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Association of Membrane and Cytoplasmic Proteins with the Cytoskeleton in Blood Platelets†

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ABSTRACT: The association of membrane and cytoplasmic proteins with the cytoskeleton of resting and activated platelets was studied. Glycoproteins were identified by labeling with ^{125}I-labeled lectins (concanavalin A, wheat germ agglutinin, and Lens culinaris). Polypeptides, which are embedded in the lipid bilayer, have been identified by their photolabeling with the lipid-soluble reagent 5-[^{125}I]iodonaphthyl 1-azide (^{125}INA). Cytoplasmic proteins were identified by their photolabeling with the intracellular probe azidofluorescein diacetate. Results indicate that the Triton X-100 residue contains the membrane-associated glycoprotein Ia, a 95 000-dalton protein, and two other acidic proteins of molecular weights of 35 000–40 000, one labeled with ^{125}INA and the other with azidofluorescein diacetate. The presence of part of these proteins in the Triton residue is dependent upon the mode of platelet activation. Glycoproteins IIb and III are embedded in the membrane lipid bilayer but sedimented with the Triton residue only after thrombin activation. Another protein with M_r 70 000, which is highly labeled by ^{125}INA in resting platelets, is found only in the Triton-soluble fraction.

The association of platelet membrane proteins with the cytoskeleton and the changes in the membrane proteins as a result of activation were the focus of a few studies in recent years (Phillips et al., 1980; George et al., 1980). Thus, Phillips et al. have recently shown that the amount of actin present in the cytoskeleton of thrombin-aggregated platelets is almost double that of actin in the cytoskeleton of unstimulated platelets. This is in accordance with observations made by Pribluda et al. (1981) and by Carlsson et al. (1979) that there is a significant mobilization of actin (decrease in DNase-available actin) as a result of platelet aggregation. A few membrane proteins were identified by Phillips et al. (1980) as being associated with the cytoskeleton, among them glycoprotein IIb and glycoprotein III. The association of actin with platelet membrane was shown morphologically by Zucker-Franklin (1970), biochemically by Taylor et al. (1976), and by immunofluorescent technique (Bouvier et al., 1977; Diggle et al., 1979). Some evidence was presented recently that platelet membrane glycoprotein III may be α-actinin (Gerard et al., 1979) and that this protein spans the plasma membrane (Phillips & Agin, 1974) and can function to anchor actin to the membrane. Thus, data accumulated so far indicated that glycoprotein III and glycoprotein IIb are associated with the cytoskeleton of aggregated platelets (Phillips et al., 1980) and that they may participate in the mediation of platelet–platelet interaction.

In this contribution we report the identification of those membrane proteins embedded in the lipid bilayer and their association with the cytoskeleton, using [^{125}I]iodonaphthyl azide (^{125}INA). We used the lectins wheat germ agglutinin (WGA), Lens culinaris lectin, and concanavalin A (Con A) to determine the association of glycoprotein Ia, glycoprotein Ib, glycoprotein IIb, and glycoprotein III with the cytoskeleton. We also identified those cytoplasmic proteins associated with the cytoskeleton by the use of the fluorescent intracellular probe azidofluorescein diacetate (Rotman & Heldman, 1980).

Materials and Methods

Platelets were prepared according to the method of Peerschke et al. (1980), with the modification that albumin was omitted from the medium. [^{125}I]Iodonaphthyl azide was

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prepared as described by Bercovici & Gitler (1978). Azidofluorescein diacetate was prepared from aminofluorescein (Sigma Chemical Co., St. Louis, MO), as described by Rotman & Heldman (1981).

Labeling with ^{125}INA. To 4 mL of gel-filtered platelets (4×10^8 cell/mL) in HBMT (Hepes buffer modified Tyrode's) buffer was added 25 μL of ^{125}INA in ethanol in the dark. The suspension was kept at room temperature for 5 min and was divided into four samples of 1 mL each. Each sample was activated in the dark for 1 min and then irradiated further for 3 min with continuous activation. In another set of experiments, ^{125}INA was first incorporated covalently by irradiation into the membrane proteins of resting platelets and only then were the platelets activated by ADP or thrombin as described. Thrombin activation was carried out with 1 unit/mL (final concentration), with or without 5 mM (final concentration) EDTA. ADP activation was carried out with 40 μM ADP and 350 μg/mL fibrinogen (final concentrations). The sample of resting platelets was irradiated for 4 min without activation. Cells were centrifuged at 12000*g* for 4 min and washed with buffer. The light source for irradiation was a Wild microscope UV source, containing a 200-W mercury arc lamp (Osram, HBO); a filter excluding light below 300 nm was used. No effect of photoactivation labeling on platelet activity was observed.

Labeling with Azidofluorescein Diacetate. Intracellular labeling with azidofluorescein was performed as follows. Azidofluorescein diacetate in acetone solution (100 mM) was added to platelet-rich plasma (PRP) to a final concentration of 0.5 mM, and the suspension was incubated at 37 °C for 15 min. The platelets were centrifuged at 750*g* for 20 min and resuspended in 4 mL of HBMT buffer. All these operations were performed in the dark. The platelet suspension was irradiated for 3 min and the platelets were gel filtered, as described by Peerschke et al. (1980). Cytoskeleton from thrombin-aggregated platelets was prepared (see below) and was separated on $NaDodSO_4$–polyacrylamide gel electrophoresis. The proteins were extracted by cutting the gels into 3-mm strips, each of which was homogenized with 3 mL of phosphate-buffered saline (PBS), pH 7.5, and incubated overnight at 37 °C. Those fluorescent fractions, containing the protein solution, were dialyzed against water, lyophilized, redissolved in 0.1 mL of PBS, and labeled with Bolton and Hunter reagent (Bolton & Hunter, 1973). Each fraction was electrophoresed to verify its purity.

Cytoskeleton structures were prepared as follows. At the desired time, a solution containing Triton X-100 in HBMT buffer containing EGTA and phenylmethanesulfonyl fluoride, 40 mM, was added to the platelet suspension until the concentrations of Triton X-100 and EGTA were 1% and 5 mM, respectively. The suspension was stirred in the aggregometer for 1 more min and then kept at 4 °C for 30 min. The suspensions were centrifuged in a Beckman microfuge (12000*g*) for 2 min, the supernatant was removed, and the pellets were washed with HBMT buffer containing 5 mM EGTA and 0.1% Triton X-100.

Labeling with Lectins. ^{125}I-Labeled WGA, Lens culinaris, or Con A was prepared by the Chloramine T method (Hunter & Greenwood, 1962) or by using the Bolton and Hunter reagent (Bolton & Hunter, 1973). The glycoproteins were labeled after separation on $NaDodSO_4$–polyacrylamide gel essentially as described by Gurd (1977). Briefly, the gels were stained, destained, and incubated in saline–Tris, 50 mM, pH 7.5, overnight at room temperature. The gels were then incubated in a solution of the lectin (10^6–10^7 cpm/mL, 10–100 μg/mL) containing 1% bovine serum albumin (BSA) for 10 h at room temperature, washed with the same buffer without BSA over 48 h, and dried. In these experiments the usual exposure time for the X-ray film, when an intensifying screen was used, was 1–5 days.

$NaDodSO_4$–polyacrylamide gel electrophoresis was carried out according to Laemmli (1970). Isoelectric focusing was done according to O'Farrell (1975). The gels were stained for proteins with Coomassie brilliant blue (de St. Groth et al., 1963) and dried. Radioactive profiles were obtained by exposing Agfa Gevaert X-Ray film to the dried gels at −80 °C for the appropriate time in each case, in the presence of a Du Pont Cronex Hi-plus intensifying screen.

Carrier-free [^{125}I]KI and Bolton and Hunter reagent were purchased from Amersham Radiochemical Center, Amersham, England.

Thrombin (human plasma), ADP, and Lens culinaris lectin were purchased from Sigma, St. Louis (MO). Wheat germ agglutinin was purchased from Makor Chemicals (Jerusalem) and used without further purification. Concanavalin A was purchased from Miles-Yeda, Rehovot. Other chemicals used were of the highest purity available.

Aggregation was measured on a Chronolog aggregometer. Fluorescence was measured in a Perkin-Elmer 1000 M fluorometer. An excitation wavelength of 474 nm and emission wavelength of 541 nm were used.

Results

Most of the studies described in this paper were carried out in parallel on four forms of platelets: resting platelets, ADP-aggregated platelets, thrombin-aggregated platelets, and thrombin-activated platelets.

Identification of Membrane Proteins Embedded in the Lipid Bilayer. The use of [^{125}I]iodonaphthyl azide (^{125}INA) as a hydrophobic probe to label membrane proteins embedded in the lipid bilayer is well documented (Bercovici & Gitler, 1978; Karlish et al., 1977; Gitler & Bercovici, 1979; Tarrab-Hazdai et al., 1980). When this reagent was incorporated in resting platelet membrane and activated photochemically, it inserted into membrane proteins, the labeling pattern of which is shown in Figure 1B. About 10 proteins are labeled, among them 3 with M_r of ~35000 (group H in Figure 1A), actin, a protein of 70000 daltons (named here as aridanin), glycoprotein IIb, glycoprotein III, a protein with M_r of 95000, and a few other polypeptides. When the probe (^{125}INA) was incorporated in resting platelets in the dark and the platelets were first activated (still in the dark) with ADP or thrombin and only then ^{125}INA was photochemically activated and hooked covalently to membrane proteins, dramatic changes in the labeling pattern were observed. According to Figure 1B,C, there is a strong decrease in the labeling of the 70000-dalton protein, and no significant change in the labeling of glycoprotein III, glycoprotein IIb, or actin is observed. In another experiment the ^{125}INA was added in the dark to resting platelets (and thus incorporated noncovalently in the membrane), the platelets were treated with 0.1% Triton X-100 (final concentration), and only then was the reagent activated photochemically. In this case, a strong labeling of actin was observed (Figure 1D).

Membrane Proteins Associated with the Cytoskeleton. For determination of which membrane proteins are associated with the cytoskeleton, resting platelets were labeled with ^{125}INA and divided into four samples: one was kept nonactivated and the others were activated with ADP, thrombin, and thrombin in the presence of EDTA, respectively. When the cytoskeleton of platelets was isolated, essentially as described by Phillips et al. (1980), Osborn & Weber (1977) and Trotter et al.

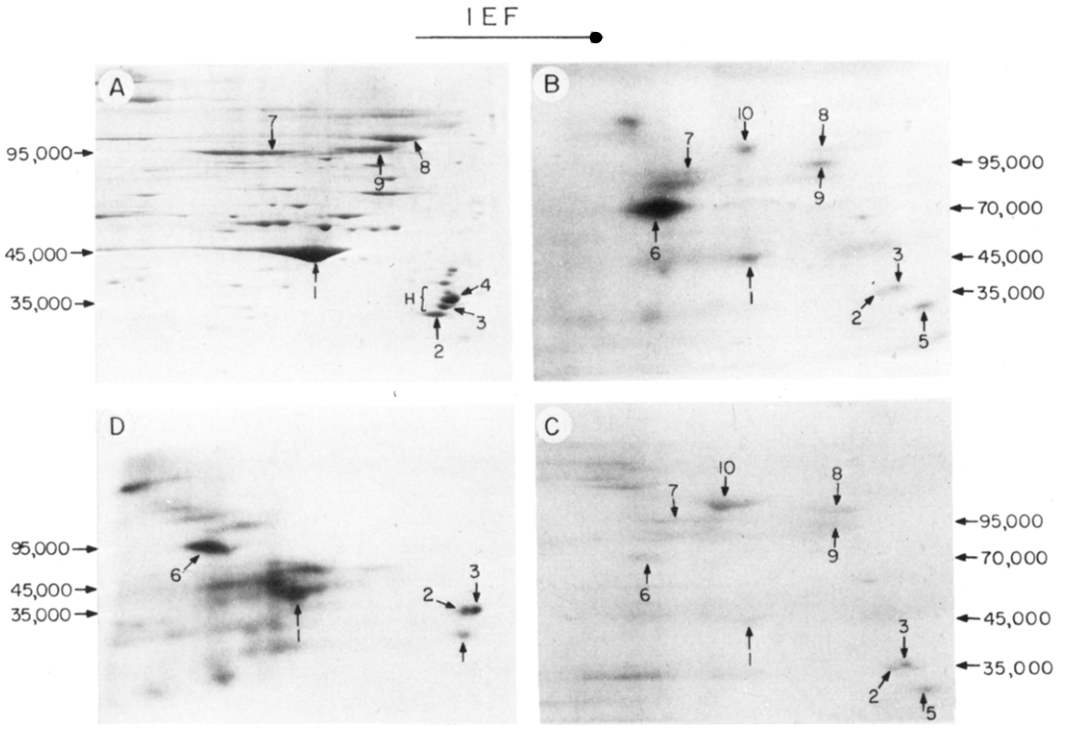

FIGURE 1: ^{125}INA labeling of washed platelets. Two-dimensional (isoelectric focusing and 5–15% NaDodSO$_4$–polyacrylamide) gel electrophoresis: (A) Coomassie blue staining, whole platelets; (B) autoradiogram, resting platelets photolabeled with ^{125}INA; (C) autoradiogram, thrombin-aggregated platelets (in the dark), photolabeled with ^{125}INA only toward the end of aggregation; (D) autoradiogram, resting platelets were treated (in the dark) with 0.1% Triton X-100 and then photolabeled with ^{125}INA. The numbers represent identified and nonidentified proteins: (1) actin; (2) nonidentified protein; (3) nonidentified protein; (4) nonidentified protein; (5) nonidentified protein; (6) 70 000-dalton protein (aridanin); (7) 95 000-dalton protein; (8) glycoprotein IIb; (9) glycoprotein III; (10) one of the forms of glycoprotein I.

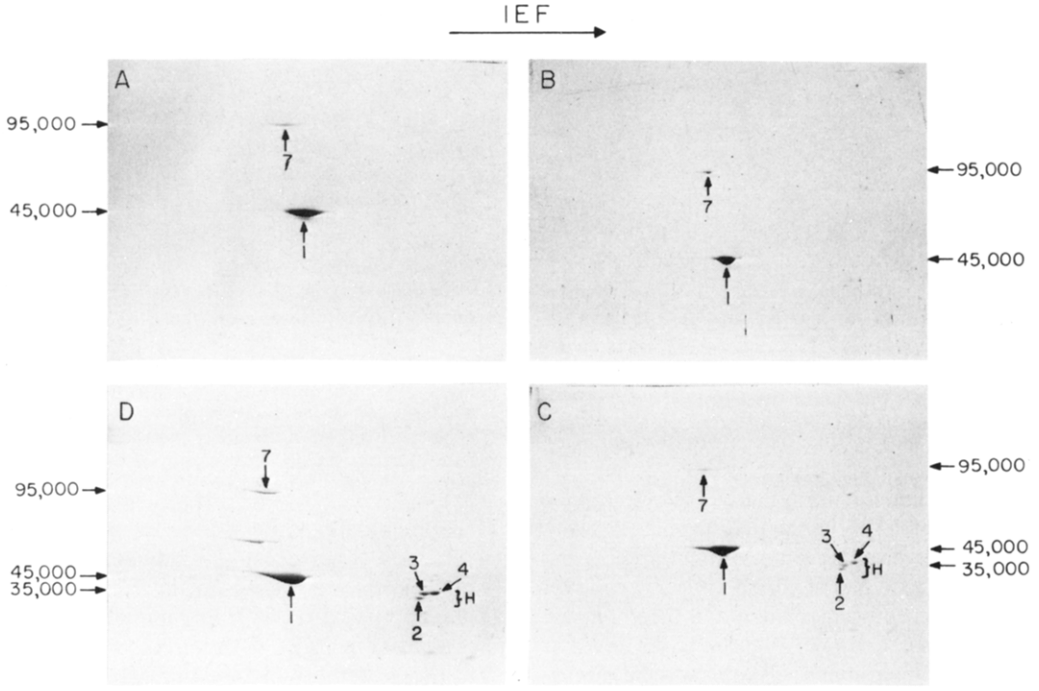

FIGURE 2: Coomassie blue staining of two-dimensional (isoelectric focusing and 5–15% gradient NaDodSO$_4$–polyacrylamide) gel electrophoresis of the Triton residue of (A) resting platelets, (B) ADP-aggregated platelets, (C) thrombin-aggregated platelets, and (D) thrombin-activated platelets (in the presence of EDTA). The numbering of protein spots is as in the legend to Figure 1. The amount of total protein in the Triton residue of thrombin-treated platelets (C and D) was about 8 times higher than that of resting platelets (A) and about 2 times higher than that of ADP-aggregated platelets (B). Therefore, so that a comparable result could be obtained, the gels were loaded with the same amount of total protein obtained from different amounts of treated platelets.

(1978), a few membrane proteins were detected as being attached to it. Figure 2 shows two-dimensional gel electrophoresis of the protein pattern of the Triton X-100 residues of the four samples described above: resting platelets (Figure 2A), ADP-aggregated platelets (Figure 2B), thrombin-aggregated platelets (Figure 2C), and thrombin-activated (but not aggregated) platelets (Figure 2D).

The main spots observed are actin, a 95 000-dalton protein, and a group of three proteins at the acidic side of the isoelectric focusing with M_r ~35 000. Myosin heavy chain usually

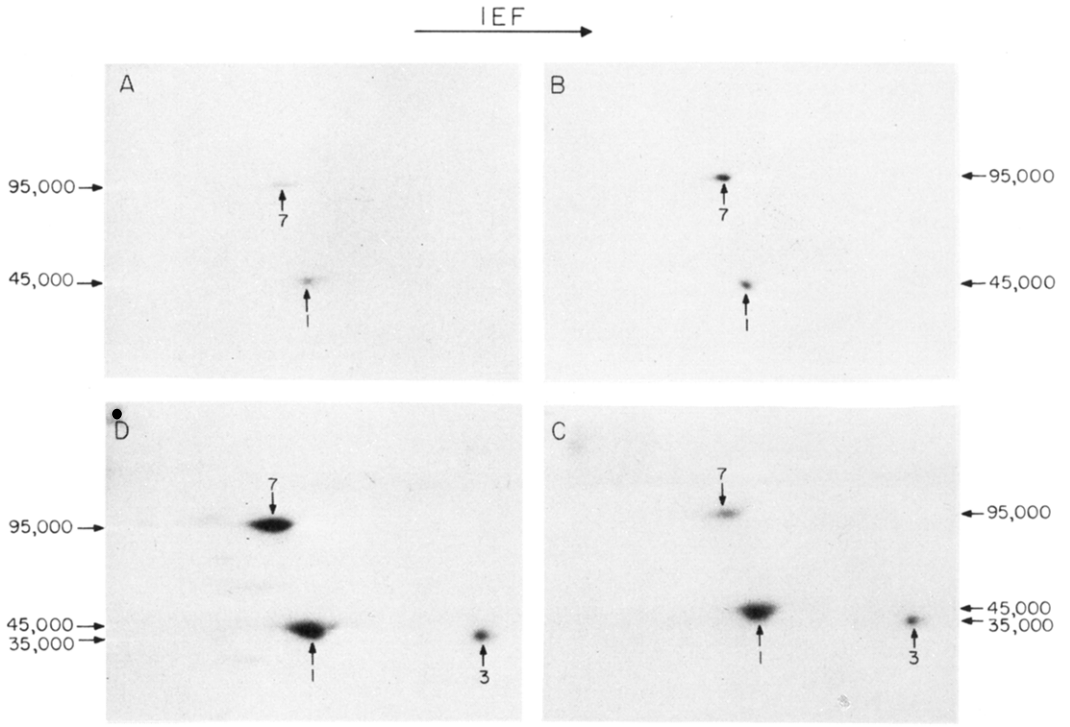

FIGURE 3: Autoradiograms of gels described in Figure 2. (Platelets were photolabeled by ^{125}INA prior to activation and Triton residue preparation.)

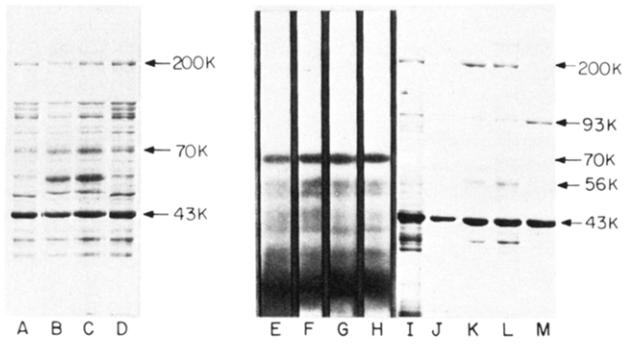

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis (5–15% gradients) of Triton-soluble fractions. (A–D) Coomassie blue staining; (A) resting platelets; (B) thrombin-activated platelets; (C) thrombin-aggregated platelets; (D) ADP-aggregated platelets; (E–H) autoradiograms of samples A–D, respectively (^{125}INA labeling); (I) whole platelets; (J–M) Triton residue of samples A–D, respectively. For the relative amount of protein in each gel, see the legend to Figure 2.

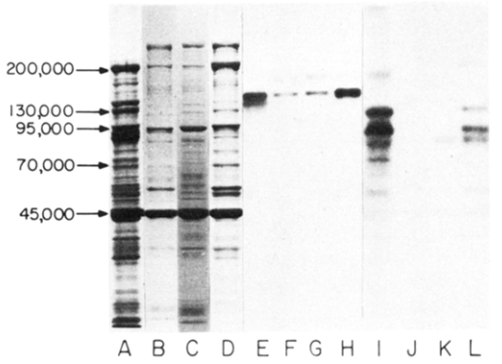

FIGURE 5: Coomassie blue staining and autoradiograms of whole platelets and Triton residues impregnated with ^{125}I-labeled WGA and ^{125}I-labeled Con A (5–15% $NaDodSO_4$–polyacrylamide gel electrophoresis): (A) Coomassie blue staining of whole platelets; (B) Coomassie blue staining of Triton residue of resting platelets; (C) Coomassie blue staining of Triton residue of ADP-aggregated platelets; (D) Coomassie blue staining of Triton residue of thrombin-aggregated platelets; (E–H) autoradiograms of gels A–D labeled with [^{125}I]WGA, respectively; (I–L) autoradiograms of gels A–D labeled with [^{125}I]Con A, respectively. Gels B–D were loaded with the same amount of total protein (see the legend to Figure 2).

present in the cytoskeleton (as observed on single-dimension $NaDodSO_4$ gel electrophoresis in rather variable amounts) is not detected in this two-dimensional system. The cytoskeleton of resting platelets and of ADP-aggregated platelets contains only small amounts of the proteins comprising group H (parts A and B of Figure 2). This group of proteins is easily observed in the cytoskeleton of thrombin-activated and thrombin-aggregated platelets (parts C and D of Figure 2). The autoradiograms of these two-dimensional gels are shown in Figure 3. The main spots labeled are actin, the 95 000-dalton protein, and spot 3 of group H (only in the case of thrombin-activated and thrombin-aggregated platelets). The Triton X-100 soluble fractions from the platelet preparations described in Figures 2 and 3 were analyzed both for protein distribution and for labeling with ^{125}INA. Results shown in Figure 4 clearly indicate that except for one band of fibrinogen (M_r 56 000) there is no significant difference in the pattern of proteins between the Triton-soluble part of the four forms of platelets studied. This 56 000 band is part of the fibrinogen subunits. Comparison of the autoradiogams of the Triton-soluble fractions (Figure 4E–H) with the autoradiograms of the Triton residue fractions (Figure 3A–D) indicates that the 70 000-dalton protein, which is the most highly labeled protein in total resting platelets (Figure 1A), is completely absent in the Triton residues (Figure 3A–D) and is the main labeled protein present in the Triton-soluble fractions (Figure 4E–H). Two other proteins labeled with ^{125}INA in whole platelets and observed in Figure 1A–D (proteins 2 and 5) are not observed in the autoradiograms of two-dimensional gels of the Triton residue (Figure 3A–D). Another protein of this group (protein 4) is not labeled at all by ^{125}INA.

The specific ^{125}INA labeling of actin in the Triton X-100 residue was usually about 8 times higher than the corresponding labeling in the Triton X-100 soluble fraction (measured from gels and autoradiograms of the Figures 2–4).

Another method to detect membrane proteins associated with the cytoskeleton was the incubation of gel electrophoresed

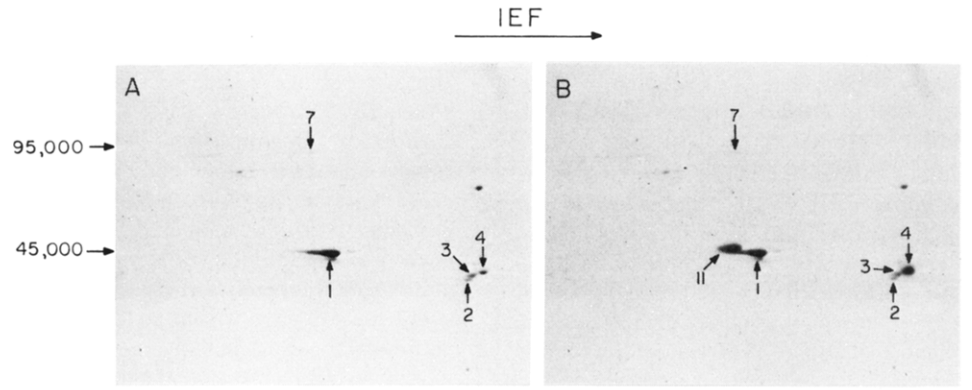

FIGURE 6: Identification of intracellular proteins. Two-dimensional isoelectric focusing (IEF)–$NaDodSO_4$–polyacrylamide gel electrophoresis of Triton residue of thrombin-aggregated platelets [(A) Coomassie blue staining] and superposition of two separate autoradiograms on the Coomassie blue staining (B). These autoradiograms were of two proteins isolated from the cytoskeleton of thrombin-aggregated platelets previously labeled with azidofluorescein diacetate (see Materials and Methods). The fluorescent proteins were labeled with Bolton and Hunter reagent, and each of them coelectrophoresed separately with a nonradioactive sample of Triton residue of thrombin-aggregated platelets.

cytoskeleton with ^{125}I-labeled WGA, Lens culinaris, and Con A. Figure 5 shows the Coomassie blue staining and autoradiograms of gels of whole platelets and Triton residue fractions of resting platelets, ADP, and thrombin-aggregated platelets incubated with ^{125}I-labeled WGA and ^{125}I-labeled Con A. Two bands (glycoproteins Ia and Ib) are labeled with ^{125}I-labeled WGA in total platelets (Figure 5E), but only one (glycoprotein Ia) is observed in the cytoskeleton fraction of resting platelets and ADP-aggregated and thrombin-aggregated platelets (parts F, G, and H of Figure 5). Similar preparations incubated with ^{125}I-labeled Con A showed three main labeled bands in the electrophoresis of whole platelets (Figure 5I) and thrombin-aggregated platelets (Figure 5L). These labeled bands have molecular weights of ~80 000, 95 000, and 120 000. All these three bands are missing in the pattern of resting platelets (Figure 5J), and only the 80 000 appears in the pattern of the Triton residue of ADP-aggregated platelets (Figure 5K). Lens culinaris lectin labeling gave a similar pattern to that of Con A. All these labelings were reconfirmed on a two-dimensional isoelectric focusing–$NaDodSO_4$–polyacrylamide gel electrophoresis (data not shown).

Cytoplasmic Proteins Associated with the Cytoskeleton. In order to identify cytoplasmic proteins that are associated with the cytoskeleton, we used the intracellular photoprobe azidofluorescein diacetate (Rotman & Heldman, 1980). This probe binds mainly to low molecular weight proteins in the cytoplasm (91%) and less than 0.5% are bound to the membrane (Rotman & Heldman, 1982). Thus, platelets were treated with this probe, and cytoplasmic proteins were labeled. The Triton residue of such platelets aggregated with thrombin was isolated, and the fluorescent proteins were isolated by $NaDodSO_4$ gel electrophoresis. Each protein was iodinated and coelectrophoresed on two-dimensional isoelectric focusing–$NaDodSO_4$–polyacrylamide gel electrophoresis with a nonradioactive sample of cytoskeleton of thrombin-aggregated platelets. Results shown in Figure 6 reveal that protein 4 (see Figures 1A and 2B,C) is a cytoplasmic protein, as well as another protein, 11, with an M_r of 46 000–47 000. These two proteins are associated with the cytoskeleton. The properties (except isoelectric point and molecular weight) and functions of these two proteins are not yet known.

Discussion

For the purpose of our study we define the Triton residue, which contains mainly actin and actin filament associated proteins, as the cytoskeleton of the cell. This method of cytoskeleton preparation by the use of Triton proved to be very useful in our studies. We are able to distinguish between those membrane proteins that are associated with the cytoskeleton and those that are not. Moreover, we are able to distinguish between different patterns of cytoskeleton–membrane association when platelets are activated by different agents, such as ADP or thrombin.

INA Labeling of Platelets. (*a*) *Labeling of Actin.* When resting platelets are labeled with ^{125}INA (Figure 1B), there is only slight labeling of actin (compare, e.g., with the 70 000-dalton protein), and this labeling does not change significantly with activation (compare part B with part C of Figure 1). There are two alternative explanations for the actin labeling: either INA penetrates into the cell and labels cytoplasmic actin or membrane-associated actin is slightly labeled. Comparison of the autoradiograms of the Triton residues and Triton-soluble fractions shows relatively high labeling of actin in the Triton pellet (cytoskeleton) compared to the labeling of actin in the Triton-soluble fractions (compare Figure 3A–D with Figure 4E–H). Thus, if the labeling of actin is due to slight penetration of INA into the cell, we could expect to find the same amount of labeling in actin in both fractions. The fact that the cytoskeletal actin is much more labeled indicates that membrane-associated actin is labeled, and it is mainly this actin that appears in the cytoskeleton. Pure muscle actin in HBMT buffer is not labeled by ^{125}INA, but when platelets were treated with 0.1% Triton X-100 and the protein was thus in micelles, there was high labeling of actin (Figure 1D). Irradiation of erythrocytes or brush border membrane of the intestinal epithelial cell with ^{125}INA revealed no labeling of actin (Sigrist-Nelson et al., 1977; Bercovici & Gitler, 1978). Thus, it seems justified to conclude that the labeling of actin in our case indicates some penetration of actin into the lipid portion of the membrane. This membrane-associated actin, which is relatively highly labeled by ^{125}INA, is probably the first to polymerize and constitutes a relatively large fraction of the actin in the Triton residue.

(*b*) *Labeling of the 70 000 Protein.* The main protein labeled with ^{125}INA in resting platelets is the 70 000-dalton protein, but its labeling decreases dramatically in activated platelets (Figure 1B,C). There are three possible explanations for this change: one, the 70 000 protein, which is highly embedded in the membrane bilayer, is moving vertically upon activation and as a result is not available to the reagent; two, this protein is moving laterally in the membrane plane and is forming aggregates which will diminish the efficiency of labeling by ^{125}INA; three, as a result of activation there is some degradation of proteolytic cleavage of this protein. We have

some preliminary results indicating that this 70 000-dalton protein is one of the main constituents of the platelet pseudopods (A. Rotman, N. Makov, and E. F. Luscher, unpublished results). Except for this protein, there is no significant change in the labeling pattern after aggregation.

(*c*) *Labeling of Other Proteins.* The other proteins labeled by ^{125}INA are glycoproteins IIb and III and a few of the proteins constituting group H (Figure 1). There was no significant change in the labeling of these proteins as a result of platelet activation, either with ADP or with thrombin. Other proteins are labeled by ^{125}INA in whole platelets and most of them are as yet unidentified by us. The group of proteins marked H in Figure 1 (Coomassie blue staining of whole platelets) consists of three sharp spots. Two proteins, 2 and 3, are labeled by ^{125}INA and there is another protein marked 5 in Figure 1B,C which is not detectable by the Coomassie blue staining but is labeled by ^{125}INA. Of these four proteins the three 2, 3, and 4 appear also in the cytoskeleton of thrombin-activated platelets (Triton residue, Figure 2C,D), but only 3 is labeled with ^{125}INA (Figure 3). Therefore, we can conclude that the protein 3 is a membrane protein embedded in the lipid bilayer and is associated with the cytoskeleton of thrombin-activated platelets. The proteins 2 and 3 are labeled by ^{125}INA in whole platelets, but 2 is not associated with the cytoskeleton. The spot 2 is probably composed of two proteins, one labeled by ^{125}INA and Triton soluble and the other (as well as protein 4) not a membrane protein but associated with the cytoskeleton. This group of proteins (group H) is barely detected in the Triton residue of resting and ADP-activated platelets (parts A and B of Figure 2) but is very intense in the residue from thrombin-activated (with EDTA) and thrombin-aggregated platelets (parts D and C of Figure 2). This is in contrast to the 95 000-dalton protein, which appears with a similar intensity relative to actin in both ADP- and thrombin-aggregated platelets. Thus, it seems that the 95 000-dalton protein is always anchored to actin (either monomeric or filamentous), and, upon activation and polymerization of actin, this protein will sediment with the polymerized actin. On the other hand, the proteins that contribute to group H are not associated with the cytoskeleton in the resting platelets but do associate upon activation.

Identification of Cytoplasmic Proteins Associated with the Cytoskeleton. The availability of the intracellular probe, azidofluorescein diacetate, enabled us to identify two proteins in the Triton residue as cytoplasmic proteins. Thus, protein 4 of group H (Figure 1) and protein 11 (Figure 6) are cytoplasmic proteins (labeled by azidofluorescein diacetate). This approach of identification of cytoplasmic proteins, with the intracellular probe azidofluorescein diacetate, has a great potential. Unfortunately, we assume that not all the proteins labeled with this probe were detected by us, due to the relatively low sensitivity of the fluorescence detection of gel extracts (e.g., compared with radioactive labels). The use of radioactive azidofluorescein diacetate might be more powerful to detect also those membrane proteins that are exposed toward the interior of the cell.

Labeling of Membrane Glycoproteins by ^{125}I-Labeled Lectins. (*a*) *Labeling with ^{125}I-Labeled WGA.* The third tool which we used to identify the association of protein with the cytoskeleton was three lectins: WGA, Con A, and Lens culinaris. WGA is known to bind glycoprotein Ia and glycoprotein Ib (Clemetson et al., 1977; Nachman et al., 1977a), as well as another polypeptide with a molecular weight of 210 000 (Nachman et al., 1979). Another component of the glycoprotein I complex, glycocalicin, which is removed from the platelet membrane by homogenization (Okumura & Jamieson, 1976b), was found to have a similar mobility in Na-DodSO$_4$–polyacrylamide gel electrophoresis to that of glycoprotein Ib (Clemetson et al., 1977; George et al., 1978; Jenkins et al., 1979; Okumura et al., 1976). As the glycoprotein I complex has been implicated in von Willebrandt factor-dependent platelet agglutination induced by ristocetin (Nurden & Caen, 1975; Nachman et al., 1977b) and glycocalicin may act as a single receptor for thrombin-induced platelet aggregation (Okumura & Jamieson, 1976a), it was important to study the presence of components of glycoprotein I in the cytoskeleton. Results shown in Figure 5 indicate that ^{125}I-labeled WGA resulted in labeling of glycoproteins Ia and Ib. This is compatible with the observation reported by Clemetson et al. (1977), concerning WGA binding to platelet membrane, and was corroborated by us in two-dimensional isoelectric focusing–NaDodSO$_4$ gel electrophoresis (data not shown). According to Figure 5, only glycoprotein Ia appears in the cytoskeleton of resting and ADP- and thrombin-aggregated platelets, while the other band (which might be glycocalicin or glycoprotein Ib) is absent in the Triton residue but present in the Triton-soluble fraction. However, glycoprotein Ia was not labeled with ^{125}INA (Figures 1 and 3) and is thus probably not embedded in the lipid bilayer and is not a transmembrane protein. It seems, therefore, that glycoprotein Ia is associated with another membrane protein which is anchored to the cytoskeleton, and candidates for such a protein are, for example, the 95 000-dalton protein or protein 2 (Figure 3).

(*b*) *Labeling with ^{125}I-Labeled Con A.* Similar labeling performed with ^{125}I-labeled Con A (Figure 5) or with ^{125}I-labeled Lens culinaris (data not shown) indicates that glycoprotein IIb and glycoprotein III are present in the Triton residue of thrombin-aggregated platelets but not in the Triton residue of resting or ADP-aggregated platelets. Thus, our results show that, although glycoprotein IIb and glycoprotein III are embedded in the membrane lipid bilayer, they are not anchored directly with actin and are possibly not transmembrane proteins. In their elegant study, Phillips et al. (1980) identified glycoprotein IIb and glycoprotein III in the Triton residue of thrombin-aggregated platelets, but these two glycoproteins were readily extracted by Triton from resting or thrombin-activated (but not aggregated) platelets. We confirmed the finding of Phillips et al. concerning the existence of these two glycoproteins in the thrombin-aggregated cytoskeleton, but our results indicate that they are not transmembrane proteins. The fact that these two glycoproteins could not be detected in the ADP-activated Triton residue but only in the thrombin-aggregated residue might be explained by the following hypothesis: glycoprotein IIb and glycoprotein III are embedded in the lipid bilayer of platelet membrane but are not transmembrane proteins. They might be connected loosely to actin via another membrane protein [a reasonable candidate is protein 3 (Figure 3)]. In the case of thrombin activation, the released fibrinogen might participate in the incorporation of the two glycoproteins into the cytoskeleton, which is not the case of ADP aggregation (less fibrin in part L of Figure 4 compared with parts J and K of Figure 4). It is interesting that the proteins constituting group H are barely detected in the Triton residue of resting or ADP-activated platelets (parts A and B of Figure 2) but are easily observed in the Triton residue of thrombin-treated platelets (Figure 2C,D). Thus, there is correlation in the appearance of glycoprotein IIb, glycoprotein III, and proteins 2, 3, and 4 in the Triton residue. If this correlation means any role for group H in anchorage of the glycoproteins, it is not yet known, but

the possibility that one or a few of them might serve as anchoring protein(s) for glycoproteins IIb and III cannot be ruled out. Protein(s) from this group might be bound loosely to the two glycoproteins in the resting and ADP-activated platelets, and, thus, none of them will appear in the cytoskeleton. Activation of platelets by thrombin might result in reorganization of proteins within the membrane, which can lead to a stronger interaction between the group H proteins and the two glycoproteins from one side and the actin from the other side. This situation will end up in the appearance of all of these proteins in the Triton residue of thrombin-activated platelets. Thus, our results do not contradict the findings of Phillips et al., as their studies were done only on thrombin-treated platelets. However, we have evidence that the interaction of platelet glycoproteins with the cytoskeleton is more complicated than was assumed before.

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