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CD63 associates with CD11/CD18 in large detergent-resistant complexes after translocation to the cell surface in human neutrophils[1]

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Abstract CD63 antibody binding to the neutrophil surface triggers a transient activation signal that regulates the adhesive activity and surface expression of CD11/CD18. Gel permeation chromatography demonstrated that all of the cell surface CD11/CD18 associated with CD63 eluted in the void volume, indicating that they were present in large detergent-resistant complexes. In contrast, the majority of the total cellular CD63, CD11 and CD18, which are largely intracellular, was not present in complexes. The data suggest that intracellular CD11, CD18 and CD63 are not in detergent-resistant complexes, but enter such complexes following translocation to the cell surface.

Key words: Inflammation; Granulocyte; Integrin; Neutrophil activation; Membrane domain; CD63

1. Introduction

In neutrophils, CD63 is an activation antigen in that its surface expression is upregulated from intracellular stores by stimulation [1,2] and CD63 antibody binding to the neutrophil surface triggers a transient activation signal that requires extracellular calcium and regulates the adhesive activity and surface expression of CD11b/CD18 (the β2-integrin subfamily member also known as HMac-1) [2]. CD63 has been found in a wide variety of cells including neutrophils, platelets, basophils, cytotoxic T-lymphocytes and the Weibel–Palade bodies of endothelial cells [1–6] CD63 is identical to the ME491 antigen, originally described as a stage-specific differentiation antigen of melanoma cells [5–7] and to granulophysin, deficient in platelets, but not leukocytes, of patients with the Hermansky–Pudlak syndrome [8]. CD63 is one of the major lysosomal membrane proteins (LAMPs) [9,10].

CD63 is a member of the tetraspan family, traversing the membrane four times and having a major extracellular loop and a small extracellular loop, with short intracellular amino and carboxy termini [3,5–10]. The major extracellular domain is heavily glycosylated and contains poly-*N*-acetyllactosamines [3,9,10]. The intracellular domains of CD63 are small and have no clear motif known to be involved in signal transmission. The previous finding of the association of CD11b/CD18 and protein kinase activity, including the src family kinases lyn and hck, with CD63 [2] suggests that these associations may be involved in signal transduction, but the nature of these associations are unclear.

Recent studies have demonstrated the existence of large non-covalent detergent-resistant complexes in cell extracts that contain important signaling molecules, including protein kinases and many glycosyl-phosphatidylinositol (GPI)-linked membrane proteins capable of transmitting signals [11–19]. In this study, gel filtration chromatography demonstrated that most of the cell surface CD11b/CD18 associated with CD63 was present in such complexes. In addition, most of the total cell surface CD11b/CD18 was present in such complexes, however, some was also detected in lower mobility fractions. In contrast, most of the total cellular CD63, CD11b and CD18, which are largely intracellular, was not present in these complexes. Thus, CD11b/CD18 and CD63 appear to associate and enter large detergent-resistant complexes following translocation to the cell surface. The colocalization of CD11b/CD18 and CD63 in these complexes may play a role in signal transmission by CD63.

2. Materials and methods

2.1. Antibodies and reagents

Normal mouse IgG and normal mouse serum (NMS) were purchased from Sigma (St. Louis, MO). The CD63 mAb AHN-16 [2], the CD45 mAb AHN-12 (IgG1) [20], and the CD11b mAb OKM1 (IgG2b) [21], have been previously described. The CD18 mAb 60.3 (IgG2a) was a gift of Dr. J. Harlan, University of Washington, Seattle, WA.

2.2. Preparation of biotinylated cell extract and immunoprecipitation

Neutrophils were prepared from heparinized (2 U/ml) human venous blood as described [22]. Neutrophils were biotinylated with sulfosuccinimidyl-6-(biotinamido)hexanoate (Pierce, Rockford, IL), treated with 10 mM diisopropylfluorophosphate (DFP) (Sigma), solubilized and used for immunoprecipitation as previously described [2,23] or analyzed by SDS–PAGE directly. Biotinylated proteins,

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Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GPI, glycosyl-phosphatidylinositol; DFP, diisopropylfluorophosphate; NMS, normal mouse serum; PBS, phosphate buffered saline, pH 7.4; Brij solubilization buffer, 20 mM Tris–HCl, pH 8.2, 150 mM NaCl, 1 mM PMSF, 2 mM $MgCl_2$, 0.02% NaN_3 and 1.0% Brij 58; Brij wash buffer, 20 mM Tris–HCl, pH 8.2, 150 mM NaCl, 1 mg/ml BSA, 0.5% Brij 58, 2 mM $MgCl_2$, 0.125 mg/ml gelatin, 1 mM PMSF and 0.02% NaN_3

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were visualized by using an enhanced chemiluminescence system after transfer to Immobilon transfer membranes (Millipore Corp., Bedford, MA) as previously described [2,24].

2.3. Gel chromatography

Gel filtration chromatography was performed as previously described [16]. Briefly, 3 ml columns (0.8×5 cm) of Sepharose 4B (Pharmacia) were equilibrated with Brij solubilization buffer at 4°C and 0.3 ml of cell extract was applied to the top of the column. The column was eluted with Brij solubilization buffer and 0.3 ml fractions were collected. The column was calibrated with blue dextran (void volume) (Sigma), which eluted in fractions 2 and 3, and IgG, which eluted in fraction 7.

3. Results

3.1. Gel filtration chromatography of biotin-labeled proteins

Recent reports suggest that some cell surface proteins may exist in detergent-resistant complexes that can be resolved by gel filtration chromatography [11,15–19]. To determine if the CD11b/CD18 associated with CD63 has similar properties, neutrophils were surface-labeled with biotin, extracted with Brij buffer and fractionated by gel permeation chromatography on Sepharose 4B as described in Section 2. Aliquots of each fraction were immunoprecipitated with the CD63 mAb AHN-16 (Fig. 1, left panel). Since CD63 is not efficiently labeled with biotin under these conditions [2,25] only biotin-labeled proteins associated with CD63 are visualized. Essentially, all of the observed biotin-labeled protein associated with CD63 is accounted for by CD11b/CD18 [2]. CD11b/CD18 is readily detected in the void volume in column fractions 2 and 3 (Fig. 1, lanes 2 and 3, left panel). At this level of detection, no CD11b/CD18 was detected in fraction 1 (not shown) or in fractions 4–8 (Fig. 1, lanes 4–8, left panel). Most of the biotin-labeled CD11b/CD18 was present in fraction 2 (in Fig. 1, 20% of the fraction 2 immunoprecipitate was loaded in lane 2 while 100% of the corresponding immunoprecipitates of fractions 3–8 were loaded on the gel). Biotin-labeled CD11b/CD18 were not detected in immunoprecipitates using NMS (right panel). Since the exclusion limit of Sepharose 4B is more than ~20 million Da, most cell-surface CD11b/CD18 that is associated with CD63 appears to be present in complexes of relatively large size.

Most neutrophil CD63 is present intracellularly, largely in the primary granules [1]. Therefore, in light of the above results, it would be predicted that less biotin-labeled CD11b/CD18 might be detected in a CD63 immunoprecipitate from whole cell extract, since the signal would be diluted by CD63 from the primary granules. Indeed, at the level of detection used in Fig. 1, very little biotin-labeled CD11b/CD18 was detected in a CD63 immunoprecipitate from unfractionated cell extract (labeled 'E' in Fig. 1, left panel) compared with that observed in the void volume in fraction 2 (lane 2) (in Fig. 1, 20% of the whole cell extract and fraction 2 immunoprecipitates were loaded on the gel in lanes E and 2, respectively). As expected, no biotin-labeled CD11b/CD18 was detected in immunoprecipitates using the control antibody NMS (Fig. 1, right panel).

The distribution of cell-surface CD11b and CD18 in detergent-resistant complexes in neutrophils was examined directly by immunoprecipitation of biotin-labeled neutrophil extract fractionated by gel permeation chromatography on Sepharose 4B (Figs. 2 and 3, respectively). CD11b, detected by immunoprecipitation with the CD11b mAb, OKM1 and associated CD18 were readily detected in the cell extract (Fig. 2, lane E) and in fractions 2 and 3, but were also detected in fractions

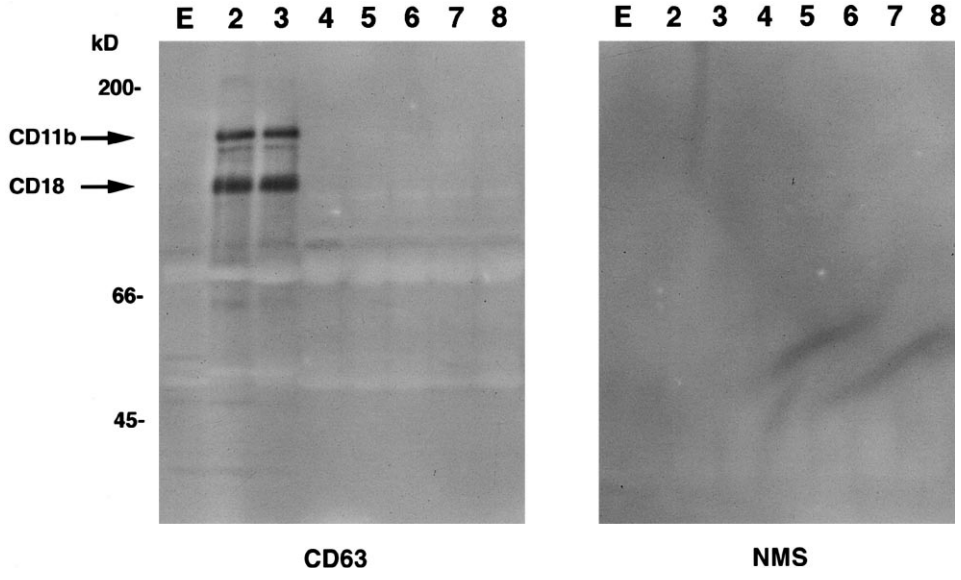

Fig. 1. Distribution of the CD63-CD11b/CD18 complexes in gel permeation chromatography fractionated neutrophil extract. Neutrophils were surface-labeled with biotin, solubilized in Brij solubilization buffer, and the extract was fractionated by gel permeation chromatography on Sepharose 4B as described in Section 2. Column fractions were immunoprecipitated with the CD63 mAb AHN-16 (left panel) or NMS (right panel), analyzed by SDS–PAGE under reducing conditions, and the surface-labeled proteins detected as described in Section 2. Lane 1 contains an immunoprecipitate from whole cell extract (E). The column fractions are indicated by the numbers at the top of the gels. Lanes E and 2 were loaded with 20% of their respective immunoprecipitates while 100% of the immunoprecipitates of fractions 3–8 were applied to their respective lanes. The arrows indicate the positions of the ~165-kDa CD11b and the ~100-kDa CD18. Blue dextran eluted in fractions 2 and 3 (void volume), while IgG eluted in fraction 7. A duplicate experiment gave similar results. Proteins used as molecular weight standards were: myosin heavy chain, 200 000; *Escherichia coli* β-galactosidase, 116 000; phosphorylase a, 97 400; bovine serum albumin, 66 000; ovalbumin, 45 000; and carbonic anhydrase, 29 000.

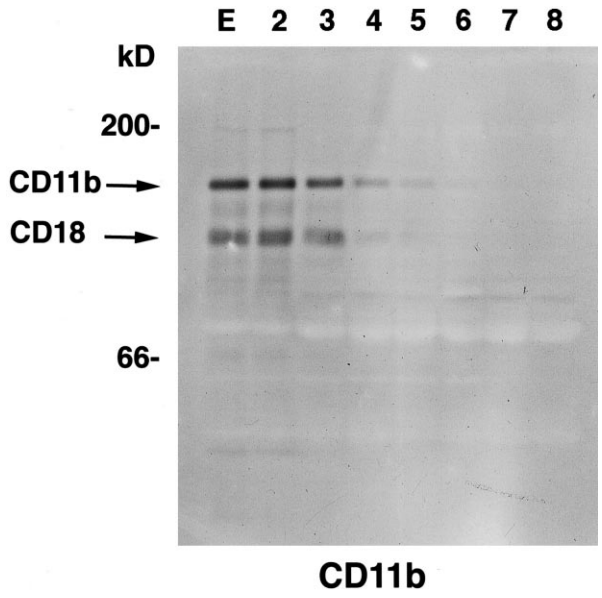

Fig. 2. Distribution of cell-surface CD11b in gel permeation chromatography fractionated neutrophil extract. Column fractions were immunoprecipitated with the CD11b mAb OKM1 and analyzed as in Fig. 1. The arrows indicates the position of CD11b and CD18.

4–6 (Fig. 2, lanes 2–6). Similarly, immunoprecipitation with the CD18 mAb 60.3, readily detected CD11b and CD18 in the cell extract (Fig. 3, lane E) and in fractions 2 and 3 (Fig. 3, lanes 2–3). However, some CD11b and CD18 were also detected in fractions 4–6 (Fig. 3, lanes 4–6). As in Fig. 1, 20% of the whole cell extract immunoprecipitate and the fraction 2 immunoprecipitate and 100% of the immunoprecipitates of fractions 3–8, were loaded on the gel in Figs. 2 and 3. NMS did not immunoprecipitate any biotin-labeled protein (Fig. 1, right panel).

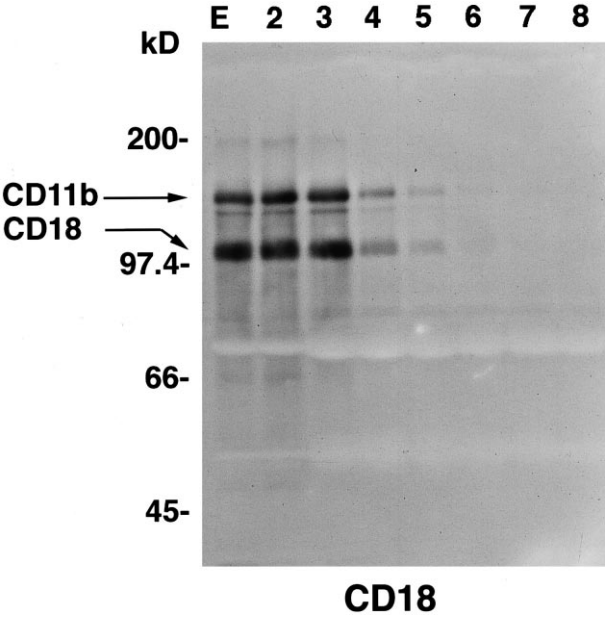

Fig. 3. Distribution of the cell-surface CD18 in gel permeation chromatography fractionated neutrophil extract. Column fractions were immunoprecipitated with the CD18 mAb 60.3 and analyzed as in Fig. 1. The arrows indicates the positions of CD11b and CD18.

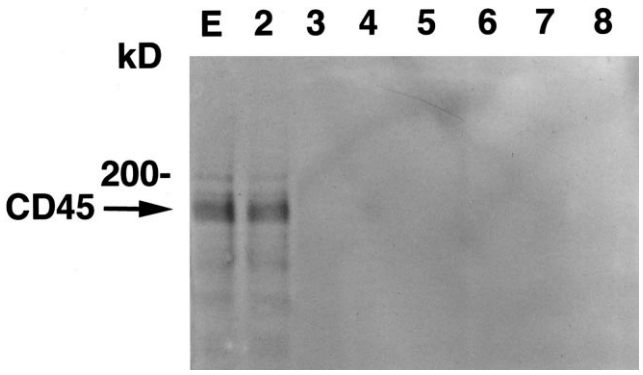

Fig. 4. Distribution of the cell-surface CD45 in gel permeation chromatography fractionated neutrophil extract. Column fractions were immunoprecipitated with the CD45 mAb AHN-12 and analyzed as in Fig. 1. 100% of the immunoprecipitates were applied to their respective lanes. The arrow indicates the position of CD45.

Since some CD45 mAbs have been reported to inhibit neutrophil chemotaxis [20], column fractions were also analyzed by immunoprecipitation with the CD45 mAb AHN-12. All detectable biotin-labeled CD45 was present in the void volume, i.e. fractions 2 and 3 (Fig. 4). No other biotinylated proteins were detectable in the CD45 immunoprecipitates.

3.2. *Gel permeation chromatography of total cell CD11b, CD18 and CD63*

Since most CD11b/CD18 is present intracellularly in secondary granules [26] we examined the distribution of total neutrophil CD18 in detergent-resistant complexes by fractionating a neutrophil extract by gel permeation chromatography on Sepharose 4B, and analyzing the fractions by SDS–PAGE and immunoblotting with the CD18 mAb 60.3 (Fig. 5). In contrast to the fractionation of cell-surface CD18 detected by biotin-labeling intact neutrophils, total cellular CD18 appeared to be evenly distributed among fractions 2–7 and could also be faintly detected in fractions 8 and 9 (Fig. 5, top panel). CD18 was not seen in parallel experiments when immunoblotting was conducted using NMS as the control antibody (Fig. 5, bottom panel).

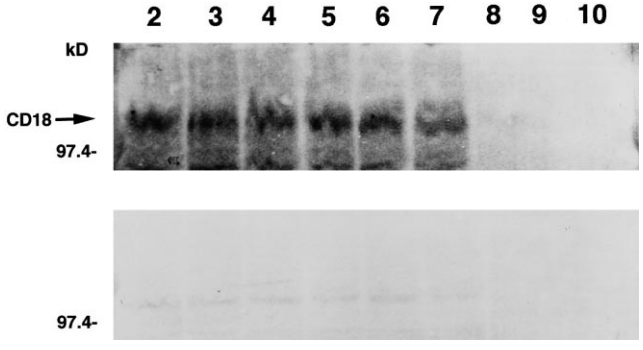

Fig. 5. Distribution of the total cell CD18 in gel permeation chromatography fractionated neutrophil extract. Neutrophils were solubilized in Brij solubilization buffer, and the extract was fractionated as in Fig. 1, separated by SDS–PAGE under reducing conditions, transferred to Immobilon-P, and CD18 was detected by immunoblotting with the CD18 mAb 60.3 (top panel). NMS was used as a control antibody for immunoblotting (bottom panel). The arrow indicates the position of CD18.

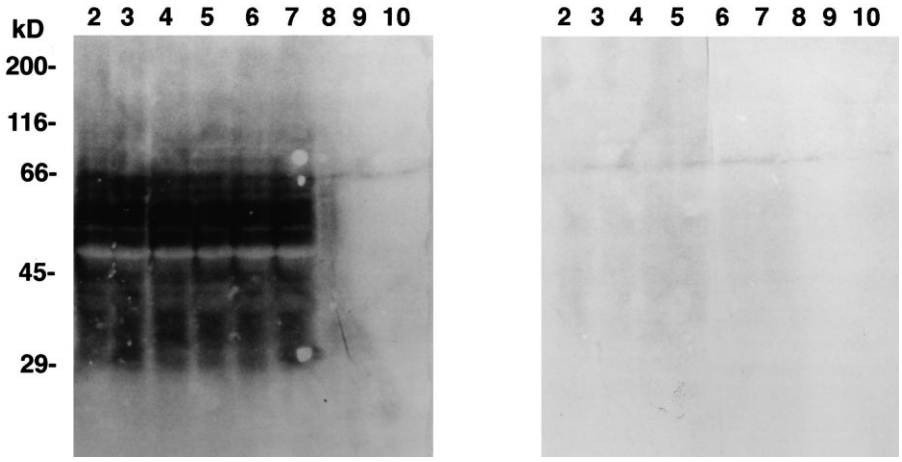

Fig. 6. Distribution of the total cell CD63 in gel permeation chromatography fractionated neutrophil extract. Neutrophils were solubilized in Brij solubilization buffer, and the extract was fractionated as in Fig. 1, and analyzed by SDS–PAGE under non-reducing conditions, transferred to Immobilon-P, and CD63 was detected by immunoblotting with the CD63 mAb AHN-16 (left panel). NMS was used as a control antibody for immunoblotting (right panel).

The distribution of total neutrophil CD63 in complexes was also examined by gel permeation chromatography and immunoblotting with the CD63 mAb AHN-16. As observed with CD18, total cellular CD63, which appears routinely as a broad band over a wide range of apparent molecular weight due to extensive and variable glycosylation, appeared to be rather evenly distributed among fractions 2–7 (Fig. 6, left panel). This band of ~30–100 kDa was not seen in parallel experiments when immunoblotting was conducted using NMS as the control antibody (Fig. 6, right panel).

4. Discussion

In this study, gel filtration of neutrophils solubilized in Brij 58 demonstrated that the cell-surface CD11b/CD18 associated with CD63 was found in large detergent-resistant complexes. In addition, the majority (but not all) of the total cell-surface CD11b/CD18 in neutrophils was also present in such complexes. In contrast, the majority of total cellular CD11b/CD18 was not restricted to these complexes. Similarly, the majority of total neutrophil CD63 was not present in such complexes. Thus, the data suggest that in neutrophils, CD63 and CD11b/CD18 are not present in large detergent-resistant complexes while intracellular, but become sequestered in membrane domains corresponding to such complexes following translocation to the cell surface.

Several studies have described the existence of very large non-covalent complexes relatively resistant to dissociation by detergent [11–19]. It has been postulated that these complexes reflect the existence of specific membrane microdomains that have a particular lipid composition and these clusters may be important in the transmembrane signaling by proteins in the complex [11–14,18]. Studies of the subcellular distribution of glycosphingolipids in neutrophils have found no major differences among the primary granules, secondary granules and plasma membranes, in the relative amounts of the five major neutrophil glycosphingolipids. However, relatively more α-2,3-sialosylneolactotetraosyl-ceramide may be present in the plasma membrane than in the primary and secondary granules [27].

Previous studies demonstrated that the binding of CD63 mAbs to the neutrophil surface results in a transient activation state during which time a signal can be transmitted to CD11b/CD18 if extracellular calcium is present, resulting in an increase in CD11b/CD18 adhesive activity and surface expression [2]. The CD11b/CD18 complex is well known to play an important role in neutrophil adhesion (reviewed in [2,28]). Thus, CD63 plays a signaling role and regulates the adhesion activity of CD11b/CD18 in human neutrophils and may play an important regulatory role in inflammation via its effects on leukocyte function. CD63 has been reported to associate with CD11b/CD18 in neutrophils [2] and α3β1 and α6β1 integrins in several cell lines [25,29,30]. However, this is the first report of the tetraspan CD63 localizing in large detergent-resistant complexes and the first demonstration of such complexes in neutrophils.

The urokinase plasminogen activator receptor in monocytes has been found to be present in a large receptor complex containing CD18 (β2 integrin) and the src kinases fyn, lyn, hck and fgr [16]. A recent report suggests that most of the cell-surface β1 integrin in ovarian carcinoma cells is present as large detergent-resistant complexes [31].

Data suggest that more than one type of large complex containing different GPI-anchored proteins exist in monocytes [16] and thymoma cells [32]. We found that most neutrophil cell-surface CD45 was also present in such complexes, but not associated with CD63 or CD11b/CD18, thus demonstrating the existence of more than one type of large detergent-resistant complex in neutrophils.

Thus, the present study demonstrates that CD11b/CD18 associated with the tetraspan CD63 on the neutrophil surface is present in large detergent-resistant complexes. This association, and entry into large detergent-resistant complexes, occurs after translocation to the cell surface, since most intracellular CD11b, CD18 and CD63 are not present in such complexes. Also, CD63 resides in a different intracellular granule population than CD11b/CD18. In addition, the majority of cell-surface CD11b and CD18, as identified by biotin labeling, was also present in large detergent-resistant complexes. The sequestration of signal transducing and effector molecules in complexes in membrane microdomains may play a role in signal transduction and effector function by

increasing the local concentration of reactive species, thus leading to potential cooperativity or other mechanisms.

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