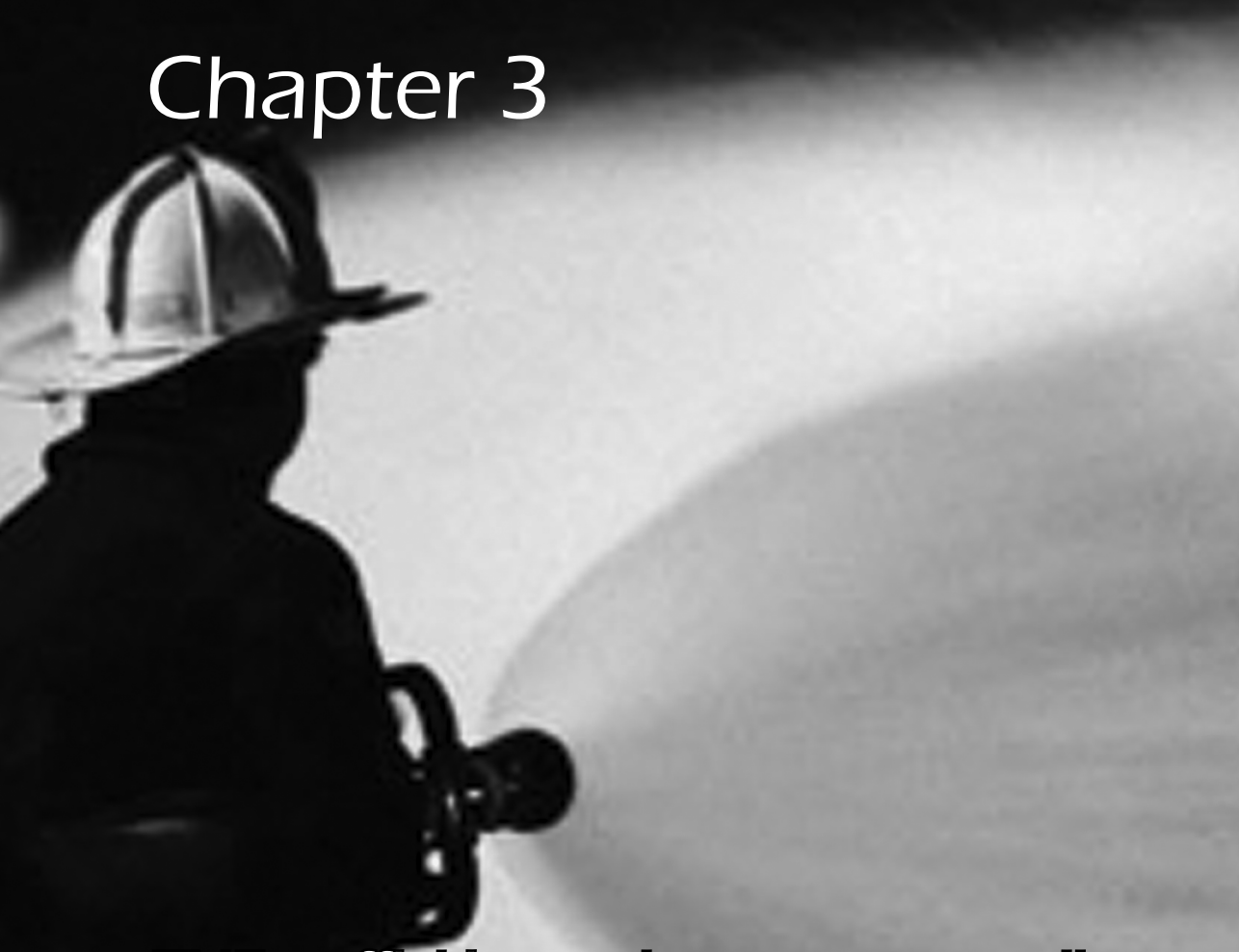
Chapter 3



TNF trafficking to human mast cell granules: mature chain-dependent endocytosis

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Abstract

Mast cells play crucial role at early stages of immune response against bacteria and parasites where their functionality is based on their capability of releasing highly bioactive compounds, amongst them TNF. Mast cells are considered the only cells storing preformed TNF which allows for immediate release of this cytokine upon contact with pathogens. We approached the question of mechanisms and amino acid motifs directing newly synthesized TNF for storage in cytoplasmic granules by analyzing trafficking of a series of TNF-EGFP fusion proteins in human mast cell lines HMC-1 and LAD2. Protein covering full TNF sequence was successfully sorted into secretory granules in a process involving transient exposure on the outer membrane and reendocytosis. In human cells, contrary to results obtained in a rodent model, TNF seems not to be glycosylated and thus trafficking is carbohydrate-independent.

In an effort to localize the amino acid motif responsible for granule targeting, we constructed additional fusion proteins and analyzed their trafficking, concluding that granule targeting sequences are localized in the mature chain of TNF and that the cytoplasmic tail is expendable for endocytotic sorting of this cytokine thus excluding direct interactions with intracellular adaptor proteins.

Introduction

Mast cells are an important element of both innate and acquired immunity. Upon appropriate stimulation cells undergo activation and subsequent degranulation, releasing and synthesizing highly bioactive, proinflammatory, vasodilatative, chemotactic and cytotoxic substances. One class of mast cell mediators are cytokines, amongst them TNF. As shown in a model of bacteria-induced inflammation, deprivation of the mast cell-derived pool of TNF leads to significantly reduced influx of neutrophils and increased mortality (1, 2). In addition, in the reverse Arthus reaction animal model the critical importance of mast cell-derived TNF was ascertained (3, 4). The important role of mast cells as a source of TNF may be explained by their unique capability of storing preformed cytokine in cytoplasmic granules, which allows for its immediate release upon stimulation (5, 6). Several hours after initial stimulation and degranulation-related release of TNF, mast cells synthesize and release additional amounts of TNF by direct vesicular transport from Golgi apparatus to the plasma membrane (7, 8). The mechanism of TNF trafficking has been studied in macrophages, revealing that TNF is synthesized as a 26 kDa transmembrane precursor protein (tmTNF) and later on proteolytically processed to 17 kDa soluble form (sTNF). Conversion of tmTNF into sTNF takes place en route from endoplasmatic reticulum to the plasma membrane (9) and is catalyzed by TNF converting enzyme (TACE/ADAM17).

The mechanisms regulating TNF trafficking in mast cells have not been studied extensively. Particularly little is known about the nature of the switch directing TNF protein either into cytoplasmic granules or directly to the cell membrane, which might depend on the cellular activation status.

We have shown previously (10) that in rodent mast cells TNF sorting to cytoplasmic granules largely depends on N-linked glycosylation and MPR (mannose-6-phosphate receptor)-based system. While major pieces of evidence for specific biological function of TNF stored in mast cell granules come from a rodent model the existence of similar pool of this cytokine in many subsets of human mast cells has been reported (11-13). There is growing body of evidence, that human mast cell-derived TNF plays a critical role in certain physiological and pathological processes including vascular diseases (14, 15), host defense (16), tissue remodeling (17, 18) and various chronic inflammatory states (19-21).

Both the pleiotropic nature of TNF and its potential as a drug target prompted us to explore the mechanisms of trafficking of this cytokine into cytoplasmic granules in human mast cells. Analysis of amino acid sequence of human TNF reveals that the N86 asparagine residue that is glycosylated in rodents is replaced by threonine and thus no N-linked glycosylation, important for trafficking of this molecule in rodent mast cells, is possible. In this work we provide evidence for existence of specific ER-entry and cytoplasmic granules targeting motifs in human TNF and show

that in human mast cells a fusion protein composed of full-length TNF fused to EGFP (Enhanced Green Fluorescent Protein) on its way to cytoplasmic granules is transiently exposed on the outer membrane.

Results

Human TNF is not modified with sialic acid

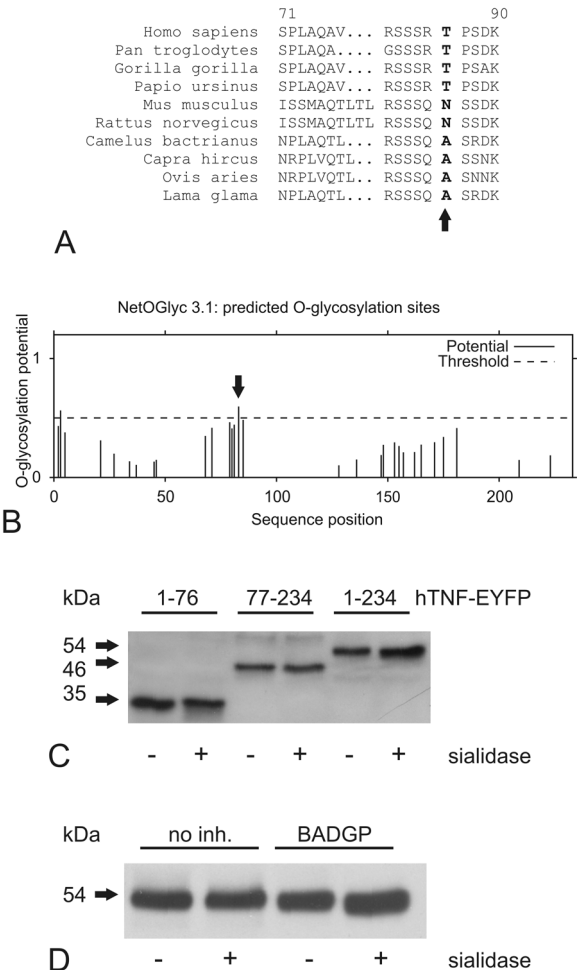


Figure 1 TNF glycosylation analysis. (A) TNF sequences were compared across species showing evolution from N-glycosylation (N86 residue in rodents) to potential O-glycosylation (T83 in primates, indicated by arrow) (B) Neural network prediction of O-glycosylation of human TNF. T83 residue indicated by arrow (C) Western blot analysis of lysates HMC-1 cells transfected with p1-76, p77-234 and p1-234hTNF-EGFP and treated with sialidase. (D) Western blot analysis of lysates HMC-1 cells transfected with p1-234hTNF-EGFP, cultured in the presence or absence of 2 mM benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside and treated with sialidase

We have shown before that in rodent mast cells TNF delivery to mast cell granules largely depends on N-linked glycosylation (10). Phylogenetic protein sequence analysis showed, however, that the N-terminal region of sTNF is one of the least conserved and that the N-linked glycosylation motif NSS is unique to rodents and in primates is replaced by RTP motif (Fig. 1A). Sequence context-based prediction indicated that in human TNF T83 residue could be glycosylated (Fig. 1B). To test this prediction HMC-1 cells were transfected with p1-76hTNF-EGFP, p77-234hTNF-EGFP or p1-234hTNF-EGFP and lysed 24 hours after transfection .The former two were used as negative controls due to lack of the glycosylation target motif and improper subcellular localization, respectively. Lysates were treated with neuraminidase and analyzed by Western blot. Proteins of apparent molecular masses of 35, 46 and 54 kDa were detected in lysates of cells transfected with p1-76-, p77-234- and p1-234hTNF-EGFP. Regardless of the lysate tested, the protein molecular weight did not change following neuraminidase treatment which suggests the protein is not sialylated (Fig. 1C). To substantiate that result HMC-1 cells were transfected with p1-234hTNF-EGFP and following transfection cultured in the presence of 2 mM benzyl

2-acetamido-2-deoxy-α-D-galactopyranoside (BADGP), which serves as a competitive substrate for N-acetyl-β-D-glucosaminyltransferase and inhibits O-linked glycosylation (25). 24 hours after transfection cells were lysed; lysates were treated with neuraminidase and analyzed by Western blot. Regardless of BADGP and neuraminidase treatment in all lysates a protein of apparent molecular mass of 54 kDa was detected (Fig. 1D). Since majority of O-linked carbohydrate chains contain sialic acid as a terminal residue, these results strongly suggest that in HMC-1 cells TNF is not O-glycosylated. This is further supported by a good agreement between calculated and observed molecular weight of TNF expressed in HMC-1 cells.

Intracellular localization TNF-EGFP fusion proteins in mast cells

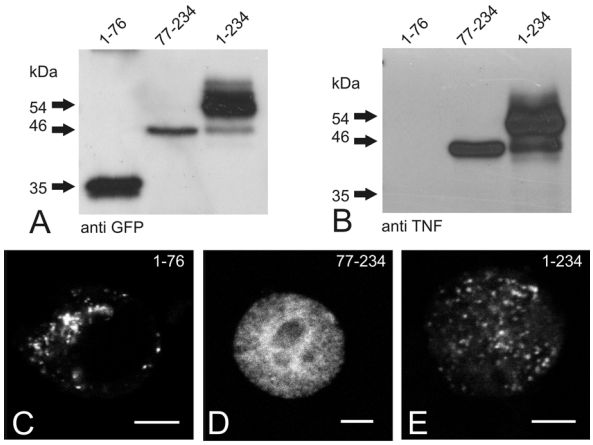
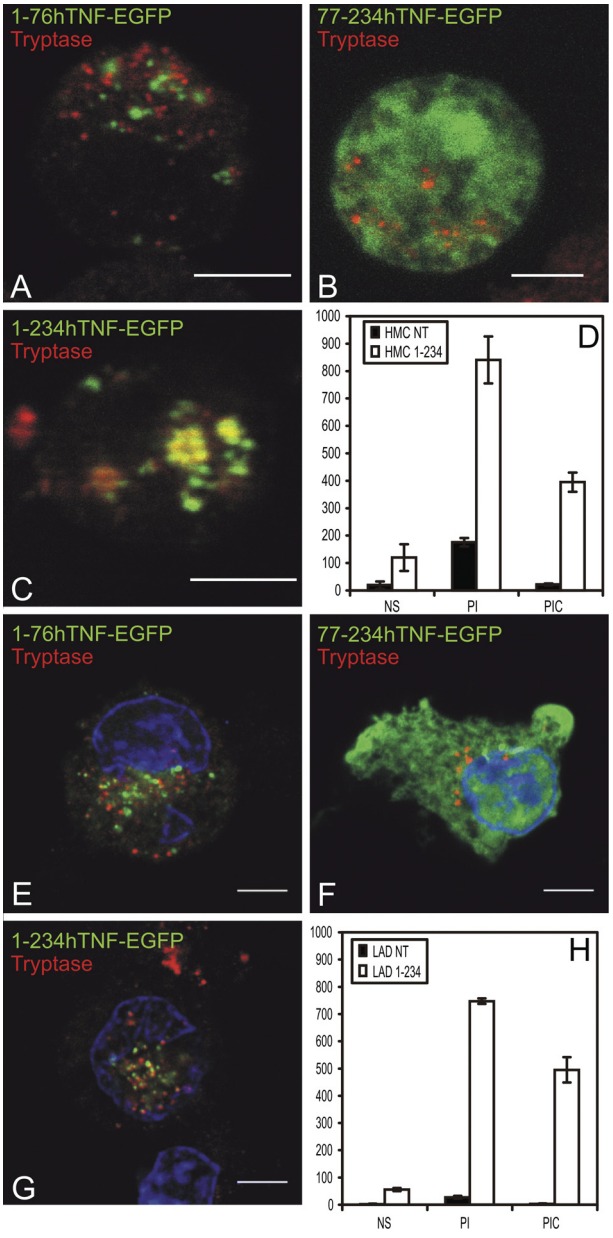


Figure 2 Expression and intracellular localization of TNF-EGFP fusion proteins. (A-B) HMC-1 cells were transfected with p1-76, p77-234 or p1-234hTNF-EGFP and lysed 24 hours post-transfection; lysates were analyzed by Western blot using anti-GFP and anti-TNF primary antibodies. (C-E) HMC-1 cells were transfected with p1-76, p77-234 or p1-234hTNF-EGFP and imaged under confocal microscope 24 hours post-transfection. Representative confocal planes are presented. Bars represent 5 μm.

HMC-1 cells were transiently transfected with p1-76hTNF-EGFP, p77-234hTNF-EGFP or p1-234hTNF-EGFP plasmids and 24 hours post-transfection cell lysates were analyzed by Western blot (Fig. 2A-B). In lysates of p1-76hTNF-EGFP-transfected cells a protein of apparent molecular mass of 35 kDa could be detected using anti-GFP antibodies whereas no bands were detected with antibodies directed against sTNF. In lysates of p77-234hTNF-EGFP-transfected cells a protein of apparent molecular mass of 46 kDa was detected with both anti-TNF and anti-GFP antibodies. In lysates of p1-234hTNF-EGFP-transfected cells a major band of apparent molecular mass of 54 kDa and minor bands of 46 kDa and 56 kDa were detected. Thus, proper expression of TNF-EGFP fusion proteins has been confirmed, additionally revealing possible proteolytic processing and posttranslational modification of 1-234hTNF-EGFP (46 and 56 kDa bands, respectively). In parallel, green fluorescence patterns in HMC-1 cells transiently transfected with p1-76hTNF-EGFP, p77-234hTNF-EGFP or p1-234hTNF-EGFP were analyzed by confocal microscopy at 3, 10 and 24 hours posttransfection. At early timepoint (3h) in both p1-76- and p1-234hTNF-EGFP-transfected cells fluorescence could be observed in a perinuclear organelle morphologically resembling Golgi apparatus, whereas in p77-234hTNF-EGFP-transfected cells fluorescence was distributed rather evenly (data not shown) and the distribution of 77-234hTNF-EGFP did not change for the whole observation period of 24 hours posttransfection (Fig. 2 D). In contrast 10 hours posttransfection in p1-76- and p1-234hTNF-EGFP-transfected cells vesicular fluorescent structures

could be observed (data not shown) and after 24 hours the number of these structures has increased while fluorescence in Golgi-resembling structure has diminished, probably due to transient transfection and decreasing expression of a transgene (Fig. 2C and E). Thus, both propeptide (1-76) and full-length (1-234) TNF sequences, as opposed to soluble TNF (77-234) were shown capable of sorting EGFP into vesicular intracellular structures.

1-234hTNF-EGFP is sorted to and released from mast cell granules



In order to establish whether fluorescent vesicular structures observed following transfection of mast cells with p1-76hTNF-EGFP and p1-234hTNF-EGFP indeed are mast cell granules, intracellular localization of the three fusion proteins and an established mast cell granule compound, tryptase, was compared. HMC-1 and LAD2 cells were transfected with p1-76hTNF-EGFP, p77-234 hTNF-EGFP or p1-234hTNF-EGFP and 24 hours after transfection stained for tryptase. Cells expressing 1-76hTNF-EGFP exhibited vesicular patterns in

Figure 3 1-234hTNF-EGFP is sorted to and released from mast cell granules. (A-C) HMC-1 cells and (E-G) LAD2 cells were transfected with p1-76, p77-234 or p1-234hTNF-EGFP (green channel) and 24 hours post-transfection fixed and stained for tryptase (red channel). Colocalization is represented by yellow color. For LAD2 cells nuclei are visualized by TO-PRO3 stain (blue channel). Representative confocal planes are shown. Bars represent 5μm. (D, H) ELISA analysis of TNF release from HMC-1 and LAD2 cells, respectively. Non-transfected cells (NT), cells transfected with p1-234hTNF-EGFP (1-234). Stimuli: non-stimulated (NS), PMA/ionomycin (PI), PMA/ionomycin/cycloheximide (PIC). Supernatants for analysis were collected 60 minutes following stimulation. For detailed description see text. The differences in TNF release resulting from transfection or treatments, measured at 1 hour time-point were found statistically significant.

both green and red fluorescence channels (EGFP and tryptase, respectively) but hardly any colocalization was observed (Fig. 3A and E for HMC-1 and LAD2, respectively). In cells transfected with p77-234hTNF-EGFP mostly a diffuse pattern of fluorescence was observed in EGFP channel whereas tryptase stain revealed vesicular pattern (Fig. 3B and F for HMC-1 and LAD2, respectively). No specific colocalization of 77-234hTNF-EGFP and tryptase was observed in any of the cell lines. Transfection of cells with p1-234hTNF-EGFP resulted in accumulation of EGFP fluorescence in vesicular structures most of which also stained positive for tryptase (Fig. 3C and G for HMC-1 and LAD2, respectively) identifying the compartment as mast cell granules.

In order to test whether the function of granules is not disturbed by directing exogenously expressed TNF-EGFP fusion protein into this compartment, TNF release from stimulated mast cells was assessed. HMC-1 and LAD2 cells were transfected with p1-234hTNF-EGFP and cultured for 24 hours; a subset of cells was then incubated with 20 μM CHX for 60 minutes. For TNF release cells were incubated for 60 minutes in culture medium alone, in culture medium containing 100 ng/ml PMA and 1 μM ionomycin, or in culture medium containing both stimuli and 20 μM CHX . Non-transfected HMC-1 cells and cells expressing 1-234hTNF-EGFP, following treatment with PMA/Ionomycin, released 176 and 841 pg of TNF /106 cells, respectively. In the presence of PMA, ionomycin and CHX measured values were 22 and 402 pg TNF /106 cells. Basal release from non-stimulated cells was 20 and 120 pg of TNF /106 cells for non-transfected and transfected cells, respectively (Fig. 3D). The amounts of TNF released by HMC-1 cells within 30 minutes from stimulation, while not in all cases significantly different between transfection groups and treatments, fit the trend that is set by the statistically significant differences measured 60 minutes of stimulation thus supporting the presence of preformed cytokine in the cells (data not shown). Non-transfected LAD2 cells and cells expressing 1-234hTNF-EGFP, following treatment with PMA/Ionomycin, released 27 and 747 pg of TNF /106 cells, respectively. In the presence of PMA, ionomycin and CHX measured values were 3 and 495 pg TNF /106 cells. Basal release from non-stimulated cells was 2 and 27 pg of TNF /106 cells for non-transfected and transfected cells, respectively (Fig. 3H).

These results demonstrated that 1-234hTNF fusion protein is indeed sorted to, and released from mast cell granules and raised the question of the trafficking route utilized for delivery of 1-234hTNF-EGFP to this compartment.

1-234hTNF-EGFP is exposed on the cell membrane on its way to granules

In absence of carbohydrate residues TNF in HMC-1 cells might not be efficiently retrieved from the secretory pathway and thus become exposed on the cell membrane and eventually re-internalized for granule delivery. To test this hypothesis HMC-1 cells were transfected with p1-234hTNF-EGFP and 12 hours posttransfection surface proteins were biotinylated. Subsequently, cells were disrupted (control) or cultured for another 8 hours and disrupted. Cellular organelles were separated by centrifugation on a Percoll gradient and β-hexosaminidase (granular enzyme) activity in these fractions was determined to localize the granule-containing fraction (Fig. 4A).

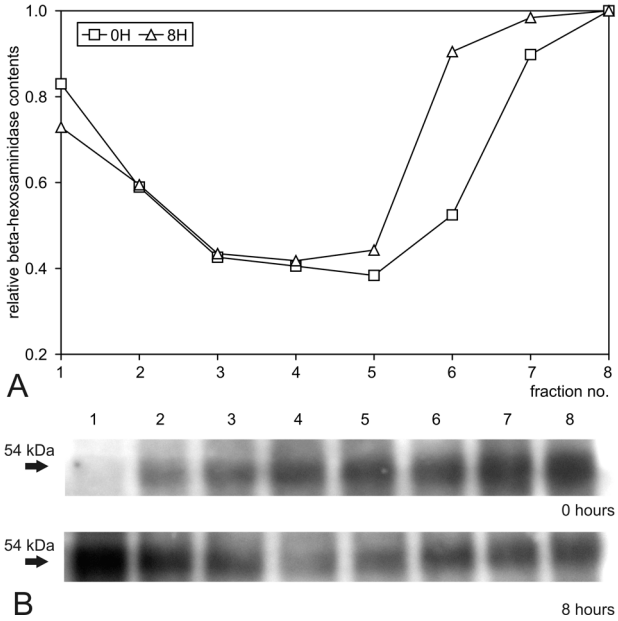


Figure 4 1-234hTNF-EGFP is exposed on the outer membrane on the way to the granules. Cell surface of HMC-1 cells transfected with p1-234hTNF-EGFP was biotinylated 12 hours post-transfection. Cells were disrupted immediately (0H) or following 8 hours chase (8H) and subject to subcellular fractionation. (A) beta-hexosaminidase activity in fractions from heaviest (1) to lightest (8); granule-associated activity in fractions 1-2. (B) anti-GFP Western blot analysis of biotinylated proteins immunoprecipitated from fractions by streptavidin beads.

Additionally, biotinylated proteins were immuno precipitated from fractions using streptavidin-sepharose and analyzed by Western blot using anti-EGFP antibodies. This allowed for detection of EGFP-containing proteins that were exposed on the outer membrane. In fractions obtained from cells disrupted directly after biotinylation, the majority of biotinylated, EGFP-containing protein was detected in light fractions (6-8) that represent cytosol and cell membrane. In fractions obtained from cells that were cultured for 8 hours before disruption, the majority of this protein was found in fractions 1 and 2, containing heavier, β-hexosaminidase-containing organelles, including mast cell granules (Fig. 4B). This indicates that during the 8 hours chase period 1-234hTNF-EGFP protein was translocated from the cell membrane into mast cell granules, though the mechanism of this translocation is not known.

N-terminal domain is dispensable for targeting of TNF to mast cell granules

We addressed the question of processes and amino acid motifs responsible for endocytosis and further trafficking of 1-234hTNF-EGFP to mast cell granules. Deletion mutants 21-234hTNF-EYFP and 46-234hTNF-EYFP were employed to localize the region responsible for endoplasmatic reticulum entry and granular targeting of TNF. Expression of 1-, 21- and 46-234hTNF-EYFP was examined by transfection of HMC-1 cells with respective plasmids and Western blot analysis of lysates with the use of both anti-GFP and anti-TNF antibodies. Both antibodies detected proteins of apparent molecular masses of 54, 52 and 49 kDa in lysates of cells transfected with p1-, 21- and 46-234hTNF-EYFP, respectively (Fig. 5A-B). In order to analyze intracellular localization of truncated mutant proteins HMC-1 cells were cotransfected with p1-234hTNF-ECFP, employed as a granular marker, and p21-234hTNF-EYFP or p46-234hTNF-EYFP or p1-234hTNF-EYFP. 24 hours posttransfection cells were imaged under confocal microscope. Both 21-234hTNF-EYFP and 1-234hTNF-EYFP exhibited granular fluorescence pattern and colocalized with 1-234hTNF-ECFP in mast cell granules (Fig. 5 C and D). In contrast cells expressing 46-234hTNF-EYFP exhibited

diffuse cytoplasmic and nuclear fluorescence with weakly fluorescent vesicles in yellow channel and granular fluorescence in cyan channel. Vesicular structures visible in yellow channel colocalized well with 1-234hTNF-ECFP which identified them as mast cell granules (Fig. 5E).

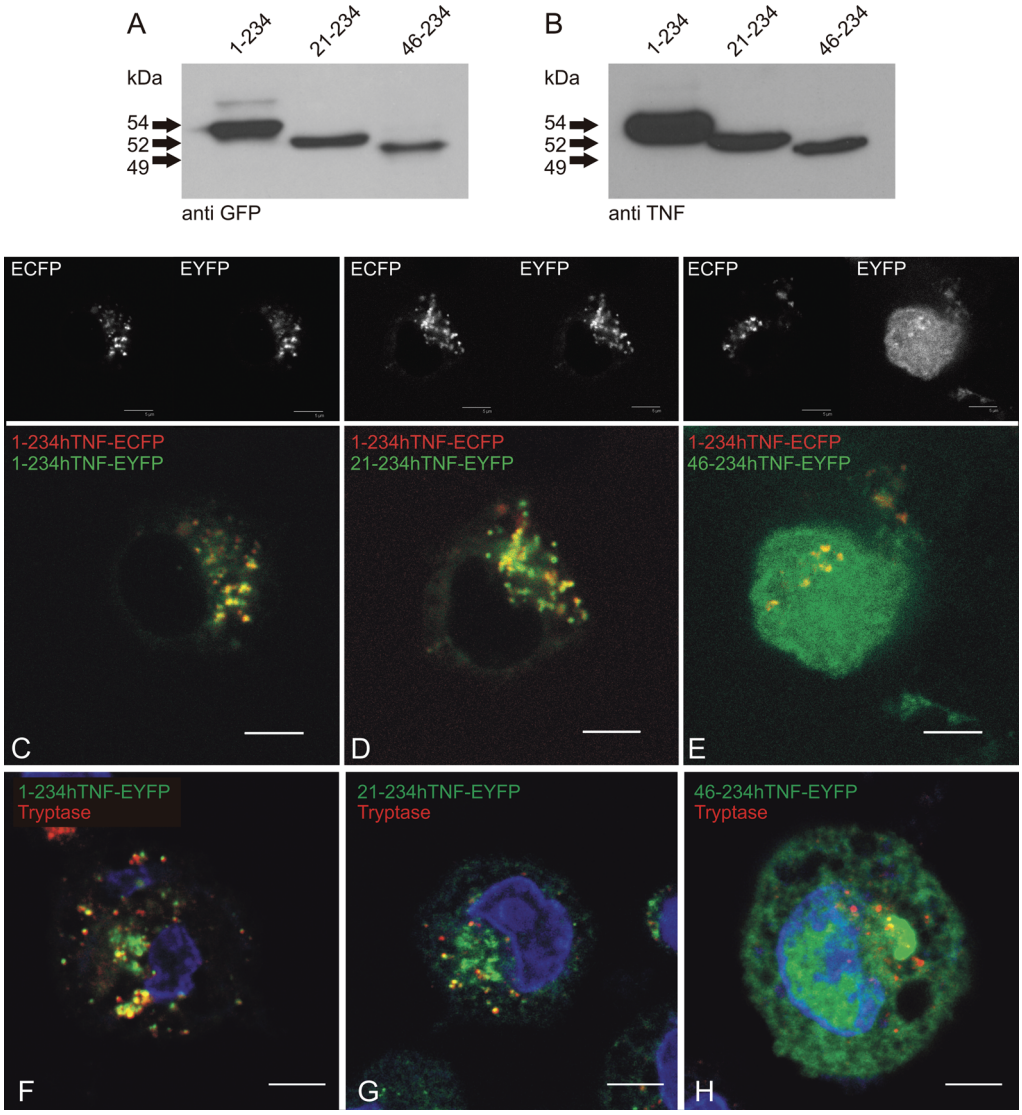


Figure 5 N-terminal domain is dispensable for granular targeting of TNF. (A-B) anti-GFP and anti-TNF Western blot analysis of lysates of HMC-1 cells transfected with p1-, 21- or 46-234hTNF-EYFP. (C-E) confocal microscopy analysis of HMC-1 cells cotransfected with p1-234hTNF-ECFP and p1-, 21- or 46-234hTNF-EYFP and imaged 24 hours post-transfection. In overlay images ECFP is represented by false color red and EYFP by false color green. Colocalization is represented by yellow color. Representative confocal sections are shown. Bars represent 5 µm. (F-H) confocal microscopy analysis of LAD2 cells transfected with p1-, 21- or 46-234hTNF-EYFP and 24 hours posttransfection stained for tryptase. In overlay images EYFP is represented by false color green, tryptase stain by false color red and nuclei are visualized by TO-PRO3 stain (blue channel). Colocalization of EYFP and tryptase is represented by yellow color. Representative confocal sections are shown. Bars represent 5 µm.

In order to ascertain these results LAD2 cells were transfected with p1-, p21- or p46-234hTNF-EYFP and 24 hours post-transfection stained for tryptase. In cells transfected with p1-234hTNF-EYFP or p21-234hTNF-EYFP vesicular pattern of fluorescence was observed in green channel (EYFP) which colocalized well with granules visualized by tryptase staining (red channel) (Fig. 5F and G). Conversely, cells transfected with p46-234hTNF-EYFP exhibited diffuse cytoplasmic and nuclear pattern in the green channel with no specific colocalization observed between 46-234hTNF-EYFP and tryptase (Fig. 5H).

These data indicate that N-terminal 20 amino acids of TNF are dispensable for delivery of this protein to mast cell granules and that 21-45 region of TNF is necessary for its efficient endoplasmatic reticulum entry and subsequent trafficking.

1-234- and 21-234- but not 46-234hTNF-EGFP are actively released from cells

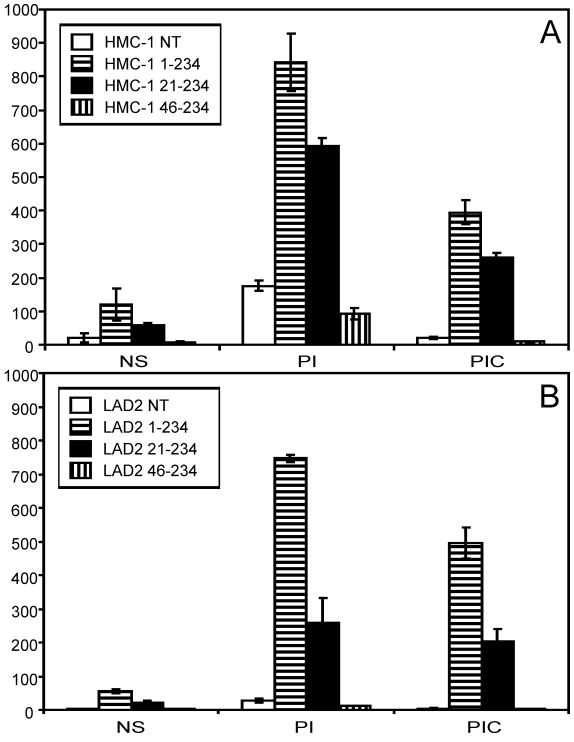


Figure 6 1- and 21-234 but not 46-234 TNF-GFP are actively released from cells. (A-B) ELISA analysis of TNF release from HMC-1 and LAD2 cells. Non-transfected cells (NT), cells transfected with p1-, 21- or 46-234hTNF-EGFP (1-234, 21-234, 46-234 respectively). Stimuli: non-stimulated (NS), PMA/ionomycin (PI), PMA/ionomycin/cycloheximide (PIC). Supernatants for TNF concentration measurements were collected from HMC-1 and LAD2 cultures 60 minutes post-stimulation (A and B, respectively). For detailed description see text. The differences in TNF release resulting from transfection or treatments, measured at 1 hour time-point were found statistically significant.

Mast cells' ability to release N-terminal deletion mutants of TNF-EGFP upon stimulation was assayed. HMC-1 and LAD2 cells were transfected with p46-234hTNF-EGFP, p21-234hTNF-EGFP or p1-234hTNF-EGFP and cultured for 24 hours; a subset of cells was then incubated with 20 μM CHX for 60 minutes. For TNF release cells were incubated in culture medium alone, in culture medium containing 100 ng/ml PMA and 1 μM ionomycin or in culture medium containing both stimuli and 20 μM CHX . After 60 minutes of stimulation and/or inhibition, culture supernatants were collected and TNF concentration was assayed. Results obtained for HMC-1 cells are shown in Fig. 6A. Cells expressing 46-234hTNF-EGFP, 21-234hTNF-EGFP, 1-234hTNF-EGFP or non-transfected cells following treatment with PMA/Ionomycin released 92, 593, 854 and 162 pg TNF /106 cells, respectively. In the presence of PMA, ionomycin and CHX measured values were 9, 262, 410 and 17 pg TNF /106 cells for respective transfectants. Basal

release from non-stimulated cells was 6, 59, 123 and 16 pg TNF /106 cells for cells expressing 46-234hTNF-EGFP, 21-234hTNF-EGFP, 1-234hTNF-EGFP and non-transfected cells, respectively. The amounts of TNF released by HMC-1 cells within 30 minutes from stimulation, while not in all cases significantly different between transfection groups and treatments, fit the trend that is set by the statistically significant differences measured 60 minutes of stimulation (data not shown). TNF concentrations measured in LAD2 culture supernatants are shown in Fig. 6B. Cells expressing 46-234hTNF-EGFP, 21-234hTNF-EGFP, 1-234hTNF-EGFP or non-transfected cells following treatment with PMA/Ionomycin released 11, 258, 747 and 27 pg TNF /106 cells, respectively. In the presence of PMA, ionomycin and CHX measured values were 3, 203, 495 and 3 pg TNF /106 cells for respective transfectants. Basal release from non-stimulated cells was 3, 22, 55 and 2 pg TNF /106 cells for cells expressing 46-234hTNF-EGFP, 21-234hTNF-EGFP, 1-234hTNF-EGFP and non-transfected cells, respectively.

These data show that both 21-234hTNF-EGFP and 1-234hTNF-EGFP are delivered to mast cell granules and released upon stimulation although in the case of 21-234hTNF-EGFP this process is less efficient as compared to 1-234hTNF-EGFP.

Discussion

Among many cytokine-releasing cells mast cells are the only ones known to store preformed TNF in their cytoplasmic granules and release it upon activation. While intracellular trafficking, processing and release of TNF were investigated in macrophages (26), the noticeable difference is that in mast cells there apparently operate mechanisms responsible for diverting TNF from the constitutive secretory pathway to mast cell granules.

We have shown recently, that in rodent mast cells TNF trafficking to cytoplasmic granules is dependent on N-linked glycosylation (10). This mechanism however does not seem to be evolutionarily conserved, since it is predominantly found in Rodentia while in Artiodactyla N86 residue is replaced by alanine which excludes any glycosylation. In contrast in Primates the N-linked glycosylation motif is replaced by potential O-linked glycosylation site (Fig. 1A). This posttranslational modification may also be involved in protein trafficking (25, 27) but its presence is more context-dependent than N-linked glycosylation. Neural network-based computer prediction (28) indicated that in human TNF T83 residue might be glycosylated (Fig. 1B). Our results obtained by two different approaches suggest opposite (Fig. 1 C-D). It has to be noted, however, that posttranslational modifications may be cell lineage-specific as O-glycosylation of TNF in human B-cell lymphoblastoid cell line BALL-1 has been reported (29). This might reflect involvement of differential, cell line-specific trafficking mechanisms for delivery of the protein cargo to the target compartment such as mast cell granules, or release to extracellular space.

EGFP fusion proteins have been successfully employed to follow intracellular trafficking of many proteins, including mast cell granular proteins CD63, VAMP-7, syntaxin-3 and phospholipase D (30-33). In this work we chose a similar strategy

and transiently transfected human mast cell line HMC-1 or LAD2 with several TNF-EGFP fusion proteins to visualize intracellular trafficking of TNF in these cells. HMC-1 cells are often utilized as a model system for investigation of mast cell biology, including cytokine physiology (34-36). It has to be mentioned, however, that these cells lack several phenotypic features that are characteristic for normal human mast cells, including high-affinity IgE receptors (34), and thus conclusions concerning human mast cells have to be drawn with caution. LAD2 is a recently developed cell line that closely resembles primary culture of CD34+-derived human mast cells (23). Cell surface marker expression, enzyme contents and functional FcεRI and FcγRI receptors make it a good model system for mast cells investigation. Low growth rate, possibly allowing LAD2 cells to exhibit a more mature phenotype, precludes experiments requiring large amounts of cells such as subcellular fractionation presented in Fig. 4.

The EGFP fused to full-length TNF, when expressed in both HMC-1 and LAD2 cells, accumulated predominantly in cytoplasmic granules, as visualized by colocalization with tryptase, a mast cell granular marker (Fig. 3C and G, respectively). This was not the case for fusion proteins consisting of either TNF propeptide or mature chain fused to EGFP (1-76hTNF-EGFP or 77-234hTNF-EGFP). The former produced vesicular pattern, but organelles it accumulated in apparently are not mast cell granules, as shown by lack of significant colocalization with tryptase (Fig. 3A and E, respectively). This suggests that the protein enters endoplasmatic reticulum, but due to lack of additional signals located in mature chain it is missorted. Fusion protein consisting of mature chain fused directly to EGFP exhibits diffuse fluorescence pattern (Fig. 3B and F, respectively) which indicates that in absence of propeptide, ER entry is impaired and hypothesized granular targeting motifs cannot act. Observed differences in amounts of TNF released by non-transfected and p1-234hTNF-EGFP-transfected cells, even in the presence of CHX, suggest that the fusion protein is not only sorted to, but also efficiently released from mast cell granules.

Because human TNF expressed in HMC-1 cells apparently is not glycosylated it cannot be retrieved from the secretory pathway by carbohydrate-dependent mechanism and might reach the outer membrane. We tested this hypothesis and showed that TNF is transiently exposed on the outer membrane, reendocytosed and ultimately delivered to mast cell granules (Fig. 4). A protein that reaches cell membrane can be reendocytosed through interactions of its cytoplasmic tail with a cytoplasmic protein (reviewed in (37)), by an extracellular receptor (38) or passively with the membrane. Endocytosis of uncleaved TNF has been demonstrated in macrophages (39) and we addressed the question of molecular features involved in reendocytosis of TNF leading to its storage in mast cell granules.

Using EYFP fusion proteins we have shown that truncation of the 20 N-terminal amino acids, constituting majority of the cytoplasmic tail of TNF, does not interfere with its transport to mast cell granules (Fig. 5D and G). Contrarily, deletion of 45 N-terminal amino acids dramatically decreases TNF ER entry although the fraction of 46-234hTNF-EGFP that is translocated into ER (only in HMC-1 cells) seems to

be sorted to mast cell granules (Fig. 5E and H). This localizes an ER-entry motif to 21-45 region of TNF which is in agreement with earlier data obtained in vitro (40). Sorting of 21-234hTNF-EYFP to mast cell granules suggests that TNF granular targeting is mediated by a mechanism that is operating on the outer side of the cell membrane, independently of the cytoplasmic tail of the cytokine. This is further supported by the observation, that 1-76hTNF-EGFP is not sorted to granules, despite its complete cytoplasmic tail. It has to be noted, however, that palmitoylation of human TNF on C30 has been reported (41) and a regulatory role for the lipid residue has been postulated with regard to endocytosis efficiency (42, 43). This reversible posttranslational modification could provide additional level of regulation of TNF endocytosis and granule delivery rate.

Functional characterization of the target compartment reached by 1-, 21- and 46-234hTNF-EYFP as compared to endogenous TNF revealed, that upon stimulation in the presence of CHX mast cells expressing 1- or 21-234hTNF-EYFP release significantly more TNF than cells that are non-transfected or transfected with 46-234hTNF-EYFP (Fig. 6). These data indicate that in absence of majority of its cytoplasmic tail TNF is still efficiently delivered to, stored in and released from mast cell granules.

In this report we present data indicating that glycosylation is not necessary for efficient sorting and release of TNF from human mast cell granules. We also identify a new trafficking route of this cytokine in human mast cells which involves transient exposure to extracellular space followed by endocytosis. The exact nature of this endocytotic mechanism is not known, but the data gathered suggest a mechanism involving recognition of mature chain by a hypothetical receptor mediating delivery to granules. Based on the data presented here it might be hypothesized that regulatory mechanism diverting TNF from constitutive secretory pathway to mast cell granules might be TNF cleavage-dependent. Resting cells express low levels of TNF converting enzyme (TACE) and uncleaved TNF is endocytosed and stored in mast cell granules. Following activation TACE is induced and released into extracellular space (44, 45). Consequently, TNF is processed as it follows the secretory pathway and also during exposure on cell membrane which results in increased release into environment.

Further studies will be required to test the above hypothesis. Identification of the motif recognized in the TNF molecule by the granule-targeting endocytotic mechanism will be particularly important, because interference with the mechanism of TNF trafficking is of potential therapeutic relevance for mast cell-derived TNF-related diseases (46, 47) and reviewed in (48)).

Materials and methods

Plasmid construction

The DNA fragment coding for TNF was obtained by RT-PCR. Briefly, 5 μg of total human RNA (isolated from HMC-1 cell line) was subject to RT reaction, using MMLV reverse transcriptase (Gibco) and random hexamer oligonucleotides in a final volume of 20 μl. cDNA was amplified using AccuTaq polymerase (Sigma) and GGAAAGGACACCATGAGCACTG and GTCCTCCTCACAGGGCAATGAT oligonucleotides

(all oligonucleotide sequences are given in 5' to 3' direction). Product of PCR reaction was cloned into pT-Adv plasmid (Clontech) resulting in plasmid pTA-hTNF. pTA-hTNF was sequenced to verify the integrity of the TNF reading frame. To obtain plasmids encoding for fusion proteins consisting of 1-234, 1-76, 77-234 fragments of TNF and EGFP respective fragments of TNF ORF were PCR-amplified from pTA-hTNF using Pvu polymerase (Fermentas MBI), digested with indicated restriction enzymes and in-frame cloned into pEGFP-N1, pECFP-N1 (enhanced cyan fluorescent protein) and pEYFP-N1 (enhanced yellow fluorescent protein) plasmids (Clontech) digested with XhoI and AgeI. The following forward/reverse oligonucleotide pairs were used: TATACTCGAGATGAGCACTGAAAGCATGATCCGGG/TGACCGGTAATTCGG CTGGTCCTCCTACCAG**(1-234,XhoI/AgeI)**,TATACTCGAGATGAGCACTGAAAGCATG ATCCGGG/ATGACCGGTGGGATTCCAGGACATAATCTGACTG**(1-76,XhoI/AgeI)** and GTCACTCGAGATGGTCAGATCATCTTCTCGAACCCCG/TGACCGGTAATTCGGCTGGTCCTC CTACCAG**(77-234, XhoI/AgeI**). Resulting plasmids were named p1-234, p1-76 and p77-234hTNF-ECFP (EGFP/EYFP), respectively, and sequenced to confirm the integrity of the fusion proteins' ORFs. To prepare p21-234hTNF-EYFP and p46-234hTNF-EYFP corresponding fragments of fusion reading frame were amplified from p1-234hTNF-EYFP, digested with indicated restriction enzymes and ligated into XhoI/NotI digested pEYFP-N1 (4 kbp fragment). The following forward/reverse oligonucleotide pairs were used: CTGCTCGAGGAGGCGCTCCCCAAGATGACA/AGTCGCGGCCGCTTTACTTGTACAG CTCGTCC**(21-234,XhoI/NotI)** and CTGCTCGAGGCAGGCGCCACCATGCTCTTC/AG TCGCGGCCGCTTTACTTGTACAGCTCGTCC**(46-234,XhoI/NotI)**. Resulting plasmids were sequenced to confirm the integrity of the fusion proteins ORFs.

Cell culture, transfection and ELISA

The human mast cell line HMC-1 (22) was a generous gift from Dr. J. Butterfield, (Mayo Clinic, Rochester, MN). Cells were maintained in Iscove's medium supplemented with 10% heat-inactivated FBS and 2 mM glutamine. At the density of ca. 1.5*106 cells/ml cells were diluted to 0.4*106 cells/ml. Cells were grown in 5% CO2 at 37°C in fully humidified atmosphere. All media components were purchased from Sigma. LAD2 cells (23) were a generous gift from Dr. A. S. Kirshenbaum, NIH, Bethesda, USA. Cells were cultured in serum-free media (StemPro-34, Life Technologies, Grand Island, NY) supplemented with 2mM L-glutamine, 100 IU/ml penicillin, 50 µg/ml streptomycin (complete SFM) and 100 ng/ml recombinant human stem cell factor. For transfection 25-50 µg DNA was added to 2-10*106 (HMC-1) or 5*105 (LAD2) cells in 400µl culture medium and electroporated in Gene Pulser II (Biorad) at 250V (HMC-1) or 300V (LAD2), 950µF. Immediately following transfection cells were diluted to 5*105 cells/ml in fresh medium. For TNF release/inhibitor studies cells were incubated, where applicable, with 20 µM cycloheximide (CHX) applied 60 minutes before stimulation. For stimulation 1 µM ionomycin and 100 ng/ml PMA were added for the indicated time. Cell suspension density was adjusted to 1*106 cells/ml. Viability of the cells subject to concentrations of CHX used in experiments was assessed by AnnexinV-PE/7-AAD staining and established as >90%. Non-inhibited cells exhibited 95% viability. TNF ELISA was performed using a commercial kit (Sanquin) according to manufacturer's instructions.

SDS-PAGE and Western blotting

For SDS-PAGE cells were collected and lysed in 0.2% Triton X-100 supplemented with protease inhibitor cocktail (Roche Applied Science) and 1 mM PMSF. For deglycosylation analysis cell lysate containing 10 µg total protein was treated with neuraminidase (New England Biolabs) according to manufacturer's instructions. For fusion protein expression analysis 10 µg of total lysate was loaded per lane of the gel. Samples were resolved on 8 or 10% polyacrylamide gels under denaturing conditions. Following electrophoresis proteins were transferred to Hybond N membrane (Amersham) in wet blotter (Biorad) at 400 mA for 60 min. Western blotting was conducted according to standard procedures using mouse monoclonal anti-GFP (Roche Applied Science) and HRP-conjugated goat anti-mouse (Dako) antibodies or goat anti-TNF and HRP-conjugated donkey anti-goat antibodies (both from Santa Cruz Biotechnology). Chemiluminescent detection was performed using an ECL kit (Amersham) according to manufacturer's protocol.

Subcellular fractionation

HMC-1 cells were transfected with p1-234hTNF-EGFP and 12 hours post-transfection cell surface proteins were biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology) according to manufacturer's instructions. Immediately after labeling or after indicated chase period cells were fractionated as described with minor modifications (24). Briefly, 5-10*107cells were suspended in 2 ml of 0.34 M sucrose, 5 mM HEPES (pH 7.3), 0.5 mM EDTA, 1 mM PMSF (supplemented with cocktail of protease inhibitors) and disrupted by 20 passages through a 21-gauge needle. Unbroken cells and nuclei were removed by centrifugation for 10 minutes at 700g. 6 mL of 20% Percoll in 15 mM HEPES pH 7.4/0.25 M sucrose was layered on top of a 1-mL cushion of saturated sucrose. The 700g supernatant of the cell homogenate was layered on top of the Percoll and centrifuged in SS-34 rotor at 16.4 krpm (32.000 g) for 45 minutes. Fractions (1 ml each) were collected from the bottom with a peristaltic pump. Beta-hexosaminidase activity assay in collected fractions was performed by mixing of 50 µl of 2 mM 4-Nitrophenyl N-acetyl-β-D-glucosaminide (Sigma) in citrate buffer pH 4.5 and 50 µl of each fraction diluted with the same buffer and incubating in 37°C for 60 minutes. Following incubation 150 µl of 0.1 M carbonate buffer pH 10 was added and absorbance measured at 405 nm. Controls showing no influence of Percoll on beta-hexosaminidase assay results were performed. For biotinylated proteins distribution analysis fraction samples were diluted with phosphate buffered saline (PBS) containing 0.1 % Triton X-100 and protease inhibitors and incubated overnight at 4°C with streptavidin-sepharose beads. The beads were washed 5 times with 0.5% Triton X-100 in PBS, boiled for 5 minutes in SDS-PAGE sample buffer and analyzed by Western Blot using anti-GFP antibodies (Roche Applied Science).

Microscopy

For live imaging HMC-1 cells were placed in LabTek chambered coverslips (Nunc). For tryptase staining cells were washed and resuspended in cold PBS and incubated on poly-L-lysine slides. Following adhesion cells were washed with PBS, fixed in 4%

formaldehyde/0.1 % glutaraldehyde/PBS. All further steps were performed in 0.05% saponin/2.5 % BSA/PBS. Briefly, cells were incubated for 1 hour at RT with 1:100 mouse monoclonal anti-human tryptase antibodies (Dako). Following thorough washing cells were incubated for 1 hour at RT with 1:200 TRITC-conjugated goat anti-mouse F(ab)2 (Sigma) and washed. After final wash cells were mounted in 5 mg/ml DABCO (Sigma)/Fluoromount G (Southern Biotech). Confocal microscopy was performed on Leica TCS SP2 AOBS microscope. Image processing was performed in Leica Confocal Software package. All monochrome images are presented in unprocessed form. For visualization purposes color restoration in multicolor images was accompanied by linear adjustments of brightness/contrast.

Sequence and statistical analysis

Predictions of 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/). TNF protein sequence alignment was performed with Vector NTI software. Two-way ANOVA followed by Holm-Sidak t-test were used to determine statistical significance of observed differences

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