MEMBRANE GLYCOPROTEIN IIb IS THE MAJOR ENDOGENOUS ACCEPTOR FOR HUMAN PLATELET ECTOSIALYLTRANSFERASE

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1. Introduction

Platelet surface glycoconjugates have been suggested to play roles in the major biological functions of this cell, namely aggregation and adhesion [1–4]. Bosmann [5] first showed that platelet suspension as well as plasma membrane fraction possessed sialyltransferase activity which would play a role in platelet aggregation. In fact, a parallel inhibition of aggregation and sialyltransferase activity by aspirin [5,6] and an apparent stimulation of sialyltransferase activity with various platelet aggregating agents such as collagen, epinephrin and ADP plus fibrinogen [7] have been noted, while cancer patients with a high incidence of thrombosis have markedly elevated levels of platelet sialyltransferase activity [6]. Despite the possible functional significance of the above studies, the presence of ectosialyltransferase activity at the human platelet surface has not been rigorously defined. This is due to possible errors introduced into most assay systems by:

(i) Precursor degradation and intracellular utilisation of the free radioactive sialic acid;
(ii) The liberation of intracellular enzymes as a result of cell lysis during the assay;
(iii) Phagocytosis of added exogenous acceptors [8].

Using exogenous non-phagocytosable acceptors [9] and the methodology developed to prove the presence of ectoglycosyltransferases at the surface of other cell types [10,11], we demonstrate here that the human blood platelet exhibits ectosialyltransferase activity and that the major endogenous acceptor is the plama membrane glycoprotein GP IIb.

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2. Materials and methods

2.1. *Preparation of platelets and platelet homogenates*

Blood from normal human donors was collected in plastic containers using an acid–citrate–dextrose (ACD) anticoagulant as in [12]. The platelets were isolated as in [12] and resuspended in 10 mM Tris–HCl (pH 7.4), containing 0.154 M NaCl, 1 mM EDTA, 0.3% (w/v) bovine serum albumin and 5 mM glucose. Lactate dehydrogenase activity was measured [13] as a control of cell lysis. Platelet homogenisation was performed at 10^{10} platelets/ml by sonication for 3 × 1 min using a MSE ultrasonicator (wavelength 14.5 μm) under constant cooling at 4°C.

2.2. *Preparation of acceptors*

Fetuin was purchased from Sigma (St Louis USA). Desialylation was performed by mild acid hydrolysis (0.1 N trifluoroacetic acid, 80°C, 35 min) and effective desialylation was checked by gas–liquid chromatography. Asialofetuin was coupled either to Sepharose 4B (Pharmacia, Sweden) by the CNBr method [14] or to Biogel P-300 (Biorad, USA) and Ultrogel AcA 44 (Industrie Biologique Française, France) [15].

2.3. *Standard sialyltransferase assays*

Washed platelets were resuspended in the washing buffer as above. All inbubations were performed in this buffer. A standard assay consisted of 100 μl final vol. containing 10^8 platelets, 100 μg asialofetuin and 2 μM CMP–[^{14}C]NeuAc (NEN, FRG; spec. act. 235 mCi/mmol). When insolubilised acceptors were used, 100 μl suspension containing 0.5 mg fixed asialofetuin/ml beads was employed; the assay volume was 200 μl and contained 6 × 10^8 platelets. After incuba-

tion at 37°C for 1 h, the integrity of the remaining substrate was examined after the separation of free [^{14}C]NeuAc from CMP–[^{14}C]NeuAc by paper chromatography in absolute ethanol, 1 M ammonium acetate (7:3, v/v) for 24 h.

2.4. *Determination of radioactivity incorporated into endogenous and exogenous acceptors*

The radioactivity incorporated into phosphotungstic acid-precipitable material was measured as in [9]. The radioactivity incorporated into the exogenous acceptors was determined by subtracting the value obtained when the assay was performed in the absence of added acceptors. When insolubilised acceptors were used, the separation of the coupled beads from platelets was achieved by low speed centrifugation in a 32% metrizamide medium [9]. Acid-precipitable or -insoluble material was collected and washed on glass fiber filters (Whatman GF 83) then the radioactivity determined in a scintillation liquid.

2.5. *Platelet desialylation*

Platelets were resuspended in washing buffer in the presence of 10 mM $CaCl_2$ at 10^9 platelets/ml. Neuraminidase (*Vibrio cholerae*; Behringwerke, FRG) was added to 10 units/ml final conc. and the platelet suspension incubated for 1 h at 37°C. The treated platelets were washed twice in the washing buffer (without $CaCl_2$) before use.

2.6. *Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE)*

Following incubation with CMP–[^{14}C]NeuAc but in absence of exogenous acceptor, the platelets were sedimented and resuspended at 10^9 platelets/ml in 0.01 M Tris–HCl (pH 7.4), 0.154 M NaCl, 3 mM EDTA and 5 mM *N*-ethylmaleimide. The SDS–PAGE pattern of platelet surface proteins was obtained by labelling of platelet suspensions with ^{125}I by the lactoperoxidase-catalysed procedure [16]. The ^{125}I-labelled platelets were washed and solubilised by heating at 100°C for 5 min in 2% (w/v) SDS. SDS–PAGE was performed using 7–12% exponential acrylamide gradient slab gels and the buffer systems in [17]. A constant proportion (2.7%) of *N,N'*-methylene-*bis*-acrylamide to acrylamide was maintained throughout the gel. Samples containing 2000 cpm ^{14}C or 10 000 cpm ^{125}I were applied to the gel, each sample being analysed with or without disulphide bond reduction which was performed by incubating the SDS-solubilised platelets for 1 h at 37°C in the presence of 5% (v/v) 2-mercaptoethanol. Molecular weight determinations were made using Bio-Rad protein standards (Bio-Rad, USA). Electrophoresis was performed overnight at a constant 25 V. The proteins were located by Coomassie blue staining [16], the gels dried and direct autoradiography performed using Kodak X-Omat MA films (1 month, room temp.). The exposed films were developed and scanned.

3. Results and discussion

3.1. *Platelet sialyltransferase activity*

Preliminary studies were performed using platelet suspensions and soluble asialofetuin as an exogenous added acceptor. Fig.1 A shows a time-dependent incorporation of [^{14}C]NeuAc both into the asialofetuin and by the cells. Although CMP–NeuAc hydrolase activity is detected as shown on fig.1 B, the incorporation was linear for at least 2 h. However, it has to be noted that since:

(i) Intracellular utilisation of the generated free sialic has not been inhibited;

(ii) The platelet suspensions contain a certain percentage of broken cells;

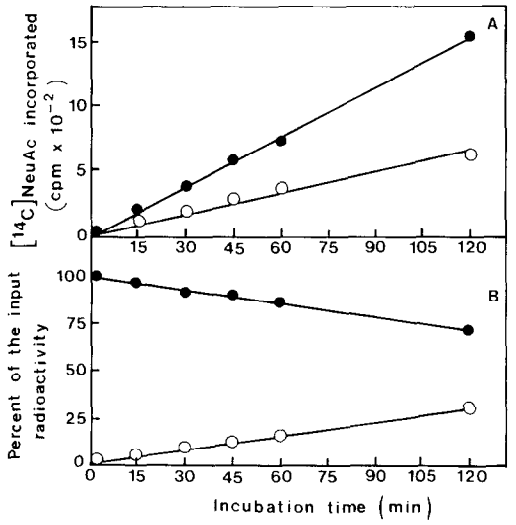

Fig.1. Kinetics of transfer of [^{14}C]NeuAc and of CMP–[^{14}C]-NeuAc degradation by whole platelet suspensions. Incubations were performed as described in the standard assay with 2 μM CMP–[^{14}C]NeuAc. (A) Transfer of [^{14}C]NeuAc to endogenous acceptors (○) and to asialofetuin (●). (B) Integrity of CMP–[^{14}C]NeuAc (●) and [^{14}C]NeuAc appearance (○) were measured for each point.

(iii) Phagocytosis of soluble asialofetuin may occur; the radioactivity bound to the cells and to the exogenous acceptor may not be related to ectosialyltransferase activity. Consequently, experimental procedures were developed to avoid these main causes of errors and demonstrate the presence of sialyltransferase activity at the surface of human blood platelets.

3.2. *Demonstration for ectosialyltransferase active on exogenous and endogenous acceptors*

Non-phagocytosable acceptors were used as in [9]. Initial studies showed that asialofetuin coupled to Sepharose 4B was a poor acceptor (table 1) despite the fact that uncoupled asialofetuin readily accumulated radioactivity (fig.1). However, increased incorporation was obtained when asialofetuin was coupled to Ultrogel AcA 44 or Biogel P 300 (table 1A). A number of control experiments determined that this incorporation did not result from a non-specific adsorption of radioactive material by the beads (table 1B). Incubation of CMP–[^{14}C]NeuAc with non-coupled beads and platelets or with asialofetuin beads and no cells resulted in little adsorption of radioactivity. This procedure allows the exclusion of a sialylation process due to phagocytosis of the acceptor.

Table 1
Incorporation of radioactivity into non-phagocytosable insoluble acceptors

	Incubation time (min)	Radioactive NeuAc transferred to asialofetuin on coupled beads (cpm)
(A) Standard assay		
+ Sepharose 4B coupled asialofetuin	60	65
+ Ultrogel AcA 44 coupled asialofetuin	60	350
+ Biogel P 300 coupled asialofetuin	60	300
(B) Standard assay		
+ Ultrogel AcA 44 coupled asialofetuin	0	30
– Coupled beads + uncoupled Ultrogel AcA 44	60	70
– Cells + Ultrogel AcA 44 coupled asialofetuin	60	50

Incubations were performed as in the standard assay with 2 μM CMP–[^{14}C]NeuAc: (A) Transfer of radioactivity to asialofetuin coupled to different support beads; (B) estimation of the non-specific adsorption to the beads. The labelled beads were separated from the platelet suspension by centrifugation in a metrizamide medium as detailed in section 2

To assign the radioactivity recovered with the cells to ectosialyltransferase activity, hydrolysis of the precursor and entry of free labelled sugar into the cell must be brought to a negligible level. As free NeuAc has been shown to enter cells [18], we tested that a 1000-fold excess of unlabelled NeuAc did not reduce the radioactivity bound to the platelets, proving that labelling of the cells was not due to entry of labelled NeuAc.

To determine whether the observed sialyltransferase activity either on exogenous insolubilized or endogenous acceptors could be due to the liberation of intracellular enzymes from the small percentage of broken cells, mixtures containing definite proportions of intact and broken platelets were incubated with CMP–[^{14}C]NeuAc in standard conditions [19]. Provided isotopic dilution brought by the release of endogenous precursors does not occur [20], extrapolation to a hypothetical level of 0% lysed platelets should represent the ectosialyltransferase activity. In the case of human platelets, the intracellular pool of CMP-NeuAc, if any, is not high enough to cause isotopic dilution of the labelled precursors as it has been demonstrated that increasing amounts of post-microsomal supernatant do not affect the amount of radioactivity incorporated by the platelet. Fig.2 shows that the extrapolation to 0% of broken cells leads to a significant incorporation of radioactivity by the platelets (fig.2A) and into the insolubilized acceptor (fig.2B) resulting from an ectosialyltransferase activity.

These extrapolations of thes sialyltransferase activity to 100% intact cells clearly demonstrates that there is an ectosialyltransferase active both on exogenous and endogenous acceptors.

In addition, if prior to incubation with CMP–[^{14}C]NeuAc, the platelet surface glycoconjugates are desialylated under conditions not affecting cell viability, the ectosialyltransferase activity appears to be stimulated (~8-fold) by the presence of an increased number of membranous acceptors at the outer surface of the platelet.

3.3. *Identification of the endogenous acceptors*

As shown in fig.3, several bands of radioactivity were observed when [^{14}C]NeuAc labelled platelets were analysed (fig.3b,d), on analysis of the ^{14}C-labelled

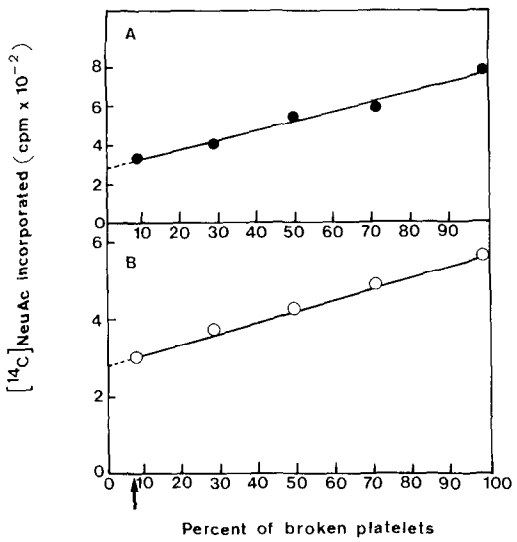

Fig.2. Sialyltransferase activity of human platelet suspension as a function of the percentage of broken cells. Mixtures containing various known proportions of whole platelet suspensions and platelet homogenates were incubated for 60 min as in section 2, with 2 μM CMP–[^{14}C]NeuAc. (A) Transfer of [^{14}C]NeuAc was measured on endogenous acceptors (•). (B) Transfer of [^{14}C]NeuAc onto asialofetuin coupled to Ultrogel AcA 44 (○). The arrow on the lower scale indicates the percentage of broken platelets in the stock platelet suspension as measured by the lactate dehydrogenase activity.

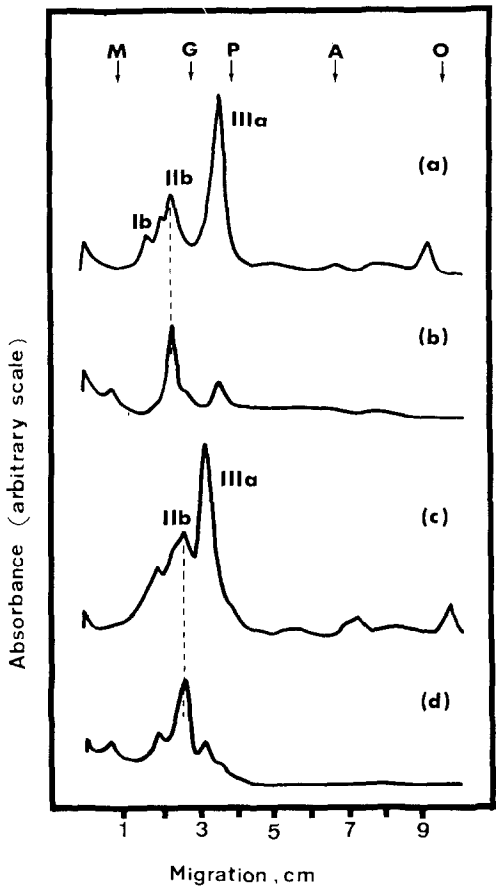

Fig.3. Identification of endogenous membrane glycoprotein acceptors for platelet ectosialyltransferase activity. Platelets were either incubated with CMP–[^{14}C]NeuAc under the standard assay conditions (b,d) or their surface proteins were labelled with ^{125}I using the lactoperoxidase-catalysed procedure (a,c). The labelled platelet proteins were then separated by SDS–PAGE as in section 2. Samples were electrophoresed both in the absence of disulphide bond reduction (a,b) and following disulphide bond reduction (c,d). Ib, IIb, IIIa correspond to the major surface glycoproteins of human blood platelets.

platelets, the most prominently labelled compound showing the characteristic mobility of GP IIb as identified by a parallel run with ^{125}I-labelled membrane proteins (fig.3a,c). This is a surface-oriented membrane glycoprotein with $M_r \sim 142\,000$, which on reduction yields an α-subunit of 132 000 M_r and a smaller β-subunit of 23 000 M_r [16]. Note that <10% of the total radioactivity bound to the cell is associated to glycolipids as assayed by a Folch extraction [21]. Our data illustrates that under the assay conditions used GP IIb is the major native endogenous acceptor for the platelet ectosialyltransferase enzyme.

4. Conclusions

We have provided evidence that ectosialyltransferase activity is present at the external surface of platelets. We have taken into account the criteria established [8] in detecting ectoglycosyltransferase activities in whole cell suspensions. We have thus eliminated:

(i) Possible phagocytosis of the acceptor;

(ii) Intracellular utilisation of free radioactive NeuAc generated by hydrolysis of the substrate;

(iii) The action of intracellular enzymes liberated from broken platelets as major contributors to our findings.

The analysis of the sialylated membrane constituents by SDS–PAGE reveals that the major 'ecto-acceptor' is the glycoprotein IIb, Bosmann [5] was the first to propose a functional role for ectosialyltransferase enzymes during platelet aggregation. GP

IIb is missing or severely decreased in platelets from patients with Glansmann's thrombasthenia [2,22], a bleeding disorder resulting from a defect in platelet aggregation. Whether the fact that GP IIb is a sialyltransferase acceptor related to the thrombasthenic platelet aggregation defect is unknown, but warrants further study.

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