Syntaxin 6 and Vti1b Form a Novel SNARE Complex, Which Is Up-regulated in Activated Macrophages to Facilitate Exocytosis of Tumor Necrosis Factor-α*

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A key function of activated macrophages is to secrete proinflammatory cytokines such as TNFα; however, the intracellular pathway and machinery responsible for cytokine trafficking and secretion is largely undefined. Here we show that individual SNARE proteins involved in vesicle docking and fusion are regulated at both gene and protein expression upon stimulation with the bacterial cell wall component lipopolysaccharide. Focusing on two intracellular SNARE proteins, Vti1b and syntaxin 6 (Stx6), we show that they are up-regulated in conjunction with increasing cytokine secretion in activated macrophages and that their levels are selectively titrated to accommodate the volume and timing of post-Golgi cytokine trafficking. In macrophages, Vti1b and syntaxin 6 are localized on intracellular membranes and are present on isolated Golgi membranes and on Golgi-derived TNFα vesicles budded in vitro. By immunoprecipitation, we find that Vti1b and syntaxin 6 interact to form a novel intracellular Q-SNARE complex. Functional studies using overexpression of full-length and truncated proteins show that both Vti1b and syntaxin 6 function and have rate-limiting roles in TNFα trafficking and secretion. This study shows how macrophages have uniquely adapted a novel Golgi-associated SNARE complex to accommodate their requirement for increased cytokine secretion.

A primary function of activated macrophages is the rapid and abundant secretion of proinflammatory cytokines such as TNFα.1 This secretion is essential to mount a successful inflammatory response but is equally the cause of severe clinical problems in acute and chronic inflammatory disease (1). The bacterial cell wall component LPS activates macrophages, eliciting the rapid synthesis of TNFα as a type II membrane precursor and subsequent proteolytic cleavage for secretion of the active cytokine (2–4). Newly synthesized TNFα precursors accumulate in the Golgi complex (5, 6); thereafter, little is known about the secretory pathway, the specific trafficking machinery or mechanisms that ensure the rapid delivery of cytokine to the cell surface for release.

SNAREs are key regulators in all fusion events occurring in the secretory pathway (7). Vesicle docking and fusion requires a specific R-SNARE on the vesicle to bind to two or three unique receptor target molecules, the Q-SNAREs, on the target membrane to form a trans-complex, thus pulling the membranes in close proximity and overcoming the energy barrier for fusion (8, 9). Four different conserved SNARE motifs (Qa, Qb, Qc and R) form an extended four-helix bundle and are sufficient for complex formation. Distinct complexes of SNARE family proteins are required to mediate vesicle docking and fusion on different membranes and at different vesicle trafficking steps (7, 10). The best characterized of the SNARE complexes functions in the fusion of synaptic vesicles at the plasma membrane. Intracellular SNARE complexes tend to be less well defined for a number of reasons; SNARES can participate in multiple fusion steps throughout the cell and as such can interact with different sets of SNARE partners. Additionally, differences in SNARE complexes and localization can exist between cell types.

Syntaxin 6 is a promiscuous SNARE partner, implicated in multiple complexes and associated with functions in both exocytic and endocytic pathways (11). It typically resides on membranes and vesicles in and around the trans-Golgi network (TGN). Vti1b is known in a much more restricted context, that of a single SNARE complex on late endosomes, where it has been implicated in late endosome homotypic fusion and in late endosome-lysosome fusion (12, 13). There is also evidence that it interacts with EpsinR, a protein involved in exocytic trafficking (14, 15).

A number of SNARE proteins have been identified in macrophages (16–18). VAMP3 has been shown to function in the formation of phagosomes (19), and we have shown that the Q-SNARE complex of syntaxin 4 and SNAP23, along with the sec1/Munc18 family (SM) protein Munc18c, functions at the plasma membrane and is required for TNFα secretion in macrophages (20). The majority of the molecules involved in regulating vesicle budding from the Golgi and the transport to and fusion with the target membrane remain to be identified in macrophages. Here we show that a novel Q-SNARE complex, located on similar Golgi-derived vesicles as TNFα, is up-regulated at a rate-limiting step in the secretion of TNFα to maximize cytokine secretion during inflammation.
Materials and Methods

Antibodies and Reagents—Anti-mouse TNFα antibodies were purchased from Genzyme, anti-syntaxin 3 antibodies were purchased from Sapphire Biosciences, and anti-β-tubulin antibodies were purchased from Molecular Probes. Antibodies specific for VAMP2, VAMP8, Stx7, Stx8, and SNAP-23 were purchased from Synaptic Systems, whereas antibodies to Vti1A, Vti1B, Stx6, GS28, Stx8, and SNAP-23 were purchased from Molecular Probes. Antibodies specific for VAMP2, VAMP8, Stx7, Stx8, and SNAP-23, along with Munc18c, are up-regulated by LPS treatment (5). SNARE proteins, including syntaxin 2 (amino acids 4–265) and syntaxin 4, are involved in the secretory process (20). We have previously shown (20) that it functions in TNF delivery to the cell surface and to the SNARE proteins Stx6 and Vti1b. B cell macrophages were activated with LPS (100 ng/ml) for 2 h with or without prior priming with IFN-γ (500 pg/ml) for 18 h, fixed, permeabilized, and immunostained for TNFα. In unprimed cells, TNFα is at the Golgi complex and on the cell surface. Priming increases the intensity of staining at the Golgi and on the cell surface, and TNFα is now also seen in endosomes after internalization of uncleaved surface TNFα (6). C, macrophages were activated with LPS for the times indicated with or without prior priming with IFN-γ for 18 h. Cell extracts were analyzed by immunoblotting using antibodies to Stx6 or Vti1b. D, bar chart, with S.E., shows that the up-regulation of Stx6 and Vti1b is shifting to the left and occurs more rapidly in IFN-γ-primed activated cells as compared with unprimed activated cells.

Results

SNARE Expression Is Quantitatively Linked to Exocytic Trafficking Requirements in Activated Macrophages—In activated macrophages, TNFα secretion is temporal in nature; the pulse of TNFα protein synthesis peaks at 2 h and ceases by 12 h (Fig. 1) (5). Identifying components of the trafficking machinery of the cell that are similarly regulated by LPS during this period provides a strategy for identifying proteins involved in the secretory process (20). We have previously shown that a plasma membrane Q-SNARE complex of syntaxin 4 and SNAP23, along with Munc18c, is up-regulated by LPS and that it functions in TNFα delivery to the cell surface (20). Microarrays were now used in a wider screen to search for genes involved in the regulation of TNFα production.
for other LPS-restricted SNARE proteins in activated macrophages (Affymetrix, 430A mouse gene chip; results published at Gene Expression Omnibus (GEO), accession number GSE1459, www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE1459). A characteristic response to LPS by these macrophages was signified by the dramatic induction of inflammatory cytokine genes, including up-regulation of TNFα itself (Fig. 1A). Two SNARE genes of interest were selected from this screen as potential regulators of TNFα trafficking, Stx6 and Vti1b, which showed significant increases in expression at both mRNA and protein levels in response to LPS (Fig. 1A), coinciding with the onset of TNFα synthesis. The data show that Stx6 is regulated at the gene level, whereas the early (2 h) rise in Vti1b protein precedes up-regulation at the gene level, suggesting some additional degree of post-translational regulation.

Murine macrophages primed with IFN-γ followed by activation with LPS make 5-fold more TNFα and secrete >2-fold more TNFα than cells activated with LPS alone (5) (Fig. 1B). Thus, in IFN-γ-primed cells, there is more TNFα synthesized, and it is trafficked to the cell surface faster than in unprimed cells. IFN-γ-primed LPS-activated macrophages were found to produce an increase in Stx6 and Vti1b protein levels (Fig. 1, C and D). Thus, in macrophages, Stx6 and Vti1b are regulated by the same signaling pathways as TNFα, and they are both up-regulated to match the need for TNFα trafficking.

Stx6 and Vti1B Partially Colocalize at the Golgi and Are Part of the Same SNARE Complex—Antibodies to Stx6 and Vti1b were used to localize the endogenous proteins in macrophages. Stx6 staining was found on the perinuclear Golgi complex, where it colocalized with newly synthesized TNFα and in scattered vesicles (Fig. 2A). Some staining of Vti1b was associated with the Golgi complex, also overlapping with TNFα, whereas additional Vti1b was on endosome-like structures (Fig. 2B). The finding that both Stx6 and Vti1b are associated with the Golgi complex suggests that both may have a possible role in the secretory pathway. We next investigated the biochemical nature of SNARE complexes in activated macrophages involving these two proteins. Typically, a complete trans-SNARE complex consists of three Q-SNARE chains (Qa,b, and c) and one R-SNARE polypeptide chain (24). Immunoprecipitation from activated macrophage extracts shows that in these cells, Vti1b is part of at least two distinct SNARE complexes (Fig. 2C). In the first, Vti1b (Qb) is able to complex with its known endosomal SNARE partner proteins Stx7 (Qa), Stx8 (Qc), and VAMP8 (R) (Fig. 2C). In the second, a novel complex, Vti1b, coimmunoprecipitates with Stx6 (Qc) and Stx7 (Qa) (Fig. 2C). A cognate R-SNARE for the Vti1b/Stx6 complex at this stage is unknown. Stx6 and VAMP8 (R) are not involved in this novel complex in macrophages (Fig. 2C). Another SNARE Vti1A (Qb) was also found to be expressed in macrophages, and it complexes with Stx6 (Qc) but not with Vti1b (Qb) as dictated by the stoichiometry of the Qa,b, and c SNARE complex. Similar complexes were also immunoprecipitated from unactivated macrophages (data not shown). Thus, Vti1b and Stx6 are components of a novel SNARE complex. Since immunostaining showed that Vti1b and Stx6 were both present in the Golgi region, this is a possible site for their interaction and function.

Stx6 and Vti1B Are Found on Similar Golgi-derived Carriers to TNFα—to test the association of Vti1B and Stx6 with the Golgi complex and secretory pathway in these cells, a biochemical approach was used. We isolated a stacked Golgi fraction from extracts of LPS-treated macrophages. Both Vti1B and Stx6 recovered in Golgi fractions (Fig. 3A). Next, Golgi membranes were incubated in vitro in the presence of cytosol and GTPγS to initiate the budding of membrane carriers. As we have previously shown, this leads to the generation of a variety of Golgi-derived vesicles that can be segregated by sucrose gradient analysis (22, 23). Vti1B and Stx6 were recovered on remnant Golgi membranes and in the fraction containing total budded vesicles. These vesicles or carrier membranes were further separated on a sucrose gradient. Effective vesicle budding was demonstrated by the de novo membrane binding of the cytosolic coatomer protein β-COP and the presence of γ-adaptin in slightly different fractions (Fig. 3B). Both Vti1B and Stx6 were detected on a subset of membrane carriers budding off the Golgi, both comigrating in the same fractions as TNFα (Fig. 3B). These results show that Vti1B and Stx6 are both associated with carriers containing TNFα that bud from the Golgi complex. Thus, both SNARE proteins are in a position to influence exocytosis.

Vti1B and Stx6 Function in the Exocytic Pathway—Since Vti1B and Stx6 are up-regulated in activated macrophages, form a SNARE complex, and are found in similar Golgi-derived
carriers to the cargo TNFα, functional studies were performed to test whether Vti1b and Stx6 operate in the exocytic pathway in macrophages. Overexpression of full-length SNARE proteins is known to enhance their function (20, 25), whereas overexpression of the cytoplasmic portions of these proteins is known to act as competitive inhibitors, blocking trans-SNARE complex formation and thereby inhibiting vesicle docking and fusion. The staining patterns of GFP-tagged full-length Stx6 and Vti1b resembled the endogenous proteins (Figs. 4 and 5). Transfected, LPS-activated cells were assayed by enzyme-linked immunosorbent assay to measure TNFα secretion into the medium (5) and by immunofluorescence to measure the effect on transport of TNFα from the Golgi complex to the cell surface (20). A 1.8-fold increase in TNFα secreted into the medium was recorded from GFP-Vti1b transfected cells (Fig. 4A). This coincided with an increase in TNFα trafficking to the cell surface, measured as increased cell surface staining of TNFα, in the majority of cells expressing the inhibitory cytoplasmic domain of Vti1b (GFP-Vti1b amino acids 1–234) (Fig. 4C). Subsequent immunostaining of permeabilized transfected macrophages revealed that TNFα was still evident at the level of the Golgi complex, indicating that its post-Golgi

FIG. 3. Stx6 and Vti1b are found on similar Golgi-derived vesicles carrying TNFα. A, macrophages activated with LPS for 2 h were fractionated into a stacked Golgi fraction (Golgi stacks) and cytosol. Golgi stacks were incubated with cytosol in an in vitro budding reaction, which resulted in the following fractions: remnant Golgi, cytosol and vesicles, remnant cytosol, and budded vesicles. Samples of these fractions were analyzed by immunoblotting. Golgi stacks are denoted by the presence of the marker GM130. Budded vesicles are enriched in the coatomer protein β-COP as compared with the original Golgi stacks and have γ-adaptin as a coated vesicle marker. Vti1b and Stx6 fractionate with isolated Golgi stacks collected during the fractionation procedure. After budding, they are both on remnant Golgi and in the budded vesicle fraction. B, Golgi-derived vesicles from the in vitro budding reaction were separated on a sucrose gradient. TNFα, Vti1b, and Stx6 separate into Golgi-derived carriers in the same subsets of fractions (F2–F5).

FIG. 4. Vti1b functions in TNFα secretion. A, RAW264.7 macrophages transiently expressing either GFP-Vti1b or GFP alone were treated with LPS for 2 h, and TNFα in the medium was quantified using an enzyme-linked immunosorbent assay. The results of three experiments are shown in the graph along with S.E. The level of TNFα secreted is increased from cells overexpressing Vti1b as compared with those expressing GFP alone. B, cells expressing GFP-Vti1b were treated with LPS for 2 h in the presence of TACE inhibitor and immunostained for surface TNFα. At least 50 transfected cells were analyzed in triplicate. GFP-Vti1b-expressing cells consistently showed increased surface TNFα. C, cells expressing the cytoplasmic domains of Stx2 and Vti1b fused to GFP were treated with LPS in the presence of TACE inhibitor and stained for surface TNFα. More than 50 transfected cells were measured in each of three experiments, and the results are shown in the graph along with S.E. Both GFP alone and the cytoplasmic domain of Stx2 had no effect on surface TNFα staining, whereas Vti1b cytoplasmic domain significantly reduced this staining. Cells expressing the cytoplasmic domain of Vti1b were immunostained after permeabilization (Perm, lower panel) to show that TNFα is still being synthesized and is present in the Golgi complex. Unperm, unpermeabilized; aa, amino acids.
Staining consistently showed increased surface TNF on GFP-labeled cells as compared with surrounding cells. At least 50 transfected cells were analyzed in each of three different experiments. had little effect on surface TNF measured in each of three experiments, and the results are shown in the graph along with S.E. Both GFP alone and the cytoplasmic domain of Stx2 did not play a role in TNF secretion. In the medium was quantified using an enzyme-linked immunosorbent assay. The results of three experiments are shown in the graph along with S.E.

Thus, by several analyses, we demonstrated for the first time a role of Stx6 and Vti1b in TNF secretion. A 1.3-fold increase in TNF secretion was observed in cells overexpressing GFP-Stx6 as compared with those expressing GFP alone. Overexpression of full-length GFP-Stx6 revealed an increase in TNFα surface staining in transfected cells as compared with surrounding untransfected cells (Fig. 5A). In contrast, when the inhibitory cytoplasmic domain of Stx6 (GFP-Stx6 amino acids 1–208) was expressed in activated macrophages, cell surface TNFα was dramatically reduced in the majority of cells as compared with surrounding untransfected cells (Fig. 5C). Thus, by several analyses, we demonstrated that Vti1B and Stx6 function in the post-Golgi secretory trafficking of TNFα. Overexpression of full-length GFP-Stx6 revealed an increase in TNFα surface staining in transfected cells as compared with surrounding untransfected cells (Fig. 5B). In contrast, when the inhibitory cytoplasmic domain of Stx6 (GFP-Stx6 amino acids 1–208) was expressed in activated macrophages, cell surface TNFα was dramatically reduced in the majority of cells as compared with surrounding untransfected cells (Fig. 5C). Thus, by several analyses, we demonstrated that Vti1B and Stx6 function in the post-Golgi secretory trafficking of TNFα to the cell surface in activated macrophages. Moreover, its expression is also rate-limiting since increasing the levels of Stx6 at the level of the Golgi enhanced the exocytosis of TNFα.

**DISCUSSION**

We show here a new site and a novel SNARE complex involved in the quantitative regulation of the secretory pathway in activated macrophages, providing these cells with increased capacity to generate vesicle carriers at the level of the TGN for post-Golgi transport of cytokine. In other hematopoietic cells, SNARE complexes on secretory granules mediate regulated secretion, for instance, the Stx2-SNAP-23 complex on platelet granules (26) and the Stx4-SNAP-23 complex on eosinophil granules (27). In macrophages, cytokines are not stored for release in granules but must be newly made upon cell activation and released in a battery of small vesicular carriers leaving the TGN. Our current data show that key SNAREs at the level of the TGN together with the cell surface SNARE complex are rate-limiting for cytokine secretion. Up-regulating SNAREs at both ends of the secretory pathway is thus a coordinated and essential process to increase vesicular traffic.

We have shown that the intracellular Q-SNAREs Stx6 and Vti1b are regulated by the same signaling pathways as TNFα and that they are both up-regulated to match the need for TNFα trafficking. The rapid increase in protein levels implies that these SNAREs may be regulated by posttranslational mechanisms as well as at a transcriptional level, as indeed is TNFα itself (6, 28). Together with our previous findings (20), it now appears that multiple SNARE proteins are subject to regulated expression during macrophage activation. Little is known about the regulation of SNARE expression in differentiated cells; one other study in melanocytes suggests that the expression of several SNARE proteins increases during cell differentiation, concomitant with melanosome formation and trafficking (29).

Experimental overexpression of each protein increases TNFα surface delivery and secretion, and the timing of expression of the endogenous proteins in IFN-γ-primed macrophages appears to be tightly linked to the timing and amount of TNFα trafficking in activated macrophages. Blocking Vti1B and Stx6 function trapped TNFα in the Golgi complex, suggesting that both are required for a step in post-Golgi exocytosis. This is the first described role for Vti1B in an exocytic pathway. The similar effects obtained by manipulating either Stx6 or Vti1B are consistent with the two proteins functioning as part of the same complex in the secretory pathway. By immunoprecipitation,
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Stx6 and Vti1b along with Stx7 were found to form a novel Q-SNARE complex. A preliminary association of Stx7 with Stx6 and Vti1b among several other SNARE coimmunoprecipitating proteins in melanocytes has previously been noted (29). Both Vti1b and Stx6 colocalize at the level of the Golgi, where they are associated with the same Golgi-derived carriers as those carrying the cargo TNFα. The most direct role foreseen for Vti1b/Stx6 is in the generation of TNFα carriers at the TGN; other possibilities include a role in regulating membrane recycling back to the TGN for carrier formation. Future studies will address these issues.

It is widely believed that trafficking proteins such as the SNAREs perform basic housekeeping functions in the cell and as such are constitutively expressed. Here we show that individual SNARE proteins are regulated to perform specialized functions in the cell. Macrophages are unique in their requirements for stimulus-coupled, temporal secretion of cytokines. Increasing essential SNARE complexes facilitates vesicle fusion required for the transport of cytokine from the Golgi to the cell surface, a process that is critical to the immune response but one with negative outcomes in inflammatory disease (1). These findings now add a new facet to the emerging knowledge that macrophages have uniquely adapted their trafficking pathways to enable dynamic roles in the immune response. Other recent and notable examples include the use of endoplasmic reticulum by the macrophage to supply additional membrane to the plasma membrane for phagosome production and to allow antigen cross-presentation to occur (30, 31). Our studies reveal an alternative mechanism that also provides additional membrane flow, in this case, in post-Golgi pathways to enable macrophages to release cytokine.

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