Subcellular Localization of a Glycoprotein Released from Human Platelets upon Stimulation by Thrombin *

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Summary

The treatment of washed, intact human blood platelets with 1 U/ml of thrombin for 5 min at 37 °C is linked to the disappearance from the particulate fraction of the homogenized cells of a protein band discernible in the SDS-polyacrylamide gel electrophoresis pattern of untreated platelets. Accordingly, this material was termed thrombin sensitive protein (TSP) by its discoverers, who also presented evidence that TSP is a glycoprotein of a molecular weight of 190,000.

The present work describes the localization of TSP in human platelets. TSP is not a membrane constituent, but associated with orgenelle fractions in the region of higher densities. It therefore is not, as originally suggested, a direct substrate for thrombin on the platelet surface, but takes part in the platelet release reaction upon a variety of external stimuli. An apparent molecular weight of 150,000 was found for TSP; this value coincides with the one reported for so-called glycoprotein I, which, different from TSP, is a membrane constituent.

Introduction

Baenziger, Brodie and Majerus [1] were the first to describe a glycoprotein, which is no longer detectable in the particulate fraction of platelets after thrombin-treatment of the intact cells. They concluded from these first studies that they were dealing with a protein which was sensitive to the proteolytic action of thrombin; hence the authors proposed the name «thrombin-sensitive protein» (TSP) for this material. Concomitant with the disappearance of TSP, a decrease in the activity of adenyl cyclase is observed in thrombin-treated platelets [2]. This enzyme is membrane-associated, possibly an integral membrane-protein, and it appeared highly suggestive that it might be linked to a thrombin substrate on the platelet membrane. The assumption that TSP was a membrane constituent, was furthermore supported by the finding that it is a glycoprotein of the relatively high molecular weight of 190,000 [3]. Such large-sized glycoproteins are in fact membrane constituents [4].

There was, however, also some evidence which quite clearly was not compatible with the hypothesis that TSP was a membrane constituent vulnerable to attack by thrombin. Thus, platelet membranes isolated by glycerol-osmotic lysis were found to be devoid of TSP [3]; furthermore, the TSP is removed by thrombin only from intact, but not from homogenized platelets [3]. Lastly, it was found by Majerus and Brodie [5] that non-proteolytic agents, such as phytohemagglutinins, will also affect TSP. These observations suggested that TSP was in fact not a membrane-bound thrombin substrate, but rather a member of the wide spectrum of substances which are released from platelets upon stimulation with a wide variety of external agents [6]. This prompted us to investigate further this material, particularly with respect to its possible localization in the platelets. Since the completion of this investigation, Hagen [7] has reported in some detail on the properties of TSP; furthermore Phillips and Agin [8] have shown that most likely another glycoprotein is the membrane-bound substrate for thrombin. This may explain why part of the findings reported here is confirmatory.

Material and methods

Blood platelets were isolated as described earlier [9] from blood collected for the Central Laboratory of the Swiss Red Cross Blood Transfusion Service. They were washed twice with the buffer proposed by Baenziger et al. [3], containing 0.102 M NaCl, 3.9 mM K_2HPO_4, 3,9 mM Na_2HPO_4, 22 mM NaH_2PO_4, and 5.5 mM glucose, *p*H 6.5. They were suspended in a Tris-buffered solution, *p*H 7.4, containing 0.14 M NaCl, 15 mM Tris/HCl, 1 mM $MgCl_2$, 4 mM KCl, and 5.5 mM glucose to a concentration of 10^{10} platelets/ml.

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Preparation of released material: Th this suspension were added either 1 U thrombin (Hoffmann-La Roche, Basel) per ml, or the corresponding volume of 0.15 M NaCl. The mixtures were incubated under mild stirring for 5 min at 37 °C; one unit of hirudin (Sigma) per ml was added and the suspension was centrifuged for 10 min at 6000 g. The supernatants were brought for 10 min to 56 °C and then again centrifuged for 30 min at 48,000 g. The clear solutions were dialyzed in bags subjected to a cold air stream, whereby their concentration was increased to about 0.2% protein. These concentrates were directly used for polyacrylamide gel disk electrophoresis (PAGE) in the presence of sodiumdodecylsulfate (SDS).

Sediments of treated platelets were resuspended in the original Tris-buffer and homogenized in the cold for 8 min at 14,000 rpm in a blendor-type MSE-homogenizer. In order to remove larger particles and undisrupted cells, the suspension was centrifuged for 5 min at 700 g. The turbid supernatants were applied directly on a density gradient for organelle-separation. For SDS-PAGE, the suspension was first sedimented at 48,000 g (30 min) and the pellets were taken up in SDS.

Preparation of a fraction rich in platelet plasma membranes

Platelets homogenized with the blendor-technique described above were centrifuged for 30 min at 33,000 g. The sediment was discarded. It consisted mainly of different types of organelles. The supernatant was submitted to high-speed centrifugation (60 min, 100,000 g). The sediment was composed to a large extent of membranes; it was taken up in a small volume of buffer *p*H 7.4 and applied to a density gradient.

Density gradients were prepared from 25 to 50% (w/w) sucrose, as described by Siegel et al. [10].

Density gradient separations were carried out in a Spinco L2 centrifuge at 2 °C; a SW 50 L "swing-out" rotor was used. The loaded gradients were spun for 90 min at 45,000 rpm (165,268 g). The obtained zones were carefully removed with a fine syringe and immediately diluted to isoosmolarity with distilled water and sedimented at 78,000 g for 30 min. The obtained pellets were resuspended in small amounts of 0.1 M phosphate buffer, *p*H 7.4, and, within one series of experiments adjusted to the same protein concentration before being used for SDS-PAGE.

Protein determinations were done according to the Biuret-method [11].

SDS-solubilization and PAGE were performed according to the method described by Baenziger et al. [3]. The following test substances were used: human IgG (Swiss Red Cross), α-amylase (Sigma), phosphorylase (Sigma), crystalized bovine serum albumin (Armour, Chicago), trypsin (1× cryst., Calbiochem) and myoglobin (Koch-Light, Colnbrook, England).

Results

1. *Identification of the "thrombin-sensitive protein"*

The comparison of the SDS-disk electrophoretic patterns of the solubilized sediments of thrombin-treated and control platelets revealed in the latter a well developed band at a distance, under our standard conditions, of 31 ± 2 mm from the origin, which is missing after thrombin-treatment (Fig. 1). The corresponding material was found in the electrophoretic pattern of the concentrated supernatants of thrombin-treated platelets. As judged from its position among the bands of proteins of known size, an apparent molecular weight of 150,000 can be estimated (Fig. 2). This value is lower than the one given originally by Baenziger et al. [1] and the question of the identy of the two proteins arises. The patterns of our gels are quite similar to those reported by these authors; however, on closer examination, their standard curve is steeper than ours in the region of high molecular weights and this may explain the discrepancy. Since TSP in our experiments is always found in very close vicinity of the dimer of serum albumin, we see no reason to revise the value of 150,000 given above.

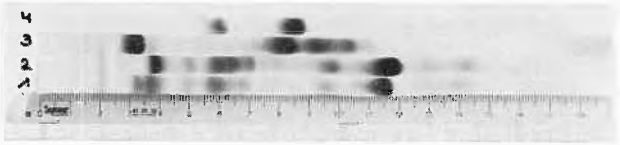

Fig. 1: SDS-PAGE-patterns of: 1. Particulate fraction of control platelets. 2. Particulate fraction of thrombin-treated platelets. 3. Supernatant of thrombin-treated platelets. 4. Standard-proteins phosphorylase and serum albumin. TSP is located at about 32 mm

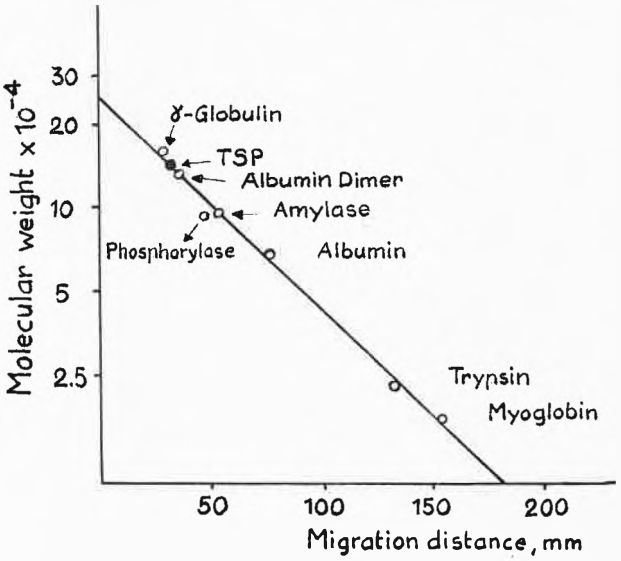

Fig. 2: Standard plot for estimation of molecular weight

2. *Subcellular localization of the TSP*

a) *Density-gradient centrifugation of the total platelet homogenate:* Fig. 3 gives a schematical representa-

tion of 2 typical separations of homogenates of platelets with and without thrombin treatment. The gradient used was continuous, beginning with a density of about 1.11. Several distinct layers were discernible which were designated, from top to bottom, with numbers from 1 to 5. In the thrombin-treated samples, the layers 4 and 5 consistently showed a floculant appearance, whereas the controls appeared finely dispersed throughout.

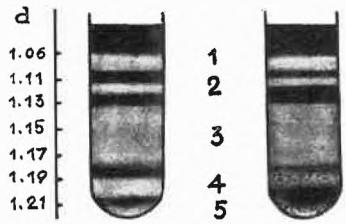

Fig. 3: Schematic representation of organelle-fractionation of untreated (left) and thrombin-treated platelets (right) on a sucrose density gradient

The SDS-disk-electrophoretic pattern of the different bands of such a density gradient separation of an untreated sample is shown in fig. 4. The TSP-band, localised at 31 mm from the top, is discernible in all fractions; however, there are significant quantitative differences: In fraction 1 to 3 it is only weakly represented, whereas it appears in fraction 4 in a concentration which corresponds roughly to the one in a non-separated sediment. In fraction 5 it even appears to be present in enriched form.

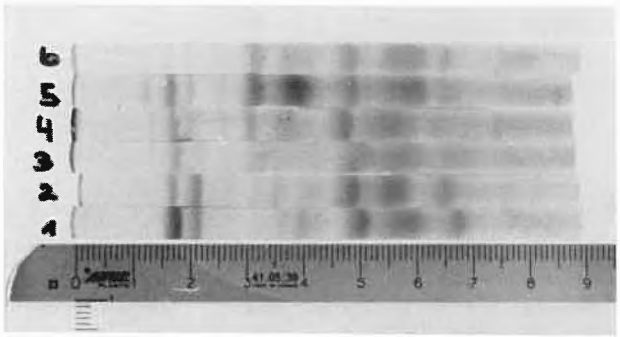

Fig. 4: SDS-PAGE-patterns of the different bands obtained by density-gradient centrifugation from untreated platelets. The numbers 1 to 5 correspond to the numbers of the bands as given in fig. 3. Nr. 6: Particulate fraction of whole, untreated platelets. TSP appears at 31 mm. Note that only upper half of gel columns is shown

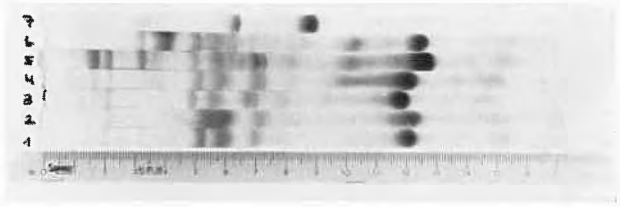

Fig. 5: SDS-PAGE-patterns of the different bands obtained by density-gradient centrifugation of thrombin-pretreated platelets. Nrs 1 to 6 correspond to those explained in fig. 4. Nr. 7: Phosphorylase and albumin. TSP appears at 31 mm

In fig. 5 are shown the patterns of the corresponding fractionation of the organelles of thrombin-treated platelets. Again, the TSP is discernible, however, the bands are much weaker; in fact, in the fractions nr. 2 and 3 it is barely visible, in fraction 4 it is represented by a very weak band, and in nr. 5, although easily discernible, it is present in greatly reduced amount as compared to the control.
These results are suggestive for the major localization of TSP in the denser granular fractions. They do not definitely answer, whether the membranes are also involved.

b) *Density-gradient centrifugation of membrane fractions*

With these experiments, an attempt at clarifying the localization of TSP in the membranes was made.

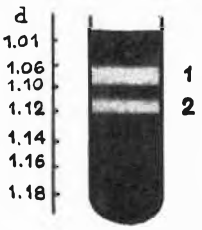

Fig. 6: Schematic representation of density-gradient separation of a fraction enriched in plasma membranes

In fig. 6 is shown the result of the density-gradient centrifugation of a 100,000 g sediment, which is enriched in membranes. Only two bands have formed with apparent densities of 1.06 to 1.10, and from 1.11 to 1.12, respectively. Both showed high Mg^{2+}-stimulated ATPase activities, which are partly inhibited by Ouabain, thus making it likely that both contain plasma membranes.

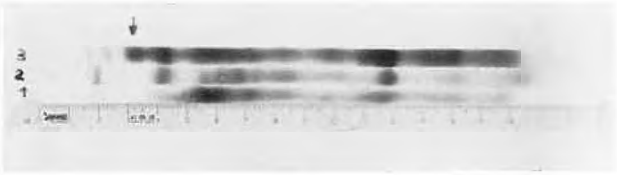

Fig. 7: SDS-PAGE-patterns of the two bands obtained after density-gradient centrifugation of a membrane-preparation. Nrs 1 and 2 correspond to bands shown in fig. 6. Nr. 3: Particulate fraction of untreated platelets, where TSP appears at 31 mm (arrow)

In fig. 7 are shown the disk-electrophoretic patterns of both these membrane-fractions. The TSP is discernible in neither of the two bands. Together with the other presented evidence, this finding makes it most unlikely that the TSP is a membrane-bound thrombin substrate.

Discussion

When working with complex biological systems involving intact cells, one of the major difficulties quite often consists in the dissociation of primary and sec-

ondary effects. The history of the TSP is an excellent illustration of this statement, since the fact that it disappears from the particulate fraction of thrombin-treated platelets synchronous with the decrease of adenyl cyclase activity led the discoverers of this effect to the assumption that TSP most likely was a direct thrombin substrate, localized in the membrane in close association with adenyl cyclase. It is well established now that TSP is in fact one of the components which are released from platelets under the influence, not only of thrombin, but most likely of any other inducer of second phase platelet aggregation and the release reaction. Our experiments clearly demonstrate the absence of TSP from the membrane preparation and its presence in the organelle fractions of higher densities. Together with da Prada and Pletscher we have recently presented evidence for the presence in platelets of a second type of secretory organelle, which contains and releases a heparin-neutralizing factor as well as fibrinogen [12]. The question whether the TSP is contained in this organelle, or in the "dense bodies", known to contain serotonin and adenine nucleotides, can as yet not be answered.

Hagen [7] in her study on TSP has found a molecular weight of 145,000 ± 1000 for the released material and of 147,000 ± 1000 for the organelle-bound material. These values are in good agreement with ours and much lower than the one reported by Baenziger et al. [3]. It is of considerable interest that the TSP obviously has a molecular weight which almost coincides with the one of membrane glycoprotein I, as revealed by surface labelling of intact platelets or by carbohydrate-staining of PAGE-separated SDS-solubilized membranes [13,14]. This means that data obtained by protein- or carbohydrate-staining of SDS-gels of whole platelet homogenates must be interpreted with the knowledge that there exists, under certain experimental conditions the possibility of a superposition of the TSP and this particular membrane glycoprotein.

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