The Protein CD63 Is in Platelet Dense Granules, Is Deficient in a Patient with Hermansky-Pudlak Syndrome, and Appears Identical to Granulophysin

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Abstract

The levels and expression of the proteins CD63 and granulophysin in platelets from control and from a Hermansky-Pudlak syndrome subject (a condition characterized by dense granule and lysosomal deficiencies and the accumulation of ceroid-like material in reticuloendothelial cells) were examined. Immunofluorescence studies indicated that anti-CD63 and anti-granulophysin antibodies recognized similar numbers of granules; coapplication of antibodies did not identify more granules than the individual antibodies. Significantly fewer granules were recognized in Hermansky-Pudlak syndrome platelets than in control using either antibody. Immunoblotting studies demonstrated that anti-CD63 and anti-granulophysin antibodies apparently recognize the same protein, which was deficient in Hermansky-Pudlak platelets. Analysis by fluorescence-activated cell sorter (FACS) showed biphasic expression of CD63 and granulophysin after thrombin stimulation of control but not Hermansky-Pudlak platelets. Anti-CD63 effectively blocked detection of the protein by anti-granulophysin using immunofluorescence, ELISA, immunoblotting, and FACS analysis. Amino-terminal sequencing over the first 37 amino acids revealed that granulophysin was homologous to CD63, melanoma antigen ME491, and pltgp40. These results suggest that granulophysin and CD63 are possibly identical proteins. This is the first report of a protein present in platelet dense granules, lysosomes, and melanocytes, but deficient in a patient with Hermansky-Pudlak syndrome. (*J. Clin. Invest.* 1993. 91:1775–1782.) Key words: platelets • CD63 • granulophysin • lysosomes • melanosomes

Introduction

When activated, platelets undergo biochemical and morphological changes that contribute to their role in thrombosis and hemostasis (1). Secretion of granule contents from activated platelets facilitates this process, resulting in enhanced interaction of stimulated platelets with each other, with other blood cells, and with vascular endothelial cells. Platelets contain four kinds of granules: α-granules, dense granules, lysosomes, and peroxisomes. Studies by Novak et al. (2, 3) have emphasized the close relationship between melanosomes and two of these organelles—dense granules and lysosomes. They described nine mouse pigment mutants which had a prolonged bleeding time owing to a deficiency in dense granules and dense granule contents, associated with altered renal lysosomal enzyme secretion. Some of the mutants have similar features to human storage pool deficiencies, the Hermansky-Pudlak and Chediak-Higashi syndromes, and are considered to be animal models for such human diseases. They hypothesized that the primary genetic defect in each of these mutants is a common gene product required for the normal function and/or structure of the three organelles: dense granules, lysosomes, melanosomes (2–7).

CD63 is a protein originally described as present in platelet lysosomes (8, 9). The melanoma associated antigen, ME491, was originally described as a stage-specific antigen of melanomas (10). However, cloning of ME491 and CD63 has shown them to be identical (9, 11). In addition, a protein initially described as a neuroglandular antigen has also been found to be identical to CD63 (12). We now report that CD63 is present in the platelet dense granule membrane and is deficient in a patient with the Hermansky-Pudlak syndrome, an autosomal recessively inherited disorder in which an absence of dense granules is associated with tyrosinase-positive oculocutaneous albinism and the accumulation of ceroid-like material in reticuloendothelial cells (13, 14). Furthermore, CD63 appears to be identical to a recently identified dense granule protein called granulophysin (15, 16), which is decreased in the Hermansky-Pudlak syndrome (15, 17). Our results thus provide the first identification of a membrane protein common to platelet dense granules, lysosomes, and melanosomes.

Methods

Monoclonal antibodies. The monoclonal antibodies (mAbs) against granulophysin used in the present study (D545, D503, and D519) have been characterized elsewhere (15, 17, 18). D541 produced in our laboratory was shown to be a mAb against P-selectin as analyzed by ELISA and by Western blotting using purified P-selectin, kindly provided by Dr. R. P. McEver (Oklahoma City, OK). Anti-CD63 antibody used for most studies was purchased from Amac Inc. (Westbrook, ME). An additional anti-CD63 antibody and FITC-anti-CD63 were kindly provided by Dr. James Hildreth and Dr. David Azorsa (Johns Hopkins University, Baltimore, MD).

Immunofluorescence studies. Platelets were stained for immunofluorescence microscopy using anti-granulophysin antibody D545 or anti-CD63 antibody, following the previously described procedure B (15). Counts of immunofluorescent granules were performed independently by two observers who were blind as to the identity of the platelets or the antibody used. The data is expressed as the mean±SEM of the total platelets counted by both observers.

Secretion of serotonin and β-glucuronidase. Blood was obtained from aspirin-free normal adult volunteers by venipuncture and drawn into acid citrate dextrose ([ACD][1] 3.8 mM citric acid, 7.8 mM tri-so-

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Received for publication 13 July 1992 and in revised form 28 October 1992.

J. Clin. Invest.

0021-9738/93/04/1775/08 $2.00
Volume 91, April 1993, 1775–1782

1. *Abbreviation used in this paper:* ACD, acid-citrate-dextrose.

Table I. Number of Platelet Granules Stained by D545 and Anti-CD63

	Number of bright granules	Number of faint granules	Total number of granules
Normal subject 1			
D545	4.38±0.43	2.35±0.33	6.72±0.42
Anti-CD63	2.78±0.41	1.70±0.31	4.47±0.39
Anti-CD63 + D545	2.85±0.45	2.97±0.43	5.82±0.44
Normal subject 2			
D545	5.12±0.48	1.28±0.28	6.40±0.36
Anti-CD63	3.55±0.41	1.18±0.20	4.72±0.39
Anti-CD63 + D545	4.40±0.45	1.55±0.31	5.95±0.36
D545-FITC	4.85±0.56	0.70±0.26	5.55±0.46
Anti-CD63 + D545-FITC	0.37±0.12	2.28±0.37	2.65±0.39
Hermansky-Pudlak syndrome patient			
D545	0.92±0.21	2.68±0.36	3.60±0.41
Anti-CD63	0.27±0.11	1.20±0.22	1.47±0.27
Anti-CD63 + D545	0.77±0.18	2.65±0.30	3.42±0.34

Values are mean±SEM granules per platelet (n = 40 platelets). Data represent three similar experiments.

dium citrate, 125 mM dextrose) anticoagulant (1.9 ml ACD/8.1 ml blood). Platelet-rich plasma was obtained by centrifugation of blood at 800 *g* for 5 min. Platelet rich plasma was aliquoted for parallel analysis of serotonin (dense granule) release, β-glucuronidase (lysosomal granule) release, and flow cytometric analysis of granulophysin. Each of these samples was washed and resuspended in a Hepes-Tyrode buffer (19) and aliquots from each were then treated as follows: (*a*) 0.5 mM colchicine for 45 min, then 5 U/ml thrombin for 5 s; (*b*) 0.5 mM colchicine for 45 min, then 5 U/ml thrombin for 30 s; (*c*) 0.4 mM RGDSP peptide and 1 mM $CaCl_2$ for 1 min, then 5 U/ml thrombin for 5 min; (*d*) no treatment. Colchicine was used to inhibit lysosomal granule secretion (20) and RGDSP peptide was used to minimize aggregation under conditions where secretion was maximum (21). Serotonin release was measured from platelets prelabeled with [^{14}C]-serotonin as previously described (22) and β-glucuronidase secretion was measured by the method of Hoehn and Kanfer (23). To facilitate comparison, all data are expressed as the mean±SEM of the respective total platelet content.

Fluorescence activated cell sorter (FACS) analysis. Samples for analysis were treated as described above, and the activation was terminated by the addition of an equal volume of ice-cold ACD. The platelets were pelleted, resuspended in Hepes-Tyrode's buffer containing 10% ACD, incubated for 60 min on ice in the dark with D545 (80 mg/ml), conjugated directly to FITC or indirectly labeled by sequential addition of D545, biotinylated goat anti-mouse IgG (1:100), and FITC-avidin (1:3,000). Unlabeled D545 was added to some samples before the fluorescent labeling. Samples were fixed with 1% paraformaldehyde and analyzed using an EPICS model 753 flow cytometer (Coulter Electronics, Haileah, FL) (500 mW, 488 nm). Fluorescence was detected at 545 nm. Forward and 90° light scatter measurements were used to establish gates for intact, viable platelets. Single parameter, 255-channel, log integral green fluorescent histograms were obtained, each based on 1×10^4 gated events.

Western blotting. The protein samples in a buffer of 2.5% glycerol, 5% SDS, 125 mM Tris HCl, pH 6.8, in the absence or presence of 5% β-mercaptoethanol, were boiled for 10 min and separated by electrophoresis on a 7.5% or 10% polyacrylamide gel with a 4% stacking gel according to Laemmli (24). After electrophoresis, proteins were transferred to a nitrocellulose sheet at 100 V for 60 min at room temperature using a bath-type transblot apparatus. The nitrocellulose was initially blocked overnight with 10% skim milk at 4°C and was then incubated with 10 μg/ml of the mAb in 0.1% Tween-TBS containing 1% BSA for 60 min at room temperature. After washing with 0.1% Tween-TBS, the nitrocellulose was incubated with goat anti-mouse IgG peroxidase-labeled (Sigma Chemical Co., St. Louis, MO, 1:3,000 dilution) for 30 min at room temperature. The reaction was developed using an enhanced chemiluminescence system (Amersham Corp., Oakville, ON).

ELISA. The activity of granulophysin was measured by the Sandwich ELISA described previously (17). Briefly, flat-bottomed, flexible microtiter plates (No. 3912, Falcon Plastics, Oxnard, CA) were coated with 150 μl/well of 20 μg/ml of the capture antibody (D519) in 0.1 M carbonate/bicarbonate buffer, pH 9.6, overnight at 4°C. After blocking the plates with 1% BSA in PBS, the samples were added to give a final volume of 100 μl. The plates were left for 75 min at room temperature. A peroxidase-conjugated second antibody (D545) at a dilution of 1:500 in PBS containing 0.1% Tween 20 and 1% BSA was then added to the wells (100 μl), and the plates were left for 60 min at room temperature. An *O*-phenylenediamine dihydrochloride tablet (Sigma

Table II. Secretion of Dense Granules and Lysosomes Compared to Granulophysin and CD63 Exposure

	Unstimulated platelets	Selective dense granule secretion: colchicine and EDTA and thrombin		Dense granule and lysosomal secretion: thrombin (600 s)
		5 s	30 s	
Normal controls				
Percentage of serotonin secretion	8.3±1.7	71.5±3.8	80.5±1.5	90.7±1.1
Percentage of β-glucuronidase secretion	0.8±0.3	2.5±0.8	4.4±0.9	31.8±4.9
Percentage of platelets expressing on their surface:				
D545 (indirect label)	5.5±2.2	23.2±7.2	30.7±6.0	51.7±4.1
CD63 (indirect label)	2.0±0.6	9.1±2.4	15.6±4.1	35.1±6.8
D545-FITC	11.7±1.1	69.6±13.6	69.2±10.5	79.3±4.4
Hermansky-Pudlak syndrome platelets				
Percentage of β-glucuonidase secretion	3.0	2.9	7.1	37.4
Percentage of platelets expressing on their surface:				
D545	0.6	0.5	0.86	2.7
CD63	0.3	0.4	0.5	4.1

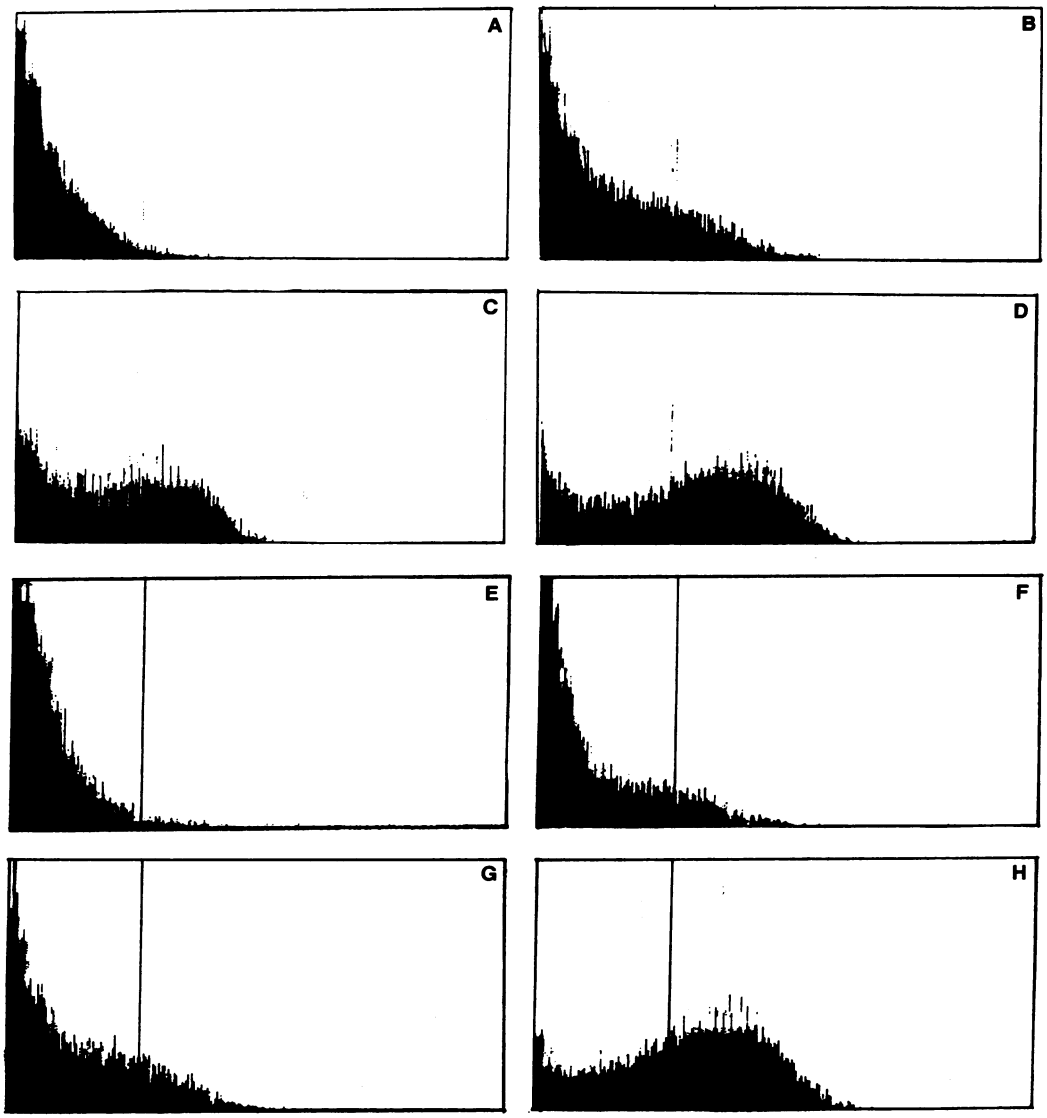

Figure 1. Expression of epitopes on control platelets after stimulation by thrombin under conditions of differential secretion. Detection antibodies: (*A–D*) D545; (*E–H*) anti-CD63. (*A, E*) Control; (*B, F*) colchicine + thrombin for 5 s; (*C, G*) colchicine + thrombin for 30 s; (*D, H*) RGDSP + $CaCl_2$ + thrombin for 5 min.

Chemical Co.) was used as a substrate and the plate was read in an ELISA reader (Flow Laboratories, McLean, VA) using a small diameter filter of 450 nm. In the case of blocking experiments using nonlabeled secondary antibodies, solubilized dense granule protein was used as the antigen and the plates were treated with different concentrations of D545, D503 (anti-granulophysins), D541 (anti-P-selectin), and anti-CD63 for 30 min. After washing the plate, peroxidase-conjugated antibody (D545) was added as described above.

Purification of granulophysin from dense granules of human platelets. Outdated platelet concentrates were obtained from normal donors by the Manitoba Red Cross. Dense granules were prepared as described previously (15) using a modification of the method of Rendu et al. (25). After washing, the platelets were resuspended in a homogenizing buffer of 25 mM Hepes buffer, pH 7.0, containing 500 mM KCl, 20 mM Na_3 citrate, 2 mM $MgSO_4$, 10 mM dextrose, 5 mM ATP, 100 mM sucrose, 1 mM PMSF, 1 mM benzamidine, and 1 μg/ml of each of the following protease inhibitors: antipain, aprotinin, chymostatin, pepstatin A, and soybean trypsin inhibitor. The cell suspension was sonicated and the homogenate was layered on top of 40% metrizamide in 350 mM KCl, centrifuged at 110,000 *g* for 30 min. The resulting pellet at the bottom of the metrizamide layer was a dense granule-rich preparation. The dense granules were solubilized with 0.2% lubrol in Tris-HCl buffer, pH 7.4, containing 150 mM NaCl and protease inhibitors as described above.

The solubilized sample was centrifuged at 100,000 *g* for 30 min at 4°C, and the supernatant was applied to a wheat germ lectin column (8 ml bed volume) at 4°C. After extensive washing with 200 ml of 20 mM Tris-buffered saline (TBS), pH 7.4, containing 0.1% lubrol, the column was transferred to room temperature and the protein was eluted with 0.5 M *N*-acetylglucosamine in TBS containing 0.1% lubrol. The fractions containing granulophysin activity were pooled and concentrated using Micro ProDiCon Model MPDC-20 (Bio-Molecular Dynamics, Beaverton, OR). The concentrated sample was applied to the monoclonal antibody bound immunoaffinity column (D545 + D503 coupled with Affigel 10, 5 ml bed volume) equilibrated with 5 mM Mops, 150 mM NaCl, pH 7.8, containing 0.1% lubrol. After washing with 150 ml of the same buffer, the protein was eluted with 80% ethylene glycol in 1 mM Mes, pH 5.6. Eluted proteins were concentrated as described above.

Protein sequencing. Affinity-purified protein was precipitated with cold acetone. The precipitate was dissolved in a small volume of 5 mM Mops containing 1% deoxycholate and electrophoresed as described above. The protein was transferred onto polyvinylidene fluoride membrane (Bio-Rad Laboratories, Mississauga, ON) in 10 mM 3-(cyclo-

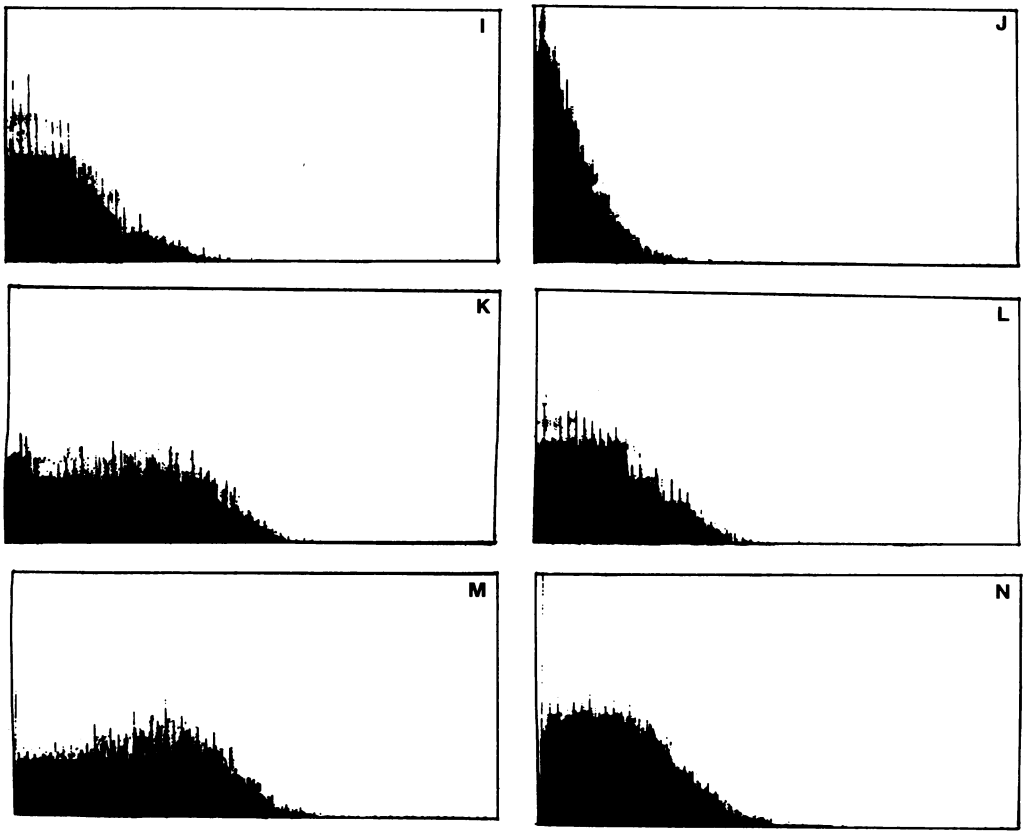

Figure 1 (Continued). Coaddition of anti-CD63 and D545. Detection antibodies: (*I, K, M*) FITC-D545; (*J, L, N*) anti-CD63 + FITC-D545. (*I, J*) Control; (*K, L*) colchicine + thrombin for 5 s; (*M, N*) colchicine + thrombin for 30 s.

hexyl-amino)-1-propanesulfonic acid buffer, pH 11, and was stained with Coomassie Brilliant Blue R-250. The protein bands of 120 kD, 200 kD, and the aggregated forms at the top of the sheet were cut and sequenced using an automated gas-phase microsequenator (Applied Biosystems Inc., Foster City, CA).

Assay of serotonin. Serotonin content of the whole-platelet and dense granule preparations was determined fluorometrically after the serotonin was derivatized with *o*-phthalaldehyde as described by Dangelmaier and Holmsen (26).

Protein determination. The protein contents were determined either by Bio-Rad Protein Assay or by Micro BCA Protein Assay Reagent Assay Kit (Pierce Chemical Co., Rockford, IL) using BSA as a standard.

Patient. The patient studied was a previously described (15) 41-yr-old man with Hermansky-Pudlak syndrome. His template bleeding time was prolonged and his platelets demonstrated impaired aggregation in response to collagen and epinephrine. The ADP content of (15) and ATP release from (17) his platelets were decreased as were his dense granule counts when assessed by whole mount, quinecrine fluorescence, and D545 immunofluorescence (17). His granulophysin level of 2.8-μg dense granule equivalents/mg platelet protein was lower than the normal values of 16.5±5.6-μg dense granule equivalents/mg platelet protein (17).

Results

Immunohistochemical study of CD63 and granulophysin in normal and Hermansky-Pudlak platelets. Immunofluorescence studies using anti-CD63 and D545 antibodies showed that they recognized 5.32±0.42 and 5.69±0.51 granules, respectively, in normal platelets. Further, staining with both antibodies concurrently did not identify more granules than staining with each antibody individually, suggesting both antibodies identified the same type of granule. This result was confirmed in samples incubated with directly labeled D545-FITC. Preincubation with unlabeled anti-CD63 antibody markedly inhibited subsequent staining by D545-FITC.

There may be two populations of granules being stained, with one population showing bright staining and one population showing weak staining (Table I). Hermansky-Pudlak platelets with extremely low levels of dense granules but with a normal lysosomal granule content (27, 28) were deficient in staining of granules by either D545 or anti-CD63 antibodies (mean values of 2.11 and 1.40 granules respectively). The deficiency was particularly evident in the number of brightly staining granules (Table I).

Staining of expressed proteins: dense granules versus lysosomes. Because it has been reported that CD63 is a lysosomal membrane protein (8, 9), we investigated the expression of CD63 and granulophysin on the plasma membrane of platelets after stimulation with thrombin using flow cytometry. A difference exists between the secretion patterns for dense granules and lysosomes. Dense granule contents are secreted very rapidly in response to thrombin, and such secretion is little affected by pretreatment of platelets with colchicine or by chelation of external calcium (8, 29, 30). In contrast, lysosomal granule secretion occurs more slowly, is greater in the presence of external calcium, and is inhibited by colchicine (Table II). When platelets were exposed to conditions with selective secretion of dense granule contents, it was apparent that a significant proportion of the epitopes recognized by D545 and anti-CD63 antibodies were present on the platelet surface in the virtual absence of lysosomal granule secretion (Fig. 1, *A–H*). On the other hand, higher levels of expression of the epitopes recognized by D545 and anti-CD63 occurred in association

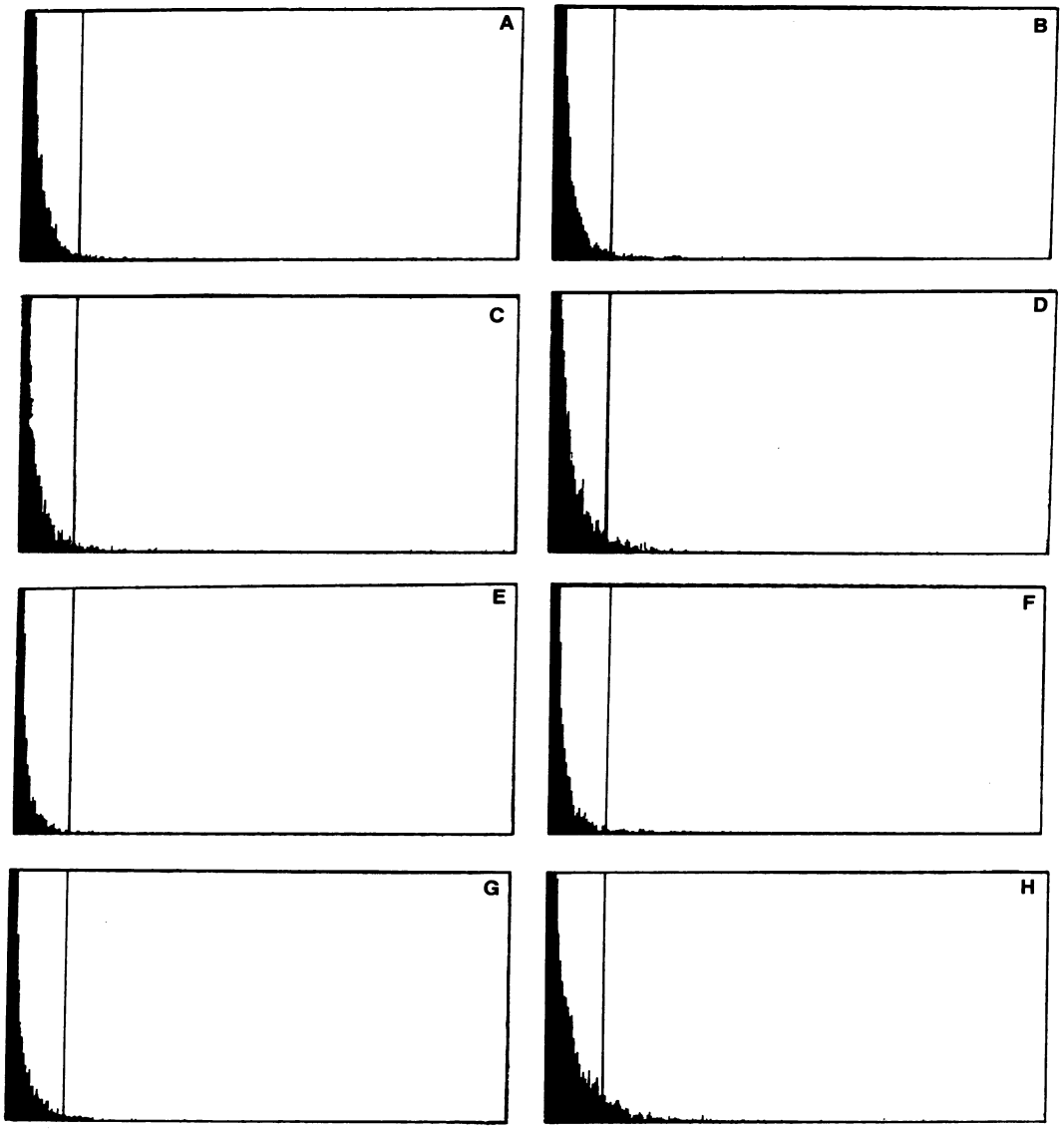

Figure 2. Expression of epitopes on Hermansky-Pudlak syndrome platelets after stimulation by thrombin under conditions of differential secretion. Detection antibodies: (*A–D*) D545; (*E–H*) anti-CD63. (*A, E*) Control; (*B, F*) colchicine + thrombin for 5 s; (*C, G*) colchicine + thrombin for 30 s; (*D, H*) RGDSP + $CaCl_2$ + thrombin for 5 min.

with maximal lysosomal enzyme secretion. Appearance of the D545 epitope occurred slightly faster than the CD63 epitope in identically prepared samples. However, preincubation with unlabeled anti-CD63 before FITC-D545 substantially inhibited labeling of the platelets using FITC-D545 suggesting that the two epitopes are closely linked if not identical (Fig. 1, *I–N*). Similarly unlabeled D545 blocked binding of FITC-anti-CD63 (data not shown).

Hermansky-Pudlak platelets showed minimal expression of CD63 and D545 epitopes in spite of substantial secretion of their lysosomal enzymes (Fig. 2; Table II).

ELISA and Western blotting. When we pretreated the plate for ELISA studies with unlabeled antibodies before the application of the secondary detecting peroxidase-labeled D545 antibody, both D545 and D503 (anti-granulophysins) inhibited the reaction in a concentration-dependent manner as observed previously (17) while D541 (anti-P-selectin) had no effect (Fig. 3). Anti-CD63 also inhibited the reaction and the inhibi-

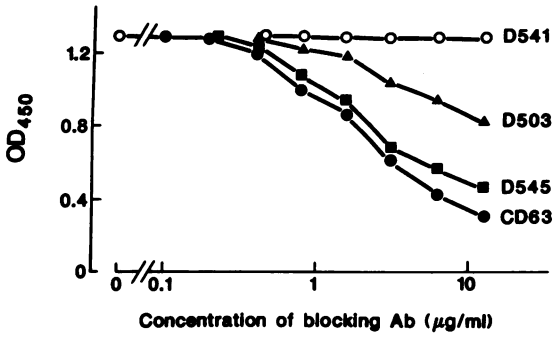

Figure 3. Effect of different unlabeled mAbs on the Sandwich ELISA assay of granulophysin detected by D545 peroxidase conjugate. The solubilized proteins of dense granules were used as antigen. The plates were incubated with serial dilutions of unlabeled D545 (■), D503 (▲), D541 (○), and anti-CD63 (●) for 30 min before applying the D545 peroxidase conjugate as described in Methods.

tory potency was as high as that of the detecting antibody D545 (Fig. 3). From the blocking experiments using different detecting and blocking antibodies (17), the epitopes recognized by our mAbs against granulophysin were shown to be closely related to each other. Therefore, it is likely that anti–CD63 also recognized the same highly antigenic epitope structure on granulophysin. We previously reported that granulophysin is deficient in platelets from patients with the Hermansky-Pudlak syndrome (15, 17). Using anti–CD63 antibody, we also observed a deficiency in CD63 protein in the Hermansky-Pudlak patient's platelets (Fig. 4) and the molecular species recognized by D545 and anti–CD63 antibodies were quite similar on Western blotting in both normal and the patient's platelets (Fig. 4). Furthermore, unlabeled anti–CD63 but not anti–P-selectin antibody inhibited the staining of granulophysin by D545-peroxidase conjugate (Fig. 5). These results strongly suggest that granulophysin and CD63 are the same protein, proteins with very high homology, or closely coupled proteins.

Purification and amino-terminal sequencing of granulophysin. By preparing dense granules from whole platelets, we achieved a 50-fold purification of granulophysin as measured by quantitative ELISA (Fig. 6). The dense granule preparation had a 50–100-fold enrichment with respect to serotonin content, a storage amine of dense granules, compared to the whole platelet homogenate. This value was comparable to the purification fold for granulophysin. Granulophysin was purified by sequential affinity chromatography using wheat germ lectin and a D545 immunoaffinity column. The enrichment of granulophysin after these chromatographic procedures was 5- and 12-fold, respectively, and a total 3,000-fold purification was achieved (Fig. 6). The efficiency of wheat germ lectin affinity chromatography strongly suggests that granulophysin is a glycoprotein as previously reported (15).

As we observed previously (15) the fully reduced form of granulophysin was not recognized by any of our mAbs using Western blotting. The nonreduced form of granulophysin in the dense granule preparation showed a wide band of staining with an apparent molecular mass of 30–60 kD but most intense at 40 kD (Fig. 7). After partial purification with a wheat germ lectin column, four or five reactive bands were observed (Fig. 7). The molecular mass of these bands corresponded to 40, 80, 120, and 160 kD, suggesting that these may be oligomeric forms of granulophysin. The possible oligomerization increased further on purification of this protein using an immunoaffinity column (Fig. 7). Under reducing conditions, we observed that these high molecular mass bands, but not the 40-kD band, were silver-stained on SDS-PAGE gels (data not shown), indicating that the formation of oligomers was not dependent on intermolecular disulfide bridge(s). After concentration of the sample with acetone the major portion of the protein transferred into the higher oligomeric forms. Therefore, we sequenced two protein samples, the combined 120- and 200-kD bands and the high molecular mass aggregated form blotted on the polyvinylidene fluoride membrane. On amino-terminal sequencing the combined 120- and 200-kD protein bands and the band containing higher molecular mass aggregated protein were identical. The results in Table III support the notion that the monomeric form is 40 kD. The tendency of granulophysin to form SDS-resistant oligomers is quite similar to pltgp40 (31). We obtained sequences on the first 37 amino-terminal amino acids of this protein and compared the predicted sequence with the SWISS-PROT protein library using the FASTA program (32). The sequences were identical to those of stage-specific melanoma antigen ME491 (11) and CD63/pltgp40 (20, 30). The blank position of signals in our sample corresponds to the cysteine residue in those proteins.

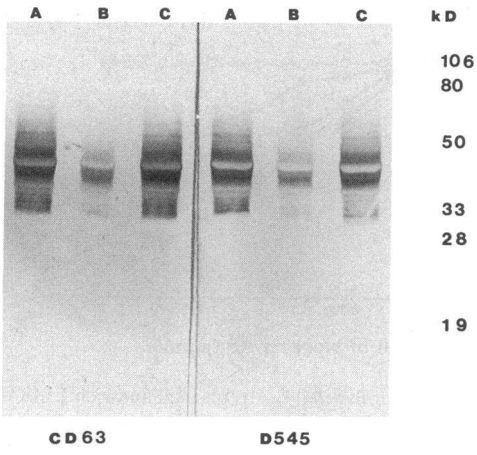

Figure 4. Western blotting of whole platelet homogenates from control subjects (lanes *A* and *C*) and HPS patient (lane *B*) using anti–CD63 antibody and anti-granulophysin antibody D545.

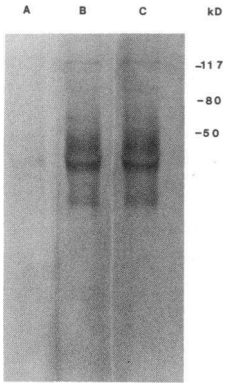

Figure 5. Western blotting of whole platelet homogenates. Lane *A*, anti–CD63 plus anti-granulophysin antibody D545 conjugated to peroxidase; lane *B*, anti–P-selectin antibody D541 plus anti-granulophysin antibody D545 conjugated to peroxidase; lane *C*, anti-granulophysin antibody D545 conjugated to peroxidase.

Discussion

Previous studies have demonstrated that CD63 is present on the lysosomal granule membrane, and that it is identical to both ME491 on melanosomes and a neuroglandular antigen (8, 9, 11, 12). In the present study we have provided evidence that CD63 is also present on dense granule membranes, and either identical to or closely coupled with the platelet dense granule protein granulophysin (15).

Western blotting analysis of platelet samples using anti–CD63 antibody and anti-granulophysin antibody D545 demonstrated a similar smeared staining pattern, suggesting that both antibodies may recognize the same protein. Moreover, blocking experiments using anti–CD63 and D545 showed that the epitopes recognized by the antibodies were closely related. The addition of unlabeled anti–CD63 prevented the recognition of the granulophysin molecule by D545 in ELISA, Western blotting, immunohistochemistry, and FACS analysis. Further, circumstantial evidence is offered by the tendency of granulophysin to form SDS-resistant oligomers after purification and concentration in a manner similar to pltgp40 charac-

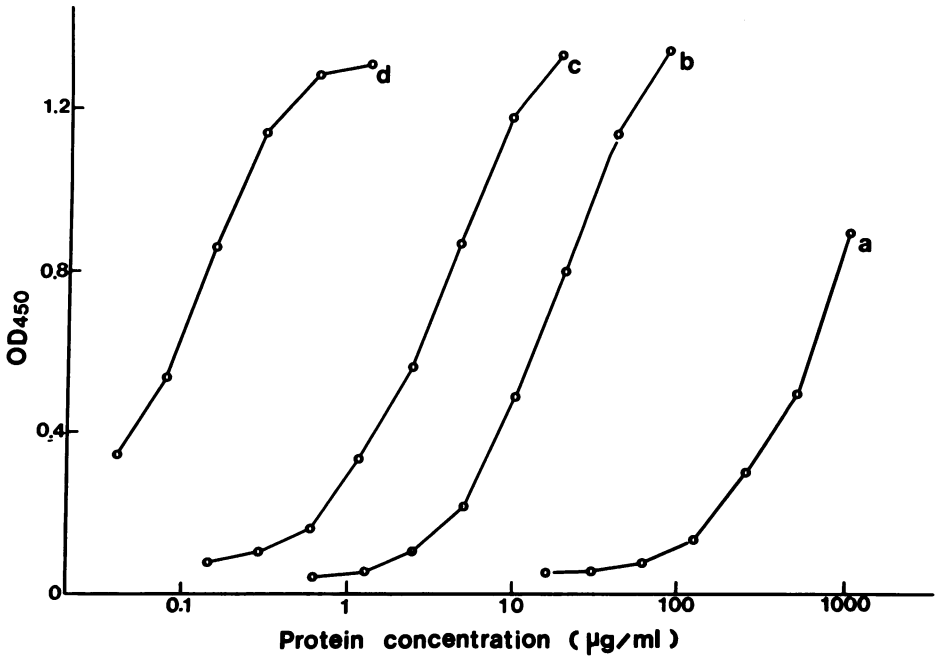

Figure 6. Quantification of granulophysin in the samples at each step of purification. The activity was determined by the Sandwich ELISA assay using serial dilutions of whole platelet homogenate (*a*), dense granule (*b*), the elute form wheat germ lectin column (*c*), and the elute from immunoaffinity column (*d*), respectively. The abscissa represents the concentration of protein in each sample and the ordinate shows the intensity of the peroxidase reaction.

terized previously (31). It was shown that the 37 amino-terminal amino acid sequence of granulophysin is the same as that of ME491/CD63/pltgp40 (9, 11, 31). Finally, Western blot and FACS analysis of platelets from a Hermansky-Pudlak patient demonstrated similar deficiencies using anti-CD63 and anti-granulophysin antibodies. However, several anti-granulophysin antibodies also recognize, to a lesser degree, other proteins such as synaptophysin (15, 18) and leukophysin (33), which have little homology to CD63.

CD63 was initially reported as a platelet lysosomal protein (8, 9), whereas granulophysin was found to be in dense granule membranes (15). The present study has taken several approaches to localize CD63 and granulophysin. Immunocytochemical analysis has demonstrated the presence of two populations of granules, termed bright and faint, using both anti-CD63 and anti-granulophysin. The number of bright granules corresponded to values reported by whole mount analysis, a technique that relies upon the calcium content specific to the dense granules. Similar immunocytochemical analysis of platelets from the Hermansky-Pudlak patient using either CD63 or D545 gave a low value for the number of bright granules, a value consistent with the low number of dense granules seen using whole mounts. In contrast, enumeration of faint granules by both anti-CD63 and D545 gave similar values in control and the Hermansky-Pudlak patient. Studies using FACS analysis clearly demonstrate the appearance of the CD63 and granulophysin epitopes on the platelet surface under conditions where there is virtually no secretion of lysosomal enzymes. As CD63 and granulophysin are clearly not present on the α-granules (8, 15, 16), the results provide strong support for the presence of the epitopes recognized by these antibodies on dense granule membranes. Studies showing a marked deficiency of both CD63 and granulophysin epitopes on the surface of thrombin-stimulated Hermansky-Pudlak platelets, platelets deficient in dense granule but not lysosomal contents (27, 28), offer additional support to the presence of both epitopes on dense granule membranes. In addition, under conditions where lysosomal as well as dense granule contents are secreted FACS analysis showed increased expression of CD63 and gran-

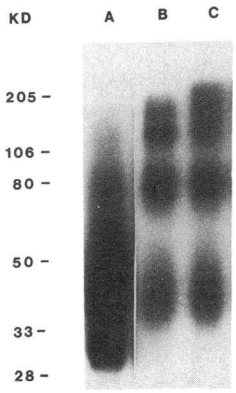

Figure 7. Western blotting of granulophysin using monoclonal anti-granulophysin antibody, D545. The samples used were solubilized dense granule (lane *A*, 10 µg protein), the elute from the wheat germ lectin column (lane *B*, 0.6 µg protein) and the elute from the immunoaffinity column (lane *C*, 0.05 µg protein) under nonreducing condition.

Table III. Amino-terminal Amino Acid Sequence of Granulophysin

	1	5	10
120-kD + 200-kD	Ala Val Glu Gly Gly Met Lys Xxx		Val Lys
Aggregate	Ala Val Glu Gly Gly Met Lys Xxx		Val Lys
CD63/gp40	Ala Val Glu Gly Gly Met Lys Cys		Val Lys
	11	15	20
120 kD + 200 kD	Phe Leu Leu Tyr Val Leu Leu Leu		Ala Xxx
Aggregate	Phe Leu Leu Tyr Val Leu Leu Leu		Ala Phe
CD63/gp40	Phe Leu Leu Tyr Val Leu Leu Leu		Ala Phe
	21	25	30
Aggregate	Xxx Ala Xxx Ala Val Gly Leu Ileu		Ala Val
CD63/gp40	Cys Ala Cys Ala Val Gly Leu Ileu		Ala Val
	31	35	
Aggregate	Gly Val Gly Ala Glu Leu Val		
CD63/gp40	Gly Val Gly Ala Glu Leu Val. . . .		

Xxx indicates that no PTH amino acid was identified.

ulophysin, consistent with the presence of both epitopes on platelet lysosomes. Evidence from subcellular fractionation studies showing some increase in expression of CD63 and granulophysin in a fraction with increased content of lysosomes also support this conclusion (data not shown). Therefore, it appears that CD63/granulophysin is present on both platelet dense granules and platelet lysosomes which are speculatively the bright and faint granules, respectively. Earlier studies failed to demonstrate the presence of CD63 on platelet dense granules (8), probably as the platelet fixation procedures for immunoelectronmicroscopy results in the disappearance of dense granule contents so that they can no longer be easily identified (16).

The present investigation provides evidence to identify the first of several proteins which Novak et al. (3) and Reddington et al. (6) suggest occur simultaneously in the membranes of platelet dense granules, lysosomes, and melanosomes. We have considered the possibility that a deficiency in CD63 may be the primary protein deficiency in the Hermansky-Pudlak syndrome. In favor of this concept is the evidence of a marked deficiency in this protein on FACS analysis of thrombin-activated Hermansky-Pudlak platelets, however there is a normal amount of granulophysin (CD63) on Hermansky-Pudlak leukocytes (15). Clarification may need to await more definitive molecular characterization of the CD63 gene in patients with the Hermansky-Pudlak syndrome.

Acknowledgments

We thank Sandra Singhroy and Catherine Robertson for their excellent technical assistance, and Dr. Arnold Greenberg for helpful discussions and comments.

Masahiro Nishibori was supported by a fellowship from the Uehara Memorial Foundation, the Manitoba Institute of Cell Biology, and the Children's Hospital of Winnipeg Research Foundation.

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