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Tyrosine sulfation is not the last modification of entactin before its secretion from 3T3-L1 adipocytes

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Tyrosine sulfation of entactin was studied by labeling of 3T3-L1 adipocytes with [^{35}S]methionine or $H_2{}^{35}SO_4$ in the presence or absence of tunicamycin or monensin. Four precursors (EN1–4) at different steps of modification were detected in addition to mature entactin. Under normal conditions, EN2 and mature entactin were intracellular species, and the latter was sulfated and secreted. Inhibition of co-translational transfer of *N*-linked oligosaccharides by tunicamycin produced EN1 and EN3 as intracellular species, and EN3 was sulfated and secreted. Interruption of protein transport from medial to trans (distal) Golgi cisternae by monensin, and consequent blockage of terminal glycosylation caused intracellular accumulation of EN4. EN4 was sulfated and of different size compared to mature entactin. These facts suggested that tyrosine sulfation of entactin occurs in medial Golgi cisternae and is not the last modification before its secretion. Our results appeared inconsistent with recent observations by Baeuerle and Huttner [(1987) J. Cell Biol. 105, 2655 2664] that tyrosine sulfation of IgM occurred within the trans (distal) Golgi cisternae as the last modification before its exit from the Golgi complex.

Tyrosine sulfation; Entactin; Golgi complex; Monensin; Tunicamycin; (3T3-L1 adipocyte)

1. INTRODUCTION

Tyrosine sulfation is a frequent modification of secretory proteins, and has recently received great deal of attention because of its potential influence on the various biological activities of proteins [1]. A possible role for tyrosine sulfation in modulating intracellular protein transport makes the identification of its intracellular site particularly important. Using IgM as a model protein, Baeuerle and Huttner [2] reported recently that tyrosine sulfation occurs in the trans (distal) Golgi cisternae and is apparently the final form of modification before exit from this compartment. Using synthesis and secretion of entactin by 3T3-L1 adipocytes as another model, however, we reached a somewhat different conclusion.

Entactin is a sulfated glycoprotein of 150 kDa, found in various basement membranes [3], including Reichert's membrane [4]. Secretion of this protein from a variety of endodermal cell lines and Swiss 3T3 cells has been reported [5–7]. Entactin contains *N*-linked oligosaccharides, which are, however, not the sites of sulfation [6]. Sulfation of entactin was later shown to be at tyrosine to yield tyrosine *O*-sulfate [8]. This protein was also suggested to contain *O*-linked oligosaccharides [6]. Due to these multiple modifications, entactin is an interesting protein for studying intracellular processing and transport events.

Taking advantage of the enhanced synthesis of entactin during adipose conversion of 3T3-L1, we studied post-translational glycosylation and sulfation of entactin. Monensin caused intracellular accumulation of a sulfated precursor with a smaller size than that of mature entactin. This indicated that tyrosine sulfation of entactin is not the last

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; MEM, Eagle's minimal essential medium; RER, rough endoplasmic reticulum

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modification before its exit from Golgi complex of 3T3-L1 adipocytes.

2. MATERIALS AND METHODS

Anti-entactin antiserum (rabbit) was a generous gift from Dr A.E. Chung (University of Pittsburgh, Pittsburgh). Monensin from Calbiochem (La Jolla, CA), protein A-Sepharose CL-4B from Pharmacia (Uppsala), tunicamycin from Wako Chemical (Osaka), [^{35}S]methionine (1200 Ci/mmol) from Amersham (Bucks, England), and $H_2{}^{35}SO_4$ (carrier-free) and En^{3}Hance from NEN Products (Boston, MA) were used. All other reagents were obtained as described [9].

Cultures on 24-well plates on day 4 of adipose conversion [9] were washed once with methionine-free MEM and incubated in 200 μl methionine-free MEM containing 0.5 mCi/ml [^{35}S]-methionine at 37°C for 4 h under humidified 5% CO_2/95% air. Labeling with $H_2{}^{35}SO_4$ (1.6 mCi/ml) was performed as above except that DMEM was used instead of methionine-free MEM. To study the effects of monensin (10 μM) or tunicamycin (10 μg/ml), cultures were preincubated for 120 min in DMEM containing fetal calf serum and drug, and labeled as above. Trichloroacetic acid-insoluble radioactivity incorporated into total cellular protein was measured by the filter-disk method [10]. Monensin reduced incorporation of [^{35}S]methionine and $H_2{}^{35}SO_4$ to 95 and 45% of the value in its absence, respectively; tunicamycin reducing these values to 70 and 55%, respectively. To normalize the effect of monensin and tunicamycin, aliquots of labeled medium or cell lysate corresponding to the same amount of [^{35}S]methionine or $H_2{}^{35}SO_4$ incorporated into total cellular protein were used for immunoprecipitation. For pulse-chase experiments with [^{35}S]methionine, cultures were preincubated with methionine-free MEM for 30 min and pulse-labeled for 15 min with the same medium containing 1.66 mCi/ml [^{35}S]methionine as above. The radiolabeled cells were 'chased' by replacing the medium with DMEM and incubation for the indicated periods. Labeled medium was collected and centrifuged before analysis and cells were lysed with 200 μl of 'immunoprecipitation buffer' containing 10 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1% Triton X-100, 0.4% SDS, 1 mM phenylmethylsulfonyl fluoride, and 0.4 M NaCl.

Aliquots of labeled medium or cell lysate diluted to 500 μl with immunoprecipitation buffer were clarified by incubation with 30 μl of a 10% (w/v) suspension of protein A-Sepharose for 30 min with shaking. The Sepharose beads were spun out and the supernatants incubated with 1 μl antiserum for 30 min, followed by further incubation with 30 μl protein A-Sepharose for 30 min with shaking. All incubations were carried out at room temperature. Packed Sepharose beads were washed three times with immunoprecipitation buffer and extracted by boiling in buffer containing 6% (w/v) SDS, 134 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 4% (v/v) 2-mercaptoethanol, and 0.006% (w/v) bromophenol blue (2× concentrated SDS sample buffer).

The discontinuous buffer system of Laemmli [11] was used with 3% (w/v) acrylamide for the stacking gel and 4% (w/v) acrylamide for the separation gel. After electrophoresis, the gels were impregnated with En^{3}Hance, dried and exposed to X-ray film at −80°C.

3. RESULTS AND DISCUSSION

Immunoprecipitation with anti-entactin antiserum from [^{35}S]methionine- or $H_2{}^{35}SO_4$-labeled medium of 3T3-L1 adipocytes gave a strong band of approx. 150 kDa on fluorograms from SDS electrophoresis. This polypeptide was included among the major bands observed upon electrophoresis of the radiolabeled medium before immunoprecipitation (not shown). Almost equal densities of bands before and after immunoprecipitation guaranteed that the procedures were quantitative.

Fig.1 summarizes the results from metabolic labeling of 3T3-L1 adipocytes with [^{35}S]-methionine (A) or $H_2{}^{35}SO_4$ (B) in the absence or presence of monensin or tunicamycin. SDS electrophoresis of immunoprecipitates from labeled cell lysates and media showed five [^{35}S]methionine-labeled bands in the range 140–150 kDa (bands EN 1–5), three of which (EN3–5) were also labeled with $H_2{}^{35}SO_4$. In the absence of tunicamycin or monensin, EN2 and EN5 were observed in [^{35}S]methionine-labeled cell lysates (fig.1A, lane 'None, C'). EN5 corresponds to mature entactin as it was secreted without any detectable processing (fig.1A,B, lanes 'None, M'), and EN2 may correspond to a precursor with extensively trimmed high-mannose oligosaccharides. The abundance of EN2 suggests that transport from the site of mannose trimming to that of further processing is a rate-limiting step in the intracellular traffic of entactin. As described by Hogan et al. [6], EN2 was not sulfated (fig.1B, lane 'None, C'). This suggests that tyrosine sulfation may follow mannose trimming, i.e. that it occurs in medial or trans (distal) cisternae. After pulse labeling for 15 min with [^{35}S]methionine in the absence of tunicamycin or monensin (fig.2), EN2 was strongly labeled. EN2 had matured to EN5 within 30–90 min chase, and EN5 was first secreted after 90 min chase. Intracellular accumulation of EN5 suggested that the exit of entactin from trans Golgi network is another rate-limiting step in the traffic of entactin.

In the presence of tunicamycin, [^{35}S]methionine-labeled EN1 and EN3 were observed in cell lysates (fig.1A, lane 'Tunicamycin, C'). These may correspond to entactin precursors to which the co-translational transfer of high-mannose oligosaccharides did not occur [12]. In the presence of

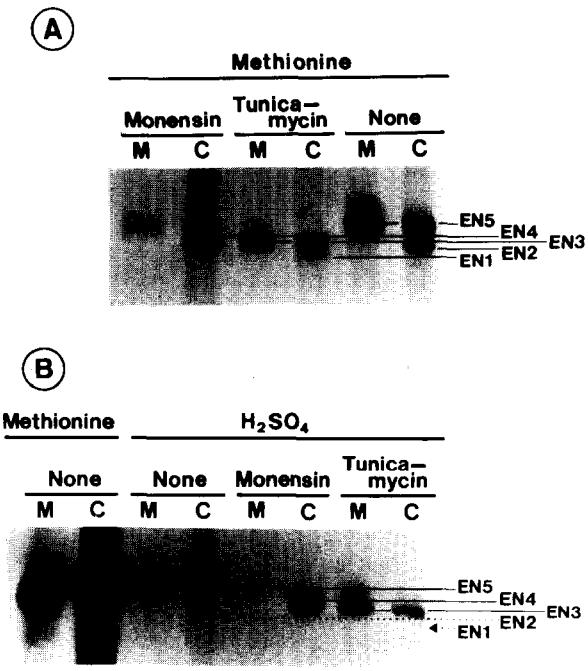

Fig.1. Intracellular precursors and mature entactin observed in 3T3-L1 adipocytes in the presence or absence of tunicamycin or monensin. 3T3-L1 cells on day 4 of adipose conversion were labeled with [^{35}S]methionine (A and a part of B) or $H_2{}^{35}SO_4$ (B) in the presence or absence of tunicamycin (10 μg/ml) or monensin (10 μM) for 4 h. Radiolabeled cell lysates (C) corresponding to the same amounts of incorporated radioactivity of [^{35}S]methionine or $H_2{}^{35}SO_4$ into total cellular protein were immunoprecipitated with anti-entactin antiserum. Radiolabeled media (M) corresponding to the same sizes of cultures as those for cell lysates were immunoprecipitated. Immunoprecipitates were applied to SDS electrophoresis and a fluorogram of the dried gel was taken. EN1–EN5, intermediates of entactin detected are numbered in order of mobility.

tunicamycin, only EN3 was found after $H_2{}^{35}SO_4$ labeling (fig.1B, lane 'Tunicamycin, C'). This suggested that *N*-linked oligosaccharide is not essential to the transport of entactin precursor from RER to the site of tyrosine sulfation. There is a difference in molecular mass of 3.5 kDa between EN1 and EN3. If this difference reflects exclusively tyrosine sulfation [8], almost all tyrosine residues in entactin [7] must be sulfated. It therefore seems more reasonable to attribute this gel mobility difference to *O*-linked oligosaccharide addition, and to postulate that tyrosine sulfation takes place after *O*-linked oligosaccharide addition. EN3 was readily secreted even in the presence of tunicamycin (fig.1A,B, lanes 'Tunicamycin, M').

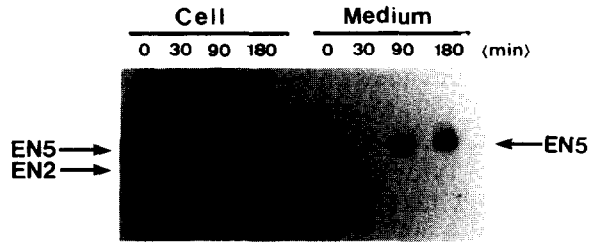

Fig.2. Pulse labeling with [^{35}S]methionine and chasing of entactin in 3T3-L1 adipocytes. 3T3-L1 cells on day 4 of adipose conversion were pulse labeled for 15 min with [^{35}S]methionine and chased for the indicated periods as described in the text. Radiolabeled media or cell lysates corresponding to the same size of cultures were immunoprecipitated as in fig.1.

This is in contrast with the tunicamycin sensitivity of secretion of another basement membrane protein, laminin [13,14], which is not sulfated. This result can be related to the hypothesis of Baeuerle and Huttner [15] that tyrosine sulfation functions as a sorting signal for IgG secretion.

In the presence of monensin (10 μM), which perturbs intracellular transport at the level of the Golgi complex [16], secretion of entactin was virtually blocked. Instead, strong [^{35}S]methionine labeling of intracellular EN4 was observed (fig.1A, lane 'Monensin, C'). This may correspond to a precursor which has had its oligosaccharide processing blocked. Continued sulfation of EN4 (fig.1B, lane 'Monensin, C') suggests that this event takes place before terminal glycosylation, and before the cytologic site of transport interruption. The site of interruption by monensin was identified, in baby hamster kidney cells, to be the movement from medial to trans (distal) Golgi cisternae [17]. If this is the case in 3T3-L1 adipocytes as well, our results with entactin show that the cytologic site of tyrosine sulfation is in medial Golgi cisternae.

Paulsson et al. showed that virtually all of the entactin-bound radioactivity from $H_2{}^{35}SO_4$ was due to tyrosine *O*-sulfate (8). This excludes the possibility that sulfation of entactin in the presence of monensin was due to ^{35}S in molecular forms other than tyrosin *O*-sulfate. Monensin reduced the incorporation of $H_2{}^{35}SO_4$ into total cellular protein to about half, and the result in fig.1B was obtained after normalization of this reduction. Rough estimation of incorporated $H_2{}^{35}SO_4$ based on the density of bands (fig.1B) suggested that

tyrosine sulfation of entactin was inhibited by monensin to one third of that in its absence. This may pose the question as to whether the tyrosine sulfation of entactin observed in 3T3-L1 adipocytes in the presence of 10 μM monensin was unsound. Tyrosine sulfation of secretogranins I and II in PC12 cells [18], and of IgM in hybridoma cells [2], was inhibited by lower concentrations (0.1 or 1 μM, respectively) of monensin. This observation was part of the evidence leading Baeuerle and Huttner to conclude that tyrosine sulfation occurs within trans (distal) Golgi cisternae [2]. One of the possible explanations for these contradictory observations may be that tyrosine sulfation in 3T3-L1 adipocytes also occurs within trans (distal) Golgi but that it is somehow more resistant to the effect of monensin than those in PC12 and hybridoma cells. Even though this was the case, one should note that terminal glycosylation of EN4 in trans (distal) Golgi cisternae was inhibited by monensin (fig.1B). The most plausible explanation for all the results available up to now is that tyrosine sulfation occurs within late medial Golgi cisternae, and is followed immediately by terminal glycosylation after rapid transport into trans (distal) Golgi cisternae. This activity within late medial Golgi cisternae may be perturbed by monensin to different extents depending on the substrate protein or cell type. Tyrosine sulfation on entactin in 3T3-L1 is more resistant to monensin than those on secretogranins I and II in PC12 cells [18] and on IgM in hybridoma cells [2].

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