Munc13-4 reconstitutes calcium-dependent SNARE-mediated membrane fusion

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Munc13-4 is a widely expressed member of the CAPS/Munc13 protein family proposed to function in priming secretory granules for exocytosis. Munc13-4 contains N- and C-terminal C2 domains (C2A and C2B) predicted to bind Ca2+, but Ca2+-dependent regulation of Munc13-4 activity has not been described. The C2 domains bracket a predicted SNARE-binding domain, but whether Munc13-4 interacts with SNARE proteins is unknown. We report that Munc13-4 bound Ca2+ and restored Ca2+-dependent granule exocytosis to permeable cells (platelets, mast, and neuroendocrine cells) dependent on putative Ca2+-binding residues in C2A and C2B. Munc13-4 exhibited Ca2+-stimulated SNARE interactions dependent on C2A and Ca2+-dependent membrane binding dependent on C2B. In an apparent coupling of membrane and SNARE binding, Munc13-4 stimulated SNARE-dependent liposome fusion dependent on putative Ca2+-binding residues in both C2A and C2B domains. Munc13-4 is the first priming factor shown to promote Ca2+-dependent SNARE complex formation and SNARE-mediated liposome fusion. These properties of Munc13-4 suggest its function as a Ca2+ sensor at rate-limiting priming steps in granule exocytosis.

Introduction

Ca2+-regulated vesicle exocytosis is essential for signaling in the nervous, endocrine, and immune systems. Mechanisms used in the regulated secretory pathway are conserved across cell types using different members from the Rab, Sec1/Munc18, CAPS (Ca2+-dependent activator protein for secretion)/Munc13, and SNARE protein families that function in the tethering, priming, and fusion of secretory vesicles. Final fusion steps are catalyzed by trans-SNARE complexes assembled between vesicle v-SNARE and plasma membrane t-SNAREs (Jahn and Scheller, 2006). After arrival at the plasma membrane and before fusion, vesicles undergo priming reactions that involve SNARE protein assembly into fusion-competent complexes. In neurons and endocrine cells, members of the CAPS/Munc13 family (Munc13-1 and CAPS-1) mediate vesicle priming reactions (Augustin et al., 1999; Jockusch et al., 2007; Liu et al., 2008). CAPS-1 and Munc13-1 interact with SNARE proteins or with SNARE protein complexes (Guan et al., 2008; Daily et al., 2010). The MHD1 region present in all CAPS/Munc13 proteins mediates SNARE protein interactions for CAPS-1 (Koch et al., 2000; Khodthong et al., 2011), whereas a larger MHD1/MHD2-containing region was reported to bind SNARE protein complexes in Munc13-1 (Guan et al., 2008).

Munc13-4 is a ubiquitously expressed member of the CAPS/Munc13 family of priming factors whose mechanism is poorly understood. Transcripts for Munc13-4 are present in many cell types (brain, heart, skeletal muscle, kidney, lung, liver, pancreas, and prostate) in which a functional role for Munc13-4 remains to be characterized (Koch et al., 2000). Munc13-4 is highly expressed in cells of the immune system, where it participates in regulated granule exocytosis in cytotoxic T lymphocytes (CTLs), mast cells, neutrophils, and platelets (Feldmann et al., 2003; Shirakawa et al., 2004; Yamamoto et al., 2004; Neeft et al., 2005; Marcenaro et al., 2006; Higashio et al., 2008; Pivot-Pajot et al., 2008). CTLs deficient in Munc13-4 from familial hemophagocytic lymphohistiocytosis (FHL) type 3 (FHL3) individuals fail to exocytose docked cytotoxic granules, which...
suggested a postdocking role for Munc13-4 in granule priming (Feldmann et al., 2003). Munc13-4 interacts with Rab27a, consistent with a tethering or priming role, although a granule localization has not been established for all cell types expressing Munc13-4 (Shirakawa et al., 2004; Neef et al., 2005; Elstak et al., 2011). Munc13-4 also localizes to endosomes in CTLs, where it likely functions in the fusion of endosomes for cyto-toxic granule maturation (Ménager et al., 2007).

Munc13-4 contains a central MHD1/MHD2 region, but Munc13-4 binding to SNARE proteins has not been reported. The breadth of cell types in which Munc13-4 may function suggests that it might interact broadly with exocytic and possibly endosomal SNARE proteins. Two C2 domains (C2A and C2B) bracket the central domain in Munc13-4, and each is predicted to bind Ca\(^{2+}\) ions (Feldmann et al., 2003), but direct Ca\(^{2+}\) regulation of Munc13-4 activity has not been demonstrated. Munc13-4 exhibited Ca\(^{2+}\)-dependent translocation to the plasma membrane in neutrophils, but it was uncertain whether this was secondary to granule translocation. Munc13-4 binding to liposomes was reported to require C2 domains but was Ca\(^{2+}\) independent (Pivot-Pajot et al., 2008). It was important to determine whether Munc13-4 exhibits Ca\(^{2+}\)-dependent activity and whether it interacts with exocytic SNARE proteins.

Understanding the mechanisms by which accessory factors regulate SNARE protein function and vesicle exocytosis has been greatly advanced by in vitro studies. Several accessory factors (synaptotagmin-1, Doc2B, Munc18-1, and CAPS-1) bind SNARE proteins and promote lipid mixing in a SNARE-dependent liposome fusion assay (Tucker et al., 2004; Shen et al., 2007; James et al., 2009; Groffen et al., 2010). In the current work, we found that Munc13-4 exhibited C2A-dependent, Ca\(^{2+}\)-stimulated SNARE binding and C2B-dependent, Ca\(^{2+}\)-stimulated membrane binding. In an apparent coupling of membrane and SNARE binding, Munc13-4 promoted SNARE-dependent liposome fusion in a Ca\(^{2+}\)-dependent manner requiring putative Ca\(^{2+}\) binding residues in both C2A and C2B domains. Munc13-4 also promoted trans-SNARE complex formation in a Ca\(^{2+}\)-dependent manner. These results clarify the mechanism of action of Munc13-4, identify it as a Ca\(^{2+}\)-dependent membrane- and SNARE-binding protein, and suggest that Munc13-4 functions as a Ca\(^{2+}\) sensor at rate-limiting priming steps in vesicle exocytosis.

Results

Munc13-4 reconstitutes Ca\(^{2+}\)-triggered exocytosis in multiple cell types

Because Munc13-4 is widely expressed, our initial studies examined the activity of Munc13-4 in several cell model systems. We previously showed that Munc13-4 stimulates Ca\(^{2+}\)-triggered serotonin secretion in permeabilized platelets (Shirakawa et al., 2004). This and other assays were used to determine whether the activity of Munc13-4 was directly Ca\(^{2+}\) regulated. Both C2A and C2B domains of Munc13-4 contain aspartate residues that align with the Ca\(^{2+}\)-binding aspartate residues in the C2 domains of synaptotagmin I (Shao et al., 1998), which suggests that Munc13-4 could be a Ca\(^{2+}\)-binding protein. To compromise this potential Ca\(^{2+}\) binding, two aspartate residues in the loop 1 region (Fig. 1 B) were mutated to asparagine in either C2A (D127N and D133N) or C2B (D941N and D947N) domains or in both (D127N, D133N, D941N, and D947N). These proteins are termed C2A* Munc13-4, C2B* Munc13-4, or C2A*B* Munc13-4, respectively. Each of the proteins purified as a major band on SDS-PAGE at the expected 120-kD size (Fig. 1 A) and exhibited similar monomer plus dimer mobility on native gels (Fig. S1). Ca\(^{2+}\) binding to purified Munc13-4 proteins was measured by monitoring Ca\(^{2+}\)-induced changes in the intrinsic fluorescence of aromatic residues (Fig. 1 C). Whereas wild-type Munc13-4 exhibited Ca\(^{2+}\)-induced increases in emission with \(<\sim 5 \mu M [Ca^{2+}]_{\text{free}}, the C2A*, C2B*, and C2A*B* Munc13-4 proteins exhibited 10-fold greater [Ca\(^{2+}\)]\(_{1/2}\) values (Fig. 1 C). These results showed that Munc13-4 is a Ca\(^{2+}\)-binding protein and that asparagine for aspartate substitutions in the C2A or C2B domains reduce Ca\(^{2+}\) binding–induced increases in the intrinsic fluorescence of Munc13-4.

Permeabilized platelets retained \(<\sim 50%\) of their Munc13-4 (Shirakawa et al., 2004), and the Ca\(^{2+}\) stimulation of serotonin release was dependent on the retained Munc13-4 based on the strong inhibition \((>75%)\) by Munc13-4 antibody (Fig. S2). However, the addition of wild-type Munc13-4 further stimulated Ca\(^{2+}\)-triggered serotonin secretion from permeable platelets twofold (Fig. 1 D), but C2A* Munc13-4 and C2B* Munc13-4 each failed to do so. Similarly, C2A*B* Munc13-4 was inactive. The results indicate that the stimulation of Ca\(^{2+}\)-triggered serotonin secretion by Munc13-4 was dependent on the putative Ca\(^{2+}\)-binding residues in the C2A and C2B domains. Thus, Ca\(^{2+}\)-dependent dense granule exocytosis in platelets was highly dependent on Munc13-4, which functioned as a Ca\(^{2+}\)-binding protein.

These studies were extended to permeable RBL-2H3 mast cells, in which similar results were obtained (Fig. 1 E). RBL-2H3 cells lost \(>90%\) of their Munc13-4 upon permeabilization (Fig. 1 E, inset). Ca\(^{2+}\)-triggered degranulation was dependent on the addition of cytosol, but purified Munc13-4 substituted for this requirement. In contrast, C2A*B* Munc13-4 was inactive, as were the C2A* and C2B* Munc13-4 proteins (Fig. 1 E). We also assessed Munc13-4 function in permeable neuroendocrine PC12 cells (Fig. 1 F). Washed permeable cells lack Munc13-4 and other soluble priming factors such as CAPS-1. Munc13-4 addition to permeable PC12 cells stimulated Ca\(^{2+}\)-triggered catecholamine secretion with an efficacy similar to CAPS-1 (Fig. 1 F). In contrast, neither C2B* or C2A*B* Munc13-4 proteins were functional in the permeable PC12 cell assay, and C2A* Munc13-4 exhibited strongly reduced activity. Thus, in three cell types, Munc13-4 exhibited activity in Ca\(^{2+}\)-triggered exocytosis that was dependent on putative Ca\(^{2+}\)-binding residues in the C2A and C2B domains. In each assay, 1–10 \(\mu M\) Ca\(^{2+}\) was optimal for triggering granule exocytosis, indicating that Munc13-4 exhibits Ca\(^{2+}\)-dependent activity over a Ca\(^{2+}\) concentration range characteristic of stimulated cells.

These experiments suggested that Munc13-4 may interact with several distinct SNARE proteins. Syntaxin-1, -2, and -4 are the major plasma membrane SNARE proteins required for evoked granule exocytosis in PC12 cells, platelets, and RBL-2H3 mast cells. These studies were extended to permeable RBL-2H3 mast cells, which were used to examine the activity of Munc13-4 in several cell model systems. We previously showed that Munc13-4 stimulates Ca\(^{2+}\)-triggered serotonin secretion in permeabilized platelets (Shirakawa et al., 2004). These experiments suggested that Munc13-4 may interact with several distinct SNARE proteins. Syntaxin-1, -2, and -4 are the major plasma membrane SNARE proteins required for evoked granule exocytosis in PC12 cells, platelets, and RBL-2H3 mast cells.
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Munc13-4 binds the H3 domain of multiple syntaxin isoforms

Previous studies used assays with membrane-integrated SNARE proteins to reveal the SNARE-binding properties of CAPS-1 and Munc13-1 (Guan et al., 2008; Daily et al., 2010; Khodthong et al., 2011). Thus, we assessed Munc13-4 binding to membrane-integrated SNARE proteins by buoyant density flotation of liposomes (Fig. 2A). Phosphatidylcholine (PC)/phosphatidylserine (PS) liposomes reconstituted with different full-length syntaxin proteins were tested. Munc13-4 bound to liposomes containing syntaxin-1, -2, and -4 but only weakly to liposomes containing syntaxin-3 (Fig. 2B, left). Munc13-4 retention on PC/PS liposomes lacking protein was minimal under these conditions (Fig. 2B, right).

Syntaxin-1 contains multiple domains (Fig. 2C), including the N-terminal Habc domain, H3 domain/SNARE motif, and transmembrane domain. To determine which domain interacts with Munc13-4, we used surface plasmon resonance with His6-tagged Munc13-4 (or His6-tagged maltose-binding protein [MBP] as a control) immobilized on a Ni2+-nitrilotriacetic acid (NTA) chip with soluble syntaxin-1 domain proteins as analytes (Fig. 2D). Munc13-4 retained the H3 domain protein syntaxin-1 (191–266) but not the Habc domain protein syntaxin-1(1–177). Munc13-4 failed to bind the full cytoplasmic domain protein syntaxin-1(1–266), likely because the Habc domain is folded over the H3 domain in this protein (Fig. 2D). These results were consistent with the finding that the related CAPS-1 and Munc13-1 proteins bind the H3 but not the Habc domain of syntaxin-1 (Daily et al., 2010; Ma et al., 2011; unpublished data).

Additional studies showed that Munc13-4 interacts with the H3 domains of syntaxin-1 and -4 but not with the H3 domains of syntaxin-3, -5, or -6 (Fig. 2E). Collectively, the results indicate that Munc13-4 selectively interacts with exocytic syntaxins (syntaxin-1, -2, and -4) that are present in cell types in which Munc13-4 can regulate exocytosis.

Because syntaxin-11 mutations give rise to an FHL phenotype (FHL4) in CTLs similar to that caused by Munc13-4 mutations (FHL3), it was proposed that Munc13-4 utilizes syntaxin-11 as its effector for cytotoxic granule exocytosis (Bryceson et al., 2007). We found that Munc13-4 does interact with the H3 domain of syntaxin-11 (Fig. 2F). Pull-down studies confirmed that Munc13-4 binds to GST–syntaxin-11 H3 but not to GST (Fig. S3). Although syntaxin-11 is required for overall cytotoxic T cell activity, this protein localizes to late endosomes and the TGN rather than the plasma membrane (Valdez et al., 1999; Arneson et al., 2007). The Munc13-4 interactions with syntaxin-11 identified here may be involved in endosome fusion (Ménager et al., 2007).

C2 domains differentially mediate Ca2+-dependent SNARE and PS binding

Munc13-4 is a Ca2+-binding protein with two C2 domains that potentially mediate the Ca2+ binding (Fig. 1, B and C). The C2 domains of synaptotagmin-1 and related proteins exhibit Ca2+-stimulated binding to acidic phospholipids as well as to t-SNAREs (Pang and Südhof, 2010). We assessed Munc13-4 for similar properties. We initially found that Munc13-4 binding to syntaxin-1–containing liposomes was stimulated by Ca2+ (Fig. 2F).
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Subsequently, we conducted buoyant density flotation experiments to determine whether Ca\(^{2+}\) enhanced the binding of Munc13-4 to t-SNARE–containing liposomes composed of PC/PS. Ca\(^{2+}\) was found to stimulate the binding of Munc13-4 to t-SNARE–containing PC/PS liposomes as well as to protein-free PC/PS liposomes (Fig. 3 A). Stimulation by Ca\(^{2+}\) was observed at free ionic concentrations as low as 10 µM (unpublished data). The Ca\(^{2+}\)-dependent binding of Munc13-4 to protein-free liposomes was selective for PC/PS liposomes, whereas binding to PC liposomes was not observed in the absence or presence of Ca\(^{2+}\) (Fig. 3 B). However, Munc13-4 exhibited a consistent 2.5-fold increase in binding to t-SNAREs in PC liposomes with Ca\(^{2+}\) (Fig. 3 B). The results revealed two Ca\(^{2+}\)-regulated properties of Munc13-4. Binding to PS-containing membranes was highly dependent on Ca\(^{2+}\) (Fig. 3 A), whereas binding to t-SNAREs in PC liposomes occurred in the absence of Ca\(^{2+}\) but was stimulated by Ca\(^{2+}\) (Fig. 3 B).

It was likely that either one or both of the C2 domains mediated the Ca\(^{2+}\) regulation of these Munc13-4 interactions. Therefore, we tested C2 domain mutant proteins for Ca\(^{2+}\)-dependent PC/PS liposome binding or for Ca\(^{2+}\)-stimulated t-SNARE binding in PC liposomes. Mutations in C2A or C2B had little effect on the binding of Munc13-4 to PC/PS liposomes in the absence of Ca\(^{2+}\) (Fig. 3 C). In contrast, C2B mutations abrogated the Ca\(^{2+}\)-dependent interaction of Munc13-4 with PC/PS liposomes in the presence of Ca\(^{2+}\) (Fig. 3 C). Binding of WT and C2A* Munc13-4 to PC/PS liposomes in the presence of Ca\(^{2+}\) was not significantly different (D). Binding of wild-type, C2A*, C2B*, and C2A*B* Munc13-4 proteins to t-SNARE-containing PC liposomes. Munc13-4 binding was normalized to syntaxin-1 (Stx1) content of liposomes. Ca\(^{2+}\)-stimulated binding of C2A* and C2A*B* Munc13-4 proteins was significantly reduced compared with wild type (*, P < 0.05; **, P < 0.002), whereas binding by C2B* Munc13-4 was not significantly different based on an unpaired t test.

In contrast, experiments in Munc13-4 binding to t-SNARE-containing liposomes in PC revealed a distinctly different basis for Ca\(^{2+}\) regulation. The stimulation of t-SNARE binding by Ca\(^{2+}\) was similar for wild-type and C2B* Munc13-4 but was largely eliminated for C2A* and C2A*B* Munc13-4 proteins (Fig. 3 D). These experiments revealed a preferential role for C2A in the Ca\(^{2+}\)-regulated interaction of Munc13-4 with t-SNARE proteins. Overall, these results demonstrated that the Ca\(^{2+}\) regulation of membrane binding and SNARE protein binding by Munc13-4 resides with distinct C2 domains.
Figure 4. Munc13-4 promotes liposome fusion in a calcium-dependent manner. (A) VAMP-2–containing PC/PS donor liposomes (with NBD-PE and Rh-PE) and t-SNARE–containing PC/PS acceptor liposomes were incubated with 1 µM Munc13-4 in the presence of EGTA or 400 µM free calcium (+Ca²⁺), as indicated. Control reactions with protein-free (Pf) liposomes replacing t-SNARE liposomes were incubated in parallel for background subtraction. Fusion was detected as increased NBD-PE fluorescence relative to maximal dequenched values. (B) Incubations similar to those of A were conducted at 100 µM Ca²⁺ at the indicated Munc13-4 concentrations (left), and initial fusion rates were determined (right). v + t, reactions with v-SNARE–containing liposomes plus t-SNARE–containing liposomes. (C) Incubations similar to those of A were conducted with 1 µM Munc13-4 at the indicated free Ca²⁺ concentrations (left), and initial fusion rates were determined (right). The EC₅₀ for Ca²⁺ was calculated to be 23 ± 4 µM. In all graphs, the curves represent single samples. Results shown are representative of two to four similar experiments.

Munc13-4 promotes SNARE-dependent liposome fusion

Previous studies showed that CAPS-1 promotes SNARE-dependent liposome fusion by using membrane interactions mediated by its Pleckstrin homology domain coupled with SNARE protein interactions mediated through its MHD1 domain (James et al., 2009; Khodthong et al., 2011). Munc13-4 contains a C2B domain that mediates membrane interactions as well as an MHD1/MHD2 region that could mediate SNARE binding (Fig. 1 A). Therefore, we determined whether the membrane- and SNARE-binding properties of Munc13-4 would enable it to promote SNARE-dependent liposome fusion. With VAMP-2 donor liposomes and syntaxin-1/SNAP-25 acceptor liposomes, Munc13-4 robustly stimulated the initial rate and final extent of lipid mixing but only in the presence of Ca²⁺ (Fig. 4 A). The Munc13-4 stimulation of lipid mixing was specific for Ca²⁺, whereas Mg²⁺, Ba²⁺, and Sr²⁺ failed to substitute (Fig. S4). Lipid mixing in this assay was previously characterized as full fusion based on contents mixing (James et al., 2009). Rates of fusion exhibited a concentration dependence for Munc13-4 in the presence of Ca²⁺ (Fig. 4 B) and were very similar to those obtained with CAPS-1 in the absence of Ca²⁺ (not depicted). The Ca²⁺ dependence for Munc13-4 stimulation of liposome fusion exhibited a half-maximal effective concentration (EC₅₀) of ~23 µM (Fig. 4 C). The Munc13-4 and Ca²⁺ concentrations needed to promote liposome fusion were higher than those required for function in cells (see Fig. 1), but this minimal fusion assay lacks proteins that may contribute to Munc13-4 localization and activity (e.g., Rab27).

Ca²⁺-stimulated liposome fusion with Munc13-4 was entirely dependent on SNARE proteins in the donor and acceptor liposomes (Fig. 4, A–C) and was strongly inhibited by the addition of a soluble syntaxin-1 protein (Fig. S5). Because previous binding experiments (Fig. 2 D) showed that Munc13-4 interacted with the H3 rather than the N-terminal Habc domain of syntaxin, we prepared acceptor liposomes reconstituted with an N-terminal–truncated syntaxin-1(191–288) plus SNAP-25 to test with Munc13-4 in liposome fusion. Munc13-4 was found to promote SNARE-dependent liposome fusion with H3/SNAP-25 liposomes in the presence of Ca²⁺ to a similar extent as with full-length syntaxin-1/SNAP-25 liposomes (Fig. 5 A). The results indicated that Munc13-4 interactions with the H3 domain of syntaxin-1 are sufficient for the function of Munc13-4 in SNARE-dependent liposome fusion.
Both C2 domains are required for Ca\textsuperscript{2+}-dependent SNARE-mediated liposome fusion

C2A and C2B domain mutations in Munc13-4 preferentially affected Ca\textsuperscript{2+}-dependent SNARE and PS binding, respectively (Fig. 3). We determined whether both of the C2 domains were essential for the Ca\textsuperscript{2+}-dependent Munc13-4 stimulation of SNARE-dependent liposome fusion. In the absence of Ca\textsuperscript{2+}, all Munc13-4 proteins failed to promote liposome fusion (Fig. 5 B). In the presence of Ca\textsuperscript{2+}, wild-type Munc13-4 promoted liposome fusion, whereas C2B\textsuperscript{*} Munc13-4 was completely inactive. This result suggested that Ca\textsuperscript{2+}-dependent C2B domain binding to PS is required for Munc13-4 activity in liposome fusion. C2A\textsuperscript{*} Munc13-4 exhibited partial but impaired activity in liposome fusion (Fig. 5 B). This result corresponds to the binding experiments (Fig. 3 D), showing a partial decrease in SNARE binding by C2A\textsuperscript{*} Munc13-4. As expected, the C2A\textsuperscript{B\textsuperscript{*}} Munc13-4 mutant was completely inactive in Ca\textsuperscript{2+}-dependent liposome fusion (Fig. 5 B). Collectively, the results indicate important roles for the putative Ca\textsuperscript{2+}-binding residues in both C2 domains in Munc13-4. They suggest that Ca\textsuperscript{2+}-stimulated SNARE binding and Ca\textsuperscript{2+}-dependent membrane binding mediated by C2A and C2B domains, respectively, are integrated for the activity of Munc13-4 in SNARE-dependent liposome fusion.

Munc13-4 promotes trans-SNARE complex assembly

CAPS-1 and Munc13-1 function in secretory granule priming steps in neural and endocrine cells (Augustin et al., 1999; Jockusch et al., 2007; Liu et al., 2008). In CTLs, Munc13-4 loss of function was associated with cytotoxic granules that docked at the immunological synapse but did not fuse, suggesting a role in granule priming (Feldmann et al., 2003). The ability of Munc13-4 to promote SNARE-dependent liposome fusion indicates that Munc13-4 can drive trans-SNARE complex assembly. We sought to confirm this prediction in two approaches to detect trans-SNARE complex formation in the absence of fusion. In the first of these, Munc13-4 was incubated with donor v-SNARE and acceptor t-SNARE liposomes on ice to arrest membrane fusion. SNARE protein complexes were detected as SDS-resistant complexes. Munc13-4 was found to promote the formation of \(\sim 70-\) and \(\sim 110\)-kD SNARE complexes (Fig. 6 A). In the presence of Ca\textsuperscript{2+}, Munc13-4 promoted the formation of \(\sim 165\)-kD SNARE complexes (Fig. 6 A). The \(\sim 165\)-kD SNARE protein complex formed by CAPS-1 on liposomes was previously characterized as a dimer of heterotrimeric SNARE complexes (James et al., 2009).

In a second approach, we determined whether v-SNARE (VAMP-2 containing) liposomes would form stable trans-SNARE complexes with t-SNAREs incorporated in a supported bilayer membrane. We found that few v-SNARE liposomes stably dock onto the t-SNARE–containing supported bilayer even when Ca\textsuperscript{2+} is provided (Fig. 6 B, a). In contrast, the addition of Munc13-4 with Ca\textsuperscript{2+} led to a substantial increase in the stable docking of v-SNARE liposomes onto the bilayer (Fig. 6 B, b). The Munc13-4 stimulation of v-SNARE liposome docking was dependent on Ca\textsuperscript{2+} rather than Mg\textsuperscript{2+} and on the presence of SNAREs in both membranes (Fig. 6 B, d and f). Moreover, the addition of an excess of a soluble syntaxin fragment to block trans-SNARE complex formation was found to fully inhibit v-SNARE liposome docking (Fig. 6 B, c). The results indicate that Munc13-4 promotes trans-SNARE complex formation in a Ca\textsuperscript{2+}-dependent manner.

Discussion

Munc13-4 plays a role in the regulated exocytosis of secretory granules, especially in cells of hematopoietic origin, but little was known about how it functions. The current work revealed several properties of Munc13-4 that are likely central to its function in regulated secretory pathways. Key properties of Munc13-4 revealed by this work include the Ca\textsuperscript{2+}-dependent activity for restoring granule exocytosis to permeable cells that use distinct syntaxins (syntaxin-1, -2, and -4), the ability to directly interact with several exocytic syntaxins (syntaxin-1, -2, and -4), interactions with SNARE proteins regulated by Ca\textsuperscript{2+} and the C2A domain of Munc13-4, binding to acidic phospholipid–containing
Munc13-4 stimulates the formation of SDS-resistant SNARE complexes. Incubations with VAMP-2– and syntaxin-1/SNAP-25–containing liposomes were conducted on ice for 30 min with 100 µM Ca\(^{2+}\) and 1 µM Munc13-4, as indicated. Reactions terminated in SDS sample buffer without boiling were analyzed by immunoblotting with monoclonal HPC-1 syntaxin-1 antibody. The arrows indicate the positions of ∼70-kD, ∼120-kD, and ∼165-kD SNARE complexes. Similar experiments indicated that VAMP-2 and SNAP-25 comigrated with these complexes. See James et al. (2009) for characterization of SNARE protein complexes. (B) Munc13-3-4 stimulates the docking of VAMP-2 liposomes onto t-SNARE-containing supported bilayer membranes. VAMP-2 liposomes with Rh-PE were incubated with t-SNARE–containing supported bilayers, and the number of liposomes stably docked for ≥2 min was monitored by total internal reflection fluorescence microscopy. Bar, 5 µm. Incubations contained 1 µM Munc13-3-4, 200 µM Ca\(^{2+}\), or 200 µM Mg\(^{2+}\), as indicated. Munc13-4–promoted liposome docking in the presence of Ca\(^{2+}\) (b) but not in its absence (c) or with Mg\(^{2+}\) (f), nor was liposome docking promoted by Ca\(^{2+}\) alone (a). t-SNAREs were required for Munc13-4–promoted docking (d), whereas preincubations of t-SNARE bilayers with 8 µM soluble syntaxin(1–266) (sol. syx.) for 30 min (e) inhibited Munc13-4–promoted docking. Mean values ± SD (n = 3) are shown.

Figure 6. Munc13-4 stimulation of trans-SNARE complex formation is Ca\(^{2+}\)-dependent. (A) Munc13-4 stimulates the formation of SDS-resistant SNARE complexes. The protein contains two C2 domains predicted in its absence (c) or with Mg\(^{2+}\) (f), but this was reported to be Ca\(^{2+}\) independent (Pivot-Pajot et al., 2008). The potential importance of C2 domains in Munc13-4 function was also suggested by the identification of missense mutations in Munc13-4 from FHL3 individuals in C2A and C2B domains, but whether these affected Ca\(^{2+}\)-dependent function was not investigated (Sieni et al., 2011).

The C2A and C2B domains in Munc13-4 both contain canonical Ca\(^{2+}\)-binding aspartate residues (Fig. 1 A) and exhibit significant homology (∼23% identity and 42% similarity) to the second (C2B) C2 domain in Munc13-1. To determine the functional significance of Ca\(^{2+}\) binding to Munc13-4, we neutralized two aspartates in the first loop region of each C2 domain to asparagine to generate potential loss of Ca\(^{2+}\) binding similar to that for other Ca\(^{2+}\)-binding C2 domains including Munc13-1 C2B (Shin et al., 2010). We found that all Ca\(^{2+}\)-dependent properties of Munc13-4 (enhanced intrinsic fluorescence, stimulation of SNARE protein binding, membrane association, and liposome fusion) were eliminated in the C2A*B*C Munc13-4 protein. Importantly, the restoration of Ca\(^{2+}\)-dependent exocytosis by Munc13-4 in permeable platelets, mast cells, and neuroendocrine cells was abolished by mutation of the putative Ca\(^{2+}\)-binding residues in the C2 domains. The results provide strong evidence that Munc13-4 is a Ca\(^{2+}\)-dependent protein for its function in granule exocytosis. Moreover, the results indicate that Munc13-4 is a Ca\(^{2+}\) sensor for a rate-limiting step in granule exocytosis in the permeable cells.

In double C2 domain proteins in which the C2 domains are tandem, such as in synaptotagmin-1, the Ca\(^{2+}\)-bound domains interact to affect the overall activity of the protein (Pang and Südhof, 2010). In contrast, the nontandem C2A and C2B domains of Munc13-4 exhibit seemingly independent roles in regulation, but these distinct roles appear to be integrated in the overall Ca\(^{2+}\)-dependent function of the protein. Thus, putative Ca\(^{2+}\) ligand mutations in C2B but not C2A exerted a strong loss of Ca\(^{2+}\)-dependent phospholipid binding. In contrast, the corresponding mutations in the C2A domain mainly affected Ca\(^{2+}\)-stimulated SNARE interactions. However, in promoting SNARE-dependent liposome fusion, both C2 domains were required. This indicates that Munc13-4 activity in the liposome fusion assay requires the integration of Ca\(^{2+}\)-dependent membrane binding (C2B mediated) with Ca\(^{2+}\)-regulated SNARE binding (C2A mediated). A similar integrated function of both C2 domains is required for Munc13-4 activity in Ca\(^{2+}\)-dependent granule exocytosis, in which putative Ca\(^{2+}\) ligand mutations in either C2 domain largely abrogated activity.

In neural and endocrine cells, a subset of docked secretory vesicles is primed for rapid exocytosis. The Ca\(^{2+}\)-triggered fusion of primed vesicles is mediated by double C2 domain proteins such as synaptotagmins and Doc2 (Pang and Südhof, 2010). However, other Ca\(^{2+}\)-dependent regulators such as Munc13-1
function upstream to control vesicle priming reactions (Augustin et al., 1999). Syntaxotagmin-2 in mast cells or syntaxotagmin-7 in CTLs is proposed to function as a Ca\(^{2+}\) sensor for the final step of Ca\(^{2+}\)-triggered granule exocytosis (Fowler et al., 2007; Melicoff et al., 2009). However, granules in these cells may not be docked or primed in advance of stimulation and would need to undergo translocation, docking, and priming after cell activation (Pores-Fernando and Zweifach, 2009). The characterization of Munc13-4 as a Ca\(^{2+}\)-dependent protein suggests that it functions as an important Ca\(^{2+}\) sensor in secretory pathways where secretory granules undergo priming after stimulation.

**Role of Munc13-4 in SNARE complex assembly**

Extensive molecular genetic studies in hemophagocytic lymphohistiocytic disorders have led to a proposed cascade of events for the docking, priming, and fusion of cytotoxic granules in CTLs (Pachlopnik Schmid et al., 2010). A subset of the proposed protein–protein interactions has been confirmed biochemically. Rab27a (mutated in Griscelli syndrome) is proposed to localize Munc13-4 (mutated in FHL3) in tethering for cytotoxic granule exocytosis (Elstak et al., 2011), and direct Rab27a–Munc13-4 interactions have been reported (Shirakawa et al., 2004; Neef et al., 2005). Munc18-2 (mutated in FHL5), potentially acting in concert with Munc13-4, is proposed to activate SNARE function by binding to a syntaxin protein. Syntaxin-11 was identified as a loss-of-function allele in FHL4 and has been shown to directly interact with Munc18-2 (zur Stadt et al., 2009) in addition to previously described interactions of Munc18-2 with syntaxin-2 and -3. Our experiments documented for the first time that Munc13-4 directly interacts with syntaxin-11 but also with syntaxin-1, -2, and -4. The role of syntaxin-11 in cytotoxic granule exocytosis by itself is uncertain, and it may function in the fusion of endosomes that participate in the multistep maturation of cytotoxic granules in CTLs after activation (Pachlopnik Schmid et al., 2010). The participation of Munc13-4 in these maturation steps upstream from cytotoxic granule priming (Ménager et al., 2007) suggests that Munc13-4–syntaxin-11 interactions could be important at these earlier steps.

The current work showed that Munc13-4 interacts directly with a broad range of plasma membrane exocytic syntaxins. In our assays, Munc13-4 bound to syntaxin-1, -2, and -4 but exhibited reduced binding to syntaxin-3. We failed to detect binding to the ER syntaxin-5 or to the TGN syntaxin-6. This specificity of binding to exocytic syntaxins was consistent with the ability of Munc13-4 to restore Ca\(^{2+}\)-regulated granule exocytosis to a range of permeable cell types that use syntaxin-1, -2, or -4. The interaction of Munc13-4 with syntaxin-1 was mediated through binding to its H3 SNARE domain rather than to the N-terminal Habc domain. CAPS-1 and Munc13-1 also interact with the syntaxin-1 H3 domain (Ma et al., 2011; unpublished data). A mode of interaction directly with helical SNARE motifs suggests that priming factors in the CAPS/Munc13 family act to stabilize SNARE complexes through direct interactions on the surface of SNARE helical bundles. For Munc13-4 in the current study (Fig. 5) and previously for CAPS-1 (Daily et al., 2010), interactions with the H3 domain of syntaxin-1 appeared to be sufficient to mediate the stimulation of SNARE-dependent liposome fusion.

For several double C2 domain proteins such as syntaxotagmins and Doc2, direct Ca\(^{2+}\)-dependent interactions of their C2 domains with SNARE proteins mediate the regulation of membrane fusion (Lynch et al., 2007; Groffen et al., 2010). In contrast, SNARE binding by CAPS-1 and Munc13-1 is mediated by MHD1- and MHD1/MHD2-containing regions of the proteins, respectively (Guan et al., 2008; Khodthong et al., 2011). The possibility that the MHD1/MHD2 region of Munc13-4 mediates SNARE binding remains to be directly assessed. MHD1 is required for Munc13-4 function based on missense mutations that have been characterized in FHL3 patients (Santoro et al., 2006). If the MHD1-containing region mediates SNARE binding in Munc13-4, the regulation of SNARE binding by Ca\(^{2+}\) may be through intramolecular C2A–MHD1 interactions. Ca\(^{2+}\) binding to C2A may reverse inhibitory interactions with MHD1 that limit SNARE binding. Alternatively, MHD1 might mediate Ca\(^{2+}\)-independent SNARE binding, as in CAPS-1, whereas the C2A domain could mediate Ca\(^{2+}\)-dependent SNARE binding, as in syntaxotagmin or Doc2. Munc13-4 C2A contains a predicted helical region in the putative Ca\(^{2+}\)-binding loop 3 similar to that in Munc13-1 C2B (Shin et al., 2010) as well as a unique B3–B4 loop insertion. Potentially, either of these insertions could participate in intramolecular regulation of SNARE binding by MHD1 or direct SNARE binding. Additional studies will be needed to determine the basis for Ca\(^{2+}\)-independent and Ca\(^{2+}\)-dependent SNARE binding by Munc13-4.

The properties of Munc13-4 revealed by these studies contribute to understanding the general features of the CAPS/Munc13 proteins for promoting vesicle priming and SNARE complex assembly. Separate domains in these proteins mediate membrane binding and SNARE protein binding, which are integrated for overall function to promote SNARE complex formation on membranes. Munc13-4 is the only priming protein in the CAPS/Munc13 family in which membrane binding and SNARE binding are each Ca\(^{2+}\) regulated. C2B may mediate direct Ca\(^{2+}\)-dependent membrane association, whereas C2A confers Ca\(^{2+}\) regulation on SNARE binding. The concerted regulation by C2A and C2B provides a strongly Ca\(^{2+}\)-dependent activity for Munc13-4 that would function as a Ca\(^{2+}\) sensor in secretory pathways where priming occurs after stimulation.

**Materials and methods**

**Materials**

1-palmitoyl, 2-oleoyl PC, 1,2-dioleoyl PS, 1,2-dioleoyl phosphatidylethanolamine (PE), NBD-PE, and N-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl PE (Rh-PE) were purchased from Avanti Polar Lipids, Inc. 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylrhodaminecarboxyanine 4-chlorobenzene-sulfonate (DiD) were purchased from Invitrogen. Accudenz was purchased from Accurate Chemical. Rabbit polyclonal antibodies generated against Munc13-4(1–262) or full-length Munc13-4 were produced as previously described (Shirakawa et al., 2004; Murata et al., 2011), and the Munc13-4 (967–980) antibody was obtained from Novus Biologicals.

**Plasmids and recombinant proteins**

Plasmid constructs pTW34 to express rat syntaxin-1A with N-terminally His-tagged mouse SNAP-25B and pTW2 to express C-terminal His-tagged
mouse VAMP-2 were provided by J.E. Rothman (Yale University, New Haven, CT) and T. Weber (Mount Sinai School of Medicine, New York, NY), and proteins were purified, as previously described (Weber et al., 1998), by Ni-NTA (QIAGEN) chromatography. pGEX-KG to express syntaxin-1A(191–266) and purified on glutathione Sepharose (GE Healthcare). PET28a to express Cterminally His-tagged syntaxin-1A(183–288) was provided by E.R. Chapman (University of Wisconsin, Madison, WI). Expressed syntaxin-1A(183–288) was purified by Ni-NTA (QIAGEN) chromatography. His-tagged human Munc13-4 protein produced in insect Sf9 cells was purified on Ni-NTA agarose (QIAGEN) and further purified by Mono Q anion exchange chromatography (GE Healthcare), as previously described (Shirakawa et al., 2004).

Preparation of proteoliposomes

Liposomes consisted of 85% PC and 15% PS (termed PC/PS) or 100% PC. For the collofattion assay, dried lipids were hydrated in 25 mM Hepes, pH 7.4, 100 mM KCl, 1% glycercoside, and 1 mM DTT (reconstitution buffer) and were forced through a 100-nm pore-sized filter using a MiniExtruder (Avanti Polar Lipids, Inc.). For syntaxin and tSNARE liposomes, 100 µl of exuded liposomes was mixed with 200 µl of protein in 25 mM Hepes, pH 7.4, 400 mM KCl, 10% glycercoside, 1% p-cholesterol, and 2 mM β-mercaptoethanol for 30 min, diluted twofold with reconstitution buffer, and then divided overnight against reconstitution buffer containing Bio-Beads (Bio-Rad Laboratories). The liposomes were purified by buoyant density centrifugation on an Accudenz step gradient (3 ml at 40%, 2 ml at 30%, and 0.5 ml at 0% in reconstitution buffer) at 45,000 rpm for 4 h in an SW50.1 rotor (Beckman Coulter). For the lipid-mixing assay, tSNARE PC/PS liposomes and VAMP-2 liposomes were prepared as previously described (Weber et al., 2003). In brief, proteoliposomes were formed by comicellization in the presence of either VAMP-2 or coexpressed syntaxin-1A and SNAP-25, as previously described (Scott et al., 2003). 167 µg SNAP-25/syntaxin-1 in elution buffer (25 mM Hepes-KOH, pH 7.4, 100 mM KCl, 500 mM imidazole-OAc, pH 7.4, and 1.0% p-cholesterol) was used to resuspend a lipid film containing 1.5 µmol PC/PS in an 85:15 mol ratio to generate liposomes that contained ~40 copies of each SNARE. For fluorescent donor liposomes, ~100 copies of VAMP-2 per liposome were incorporated with a lipid mixture of PC/PS/Rh-PE/NBD-PE at an 82:15:1:1.5 mol ratio. Lipid mixtures were spiked with 2 µl (H2O)1.2-dipalmitoyl PC ([2–x] 109 cpm/ml, DuPont) to determine lipid recoveries and to standardize fusion reactions. In some experiments (e.g., Fig. 5 A), we used alternative fluorescent lipid labeling approaches to study the effects of non-SNARE-dependent Munc13-4 (Smith et al., 2011). VAMP-2 liposomes (100 copies/liposome) composed of PC, PS, and Dl at a 76:12:10:2.2 mol ratio and tSNARE liposomes with ~40 copies of syntaxin-1A/SNAP-25 or H3(183–288)/SNAP-25 in PC, PS, DP, and Dil lipid mixtures at a 90:3:2 mol ratio were made by comi-
cellization. The Dil/Dn/DND/Rh assays were performed as previously described (Weber et al., 2003). Proteoliposomes with PS/PC were typically ~30 ± 13 nm, as measured by dynamic light scatter-
ing in an NS1 submicrometer particle size analyzer (Beckman Coulter). Lipo-
somes were flash frozen in liquid nitrogen and stored at ~80°C.

Coflotation assay

Liposome coflotation assays were performed as previously described (Weber et al., 1998), with modifications. Liposomes were incubated with Munc13-4 for 30 min at room temperature in 75 µl of reconstitution buffer. Binding re-
tentions to test for Ca2+ dependence, a standard 3 min Munc13-4 binding to tSNAREs was performed in the absence of free Ca2+. The absence of free Ca2+ (0.2 mM EGTA) or presence of the indicated concentrations of free Ca2+ (0.2 mM EGTA) or presence of the indicated concentrations of free Ca2+. 75 µl of 80% Accudenz was added to the binding reaction to yield a final concentration of 40% Accudenz. 30% Accudenz and reconstitution buffers were then layered on top and centrifuged for 4 h in an SW50.1 rotor at 45,000 rpm. Either the floated fraction or all fractions were collected and run on SDS-PAGE and analyzed by Western blotting or SYPRO ruby staining (ImageQuant software (GE Healthcare) was used to quantify protein band.

Lipid-mixing fusion assay

The lipid-mixing assay between fluorescent donor liposomes and nonflu-
orescent acceptor liposomes was performed as previously described (Weber et al., 1998), with the following modifications. Lipid mixing was reported by the loss of fluorescence resonance energy transfer between fluorescent lipids (NBD-PE and Rh-PE) concentrated in the VAMP-2 liposomes that occurs upon fusion with the nonfluorescent syntaxin-1A/SNAP-25 liposomes. The standard assay used 0.45 mM of acceptor and 0.225 mM of donor lipo-
somes in a total volume of 75 µl of reconstitution buffer without glycerol supplemented with 0.1 mM EGTA. Munc13-4 protein was added at the concentrations indicated in the figure legends. For the stimulation of Munc13-4 acceleration of SNARE-dependent lipid fusion, calcium was added at concentrations, as specified in the figure legends. Blank re-
actions were performed for each condition by substituting syntaxin-1A/SNAP-25 for the acceptor liposomes with protein-free liposomes to detect non-SNARE- mediated lipid mixing. Reactions were assembled on ice and mixed before addition to 96-well Fluraoreuroplate. Lipid mixing was detected by mea-
suring dequenching of NBD fluorescence (excited at 460-nm emission at 538 nm) every 90 s (shaken before each reading) for 2 h at 35°C in a SPECTRAMAX GEMINI-XS fluorospectrometer (Molecular Devices). At the end of 2 h, fusion reactions were solubilized with 0.5% wt/vol dodecyl-
 maltoside, and fluorescence readings were recorded for an additional 10 min. Quantification of lipid mixing is expressed as a percentage of a maximal fluorescent signal determined by detergent solubilization normal-
ized by fluorescence of parallel reactions with protein-free liposomes sub-
stitution of SNAP-25. SNAP-25/syntaxin-1A/SNAP-25 liposomes were used as a control. All lipids were purchased from Avanti Polar Lipids (Alaska) unless otherwise noted.

Surface plasmon resonance

Equimolar amounts of His-tagged MBP and Histagged Munc13-4 were immobilized on separate flow cells of an Ni-NTA surface plasmon resonance chip (GE Healthcare). The analyte proteins (20 µM) were flowed over the surface of the chip in reconstitution buffer containing 10 mM imid-
azo1e using the Biacore 2000 instrument (GE Healthcare). Protein fluorescence of native aromatic residues was measured in purified samples using the Biacore 2000 instrument (GE Healthcare) and analyzed using the same buffer and liposome concentrations as in the aforemen-
tioned standard assay. Munc13-4 protein and Ca2+ were added at the concentrations indicated in the figure legends. Lipid mixing was observed as an increase in fluorescence resonance energy transfer from Dil to Dil ID labels by measuring Dil (acceptor) fluorescence at 700 ± 5 nm during Dil (donor) excitation at 514 ± 5 nm every 90 s over 2 h at 35°C using the SPECTRAMAX GEMINI-XS fluorospectrometer.

Liposome docking assay

Supporting bilayers were created on glass slides cleaned by plasma argon sputtering for 3 min followed by overnight soaking in NANO-STRIP buffer (Cyanet Inc.). Slides were rinsed with an excess of 0.2 µm of filtered Milli-Q water (Millipore) and assembled into Sykes-Moore chambers (BELCO GLASS). 6 µl of tSNARE liposomes [1:1 syntaxin1a/SNAP-25 at a lipid/ protein ratio of 900, 5% PIP2, and 93.5% PC] labeled with 1.5% dansyl-
peptide 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[3-dimethylamino-
1-naphthalenesulfonyl] was incubated on the glass for 1 min at 37°C and washed with 5 ml of buffer (25 mM Hepes and 100 mM KCl, pH 7.4). Lipid recovery was confirmed for each bilayer by monitoring fluorescence recovery of dansyl-peptide after photobleaching. tSNARE or protein-free liposo-
es [85:15.5% mol PC/PS] labeled with 1.5% mol Rh-PE diluted to 0.8 µM in reconstitution buffer were added to tSNARE-containing planar-
monolayers with a density of 1000 liposomes as indicated, and incubated at room temperature. Bilayers were imaged at room temperature on an inverted microscope (TE2000-U; Nikon) using an Apo total internal reflection fluo-
rescein 100X NA 1.45 objective lens. Images were acquired at 250-ms intervals with an EM charge-coupled device camera (Evolve, Photometrics). Image analysis used MetaMorph software (Universal Imaging).

Intrinsic protein fluorescence

Protein fluorescence of native aromatic residues was measured in purified NBD-labeled wild-type, C2A*, C2B*, and C2A*B Munc13-4 proteins. Fluores-
creoence measurements with 2 µM Munc13-4 proteins were obtained on an Infinite M1000PRO fluorospectrometer (Tescan Group Ltd.) in 0.02 M Hepes, pH 7.4, 0.1 M NaCl, and 0.00005 M EGTA. Ca2+ titrations were performed with a buffered stock solution of CaCl2, and free Ca2+ concen-
trations were determined using WEBMVCX (created by C. Patton, Stanford University, Stanford, CA).

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GST pulldown

Binding studies were conducted in 100-µl reactions containing reconstitution buffer, 1% Triton X-100, and 1% cold fish skin gelatin (Sigma-Aldrich). GST and GST-syntoxin-11 H3 domain were bound to glutathione agarose beads overnight at 4°C. 1 µM Munc13-4 was incubated with 1 µM GST or GST-syntoxin-11 H3 domain immobilized on glutathione Sepharose beads. After a 2-h incubation at 4°C, beads were recovered by centrifugation, washed, eluted in sample buffer, and analyzed by SDS-PAGE and Western blotting. GST and GST-syntoxin-11 H3 domain were detected with GST antibody (Sigma-Aldrich), and Munc13-4 was detected with Munc13-4(1–262) antibody and Munc13-4(967–980) antibod

(Fig. S5 shows that Munc13-4 stimulation of liposome fusion is dependent on

Fig. S3 shows pulldown of Munc13-4 binding to GST–syntaxin-11.

Online supplemental material

This research was supported by the National Institutes of Health grant DKO26452 (to T.F. Martin), American Heart Association fellowships (to T.F. Martin). This research was supported by the National Institutes of Health training grant DK025861 (to T.F.J. Martin), American Heart Association fellowships (to T.F. Martin).

Submitted: 28 September 2011
Accepted: 20 March 2012

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