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Purification and partial characterization of CD9 antigen of human platelets

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CD9 antigen (p24) was purified from human platelets and partially characterized. The yield was 75 μg from 10 units of platelet concentrates. p24 (38 000 copies/platelet) has intramolecular disulfide bond(s) and, in SDS-PAGE, consists of major 24-kDa molecule and minor 26- to 31-kDa molecules. The N-terminal sequence of p24, PVKGGTKXIKYLLFGFNFIF, indicates that the protein has not previously been characterized and amino terminus (position 12–20) is hydrophobic.

CD9; Platelet; N-terminal sequence

1. INTRODUCTION

CD9 antigen (p24) was first described on common acute lymphoblastic leukemia (cALL) cells [1] but is also found on other hematopoietic cells including platelets and megakaryocytes [2–4] as well as non-hematopoietic cells [3]. We and others previously reported that monoclonal antibodies against p24 (CD9 cluster) caused direct activation of human platelets leading to aggregation and granule secretion [2,5–7] or intrinsic protein phosphorylation [8,9]. Although several groups reported that p24 is involved in Ca^{2+} flux [10] and that it has protein kinase activities [11–13], the precise functional role of this protein still remains unknown.

One of our concerns is whether or not p24 has transmembranous portion since TP82 antibody against p24 causes the signal transduction in platelets, i.e. diacylglycerol formation [3] and increases intracellular Ca^{2+} (Yatomi, Y., unpublished data). Newman et al. [14] reported that p24 is a non-integral plasma protein by using a lipophilic labeling technique [5]. LeBien et al. [15] and Seehafer et al. [16,17] proposed that palmitylation of p24 may play a role in its function perhaps by alteration its positional configuration to remain attached to the cell surface. To gain further insight into the molecular characteristics of this protein, we purified p24 from human platelets using TP82 affinity column and analyzed certain biochemical properties including N-terminal amino acid sequence.

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2. EXPERIMENTAL

2.1. *Materials*

Triton X-114, NaBr-activated Sepharose 4B, ovomucoid trypsin inhibitor, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. *Preparation of TP82 monoclonal antibody and immunoaffinity column*

TP82 antibody (IgG_1) obtained from murine ascites was purified by ammonium sulfate precipitation followed by DEAE chromatography [2]. Purified TP82, MM10 monoclonal antibody (anti-smooth muscle myosin antibody, IgG_1) [18] and mouse IgG (Sigma) were dialyzed against 0.2 M $NaHCO_3$ (pH 9.5) and coupled to NaBr-activated Sepharose 4B as previously described [18].

2.3. *Purification of CD9 antigen*

Twenty units of washed platelets were resuspended in 1 vol. of buffer A (0.5 M KCl, 0.15 M potassium phosphate, pH 6.8, 2 mM EGTA, 2 mM EDTA, 0.2 mg/ml trypsin inhibitor, 50 μg/ml PMSF, 3 mM DTT) and sonicated for 5 min. Two vols of buffer A were then added. After 1 h of extraction, the sample was centrifuged at $48000 \times g$ for 120 min at 4°C. The pellet was resuspended with buffer B (0.15 M NaCl, 30 mM Tris-HCl, pH 7.5, 1 mM EGTA, 0.1 mg/ml trypsin inhibitor, 50 μg/ml PMSF) with 1% Triton X-114 using glass homogenizer and dialyzed against buffer B overnight. After spin at $30000 \times g$ for 30 min, the supernatant was first chromatographed on the mouse polyclonal IgG column (8.5 mg, 10 ml) connected with MM-10 column (5 mg, 5 ml), then subjected to TP82 column (12 mg, 5 ml). The TP82 column was washed with 3 vols of buffer C (0.15 M NaCl, 30 mM Tris-HCl, pH 7.5, 1 mM EGTA), and then 2 vols of buffer C with 1 M NaCl to remove the non-specific binding molecules. The bound proteins (crude p24) was eluted with 3 M NaSCN, 30 mM Tris-HCl, pH 7.5. Enzyme-linked immunosorbent assay (ELISA) revealed that p24 was undetectable in both flow-through fractions and 1 M NaCl fractions. CD9-positive fractions (~16 ml) were collected and dialyzed overnight against 20 mM NaCl, Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM DTT overnight. The samples were filtrated through 0.45 μm Millipore filter, applied to a TSK-DEAE-5PW column (7.5 × 75 mm) equilibrated with the dialysis buffer, and eluted with a linear gradient of

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0.02–0.50 M NaCl, 2 × 16 ml, using Toso HPLC system. CD9-positive fractions (0.3 – 0.4 M NaCl) judged from ELISA were collected, dialyzed against buffer D (0.4 M NaCl, 30 mM Tris-HCl, pH 7.5, 1 mM EDTA), applied to TSK-4000 SW HPLC column equilibrated with buffer D. CD9-positive fractions judged from ELISA were collected, dialyzed against 0.15 M NaCl, 30 mM Tris-HCl, pH 7.5 and stocked at 4°C.

2.4. *Binding assay*

Purified TP82 was labeled with $Na^{125}I$ (Amersham Corp.) to a specific activity of 10 μCi/μg protein by the chloramin-T method [19]. Free and cell-bound ^{125}I-TP82 were separated by centrifugation through 25% sucrose. Non-specific binding was determined by adding a 100-fold excess of unlabeled TP82 antibody and Scatchard analysis was performed [20].

2.5. *N-terminal sequence*

Fifty μl of TCA was added to 200 μl purified p24 (200 μg/ml). After spinning, the pellet was dissolved in 30 μl of 0.1% SDS and applied onto an Applied Biosystems 477A Sequencer. PTH amino acids obtained from the sequencing were analyzed with an Applied Biosystems 120A.

2.6. *Phase extraction*

Detergent phase extraction was performed according to the method of Bordiger et al. [21] modified by Newmann et al. [22]. Briefly, 5×10^9 platelets were solubilized with 0.15 M NaCl, 30 mM Tris-HCl, pH 7.5, 1% Triton X-114 and centrifuged at 100000 × *g* for 60 min at 4°C. 200 μl of the supernatant (whole lysate) was carefully applied to the top of a 300 μl 6% sucrose cushion in a 1 ml polystyrene Fisher centrifuge tube [22]. The tube was warmed for 5 min at 37°C, and then centrifuged for 5 min at 2000 × *g*. The aqueous phase was removed, cooled to 0°C, re-extracted by 1% Triton X-114 and centrifuged as above. The detergent phases from both tubes were combined and the volume adjusted to 200 μl by adding Hepes-Tyrode's buffer. The detergent phase and whole lysate were coated with appropriate dilution to the 96-well plates and measured for p24 titer by ELISA.

2.7. *Others*

ELISA was performed as described previously [18]. The absorption at 492 nm was measured using Immuno-Reader MR700 (Dainatech Laboratories Inc.). Immunoprecipitation was performed as previously described [2]. Protein concentrations were estimated by the method of Bradford [23] using bovine serum albumin (Bio-Rad) as a standard.

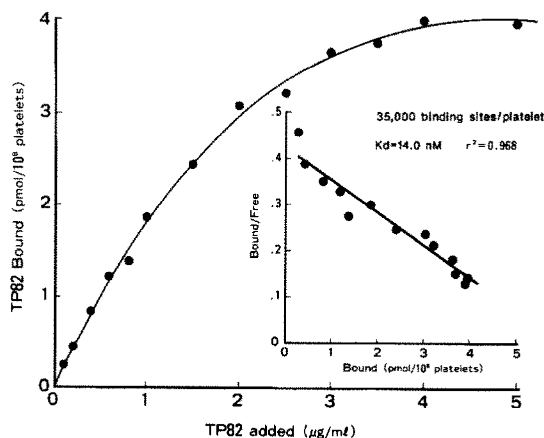

Fig. 1. ^{125}I-TP82 IgG binding on human platelets. 5×10^7 platelets were incubated with ^{125}I-TP82 antibody for 30 min at 22°C. The inset represents a Scatchard plot of the binding data.

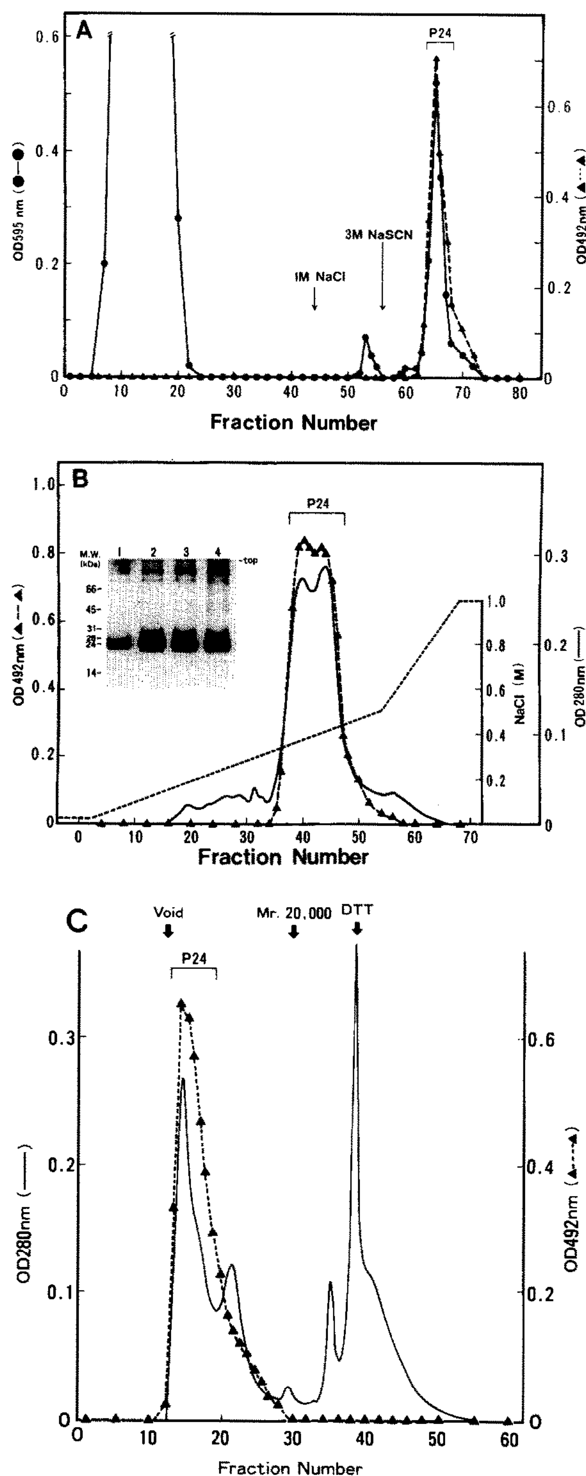

Fig. 2. Purification of p24 from human platelets. (A) Elution profile of TP82 affinity column. The flow rate was 25 ml/h. (B) Elution profile of DEAE-5PW HPLC column (0.49 ml each). The flow rate was 0.7 ml/min. Inset shows SDS-PAGE (15%) of fractions from DEAE-HPLC column. Lanes 1–4 represent the fractions 38, 40, 42 and 44, respectively. (C) Elution profile of TSK-4000SW HPLC column. The flow rate was 0.7 ml/min. Ten-fold diluted samples from each fraction were coated onto the plates and ELISA was performed (▲---▲, OD492 nm). The protein concentration was determined by the method of Bradford (●——●, OD595 nm) or OD280 nm (——).

3. RESULTS

3.1. *Binding of TP82 to platelets*

Fig. 1 shows representative data of the specific binding of ^{125}I-labeled TP82 to platelets. The binding was complete after 20 min and reached a plateau at 3–4 μg per 10^8 platelets. The Scatchard analysis indicated that TP82 binds to 38000 ± 4200 sites/platelet (mean ± SD, n = 4) with a dissociation constant (K_d) of 15.5 ± 2.5 nM/l (mean ± SD, n = 4) of a single class of binding site.

3.2. *Purification and biochemical characterization of CD9 antigen (p24)*

TP82 affinity column (Fig. 2A) followed by DEAE-5PW HPLC column (Fig. 2B) were used to purify p24. Further purification was performed by using TSK-4000SW HPLC column (Fig. 2C). p24 was eluted near the void volume probably because of the micelle formation. The yield was about 75 μg from 10 units of platelets. The purity was over 90% judged by densitometric scanning of SDS-PAGE. The purified p24 inhibited TP82-induced platelet aggregation and ATP release in a dose-dependent manner and this inhibition was overcome by increasing the concentration of antibody (data not shown).

An interesting finding is that purified p24 is always accompanied by a broad band of 26- to 31-kDa proteins. The silver staining revealed that this minor band consists of 3–4 small bands (Fig. 3A). However, early portions of p24-positive fractions of DEAE contain much of 26-kDa protein as compared with later portions which contain major 24-kDa and minor 29- to 31-kDa proteins (inset in Fig. 2B), indicating that these 24-kDa, 26-kDa and 29- to 31-kDa proteins have different affinity to DEAE-gel.

In a previous report [2], we showed that the molecular mass of iodinated p24 immunoprecipitated with TP82 was 23 kDa under reduced and 24-kDa under non-reduced conditions. Purified p24 also showed a small apparent increase in mobility (0.5–1.0 kDa) under reduced conditions (Fig. 3B), suggesting the presence of intramolecular disulfide bond(s). The diffuse appearance (26- to 31-kDa) detected by a longer exposure of immunoprecipitated molecules (Fig. 3B) is consistent with minor bands of silver-stained p24 (Fig. 3A).

N-terminal sequence (20 amino acids) was analyzed in two samples prepared separately. The results were identical (Fig. 4). No PTH amino acid was identified for portion 8. Computer searches (NBRF Protein Data Bank) revealed no significant homology with known proteins, although there was 40% homology in N-terminal sequence (20 amino acids) with that of melanoma-associated antigen [24,25] (Fig. 4). An interesting point is that N-terminal sequence has a hydrophobic region (hydropathy index [26] is −0.84 (position 1–7); 1.56 (position 9–20); 2.16 (position

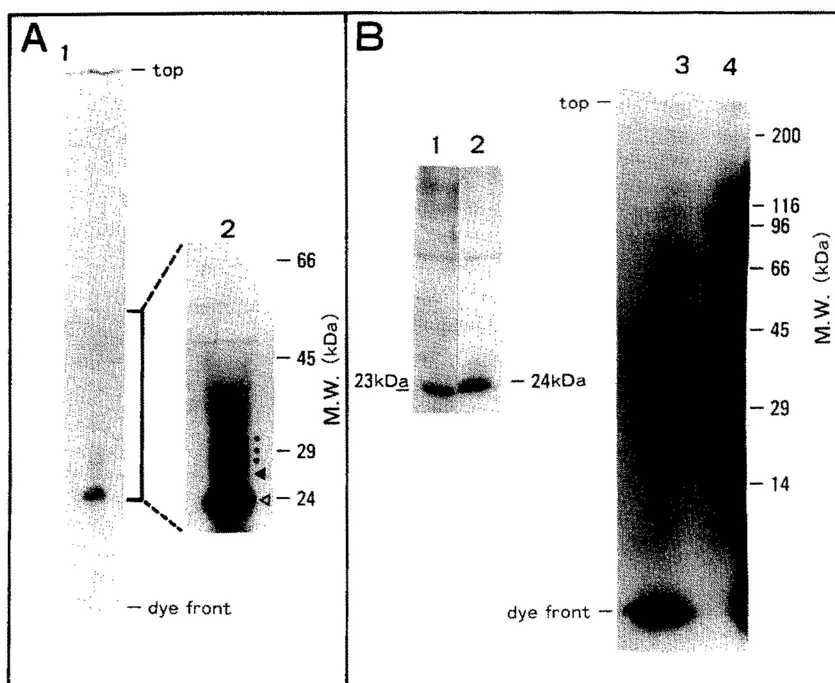

Fig. 3. SDS-PAGE of purified p24. (A) SDS-PAGE (12.5–20% gradient) of purified p24 (10 μg). Lane 1, Coomassie blue staining (non-reduced). Lane 2, silver staining of lane 1. p24 consists of major protein of 24 kDa (△) and minor proteins of 26 kDa (▲) and 29 to 32 kDa (●). (B) SDS-PAGE (12.5–20% gradient) of purified and immunoprecipitated p24. Lanes 1 and 2, purified p24 (silver staining); lanes 3 and 4, autoradiogram of immunoprecipitated p24; lanes 1 and 3, reduced; lanes 2 and 4, non-reduced. In lanes 3 and 4, there are a major band (▲) and diffuse minor band (△) as well as a few non-specific bands including 66 kDa proteins.

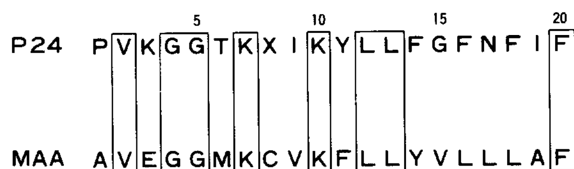

Fig. 4. Alignment of the N-terminal amino acid sequence of p24. The sequence of the 20 amino acids of p24 is compared with that of melanoma-associated antigen (MAA). Boxed residues are identical.

12–20)), suggesting that at least position 12–20 of N-terminal amino acids is a part of putative transmembrane domain, although hydrophobicity plot analysis [26] could not be performed at present. To confirm the hydrophobicity of p24, we applied the phase extraction technique using the non-ionic detergent Triton X-114 following a temperature-dependent phase separation [21,22]. Most of p24 (85 ± 6% (mean ± SD, $n = 5$) was fractionated into the Triton X-114 fraction (detergent phase). Purified p24 was dissolved in 1% Triton X-114 solution and analyzed in the same way. Similar extent of p24 (79 ± 5% (mean ± SD), $n = 7$) was fractionated into detergent phase.

4. DISCUSSION

The number of binding sites of TP82 IgG is 38000/platelet (Fig. 1) which is similar with other CD9 clusters such as PMA IgG (46000/platelet) [6] and AG-1 Fab (65000/platelet) [7]. The high copy numbers of binding sites were also reported with major platelet membrane glycoproteins such as GPIb (25000–34000) [27,28] or GPIIb/IIIa (40000–50000) [29–31].

There are a few reports that no change in molecular weight of p24 was found on reduction, implying that there are no disulphide bonds [1,14]. However, our data show that there is an apparent difference in molecular mass of both purified CD9 antigen and immunoprecipitated antigen (Fig. 3B); 23000 under reduced and 23500–24000 under non-reduced conditions. These results agree with earlier reports [2,11,15]. A failure in immunoblotting under reduced conditions (not shown) confirmed the existence of intramolecular disulfide bond(s) as suggested by others [6,7]. The diffuse appearance of the minor molecules (26- to 31-kDa) detected by silver-stained SDS gel (Fig. 3A) and autoradiograms of immunoprecipitation (Fig. 3B) suggested the varying extent of glycosylation. The difference in the binding property of the minor molecules to DEAE-gel (Fig. 2B) might be derived from the difference in glycosylation.

Although it was shown that the p24 molecule is an external membrane protein having no transmembranous moiety [5,15–17], our results are against such a notion. Affinity to Triton X-114 and the hydrophobicity of N-terminal sequence (position 12–20) (Fig. 4) suggest that p24 has transmembrane domain(s). Melanoma-associated antigen [24,25] which has 40% homology with p24 in N-terminal sequence (20 amino acids), was recently cloned and shown to have 4 transmembrane amino acid sequences including N-terminal region [25]. CD10 antigen (CALLA) was initially thought to have no transmembranous moiety as shown by photoaffinity labeling using a lipophilic probe [32]. However, the cloning of CALLA by two groups revealed that it has a transmembrane hydrophobic amino acid sequence [33,34]. Cloning of p24 from human platelets is currently under investigation in our laboratory to further characterize the structure and function of this protein.

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