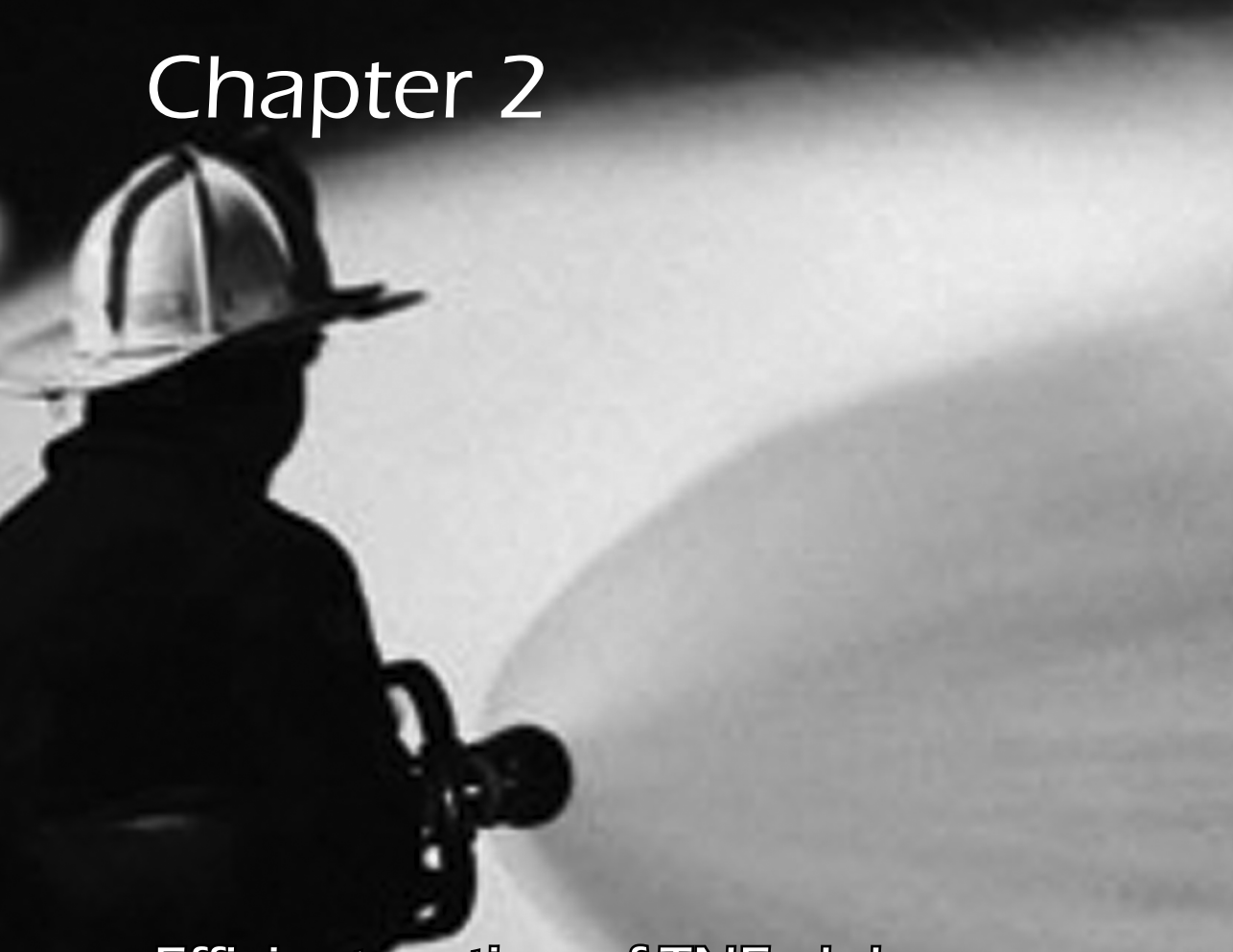
Chapter 2



Efficient sorting of TNF-alpha to rodent mast cell granules is dependent on N-linked glycosylation

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Abstract

Mast cells play an important role at the early stages of immunological response to bacterial infections and parasite infestations. One of the major mast cell pro-inflammatory mediators is TNF-alpha. Mast cells are considered the only cells capable of storing TNF-alpha in cytoplasmic granules and rapidly releasing it upon activation. To determine what pathway is utilized to direct TNF-alpha to cytoplasmic granules and what motifs are responsible for the sorting process, we constructed a fusion protein covering the full sequence of TNF-alpha, N-terminally fused to enhanced green fluorescent protein (EGFP). In rodent mast cells, such protein was sorted to secretory granules, and this process was inhibited by both brefeldin A and monensin. Considering the relationship between lysosomes and secretory granules and following TNF-alpha sequence analysis, it was determined whether TNF-alpha is sorted through the mannose-6-phosphate receptor (MPR)-dependent pathway. We observed that ammonium chloride and tunicamycin blocked TNF-alpha-EGFP fusion protein delivery to secretory granules. In situ mutagenesis experiments confirmed the necessity of N-linked glycosylation for efficient sorting of TNF-alpha into rodent mast cell granules. In this work we established that TNF-alpha travels from the ER to mast cell granules via a brefeldin A- and monensin-sensitive route, utilizing the MPR-dependent pathway, although this dependency does not seem to be absolute.

Introduction

Mast cells play an important role in both innate and acquired immunity. Antigen cross-links IgE bound to its receptors, leading to cell activation and subsequent degranulation and induction of synthesis of many chemotactic, proinflammatory, vasodilatative and cytotoxic substances. Some of the mediators released are cytokines, amongst them TNF-alpha. In a model of bacteria-induced inflammation the lack of mast cell-derived TNF-alpha was associated with drastically reduced neutrofil influx, and significantly higher mortality (1, 2). Similar data suggesting the critical role played by mast cell-derived TNF-alpha in certain types of inflammatory response were obtained using reverse Arthus reaction as an animal model of inflammatory state (3, 4). The special role for mast cells as a source of TNF-alpha might be explained by the presence of preformed cytokine protein in cytoplasmic granules that allows for its immediate release upon stimulation (5). This is supported by the two-stage kinetics of TNF-alpha release from murine mast cells (6). Despite temporal coordination of degranulation and preformed TNF-alpha release, these processes are differentially sensitive to some inhibitors, *e.g.,* azelastin (7). The presence of preformed cytokine protein in cytoplasmic granules is also confirmed by the observation that mouse mast cells are capable of TNF-alpha release following treatment with the transcription inhibitor actinomycin D (6). Within few hours from the release of preformed TNF-alpha, mast cells synthesize and release additional amount of this cytokine in a process that, as in other TNF-alpha-producing cell types, involves direct vesicular transport from the Golgi apparatus to the cell membrane (8, 9). This process was extensively studied in macrophages, where TNF-alpha is synthesized as a transmembrane protein (26-kDa, tmTNF-alpha) and cleaved to soluble form (17 kDa, sTNF-alpha). The majority of TNF-alpha exposed to the environment exists in the sTNF-alpha form, and the conversion of tmTNF-alpha into sTNF-alpha begins at some post-ER stage and is closely associated with TNF-alpha movement to the cell surface (10). The enzyme catalyzing this reaction, TNF-alpha converting enzyme (TACE, ADAM17) belongs to the metalloprotease family and is mainly found in a perinuclear compartment morphologically resembling the Golgi apparatus, and on the surface of the cell (11, 12).

Unlike that seen in macrophages, TNF-alpha trafficking and processing in mast cells has not been extensively investigated. One of the unanswered questions is what processes are responsible for the unique trafficking of TNF-alpha to cytoplasmic granules. Analysis of composition of mast cell granules suggests they are related to lysosomes (13, 14), although unlike lysosomes they are released following stimulation. Mechanisms directing proteins to lysosomes are diverse, based on both amino acid sequence and glycosylation of sorted proteins (15-17). Sequences present in cytoplasmic tails of type I transmembrane lysosomal proteins have been identified (reviewed in(18)) and used to direct fusion proteins to mast cell granules (19, 20). TNF-alpha, however, is a type II transmembrane protein and none of the aforementioned signals is found in its cytoplasmic tail. Alternative sorting of lysosomal proteins depends on recognition of the mannose-6-phosphate residues of

the glycan chain of the glycoprotein being sorted by one of the two distinct receptors, cation-dependent and cation-independent mannose-6-phosphate receptor (CD-MPR or CI-MPR, respectively). The cytoplasmic tails of these receptors contain sorting signals similar to those present in transmembrane lysosomal proteins (reviewed in (21, 22)). It has been reported that a MPR-dependent pathway is engaged in sorting of cathepsin D into cytoplasmic granules in mast cells (16). It is possible that TNF-alpha sorting to cytoplasmic granules might also utilize one of the MPR-dependent pathways. This possibility is supported by the fact that TNF-alpha contains a putative N-glycosylation site (N86 in murine TNF-alpha) and exhibits some glycosylation pattern (23). However, at present it has not been established whether TNF-alpha is N-glycosylated on N86 and whether this glycosylation is of any importance for the sorting process.

We employed transfected rodent mast cells expressing different TNF-alpha-enhanced green fluorescent protein (EGFP) fusion proteins to study mechanisms trafficking TNF-alpha into cytoplasmic granules. We report here that the full TNF-alpha sequence efficiently directs EGFP to cytoplasmic granules. This process is sensitive to brefeldin A (BFA) and monensin, and requires MPR and N-linked glycosylation of TNF-alpha, although this dependency does not seem to be absolute.

Results

Expression of 1-235TNF-EYFP

Microscopic observations were performed in RBL-2H3 cells transiently cotransfected with the following pair of plasmids: p1-235TNF-EYFP-N1 and pECFP-Golgi. Fluorescence pattern in cells was observed after 2, 6 and 16 h. In cells cotransfected with the pECFP-Golgi/ p1-235TNF-EYFP-N1 plasmids pair, both ECFP fluorescence and EYFP fluorescence were visible in the Golgi apparatus 2 h after transfection. While the pattern of ECFP fluorescence remained mostly unchanged over the following 14 h, EYFP fluorescence gradually diminished in the Golgi apparatus and accumulated in peripheral vesicular structures, which, in their number, size and distribution, resembled mast cell granules. After 16 h, this process was apparently complete (Fig. 1A-I). A similar fluorescence pattern was observed in BM-derived mast cells (BMMC) and C57.1 cells transfected with p1-235TNF-EYFP-N1 (data not shown). In cells cotransfected with pEYFP-N1/p1-235TNF-ECFP-N1 plasmids, EYFP fluorescence was present in cytoplasmic and nuclear compartments, but not in apparently vesicular structures at all time points tested. ECFP fluorescence was first observed in ER/ Golgi compartment (2-h time point), and over following 14 h localized in vesicular structures negative for EYFP (data not shown).

1-235TNF-EYFP fusion protein is localized in mast cell secretory granules

To test whether ECFP/EYFP-positive vesicular structures appearing in the cells transfected with p1-235TNF-ECFP/EYFP-N1 are indeed mast cell granules, we compared intracellular localization of the 1-235TNF-EGFP fusion protein with the localization of some established mast cell granule markers. We have stained the cells for 5-hydroxytryptamin (5-HT, serotonin), which is found selectively in mast

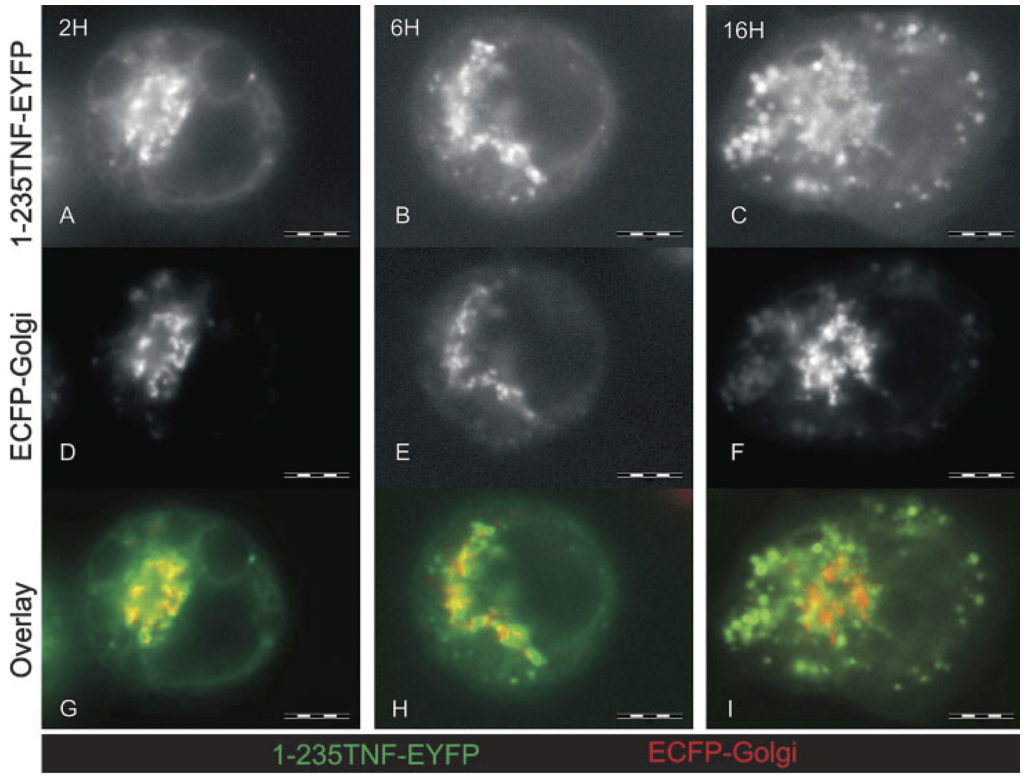

Figure 1. TNF-EYFP fusion protein undergoes intracellular sorting following transfection into rat mast cells. (A–I) RBL-2H3 cells were cotransfected with p1–235TNF-EYFP-N1 and pECFP-Golgi plasmids and subjected to microscopy at the indicated time points. Images A–C depict localization of 1–235TNF-EYFP fusion protein 2, 6 and 16 h post-transfection, images D–F depict localization of ECFP-Golgi protein 2, 6 and 16 h post-transfection. In overlay images (G–I), EYFP and ECFP are represented by false colours green and red, respectively. Bars represent 5 μm on all images

cell secretory granules after its uptake from the medium (24), and for RMCP-II, which is rat mast cell granule-specific protease. We have also used a fluid-phase endocytic marker Texas Red-Dextran conjugate (TR-D), which is known to accumulate in lysosomes and mast cell granules (16). For 5-HT and TR-D studies, cells were allowed to internalize the respective compound overnight; they were then thoroughly washed, transfected with p1-235TNF-EGFP-N1 (5-HT and TR-D-fed cells) and left overnight to attach. For RMCP-II staining, cells were transfected with p1-235TNF-EGFP-N1 and left overnight to attach. Staining revealed a vesicular pattern for both 5-HT and RMCP-II, significantly overlapping with fusion protein fluorescence (Fig. 2A-C and D-F, respectively). Colocalization of 5-HT and 1-235TNF-EGFP was also significant in C57.1 murine mast cells (data not shown). For TR-D and fusion protein colocalization, overlap was even greater, probably due to live imaging facilitating protein fluorochrome fluorescence (Fig. 2G-I). To establish what fraction of endocytosed TR-D is localized in mast cell granules, cells were allowed to internalize the compound overnight and stained for RMCP-II. As shown in Fig. 2J-L, a major fraction of TR-D was localized in a RMCP-II-containing compartment, thus validating its use as a mast cell granule marker.

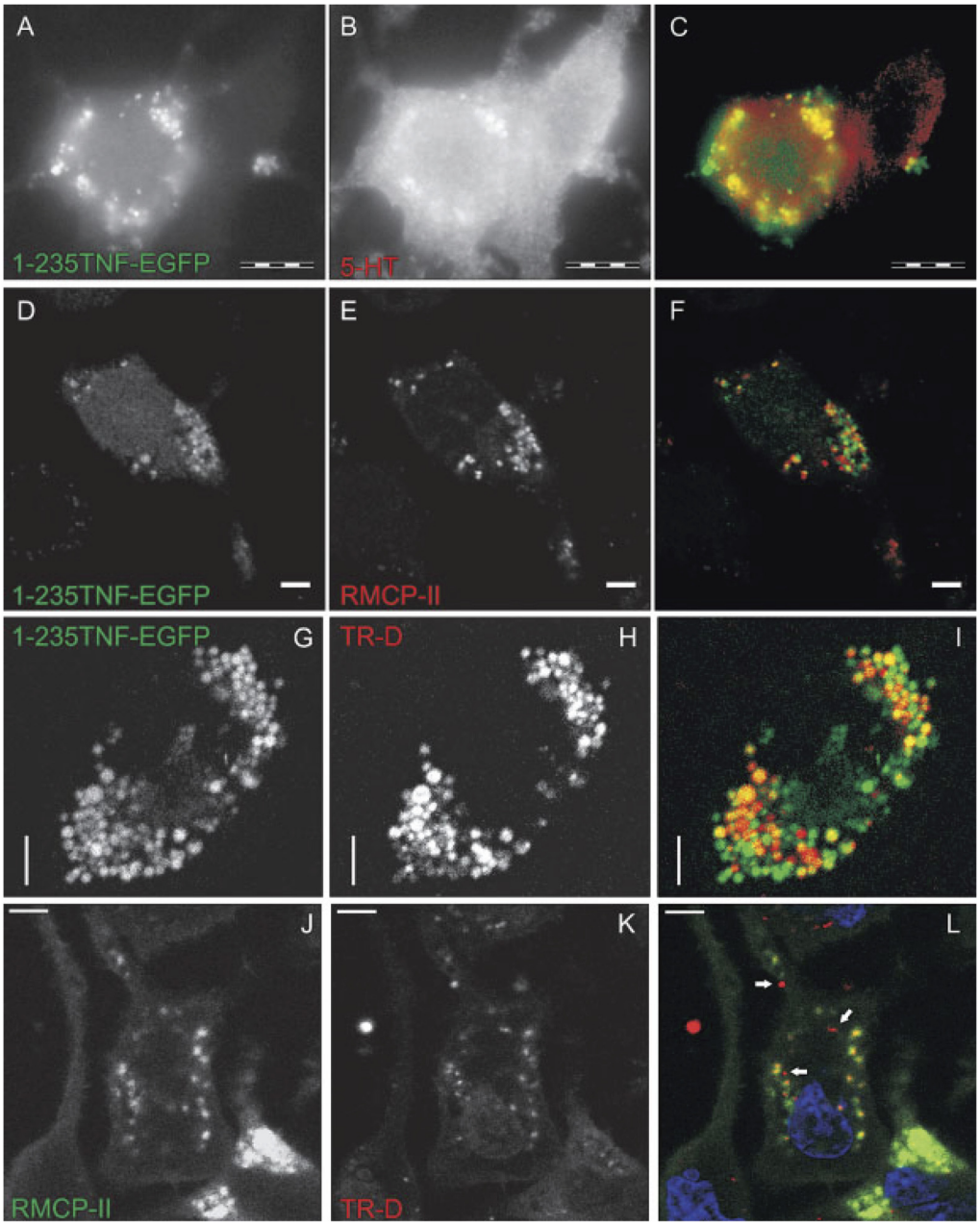

Figure 2. TNF-EGFP fusion protein localizes to mast cell granules. (A–F). RBL-2H3 mast cells were transfected with p1–235TNFEGFP-N1 (A, D) and stained against 5-HT (B) or RMCP-II (E). Images C and F represent colocalization of TNF-EGFP (false colour green) and 5-HT (C) or RMCP-II (F) (both false colour red). (G–I). RBL-2H3 cells were allowed to internalize TR-D and transfected with p1–235TNF-EGFP-N1. Colocalization of TNF-EGFP (G, false colour green) and TR-D (H, false colour red) is shown on image I. TNF-EGFP is represented by false colour green, and TR-D is represented by false colour red. (J–L) RBL-2H3 cells were allowed to internalize TR-D and stained for RMCP-II. Image L represents colocalization of RMCP-II (J, false colour green) and TR-D (K, false colour red). Nuclear stain (TO-PRO-3) is represented in blue. Cells were imaged under a fluorescence (A–C) or confocal (D–L) microscope. In the latter case, representative confocal sections are presented. In (L), arrows indicate lysosomes, which are RMCP-II negative, TR-D-positive structures. Bars represent 5 μm on all images.

TNF-EYFP is properly expressed in and can be released from mast cells

To confirm proper expression of 1-235TNF-EGFP under the experimental conditions employed, lysates from RBL-2H3 cells transfected with 1-235TNF-EGFP were analyzed by Western blot (Fig. 3B). Using both anti-TNF-alpha and anti-GFP antibodies, protein of an apparent molecular mass of 59 kDa was detected with additional band of 49 kDa visible on anti-GFP-probed membrane, probably due to stronger overall signal. Thus, double immunogenicity of 1-235TNF-EGFP protein has been shown, confirming its identity and revealing possible proteolytic processing.

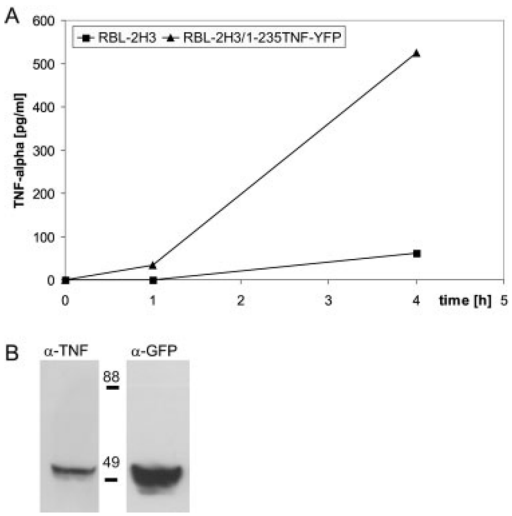

Figure 3. TNF-EYFP is properly expressed in, and can be released from, mast cells. (A) RBL-2H3 cells and RBL-2H3 cells transfected with p1-235TNF-EYFP-N1 were stimulated with 100 ng/mL PMA and 1 μM ionomycin. Concentration of TNF-alpha in culture supernatants was determined by ELISA at the indicated time points. Results of a representative experiment are shown. (B) 1–235TNF-EYFP expression pattern in RBL-2H3 cells was analyzed byWestern blot. Lanes represent analysis of lysates from RBL-2H3 transfected with p1–235TNF-EYFP-N1. Membrane was probed with anti-TNF antibodies, stripped and reprobed with anti-GFP antibodies.

To establish whether 1-235TNF-EYFP fusion protein can be released from mast cells in response to stimuli triggering release of endogenous TNF-alpha, RBL-2H3 cells were transfected with p1-235TNF-EYFP and left overnight to attach. For TNF-alpha release, cells were incubated in culture medium alone or culture medium with 100 ng/ml PMA and 1 μM ionomycin. In supernatants from non-stimulated cultures, no TNF-alpha was detected, regardless of transfection (data not shown). After 1 h of stimulation, 34 pg/ml TNF-alpha was detected in supernatants from transfected cells, but none in the case of non-transfected cells. After 4 h of stimulation, 62 pg/ml TNF-alpha was detected in supernatants from non-transfected cells, and 525 pg/ml in supernatants from transfected cells. These results showed that fusion protein covering full sequence of TNF-alpha is properly expressed, targeted specifically to mast cell secretory granules and can be released from cells upon stimulation (Fig. 3A).

1-235TNF-EYFP is retrieved from secretory pathway at the stage of late Golgi/ trans-Golgi network

Since it was confirmed that 1-235TNF-ECFP/EYFP fusion protein is sorted to secretory granules, the question arose at what stage of the secretory pathway this sorting occurs. To resolve this, RBL-2H3 cells were transfected with p1-235TNF-EYFP-N1 and incubated with 1 μg/ml BFA (anterograde ER-to-Golgi transport inhibitor) or 1 mM monensin [trans-Golgi network (TGN) export inhibitor] from the time of transfection. At 2 h post transfection, cells incubated with BFA exhibited a reticular pattern of EYFP fluorescence with a concentration of EYFP fluorescence in perinuclear

area, and this did not change over after 24 h (Fig. 4A-C). In cells incubated with monensin EYFP fluorescence at first (after 2 h) concentrated in vesicular structures in central area of the cell. These vesicular structures increased over time, closely resembling swollen TGN observed in light microscopy (data not shown). During this time EYFP fluorescence localization changed from mostly membrane bound to mostly luminal, suggesting possible proteolysis of the fusion protein (Fig. 4D-F). When cells were fed with TR-D, transfected with p1-235TNF-EYFP-N1 and incubated with BFA or monensin, there was no colocalization of EYFP and Texas Red fluorescence (Fig. 4H, I). Contrary to BFA and monensin-treated cells, control cells at 24 h after transfection exhibited concentration of EYFP in mast cell secretory granules (Fig. 4G). To exclude the possibility that the change in fluorescence pattern observed in cells incubated with BFA or monensin is an effect of improper protein processing or glycosylation, cell lysates were analyzed by Western blot. The majority of the fusion protein appears as a band of 59 kDa with minor band at 49 kDa. Following peptide: N-glycosidase F (PNGase F) treatment bands of 55 and 45 kDa were observed (Fig. 4J). This is in agreement with the calculated molecular masses of transmembrane and soluble forms of the 1-235TNF-EYFP fusion protein, respectively. This also indicates that 1-235TNF-EYFP undergoes N-glycosylation, and that a fraction of this protein is proteolytically processed, in a way similar to endogenous TNF-alpha. Thus, we observed that 1-235TNF-EYFP is expressed as a transmembrane glycoprotein and that BFA and monensin interfere with its trafficking, but not its post-translational modifications.

1-235TNF-EGFP sorting to mast cell granules is sensitive to NH_4Cl and tunicamycin

Since it was established that 1-235TNF-EYFP is a glycoprotein we decided to elucidate whether MPR-dependent pathway is used for its sorting to mast cell granules. RBL-2H3 cells were allowed to internalize TR-D overnight and were then incubated for 4 h with 10 mM NH_4C1, which is known to inhibit the MPR-mediated transport. Following incubation, cells were transfected with 1-235TNF-EYFP and examined under a fluorescent microscope. At early stages post-transfection, similar reticular fluorescence patterns were observed in cells incubated with NH_4Cl and control cells (data not shown), but after 16 h NH_4Cl-treated cells, but not control cells, exhibited EYFP fluorescence in large vesicular structures located near the center of the cell. At the same time, there was no significant overlap between 1-235TNF-EYFP and TR-D, while the morphology of the structures containing TR-D (lysosomes and mast cell granules) was not seriously disturbed (Fig. 5A-C).

Thus, NH_4Cl causes retention of the fusion protein in some compartment other than granules. To determine whether 1-235TNF-EYFP sorting to mast cell granules requires N-linked glycosylation, TR-D-fed RBL-2H3 cells were incubated with 2 μg/ml tunicamycin for 4 h prior to transfection with p1-235TNF-EYFP-N1. At 16 h post-transfection, cells exhibited pattern comparable to that of NH_4Cl-treated cells in the green fluorescence channel, and the vesicular pattern in the red fluorescence channel, indicating that whereas granule morphology was not disturbed, fusion protein was absent from secretory granules (Fig. 5D-F).

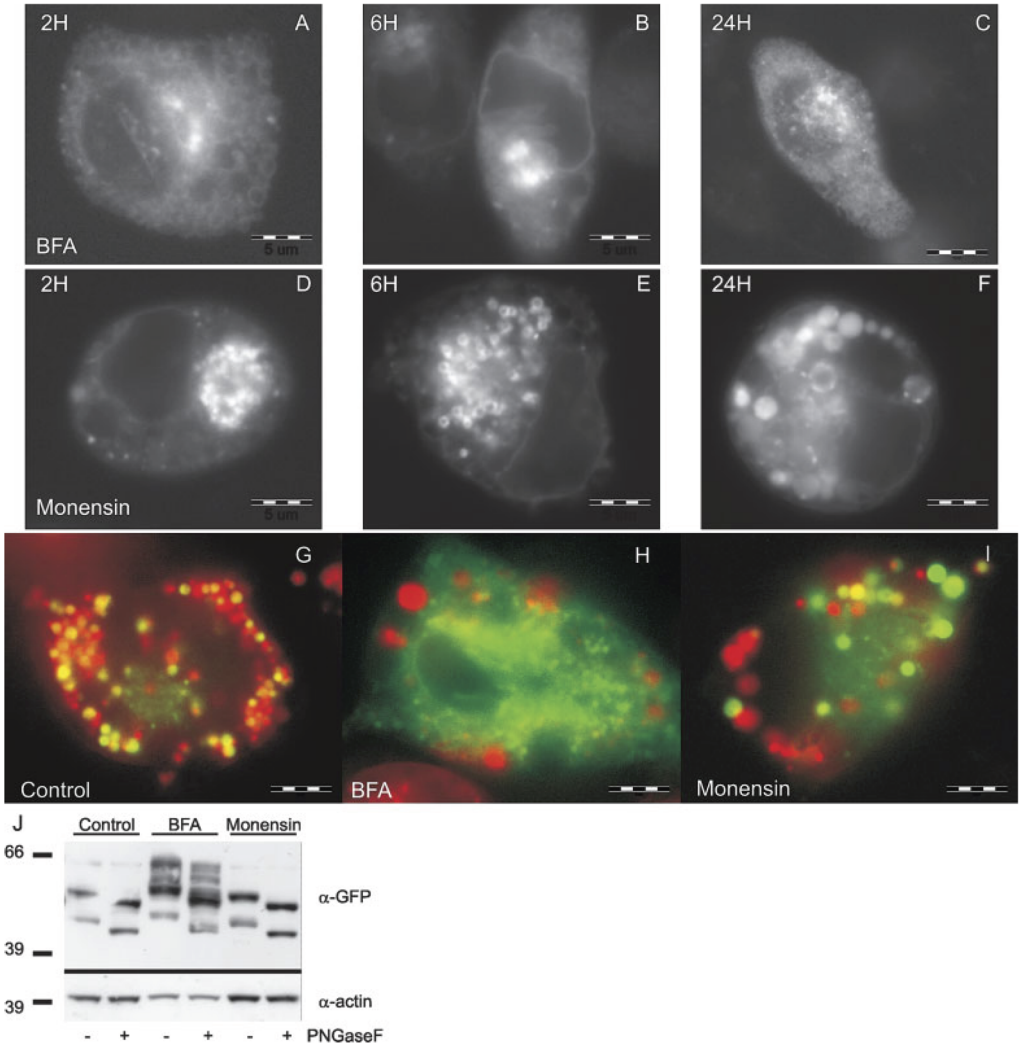

Figure 4. TNF-EYFP sorting is a BFA- and monensin-sensitive process. (A-F) RBL-2H3 mast cells were transfected with p1-235TNF-EYFP-N1, incubated with 1 µg/ml BFA (A-C) or 1 µM monensin (D–F) from the time of transfection, and imaged at the indicated time points.(G–I) RBL-2H3 cells were allowed to internalize TR-D and transfected with p1-235TNF-EYFP-N1. After transfection cells were incubated in culture medium alone (G) or medium containing 1 µg/ml BFA (H) or 1 µM monensin (I). Following attachment, cells were imaged. In all overlay images EYFP and Texas Red are represented by false colors green and red, respectively. Cells were imaged under fluorescence microscope. Bars represent 5 µm on all images. (J) 1–235TNF-EYFP expression pattern in cells imaged above was analyzed by Western blot. Lanes represent analysis of lysates from cells transfected with p1–235TNF-EYFP-N1 and treated with medium alone (Control), BFA or monensin; lysates were not treated (-) or treated (+) with PNGase F. Proteins were detected with anti-GFP antibodies (upper panel) or anti-actin antibodies (lower panel).

To investigate the effect of NH_4Cl and tunicamycin on expression of 1-235TNF-EGFP fusion protein, cell lysates from cells treated with NH_4Cl or tunicamycin for 16 h from transfection were analyzed by Western blot. As shown in Fig. 5G, the pattern of 1-235TNF-EGFP fusion protein expression in NH_4Cl-treated cells was comparable to controls, while incubation with tunicamycin resulted in appearance of additional protein bands of 55 and 45 kDa that were resistant to PNGase F treatment. This

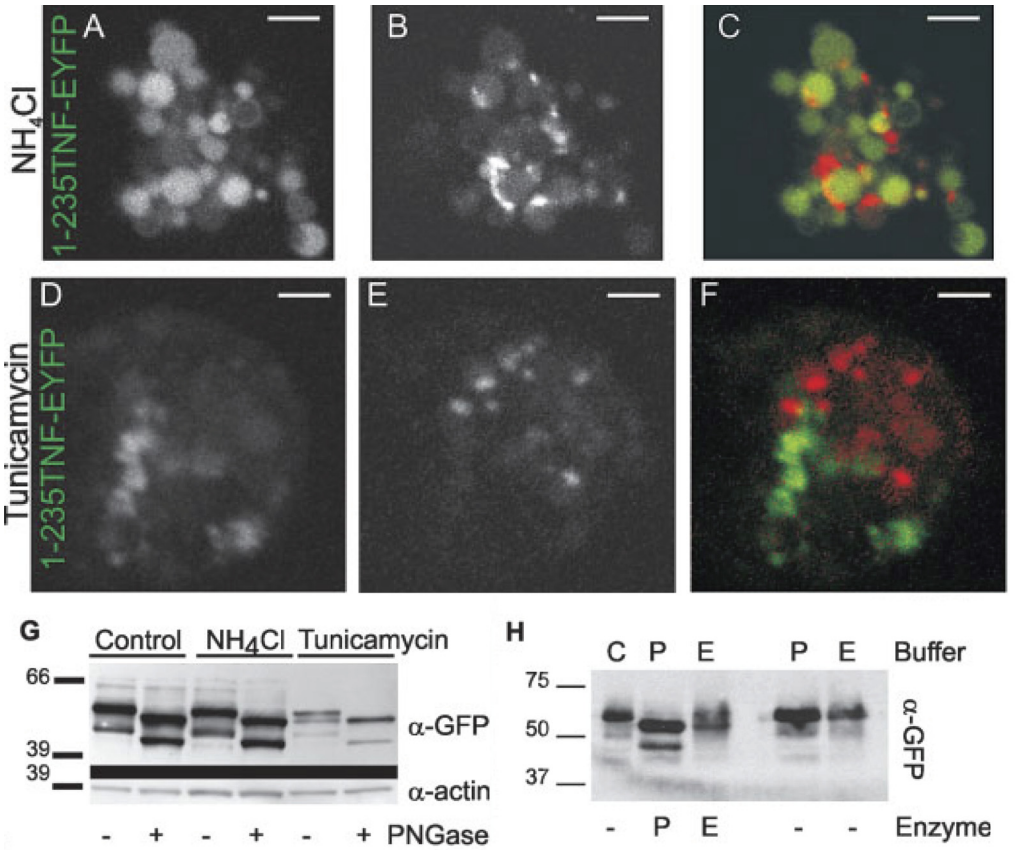

Figure 5. TNF-EYFP sorting to mast cell granules is an NH4Cl- and tunicamycin-sensitive process. (A–F) RBL-2H3 cells were allowed to internalize TR-D and incubated in culture medium containing 10 mM NH4Cl (A-C) or 2 μg/mL tunicamycin (D–F) for 4 h before transfection and following transfection. Cells were imaged at 16 h after transfection with p1–235TNF-EYFP-N1. Images A and D represent fusion protein fluorescence, images B and E represent TR-D fluorescence. In overlay images (C and F), EYFP and Texas Red are represented by false colours green and red, respectively. Cells were imaged under confocal microscope and representative confocal sections are presented. Bars represent 5 μm on all images. (G) 1–235TNF-EYFP expression pattern in cells imaged above was analyzed by Western blot. Lanes represent analysis of lysates from cells transfected with p1–235TNF-EYFP-N1 and treated with medium alone (Control), NH4Cl or tunicamycin; lysates were not treated (-) or treated (+) with PNGase F. Proteins were detected with anti-GFP antibodies (upper panel) or anti-actin antibodies (lower panel). (H) Composition of a N-linked glycan was analyzed by Western blot. Lanes represent analysis of lysates from cells transfected with p1–235TNF-EYFP-N1; lysates were incubated with indicated buffers and enzymes. C, PBS; P, PNGaseF; E, endoglycosidase H.

indicates that tunicamycin inhibited N-linked glycosylation of 1-235TNF-EGFP fusion protein. To further confirm the possibility of involvement of MPR-based system in trafficking of TNF-alpha, lysates from cells transfected with 1-235TNF-EYFP were subject to endoglycosidase H treatment. As shown in Fig. 5H, treatment with endoglycosidase H resulted in appearance of a protein band of 55 kDa in addition to the original 59 kDa. This shows that a fraction of 1-235TNF-EYFP is modified with mannose-rich glycan. The lack of the putative processed form can probably be attributed to generally lower signal in samples incubated in endoglycosidase H buffer (as seen in control lane). This could be a result of general instability of proteins incubated in relatively low pH buffer over prolonged period of time.

N86 is critical for efficient sorting of 1-235TNF-EYFP to mast cell granules

To further elucidate features of 1-235TNF-EYFP fusion protein required for its efficient sorting to secretory granules, a point mutation was introduced into the sequence of p1-235TNF-EYFP-N1, resulting in N86S substitution disrupting the N-glycosylation site in 1-235TNF-EYFP RBL-2H3 cells were transfected with p1-235TNF-EYFP-N1 or p1-235-N86S-TNF-EYFP-N1 and left overnight to adhere. At 24 h after transfection, cells transfected with p1-235TNF-EYFP-N1 developed a vesicular pattern of fluorescence with no fluorescence in cytoplasm. At the same time point, in cells transfected with p1-235-N86S-TNF-EYFP-N1 some fluorescence in vesicular structures was also observed, but a significant fraction of the mutant fusion protein was observed in cytoplasm (data not shown). To examine spatial and temporal relations of control and mutant fusion proteins more closely, RBL-2H3 cells were fed with TR-D, co-transfected with plasmids encoding for the 1-235-N86S-TNF-EYFP and 1-235TNF-ECFP proteins and subjected to microscopy After 48 h 1-235-N86S-TNF-EYFP fluorescence in granules increased, with majority still detected in cytoplasm, whereas 1-235TNF-ECFP and TR-D fluorescence patterns remained unchanged (Fig. 6A-F). However, the fraction of fluorescence of 1-235-N86S-TNF-EYFP in mast cell granules was lower than that of 1-235TNF-ECFP, suggesting that N86 is important for the transport of fusion protein to mast cell granules. Western blot analysis of the expression pattern of 1-235-N86S-TNF-EYFP revealed a pair of PNGase-resistant proteins of 55 and 45 kDa, which is equivalent to PNGase-treated 1-235TNF-EYFP expressed in control cells and 1-235TNF-EYFP expressed in tunicamycin-treated cells (Fig. 6G). In addition, ratiometric analysis of the given fusion protein expression level *versus* beta-actin performed on Western blots showed that in cells transfected with equal amounts of p1-235TNF-EYFP-N1 and p1-235-N86S-TNF-EYFP-N1, DNA mutant and control fusion proteins expression levels were comparable (data not shown). Thus, the mutant fusion protein deficient in N-linked glycosylation expressed in mast cells was not efficiently sorted to mast cell granules. It is of note, however, that even in the absence of N-linked glycans, a fraction of the mutant protein was sorted to secretory granules, indicating that another sorting system partly compensates for non-functional MPR-dependent sorting.

Discussion

The unique feature of mast cells among other TNF-alpha-expressing cells is their ability to store preformed cytokine protein and release it upon activation. While the mechanism of intracellular trafficking of TNF-alpha engaged in the process of secretion of *de novo* synthesized cytokine in macrophages has been partially delineated (25), the mechanism of TNF-alpha storage in mast cell cytoplasmic granules is still mostly unknown. EGFP-containing fusion proteins were successfully applied to decipher a variety of intracellular protein trafficking pathways. This approach was also effective in visualizing the trafficking of mast cell secretory granule proteins, including CD63, phospholipase D, VAMP-7 and syntaxin-3 (26-29). We have employed a similar strategy and transiently transfected RBL-2H3 mast cells with DNA constructs coding for TNF-alpha-EGFP fusion protein to gain insight into the process of intracellular

TNF-alpha trafficking in mast cells. One limitation of such approach is the fact that this mast cell line differs in the phenotype from peritoneal mast cells, which are known to store endogenous TNF in the granules *in vivo* (6).

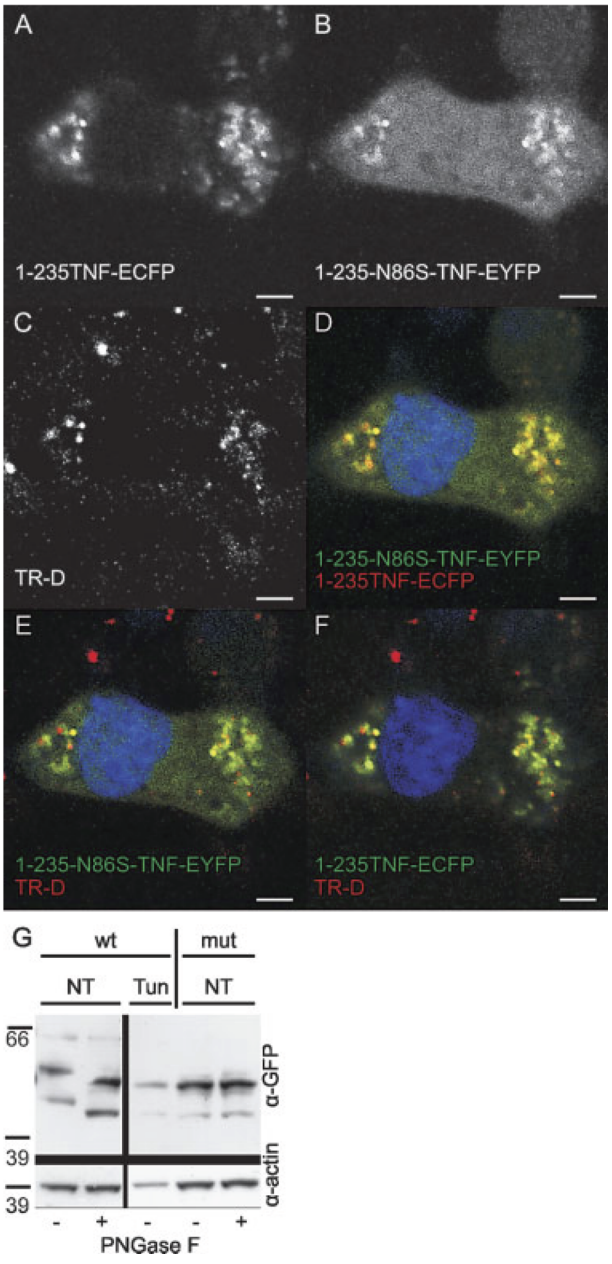

Figure 6. N86 is critical for TNF sorting into mast cell granules. RBL-2H3 cells were allowed to internalize TR-D and cotransfected with p1–235TNF-ECFP-N1 and 1–235-N86S-TNF-EYFP-N1 plasmids. Cells were imaged at 48 h after transfection. Representative confocal sections for fluorescence in ECFP, EYFP and Texas Red channels are shown on images A–C. Overlay images show colocalization of the following fluorochrome pairs (and their respective false colours): EYFP (green)/ECFP (red) (D), EYFP (green)/Texas Red (red) (E) and ECFP (green)/Texas Red (red) (F). Nuclear stain (TO-PRO-3) is represented by blue colour. Bars represent 5 μm on all images. (G) 1–235TNFEYFP and 1–235-N86S-TNF-EYFP expression patterns in cells imaged as above were analyzed by Western blot. Lanes represent analysis of lysates from cells transfected with p1–235TNF-EYFP-N1 (wt) or p1–235-N86S-TNF-EYFP-N1 (mut), treated with medium alone (NT) or tunicamycin (Tun); lysates were not treated (-) or treated (+) with PNGase F. Proteins were detected with anti-GFP (upper panel) or anti-actin antibodies (lower panel).

The EGFP fusion protein containing the entire sequence of the tmTNF-alpha, when expressed in mast cells, accumulated predominantly in cytoplasmic secretory granules (Fig. 1). The unequivocal identification of the compartment in which TNF-alpha fusion protein accumulated is supported by colocalization with three independent markers for secretory granules (Fig. 2). Accumulation of ectopically expressed TNF-alpha fusion protein is consistent with reported observation of immunogold detection TNF-alpha in this compartment (5). The kinetics of appearance of 1-235TNF-ECFP/EGFP fusion protein in transfected mast cells supports the hypothesis that it is sorted into cytoplasmic granules via a ER/Golgi secretory pathway (Fig. 1).

Observed differences in amounts of TNF-alpha released from non-transfected and p1-235TNF-EYFP-transfected mast cells (Fig. 3A) suggest that ectopically expressed 1-235TNF-EYFP fusion protein is not only sorted to mast cell granules, but also efficiently released. Biphasic kinetics of appearance of TNF-alpha in supernatants of stimulated cultures may suggest that relatively lower amounts of this cytokine are released from granule-stored pool within 1 h, while increased release in the following hours is a result of *de novo* synthesis. The apparent lack of release of TNF-alpha by non-stimulated cells, regardless of transfection, is a feature of mast cells, including this cell line(30).

This hypothesis is further supported by the retention of fusion protein in reticular structures or in tubulo-vesicular structures following treatment of transfected cells with BFA or monensin, respectively (Fig. 4). These observations are consistent with reported inhibition of TNF-alpha secretion by these compounds in activated macrophages and T cells (30-32). It is also consistent with current knowledge of the mechanisms of expression and secretion of cytokine proteins in immune cells (33, 34). One of the possible routes that allow for transport of proteins from the TGN to cytoplasmic granules is the MPR-dependent system. The observation that accumulation of TNF-alpha fusion protein in cytoplasmic granules is prevented by NH_4Cl treatment (Fig. 5) suggests that this process is largely mediated by a receptor-dependent route. Involvement of the MPR in this process is consistent with the notion that mast cell cytoplasmic granules are phylogenetically and functionally related to lysosomes (14, 35), as MPR-dependent pathways deliver proteins to lysosomes (36). This is also in agreement with previous reports that trafficking of cathepsin D to mast cell secretory granules partially depends on the MPR system (16). Interestingly, certain structural features of murine TNF-alpha are consistent with the hypothesis that this cytokine may be sorted via an MPR-dependent pathway. Whereas analysis of amino acid sequence of murine TNF-alpha did not reveal any known sorting signals, it did reveal N-linked glycosylation motif in position 86, and glycosylation of TNF-alpha protein has been reported (23). We have shown that the TNF-EGFP fusion protein is indeed glycosylated on N86 residue (Fig. 5G, 6G), and a significant fraction of the glycan attached is the mannose-rich type (Fig. 5H). Thus, the TNF-alpha molecule provides carbohydrate moieties necessary for interaction with MPR. Furthermore, TNF-alpha is a type II transmembrane protein, which renders the N86 residue available for recognition by MPR. In agreement with the involvement of carbohydrate part of TNF-alpha molecule in the sorting process, inhibition of N-glycosylation with tunicamycin prevented sorting of TNF-alpha fusion protein to cytoplasmic granules (Fig. 5). The dependency of TNF-alpha trafficking to cytoplasmic granules on N-linked glycosylation is further supported by the observation that TNF-alpha fusion protein deficient in N-glycosylation motif (N86S substitution), and thus is not N-glycosylated (Fig. 6G), was sorted to cytoplasmic granules with lower efficiency as compared to control protein (Fig. 6). It is of note, however, that this sorting was not completely abolished, even in the absence of N-linked glycans, clearly indicating that another, complementary sorting mechanism is in operation. This is consistent with our observation that a fraction of the glycan attached to TNF-alpha fusion protein is not

of the mannose-rich type, which precludes MPR involvement. Data obtained with the mutated protein confirm that N86 is utilized as a signal for N-linked glycosylation of TNF-alpha and is important for efficient sorting to mast cell secretory granules.

Relatively higher concentration of 1-235-N86-TNF-EYFP protein in cytosol (as compared to 1-235TNF-EYFP) may result not only from deficiency in carbohydrate-dependent retrieval from TGN. It cannot be excluded that once the non-glycosylated protein reaches its target compartment it cannot be stably maintained within granules, and a slow leak into cytoplasm occurs. Another possibility is that N86S mutation impairs trafficking at the stage of ER entry, thus rendering it unavailable for sorting systems operating further downstream. Thus, N86 is important for TNF-alpha sorting to mast cell secretory granules, but its role in this process may extend beyond N-linked glycosylation.

We present here what we believe to be the first observation of cytokine protein being retrieved from secretory pathway by MPR and stored in secretory granules. The hypothesis that TNF-alpha is transported from TGN to cytoplasmic granules via MPR-dependent pathway emphasizes the importance of glycosylation motif in TNF-alpha protein for formation of specific granular pool of TNF-alpha, which is functionally different from *de novo* synthesized cytokine (4, 6, 37). One of the unanswered questions arising from this hypothesis is the nature of the regulatory mechanism directing the cytokine either to cell membrane or to secretory granules as mast cells secrete both preformed and *de novo* synthesized TNF-alpha (6).

Materials and methods

Plasmid construction

The DNA fragment coding for TNF-alpha was obtained by RT-PCR. Briefly, 5 µg total murine RNA (isolated from C57.1 cell line, (38)) was subject to RT reaction using MMLV reverse transcriptase (Gibco) and oligonucleotide 5'-CCTTCACAGAG-CAATGACTC-3' in a final volume of 20 µL. cDNAwas amplified using Taq polymerase (Fermentas MBI) and 5'-TCCAGAAAAGACACC**ATG**AG-3' and 5'-CCTTCACAGAGCAATGACTC-3' as sense and antisense primer, respectively (start codon in bold).

Product of PCR reaction was cloned into pT-Adv plasmid (Clontech) resulting in plasmid pTA-TNF. pTA-TNF was sequenced to verify the integrity of the TNF-alpha reading frame. To obtain plasmid encoding for fusion protein consisting of full TNF-alpha and EGFP, full TNF-alpha ORF was amplified from pTA-TNF plasmid using 5'-GTGATGGATATCTGCA**GAATTC**GGCTTTCC-3' and 5'-CATTGCTCTGTGAA GGAAGCC**GAATTC**CAGC-3' primer pair (EcoRI sites in bold). Reverse primer was used for concomitant disruption of TNF-alpha STOP codon. Amplified fragment was digested with EcoRI, gel-purified and ligated into EcoRI-digested pECFP-N1, pEGFP-N1 and pEYFP-N1 plasmids (Clontech), resulting in series of plasmids encoding for fusion proteins containing full TNF-alpha ORF, 20 random amino acid linker and full ECFP or EGFP or EYFP ORF, i.e., p1-235TNF-ECFP/EGFP/EYFP-N1. The resulting plasmids were sequenced to confirm the integrity of fusion proteins reading frames. Mutagenesis *in situ* was performed with the use of Quick-change XL kit (Stratagene)

using 5'-CAGATCATCTTCT-CAAAGTTCGAGTGACAAGCCTG-3' and 5'-CAGGCTTGT-CACTCGAACTTTGAGAAGATGATCTG-3' primer pair and p1-235TNF-EYFP-N1 plasmid as a template. Plasmids isolated from three independent bacterial clones were sequenced and verified for the expression of the protein of proper molecular weight in C57.1 and RBL-2H3 cells. Plasmid coding for fusion protein carrying N86S mutation (with respect to TNF-EYFP ORF) was called p1-235-N86S-TNF-EYFP-N1.

Plasmid pECFP-Golgi was purchased from Clontech and encodes for human beta 1,4-GT-ECFP fusion protein, containing N-terminal 81 amino acids of human beta 1,4-galactosyltransferase

Cell culture, transfection and ELISA

The rat mast cell line RBL-2H3 was a gift from Dr. U. Blank (Paris, France). Cells were maintained in DMEM medium supplemented with 10% heat-inactivated FBS, 2 mM gluta-mine, nonessential amino acids, 25 mM HEPES, 100 µg/ml penicillin/streptomycin and sodium pyruvate. At approximately 80% confluence, cells were trypsinized and subcultured at $4x10^4$ cells/cm^2. C57.1 cells were grown in DMEM medium supplemented with 10% heat-inactivated FBS, 25 mM HEPES, 100 µg/ml penicillin/streptomycin and 4 mM glutamine. Density of the cells was maintained between $2x10^5$ and $1x10^6$ cells/ml. Murine BMMC were obtained in 4-6-week culture of cells from bone marrow of BALB/c mice in RPMI 1640 supplemented with 20% WEHI-3-conditioned medium as a source of IL-3, 10% FCS, 4 mM L-glutamine, 25 mM HEPES, and 100 µg/ml penicillin/streptomycin. All cells were grown in 5% CO_2 at 37°C in fully humidified atmosphere. All media components were purchased from Sigma.

For transfection, 25-50 µg DNA was added to $2x10^6$-$10x10^6$ cells in 400 µl culture medium and electroporated in Gene Pulser II (Bio-Rad) at 250 V, 950 µF. Immediately following transfection, cells were diluted to $2.5x10^5$ cells/ml in fresh medium.

For inhibitor studies, cells were incubated with 1 µg/ml BFA or 1 µM monensin applied at the time of transfection, or with 10 mM NH_4Cl or 2 µM tunicamycin applied 4 h prior to transfection or at the time of transfection. The viability of the cells subject to concentrations of BFA, monensin, NH_4Cl and tunicamycin used in experiments was assessed by annexinV-PE/7-AADstaining, and established as 60%, 75%, 88% and 95%, respectively. Non-inhibited cells exhibited 95% viability. For microscopic analysis only the cells exhibiting viable morphology have been chosen.

For TNF-alpha-release determination, cells transfected with p1-235TNF-EYFP, following removal of non-viable floating cells, were seeded at $8x10^4$ cells/cm^2. Medium volume was adjusted to obtain final culture density of $2.5x10^5$ cells/ml. TNF-alpha ELISA was performed using commercial kit (Biosource, Nivelles, Belgium) according to manufacturer's instructions.

SDS-PAGE and Western blotting

For SDS-PAGE, cells were collected and heated for 10 min in 99° C in PNGase F glycoprotein denaturing buffer (New England Biolabs) or in 0.1 M acetate buffer pH 5.5 containing 0.2% SDS, and immediately processed or stored in -20°C. Total lysates prepared in PNGase F glycoprotein denaturing buffer were deglycosylated with PNGase F (NEB) according to manufacturer's instructions. Lysates prepared in 0.1 M acetate buffer pH 5.5 containing 0.2% SDS were diluted 1:1 with 0.1 M acetate buffer pH 5.5 containing 2% Triton X-100 and deglycosylated with endoglycosidase H (Roche Applied Science) according to manufacturer's instructions. Samples were resolved on 4-12% NuPage gels (Invitrogen) in MOPS or MES buffer system, under denaturing conditions. Following electrophoresis proteins were transferred to Hybond N membrane (Amersham) in semi-dry blotter (Bio-Rad) at 15 V for 30 min. Western blotting was conducted according to standard procedures using rabbit anti-GFP and horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies or goat anti-actin and HRP-conjugated donkey anti-goat antibodies (Santa Cruz Biotechnology). Chemiluminescent detection was performed using ECL kit (Amersham) according to manufacturer's protocol.

Microscopy

RBL-2H3 cells were seeded in LabTek chambered coverslips (Nunc) at the density of $5x10^4$ cells/cm^2. For fixation medium was removed, and adhered cells were fixed in 4% formaldehyde/PBS. For anti-RMCP-II staining all steps were performed in 0.05% saponin/3% BSA/PBS. Briefly, cells were pre-permeabilized for 15 min at room temperature and incubated for 1 h at room temperature with 1:100 sheep anti-RMCP-II polyclonal antibodies (Moredun Scientific). Following thorough washing, cells were incubated for 1 h at room temperature with 1:200 Texas Red-conjugated rabbit anti-sheep $F(ab')_2$ (Jackson ImmunoResearch) and washed. For colocalization with TR-D cells were incubated for 1 h at room temperature with 1:200 goat anti-sheep HRP-conjugated antibodies followed by incubation with Alexa 488-coupled tyramide (Molecular Probes). For 5-HT staining, cells were incubated with 200 μM 5-HT for 16 h, fixed and stained in 0.1% Triton X-100/5% FBS/PBS, 1:1000 rabbit anti-serotonin (Sigma) and 1:5000 Texas Red-conjugated goat anti-rabbit $F(ab')_2$ (Jackson ImmunoResearch) and washed. For TR-D (Molecular Probes) live observations, cells were incubated in medium with dextran (100-500 ng/ml) for the indicated times, washed and resuspended in fresh phenol red-free medium. Nuclei were stained by 10-min incubation in 1 μM TO-PRO-3 (Molecular Probes).

C57.1 cells were stained in suspension following the above procedures, centrifuged, resuspended in PBS, transferred to LabTek chambers and subjected to microscopy. After final washing, cells were mounted in 5 mg/ml DABCO (Sigma)/ Fluoromount G (Southern Biotech). Fluorescence microscopy, image acquisition and analysis was performed using Olympus IX70 inverted microscope (UPLAPO 100x oil immersion objective), FView II cooled CCD monochrome camera (Soft Imaging System GmbH, Germany), bandpass filter sets for CFP, GFP, YFP and Texas Red (Chroma) and AnalySIS 3.2 software (Soft Imaging System). Multicolour reconstruction was also

performed in AnalySIS 3.2 software. Confocal microscopy was performed on Leica TCS SP2 AOBS microscope. Image processing was performed in Leica Confocal Software package. Additional processing was performed using NIH ImageJ software.

All monochrome images with exception of images in Fig. 6A-C are presented in unprocessed form. In processed images, linear transformation of brightness/contrast was performed to enhance details. For visualization purposes, colour restoration in multicolour images was accompanied by linear adjustments of brightness/contrast.

Sequence analysis

Predictions of N-linked glycosylation and O-linked glycosylation sites were performed using software hosted on Center of Biological Sequence Analysis, Technical University of Denmark DTU (http://www.cbs.dtu.dk/services).

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References

1. Echtenacher, B., D. N. Mannel, and L. Hultner. 1996. Critical protective role of mast cells in a model of acute septic peritonitis. *Nature 381:75*.
2. Malaviya, R., T. Ikeda, E. Ross, and S. N. Abraham. 1996. Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. *Nature 381:77*.
3. Zhang, Y., B. F. Ramos, and B. A. Jakschik. 1991. Augmentation of reverse arthus reaction by mast cells in mice. *J Clin Invest 88:841*.
4. Zhang, Y., B. F. Ramos, B. Jakschik, M. P. Baganoff, C. L. Deppeler, D. M. Meyer, D. L. Widomski, D. J. Fretland, and M. A. Bolanowski. 1995. Interleukin 8 and mast cell-generated tumor necrosis factor-alpha in neutrophil recruitment. *Inflammation 19:119*.
5. Beil, W. J., G. R. Login, M. Aoki, L. O. Lunardi, E. S. Morgan, S. J. Galli, and A. M. Dvorak. 1996. Tumor necrosis factor alpha immunoreactivity of rat peritoneal mast cell granules decreases during early secretion induced by compound 48/80: an ultrastructural immunogold morphometric analysis. *Int Arch Allergy Immunol 109:383*.
6. Gordon, J. R., and S. J. Galli. 1990. Mast cells as a source of both preformed and immunologically inducible TNF-alpha/cachectin. *Nature 346:274*.
7. Hide, I., N. Toriu, T. Nuibe, A. Inoue, M. Hide, S. Yamamoto, and Y. Nakata. 1997. Suppression of TNF-alpha secretion by azelastine in a rat mast (RBL-2H3) cell line: evidence for differential regulation of TNF-alpha release, transcription, and degranulation. *J Immunol 159:2932*.
8. Baumgartner, R. A., K. Yamada, V. A. Deramo, and M. A. Beaven. 1994. Secretion of TNF from a rat mast cell line is a brefeldin A-sensitive and a calcium/protein kinase C-regulated process. *J Immunol 153:2609*.
9. Decoster, E., B. Vanhaesebroeck, P. Vandenabeele, J. Grooten, and W. Fiers. 1995. Generation and biological characterization of membrane-bound, uncleavable murine tumor necrosis factor. *J Biol Chem 270:18473*.
10. Solomon, K. A., M. B. Covington, C. P. DeCicco, and R. C. Newton. 1997. The fate of pro-TNF-alpha following inhibition of metalloprotease-dependent processing to soluble TNF-alpha in human monocytes. *J Immunol 159:4524*.
11. Schlondorff, J., J. D. Becherer, and C. P. Blobel. 2000. Intracellular maturation and localization of the tumour necrosis factor alpha convertase (TACE). *Biochem J 347 Pt 1:131*.
12. Gearing, A. J., P. Beckett, M. Christodoulou, M. Churchill, J. Clements, A. H. Davidson, A. H. Drummond, W. A. Galloway, R. Gilbert, J. L. Gordon, and et al. 1994. Processing of tumour necrosis factor-alpha precursor by metalloproteinases. *Nature 370:555*.
13. Schwartz, L. B., and K. F. Austen. 1980. Enzymes of the mast cell granule. *J Invest Dermatol 74:349*.
14. Dell'Angelica, E. C., C. Mullins, S. Caplan, and J. S. Bonifacino. 2000. Lysosome-related organelles. *Faseb J 14:1265*.
15. Kuliawat, R., J. Klumperman, T. Ludwig, and P. Arvan. 1997. Differential sorting of lysosomal enzymes out of the regulated secretory pathway in pancreatic beta-cells. *J Cell Biol 137:595*.
16. Dragonetti, A., M. Baldassarre, R. Castino, M. Demoz, A. Luini, R. Buccione, and C. Isidoro. 2000. The lysosomal protease cathepsin D is efficiently sorted to and secreted from regulated secretory compartments in the rat basophilic/mast cell line RBL. *J Cell Sci 113 (Pt 18):3289*.
17. Marks, M. S., L. Woodruff, H. Ohno, and J. S. Bonifacino. 1996. Protein targeting by tyrosine- and di-leucine-based signals: evidence for distinct saturable components. *J Cell Biol 135:341*.
18. Bonifacino, J. S., and L. M. Traub. 2003. Signals for sorting of transmembrane proteins to endosomes and lysosomes. *Annu Rev Biochem 72:395*.
19. Gao, Y., H. Rosen, E. Johnsson, J. Calafat, H. Tapper, and I. Olsson. 2003. Sorting of soluble TNF-receptor for granule storage in hematopoietic cells as a principle for targeting of selected proteins to inflamed sites. *Blood 102:682*.
20. Hansson, M., S. Jonsson, A. M. Persson, J. Calafat, H. Tapper, and I. Olsson. 2003. Targeting proteins to secretory lysosomes of natural killer cells as a principle for immunoregulation. *Mol Immunol 40:363*.
21. Le Borgne, R., and B. Hoflack. 1998. Protein transport from the secretory to the endocytic pathway in mammalian cells. *Biochim Biophys Acta 1404:195*.
22. Dell'Angelica, E. C., and G. S. Payne. 2001. Intracellular cycling of lysosomal enzyme receptors: cytoplasmic tails' tales. *Cell 106:395*.
23. Sherry, B., D. M. Jue, A. Zentella, and A. Cerami. 1990. Characterization of high molecular weight glycosylated forms of murine tumor necrosis factor. *Biochem Biophys Res Commun 173:1072*.
24. Jansson, S. E. 1971. Uptake of 5-hydroxytryptamine by mast cell granules in vitro. *Acta Physiol Scand 82:35*.
25. Shurety, W., A. Merino-Trigo, D. Brown, D. A. Hume, and J. L. Stow. 2000. Localization and post-Golgi trafficking of tumor necrosis factor-alpha in macrophages. *J Interferon Cytokine Res 20:427*.

26. Amano, T., T. Furuno, N. Hirashima, N. Ohyama, and M. Nakanishi. 2001. Dynamics of intracellular granules with CD63-GFP in rat basophilic leukemia cells. *J Biochem (Tokyo) 129:739*.
27. Brown, F. D., N. Thompson, K. M. Saqib, J. M. Clark, D. Powner, N. T. Thompson, R. Solari, and M. J. Wakelam. 1998. Phospholipase D1 localises to secretory granules and lysosomes and is plasma-membrane translocated on cellular stimulation. *Curr Biol 8:835*.
28. Demo, S. D., E. Masuda, A. B. Rossi, B. T. Throndset, A. L. Gerard, E. H. Chan, R. J. Armstrong, B. P. Fox, J. B. Lorens, D. G. Payan, R. H. Scheller, and J. M. Fisher. 1999. Quantitative measurement of mast cell degranulation using a novel flow cytometric annexin-V binding assay. *Cytometry 36:340*.
29. Hibi, T., N. Hirashima, and M. Nakanishi. 2000. Rat basophilic leukemia cells express syntaxin-3 and VAMP-7 in granule membranes. *Biochem Biophys Res Commun 271:36*.
30. Baumgartner, R. A., V. A. Deramo, and M. A. Beaven. 1996. Constitutive and inducible mechanisms for synthesis and release of cytokines in immune cell lines. *J Immunol 157:4087*.
31. Tartakoff, A., P. Vassalli, and M. Detraz. 1978. Comparative studies of intracellular transport of secretory proteins. *J Cell Biol 79:694*.
32. Zhou, P., K. Devadas, D. Tewari, A. Jegorow, and A. L. Notkins. 1999. Processing, secretion, and anti-HIV-1 activity of IL-16 with or without a signal peptide in CD4+ T cells. *J Immunol 163:906*.
33. Zhu, F. G., K. Gomi, and J. S. Marshall. 1998. Short-term and long-term cytokine release by mouse bone marrow mast cells and the differentiated KU-812 cell line are inhibited by brefeldin A. *J Immunol 161:2541*.
34. Kurys, G., Y. Tagaya, R. Bamford, J. A. Hanover, and T. A. Waldmann. 2000. The long signal peptide isoform and its alternative processing direct the intracellular trafficking of interleukin-15. *J Biol Chem 275:30653*.
35. Suarez-Quian, C. A. 1987. The distribution of four lysosomal integral membrane proteins (LIMPs) in rat basophilic leukemia cells. *Tissue Cell 19:495*.
36. Pohlmann, R., M. W. C. Boeker, and K. von Figura. 1995. The Two Mannose 6-Phosphate Receptors Transport Distinct Complements of Lysosomal Proteins. *J. Biol. Chem. 270:27311*.
37. Zhang, Y., B. F. Ramos, and B. A. Jakschik. 1992. Neutrophil recruitment by tumor necrosis factor from mast cells in immune complex peritonitis. *Science 258:1957*.
38. Young, J. D., C. C. Liu, G. Butler, Z. A. Cohn, and S. J. Galli. 1987. Identification, purification, and characterization of a mast cell-associated cytolytic factor related to tumor necrosis factor. *Proc Natl Acad Sci U S A 84:9175*.