1 ± 2.0% when averaged over the RATIO interval of
DISCUSSION

In addition to the cyclical formation and disassembly of SNARE protein complexes, the pathways regulating membrane fusion and exocytosis require the involvement of SM proteins. Despite the critical importance of SM proteins and the existence of several proposed models that indicate a critical role in the regulation of protein-protein interactions, the exact molecular mechanism by which SM proteins act to regulate the exocytotic pathway remains enigmatic. Although biochemical properties of the syntaxin1A/Munc18-1 interaction have been characterized and the crystal structure of the heterodimer complex has been determined (22), little is known of the temporal and spatial aspects of the interaction or of its regulation in intact cells. The investigations of the present study sought unambiguous evidence that FRET could be used to visualize and quantify temporal and spatial aspects of the bimolecular Munc18-1/syntaxin1A interaction in intact cells. FRET between donor and acceptor labeled molecules results in several measurable parameters, including sensitized acceptor fluorescence, quenching of donor fluorescence, and a decrease in donor lifetime. In the present study, we have measured FRET between N-terminal fusions of CFP and cYFP to Munc18-1 and syntaxin1A, respectively, in intact HEK293-S3 and adrenal chromaffin cells using both a sensitized acceptor fluorescence stoichiometry method and by dequenching of donor fluorescence when the acceptor fluorophore was photobleached. These independent measures of FRET resulted in highly complementary results and unambiguously validated the signal measured in both cell types as FRET. The measured FRET efficiency was comparable with that obtained on expression of a directly linked CFP-cYFP fusion protein that assumes a FRET configuration.

0.9 to 1.1). Taken together, the results showed that FRET between CFP-Munc18-1 and cYFP-syntaxin1A can be quantified in neuroendocrine chromaffin cells and, potentially, can be used to evaluate dynamic changes in the interactions of these proteins.
strained into a constitutively open conformation and that fails to exhibit measurable binding interactions in vitro (28) retained the capacity for interaction when coexpressed with CFP-Munc18-1 within intact cells. These latter results demonstrate that in vitro binding data between Munc18-1 and mutants of syntaxin1A may quantitatively differ from what occurs within the cellular environment and suggest caution using in vitro binding data to interpret secretory effects resulting from expression of Munc18-1 or syntaxin1A mutants in eukaryotic cells and extrapolation of the interpretation to mechanistic models of the secretory pathway.

Determination of apparent FRET efficiency for each pixel of an image allows a subcellular mapping of energy transfer efficiency for a cell. However, because FRET between donor and acceptor fluorophore-tagged proteins within cells results from a bimolecular binding interaction, the measured FRET efficiency can vary from cell to cell and between experiments based on differences in the stoichiometry of expression of the donor and acceptor labeled proteins and on the degree of saturation of the binding reaction for the interacting proteins. These variables have often resulted in the determination of relative rather than absolute FRET efficiency values, thereby hindering direct and quantitative comparisons of FRET efficiency between cells and treatment groups. The present investigations implemented a recently developed FRET stoichiometry approach that improves the quantification of FRET in living cells to determine the molar concentration ratios (acceptor/donor) and fractions of interacting molecules throughout a cell image (48, 63). Indeed, a hyperbolic relationship was found between apparent FRET efficiency and the ratio of acceptor to donor-labeled protein (Fig. 6). That is, the apparent efficiency was a similar function of the inverse of the molar ratio. Using this information, it was possible to directly compare apparent FRET efficiency ($E_A$, $E_D$) between cells and across treatments at given molar ratios. Yet, $E_A$ and $E_D$ are defined as the product of characteristic efficiency of the interaction ($E_C$) and the fraction of acceptor ($f_A$) or donor ($f_D$) in complex. Therefore, changes in $E_A$ and $E_D$ between treatments could result from both changes in $E_C$ and/or $f_A$ and $f_D$, even when considered at identical molar ratios. However, an estimation of $E_C$ is given by the level at which the $E_A$ versus 1/(molar ratio) or $E_D$ versus molar ratio relationships saturate. By this estimate, the reduction in apparent efficiency for the syntaxin1A/Munc18 (R39C) treatment from control likely primarily resulted from a change in the fraction of molecules in complex, because the $E_C$ values for both conditions were similar. By comparison, strongly reduced FRET efficiencies for syntaxin1A (L165A/E166A) and syntaxin1A (I233A) conditions from control were accompanied by corresponding decreases in $E_C$, indicating that the change in the fraction of molecules in complex with these mutants may not be as great as that for the Munc18 R39C mutant condition. Recently reported effects of these syntaxin1A mutants to increase quantal size and slow kinetics of release from transfected adrenal chromaffin cells (64) are consistent with the observed reduction in apparent FRET efficiency. It should also be noted that at a molar RATIO of 1, the averaged $E_A$ and $E_D$ values for each treatment were not statistically different, reflecting the 1:1 binding stoichiometry between Munc18-1 and syntaxin1A and arguing against significant homo-oligomerization via the H3 domain SNARE motifs of the expressed cYFP-syntaxin1A, when coexpressed with CFP-Munc18-1.

In this study, we have also used spatial information contributed by imaging of FRET efficiency to map subcellular regions of CFP-Munc18-1/cYFP-syntaxin1A interactions. The confocal images of FRET efficiency demonstrated prominent interac-
tions at the plasma membrane regions for both cotransfected HEK293-S3 and chromaffin cells. This contrasted strongly with a FRET efficiency signal approaching background for most of the cytoplasm, with the exception of occasional densities of intense FRET that are likely related to the Golgi and/or membrane trafficking vesicles in the cytoplasm. The resolution of subcellular and regional differences was, as expected, lower in apparent FRET efficiency images using sensitized emission methods on a conventional fluorescence microscope as a result of effects of out-of-field focus fluorescence combined with FRET calculations based in part on a ratio of fluorescence intensities. However, even FRET efficiency images from sensitized emission showed localized regions within the cytosol, and particularly at the plasma membrane, of heightened FRET efficiency. We have also demonstrated, with shorter periods of expression after transfection, that FRET between CFP-Munc18-1 and cYFP-syntaxin1A colocalized with immunoreactivity against GS27, a Golgi marker protein. In addition, FRET efficiency was similar at the Golgi as that seen in the plasma membrane region. The importance of Munc18-1 in trafficking syntaxin1A to the plasma membrane has been previously well documented (19, 49). Our observations suggest that interactions between Munc18-1 and syntaxin1A that form at the Golgi complex are maintained at nearly equivalent levels during transit and delivery to the plasma membrane.

By itself, syntaxin1A switches between a closed, SNARE pairing, inactive configuration and an open, SNARE pairing competent configuration with <1 ms of relaxation time (32). Therefore, regulatory proteins such as Munc18 have been proposed as necessary to stabilize syntaxin1A in a configuration, consistent with the activation state of the secretory mechanism (27–29, 65). Knock-out of Munc18-1 gene expression in mice (33) and loss-of-function mutations for SM proteins in C. elegans (13, 21) and Drosophila (11, 12) result in an elimination or strong reduction in evoked exocytic responses that underlie synaptic neurotransmission. Although an interaction between syntaxin1A and Munc18-1 is believed essential to regulated exocytosis, there are studies indicating that each protein may subserve different roles and exert functions at different steps in the secretory pathway. For example, Munc18-1 function is critical to docking of secretory vesicles in C. elegans neurons (21) and in bovine adrenal chromaffin cells (20). By comparison, botulin toxin-mediated ablation of syntaxin1A did not disrupt vesicle docking (66, 67) (except (68)), yet regulated secretion was strongly reduced (69, 70), consistent with a role of syntaxin1A as a SNARE. Thus, Munc18 and syntaxin1A may have multiple roles in the secretory process, with certain functions requiring direct interactions between these proteins, such as syntaxin1A trafficking to the plasma membrane, whereas others, such as vesicle docking, may require independent action.

On the basis of binding studies, interactions between Munc18-1 and syntaxin1A are regulated by PKC and Cdk5 phosphorylation of Munc18. Phosphorylation by PKC inhibits Munc18-1 binding to syntaxin1A in vitro (34) and in rat brain nerve terminals in situ (36). Phosphorylation by Cdk5 has likewise been reported to inhibit the Munc18-1/syntaxin1A interaction in vitro, and pharmacological block of Cdk5 activity reduced secretory responsiveness in adrenal chromaffin cells (35, 38). Moreover, although a high constitutive phosphatase activity in rat brain nerve terminals is, therefore, likely to be critical not only for limiting rapid effects of Munc18-1 on exocytosis but also in allowing continued trafficking of syntaxin1A to the plasma membrane. This is critical as the Munc18-1/syntaxin1A interaction is also important in secretory granule docking (20, 21) and may also facilitate priming processes required for assembly of SNARE complexes (8, 71).

**In vivo** FRET analysis of Munc18-1/syntaxin1A interactions should promote further understanding of the precise timing at which these complexes assemble and function within the regulated exocytotic pathway in neurons and neuroendocrine cells.

**Acknowledgments**—We thank Adam Hoppe and Joel Swanson for advice and helpful discussions on FRET stoichiometry implementation and analysis.

**REFERENCES**

1. Sudhof, T. C. (1995) *Nature* **375**, 645–653.
2. Zahn, R., Lang, T., and Sudhof, T. C. (2003) *Cell* **112**, 519–533.
3. Lin, R. C., and Scheller, R. H. (2000) *Annu. Rev. Cell Dev. Biol.* **16**, 19–49.
4. Sollner, T., Bennett, M. K., Whiteheart, S. W., Scheller, R. H., and Rothman, J. E. (1993) *Cell* **75**, 409–418.
5. Sollner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geronimos, S., Tempst, P., and Rothman, J. E. (1993) *Nature* **362**, 318–324.
6. Rizo, J., and Sudhof, T. C. (2002) *Nat. Rev. Neurosci.* **3**, 641–653.
7. Gundelfinger, E. D., Kessels, M. M., and Schlüchtermann, O. (2003) *Nature Rev. Mol. Cell Biol.* **4**, 127–139.
8. Toonen, R. F., and Verhage, M. (2003) *Trends Cell Biol.* **13**, 177–186.
9. Novick, P., Field, C., and Schekman, R. (1988) *Cell* **51**, 205–215.
10. Schekman, R. (1992) *Curr. Opin. Cell Biol.* **4**, 587–592.
11. Harrison, S. D., Brodie, K., van de Goor, J., and Rubin, G. M. (1994) *Neuron* **13**, 555–566.
12. Wu, M. N., Schuler, K. L., Lloyd, T. E., and Bellen, H. J. (2001) *Eur. J. Cell Biol.* **13**, 19–49.
