Multimerin Is Found in the α-Granules of Resting Platelets and Is Synthesized by a Megakaryocytic Cell Line

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Abstract

In this report, we describe the intracellular localization of multimerin in platelets and its biosynthesis by Dami cells, a megakaryocytic cell line. Immunoelectron microscopy was used to examine frozen thin sections of resting and activated platelets. Multimerin was localized within the platelet α-granule in an eccentric position. Within activated platelets, multimerin was found in the surface-connected open cannalicular system and on the external plasma membrane. Light microscopic immunocytochemistry demonstrated multimerin in normal megakaryocytes and in Dami cells after stimulation with PMA. Confirmation of multimerin biosynthesis by Dami cells was obtained by metabolic labeling studies. Both platelet and Dami cell multimerin demonstrated several subunit sizes on reduced SDS-PAGE. However, peptide mapping confirmed structural homology between the different multimerin subunits. Glycosidase digestion demonstrated that multimerin is heavily glycosylated with mainly complex, N-linked carbohydrate. In contrast to the multimerin isolated from platelets, cultured Dami cells secreted mainly smaller multimers of the protein. Biosynthesis of multimerin by a megakaryocytic cell line supports endogenous biosynthesis by megakaryocytes as the origin of this platelet α-granule protein. (*J. Clin. Invest.* 1993. 91:2630–2639.) Key words: Dami cell • multimer • von Willebrand factor • bone marrow • immunocytochemistry

Introduction

Multimerin is an extremely large, soluble platelet glycoprotein that is expressed on the surface of activated platelets (1, 2). This protein exists as variably sized multimers that range from < 450 kD to many million daltons (1, 2). Multimerin and von Willebrand factor share a similar complex multimeric composition and our previous studies demonstrated that multimerin and von Willebrand factor are the two largest proteins in platelets (2). The individual multimers of platelet multimerin are comprised of p-155 and p-170 subunits, linked by disulfide bonds (2). During platelet activation, multimerin moves from the platelet interior and becomes expressed on the platelet surface (1). The largest multimers of this protein remain bound to the platelet surface after activation and the smaller multimers are released (2).

The intent of the current study was to investigate the location and origin of platelet multimerin. Soluble proteins contained within platelets can originate from two sources: endogenous biosynthesis by megakaryocytes or endocytosis from plasma (3). In general, proteins synthesized by the megakaryocyte are present at a higher concentration in platelets than in plasma, whereas plasma-derived platelet proteins are present at a greater concentration in plasma than in platelets. Fibrinogen is the only known exception (3). Because we failed to detect multimerin in plasma, we postulated that this protein might be synthesized by megakaryocytes and stored within platelet granules for release upon activation. In this report, we describe the intracellular location of multimerin in platelets. Additionally, we demonstrate the presence of multimerin in normal megakaryocytes and its biosynthesis by Dami cells (4), a malignant megakaryocytic cell line.

Methods

Antibodies. Monoclonal antibody JS-1 and polyclonal rabbit antibodies against multimerin were used for immunoprecipitation and immunohistochemistry (1). Immunocytochemistry studies were performed using polyclonal antimultimerin and both monoclonal and polyclonal antibodies to von Willebrand factor (Dako Corp., Carpinteria, CA). These polyclonal antisera to multimerin and von Willebrand factor are non-cross-reactive and recognize reduced platelet proteins of 155 and 220 kD, respectively (1, 2). Additionally, using immunoblot studies, the polyclonal anti–von Willebrand factor does not recognize purified multimerin (1) and polyclonal antibodies to multimerin do not react with purified von Willebrand factor.

Platelet preparation. Washed platelets (1×10^8/ml) were activated, without agitation, using bovine thrombin (1 U/ml; Sigma Immunochemicals, St. Louis, MO). After 10 min, recombinant hirudin (Sigma), 4 U/ml, was added. Activated platelets (1×10^9/ml) were surface radiolabeled using ^{125}I and lactoperoxidase, washed, lysed, and used for immunoprecipitation (1, 5).

Dami cell preparation. Dami cells (4) were a generous gift of Dr. Sheryl M. Greenberg, Hematology Division, Brigham and Women's Hospital, Boston, MA. The cells were obtained from the American Type Culture Collection (CRL 9792), Rockville, MD. Cells were grown in RPMI 1640 medium supplemented with 5% fetal calf serum, 10 mM sodium bicarbonate, 10 mM Hepes, and 2 mM glutamine. For some experiments, Dami cells were activated by incubating the cells with 5 nM PMA. Cytochemistry studies were performed on Dami cells that were cultured on glass coverslips.

Dami cells (5×10^5/ml) were surface labeled with ^{125}I (1, 5), washed, and solubilized in lysing buffer containing proteolytic inhibitors (1% Triton X-100, 0.1% SDS, 20 mM Tris, 100 mM NaCl, pH 7.4, with 10 mM EDTA, 0.1 μM leupeptin, 0.2 mM PMSF, 0.02 mg/liter soybean trypsin inhibitor, and 5 mM *N*-ethyl maleimide; 1×10^5 cells/ml).

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Metabolic labeling was performed using [^{35}S]methionine (NEN/Dupont Canada Inc., Mississauga, Canada) in methionine-free medium. For 18-h labeling studies, 5 ml vol of medium containing 0.1 mCi/ml of [^{35}S]methionine were used. For pulse-chase experiments, 1 ml of culture medium containing 0.5 mCi of ^{35}S methionine was used.

Cleveland mapping of the Dami cell multimerin was performed using supernatant from an 18-h labeling with Trans ^{35}S-label (0.5 mCi/5 ml; ICN Biomedicals Canada, Ltd., Montreal, Canada) and methionine-free, cysteine-free media. For some experiments, tunicamycin (1 μg/ml; Boehringer Mannheim Canada, Laval, Quebec) was added to culture medium 3 h before labeling. The labeled Dami cells were solubilized in lysing buffer to the same final volume as the culture supernatant ($\sim 1 \times 10^5$ cells/ml).

Immunocytochemistry. Resting platelets and thrombin stimulated (2 U/10^9 platelets in Tyrode's buffer containing 1 mM calcium, 5 min, 37°C.) platelets were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 for 3 h at 4°C. Cells were embedded in 2.3 M sucrose and frozen thin sections were cut from the cell block. Immunocytochemistry was performed as described (6, 7) using polyclonal rabbit antimultimerin (1:50) and goat anti-rabbit gold-10 (Amersham Corp., Arlington Heights, IL). A double-labeling experiment to localize multimerin and von Willebrand factor was performed using protein A, as described by Slot and Geuze (8). Rabbit antibody against multimerin was applied (dilution 1:10) and labeled with protein A-10. Before the addition of the second antibody, free protein A (0.05 mg/ml) was applied. Subsequently, rabbit antibodies to von Willebrand factor were added at a 1:10 dilution followed by protein A-5. A second experiment was also performed using the polyclonal multimerin antibody and goat anti-rabbit gold-5 and a monoclonal antibody against von Willebrand's factor (dilution 1:10) and goat anti-mouse gold. Controls consisted of a primary incubation with normal rabbit serum.

Immunocytochemistry was performed on normal bone marrow smears and on resting and PMA-activated Dami cells. Air-dried smears were fixed in acetone at 4°C for 15 min before blocking endogenous peroxidase activity with 0.5% hydrogen peroxide in methanol for 20 min. After further air drying, the slides were rinsed in Tris-buffered saline, pH 7.6 (TBS)[1] and incubated for 1 h in TBS with JS-1 (monoclonal antimultimerin; 1:100–1:1,000 dilution) or TBS alone (negative control). The slides were rinsed in TBS and incubated in biotinylated rabbit anti-mouse immunoglobulins (10 min) and streptavidin peroxidase conjugate (5 min; Histostain SP kit; Zymed Laboratories Inc., South San Francisco, CA). Peroxidase was demonstrated using aminoethylcarbazole in acetate buffer, pH 5.0. Nuclei were counterstained with hematoxylin.

Radioimmunoprecipitation. Immunoprecipitations of 1-ml vol of radiolabeled cell lysates or culture supernatant were performed using protein A sepharose beads (50 μl) and either monoclonal (10 μl JS-1) or polyclonal antibodies (50 μl) against multimerin (1). To reduce nonspecific binding, samples from 18-h metabolic labeling experiments were subjected to two consecutive immunoprecipitations (9), using polyclonal antimultimerin. Beads were washed five times with lysing buffer (containing 0.2% Triton X-100), eluted with 100 μl of lysing buffer containing 2% SDS, 2% Triton X-100, and 2% sodium deoxycholate, and boiled. Eluates were collected, diluted 10-fold with lysing buffer (containing 0.2% Triton X-100) and a second immunoprecipitation was performed. Immunoprecipitates were used for subunit and multimer analyses (1, 2). For pulse-chase experiments, single immunoprecipitations were performed using polyclonal antimultimerin.

Subunit analysis. Immunoprecipitates were analyzed by reduced SDS-PAGE and autoradiography or fluorography (1).

Multimer analysis. Immunoprecipitates were eluted with agarose/acrylamide sample buffer (final concentrations: 10 mM Tris, 2% SDS, 8 M urea, 0.005% bromophenol blue; pH 8.0). Samples were analyzed by nonreduced, agarose/acrylamide gel electrophoresis using 1.25% agarose gels (SeaPlaque agarose; FMC Corp., FMC BioProducts, Rockland, ME) containing 1.5% acrylamide (2). Thrombospondin was used as a reference for multimer size (2). Individual multimer subunit composition was assessed by two-dimensional, nonreduced/reduced electrophoresis as described (2).

Protease mapping. Cleveland mapping was used to compare the different multimerin subunits (10). Multimerin immunoprecipitates were prepared, reduced, and alkylated (1), followed by subunit separation using SDS-PAGE. The radiolabeled proteins were isolated from dried gels and protease digestion was performed in the stacking gel using V8 protease (0.05, 0.1, 0.5, and 5.0 μg/lane), chymotrypsin (1, 10, and 50 μg/lane) and trypsin (1, 10, and 50 μg/lane). Peptides were resolved using 7–15% SDS-PAGE.

Carbohydrate analysis. The carbohydrate content of multimerin was assessed by exo and endoglycosidase digestions (9, 11). Multimerin immunoprecipitates were eluted with 0.1% SDS, 0.2% 2-mercaptoethanol containing protease inhibitors (final concentrations: 0.1 mg/ml benzaminidine, 1 mM PMSF, 1 μg/ml aprotonin, 10 μg/ml leupeptin). The test samples were incubated with the different glycosidases, then analyzed by reduced SDS-PAGE. To exclude proteolytic degradation during the glycosidase incubations, control samples were incubated in buffer without glycosidase. Glycosidases used included: endoglycosidase H (Boehringer Mannheim; final concentrations: 50 mU/ml in 100 mM sodium phosphate buffer, pH 6.0, 18 h, 37°C), endoglycosidase F/*N*-glycosidase F-free and endoglycosidase F/*N*-glycosidase F (Boehringer Mannheim, for both glycosidases, final concentrations: 15 U/ml in 20 mM sodium phosphate buffer, pH 7.0, 18 h, 37°C), neuraminidase (Boehringer Mannheim, final concentrations: 2 U/ml in 20 mM sodium acetate buffer, pH 5.5, 1 h, 37°C) and *O*-glycanase (Genzyme Corp., Cambridge, MA; final concentrations: 30 mU/ml added to the neuraminidase treated protein, 5 h, 37°C). After incubation with the glycosidase, 100 μl of 2× reducing sample buffer was added to all tubes, followed by reduced SDS-PAGE. For some studies, both N- and O-linked carbohydrates were removed by treating the immunoprecipitates with *N*-glycosidase F followed by neuraminidase and *O*-glycanase.

Results

Intracellular localization of multimerin in platelets. Immunoelectron microscopy performed on frozen thin sections of platelets demonstrated the presence of multimerin in an eccentric position within the α-granule of resting platelets (Fig. 1, *a* and *b*). Only small amounts of multimerin were detected on the plasma membrane of resting platelets. Using double labeling experiments with antibodies (either monoclonal or polyclonal) to von Willebrand factor and multimerin and two different sizes of gold, multimerin and von Willebrand factor colocalized to the same region of the α-granule (Fig. 1 *a, inset panel,* and *b*). No labeling of α-granules was observed in the negative controls.

Studies performed on frozen thin sections of thrombin-activated platelets demonstrated that the multimerin was located on the external plasma membrane and within the surface-connected cannalicular system after platelet activation (Fig. 1 *c*). There was no significant labeling of the surface-connected cannalicular system or plasma membrane in the negative controls.

Demonstration of multimerin in megakaryocytes and in Dami cells. Normal bone marrow megakaryocytes and resting and PMA-activated Dami cells were examined using immunocytochemical techniques and the monoclonal antibody to multimerin (Fig. 2). Normal megakaryocytes and platelets stained intensely for multimerin in a granular pattern. Other bone marrow cells were not multimerin immunoreactive. While we did not detect the presence of multimerin in resting Dami cells,

1. *Abbreviation used in this paper:* TBS, Tris-buffered saline.

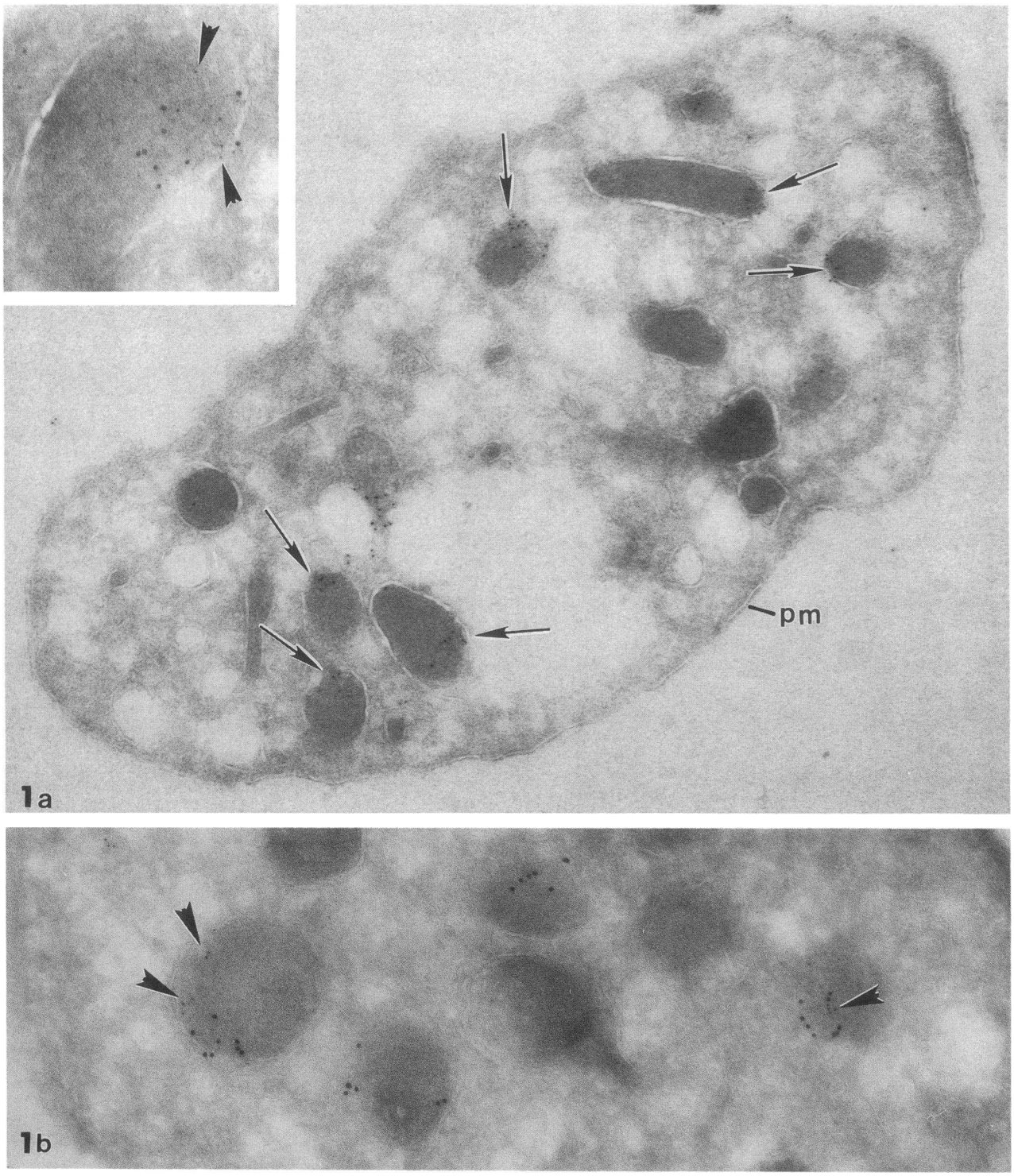

Figure 1. Immunogold labeling of multimerin in frozen thin sections of resting and activated platelets. In resting platelets, labeled using polyclonal antibodies to multimerin and goat anti-rabbit gold-10 (panel *a*, ×50,000), the gold particles were found within the alpha granule matrix, usually in an eccentric position (*large arrows*). Rare label was detected on the plasma membrane (*pm*). Double labeling studies of resting platelets (inset of an alpha granule, panel *a*, ×98,000) were performed using polyclonal antibodies to multimerin (detection with protein A gold-10) and to von Willebrand factor (detection with protein A gold-5; *small arrows*). Multimerin (the large gold) and von Willebrand factor (the small gold) colocalized to the same region of the alpha granules. In panel *b* (×60,000), von Willebrand factor was detected with a monoclonal antibody and goat anti-mouse gold-10, and multimerin was detected with a polyclonal antibody and goat anti-rabbit gold-5. The results are similar to panel *a*, and short arrows point to the granules with eccentric double-labeling.

Thrombin-stimulated platelets (panel *c*, ×42,000) exhibited shape change and aggregation. Immunocytochemistry using polyclonal antibodies to multimerin and goat anti-rabbit gold-10 demonstrated presence of gold in large vacuoles (*v*) many of which are probably sections of the surface-connected cannalicular system. Smaller amounts of the label were localized to the external plasma membrane. No label was detected in the tight "contact zones" (*CZ*).

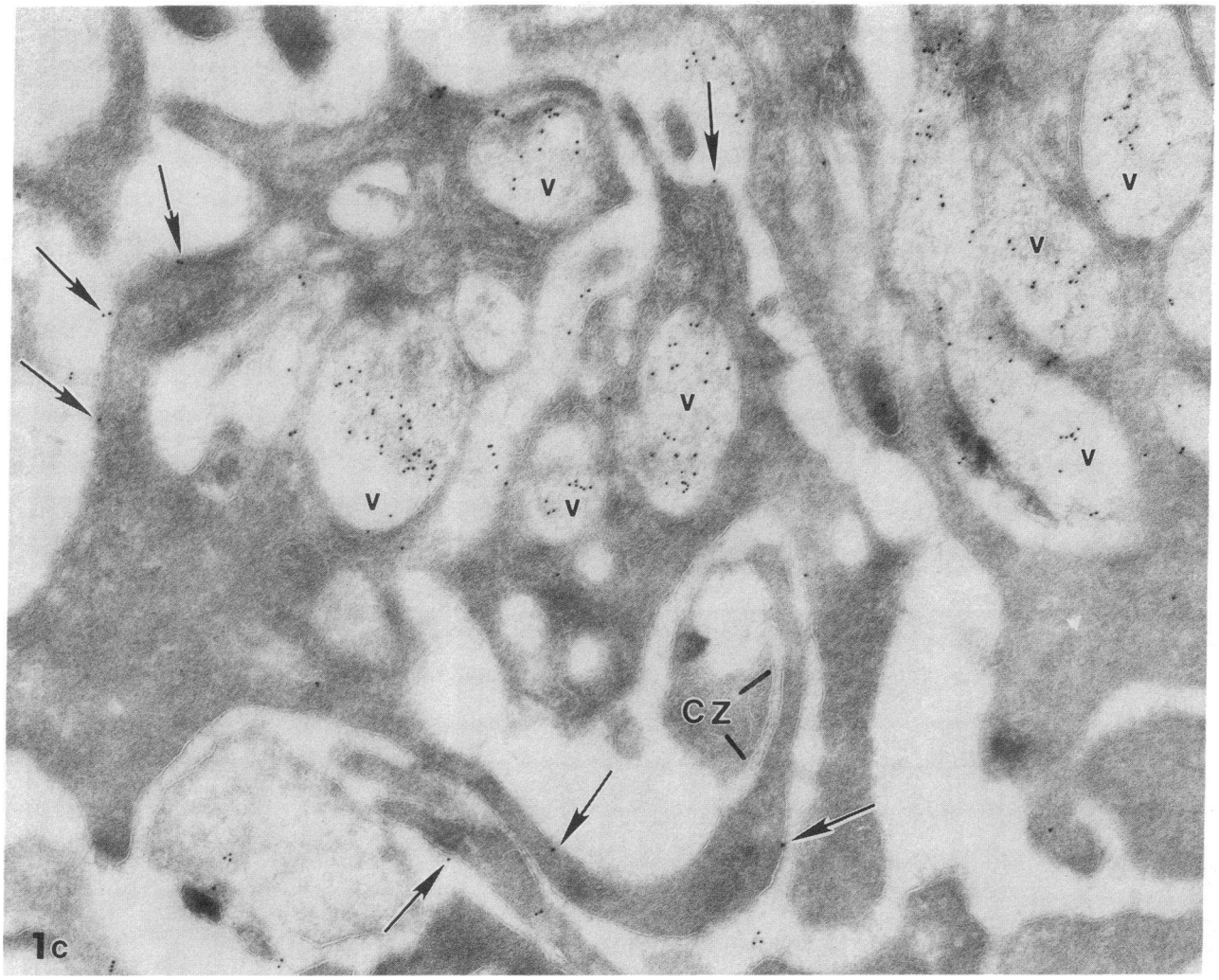

Figure 1 (Continued)

PMA-activated Dami cells showed a granular distribution of the protein. Background staining suggestive of an extracellular matrix distribution was observed for the PMA-activated Dami cells. Compared to the normal megakaryocytes, the PMA-activated Dami cells stained less intensely for multimerin. No staining was observed in the control slides processed without the monoclonal antibody.

To determine if endogenous biosynthesis was the source of multimerin in Dami cells, metabolic labeling studies were performed. Untreated and PMA-stimulated cells were metabolically labeled (18 h, [^{35}S]methionine) during the 1st, 2nd, and 3rd day of PMA stimulation. Multimerin immunoprecipitates were prepared from culture supernatants and cell lysates. Multimerin was not detected in the cell lysate or culture supernatant of resting Dami cells (Fig. 3). After stimulation with PMA, Dami cells synthesized and secreted multimerin. After 1 d of PMA stimulation, multimerin was detected both in the Dami cell lysate and culture supernatant and continued to be synthesized and secreted over the next several days of PMA stimulation. Greater quantities of multimerin were present in the culture supernatant compared to the cell lysate, indicating that the majority of the protein was secreted. Multimerin was not detected on the surface of resuspended, washed, PMA-stimulated Dami cells (Fig. 3).

When the intracellular and secreted multimerin from an 18-h metabolic labeling were compared, the proteins differed in their reduced subunit size. The 170-kD band was the predominant protein in the cell lysate (Fig. 3). The secreted protein was comprised of 196- and 165-kD subunits, with a greater proportion of the larger subunit (Fig. 3). Both monoclonal and polyclonal antibodies to multimerin immunoprecipitated the two protein subunits from the culture supernatant. Comparison of the Dami cell multimerin with the platelet multimerin subunit revealed that the protein synthesized and secreted by the Dami cells had a larger subunit size (reduced) than the mature platelet protein.

Cleveland mapping of multimerin subunits. Cleveland mapping was used to investigate if the different subunits present in the reduced multimerin immunoprecipitates originated from a common precursor protein. Protease digestion of platelet p-155 and p-170 generated identical peptides (Fig. 4). Trypsin, chymotrypsin, and V8 protease digests showed extensive, but not complete, peptide homology between the p-155 platelet multimerin subunit and the p-196 Dami cell multimerin sub-

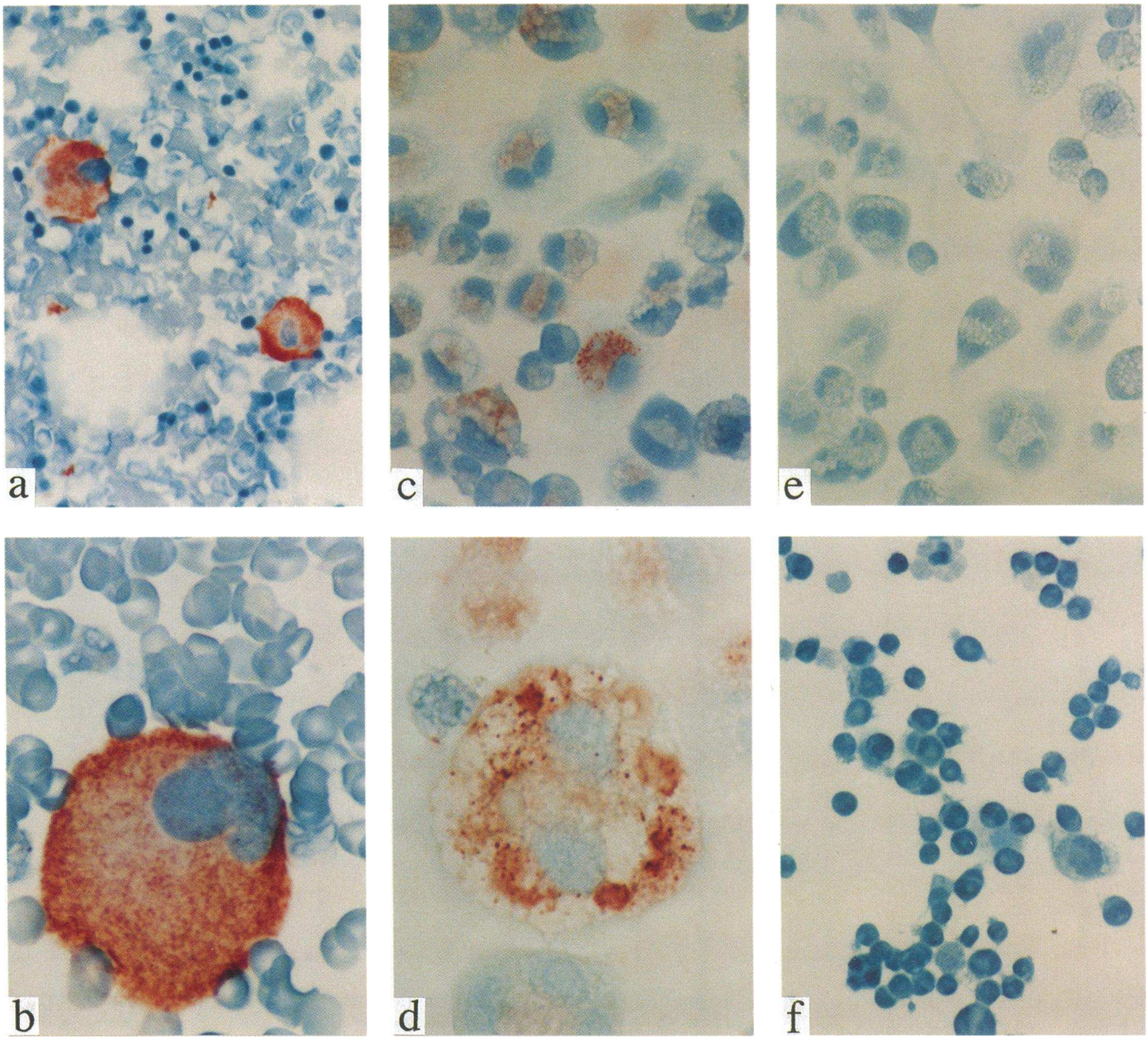

Figure 2. Immunocytochemistry demonstrating multimerin in megakaryocytes and in Dami cells. Immunocytochemistry was performed on normal bone marrow and on resting and PMA-stimulated Dami cells using JS-1 (monoclonal antimultimerin). Normal megakaryocytes exhibited intense staining for multimerin with a granular distribution (panel *a*, ×320; panel *b*, ×800). Only megakaryocytes and platelets were labeled. Resting Dami cells did not exhibit any staining with antibodies to multimerin (panel *f*, ×320); however, after PMA stimulation, multimerin was evident in Dami cells (panel *c*, ×320; panel *d*, ×800). In most cells, a granular pattern of staining was observed with some evidence of extracellular staining, suggestive of a matrix distribution. Control slides, processed without JS-1, did not show any staining (panel *e*, ×320).

unit (Fig. 4). The observed differences may be due to a longer peptide sequence in p-196, differences in glycosylation between the protein synthesized in vivo and in vitro, or the distribution of the different radiolabels on the individual peptides.

Carbohydrate composition of multimerin. Glycosidase digestions were performed to determine the carbohydrate composition of multimerin and to further investigate the differences in subunit sizes (Fig. 5). The platelet p-155 and p-170 multimerin subunits contained similar quantities of N-linked carbohydrate (Fig. 5, lane *4*) and sialic acid (Fig. 5, lane *2*). Removal of all N-linked carbohydrate by *N*-glycosidase F from p-170 and p-155 resulted in proteins with mobilities of 120 and 105 kD, respectively (Fig. 5, lane *4*). Resistance of p-155 and p-170 to endoglycosidase F and endoglycosidase H (Fig. 5, lanes *6* and *11*), indicated that p-155 and p-170 contain only the complex forms of N-linked carbohydrate. In contrast to the results obtained using *N*-glycosidase F, *O*-glycanase treatment resulted in only a minor shift in mobility of the p-155 subunit (Fig. 5, lane *21*). Together, these results demonstrate that p-155 and p-170 multimerin subunits are highly glycosylated proteins that contain mainly complex N-linked carbohydrate. The failure of the deglycosylated subunits to comigrate (Fig. 5, lane *4*) indicates that p-170 contains a larger polypeptide component than p-155.

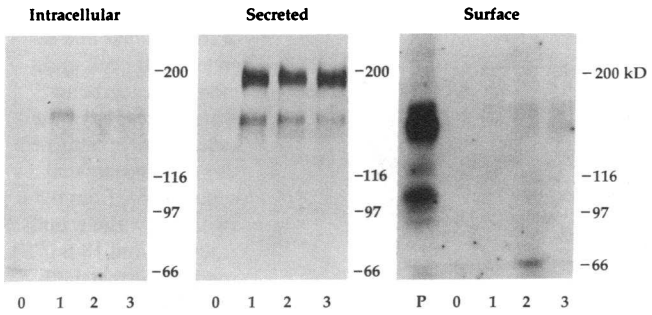

Figure 3. Dami cells synthesize multimerin after stimulation with PMA. Multimerin immunoprecipitates were prepared from ^{125}I surface radiolabeled Dami cells and [^{35}S]methionine metabolically labeled Dami cells. Cells were labeled during 0, 1, 2, or 3 d (lanes designated *0, 1, 2, 3*) of PMA stimulation. Samples were analyzed by SDS-PAGE. For comparison, the multimerin from surface radiolabeled, activated platelets is shown in lane *P*. This study demonstrates that Dami cells synthesize and secrete multimerin after stimulation with PMA. Compared to activated platelets, PMA-stimulated, resuspended Dami cells do not express detectable amounts of multimerin on their surface. Bands below 155 kD in the platelet sample may represent coprecipitated proteins or degradation products.

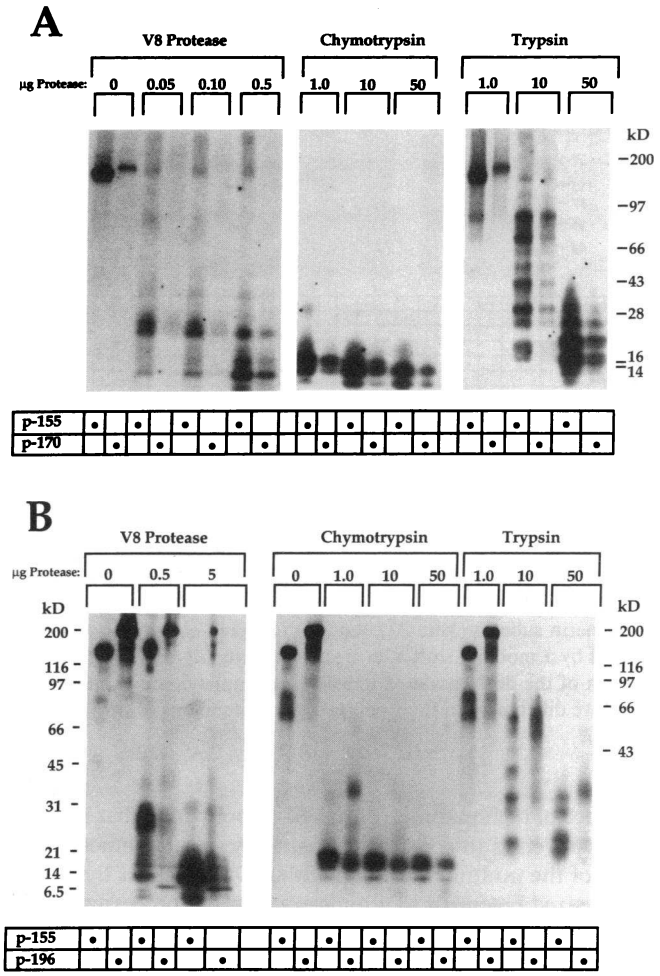

Figure 4. Peptide mapping of multimerin subunits. Multimerin immunoprecipitates prepared from ^{125}I surface labeled, activated platelets and from the culture supernatant of 18-h ^{35}S metabolically labeled, day 2 PMA-stimulated Dami cells were used to isolate the subunit proteins. Peptide mapping was performed by the method of Cleveland using V8 protease (0.05, 0.1, 0.5, and 5.0 µg/lane), chymotrypsin (1, 10, and 50 µg/lane) and trypsin (1, 10, and 50 µg/lane). (*A*) Comparison of platelet p-155 and p-170 multimerin subunits. This study demonstrates that the platelet multimerin subunits are highly related proteins with complete peptide homology. (*B*) Comparison of platelet p-155 and Dami cell p-196 multimerin subunits. This study demonstrates extensive peptide homology between the platelet p-155 and the larger Dami cell p-196.

Similar to platelet multimerin (Fig. 5, lane *15*), the secreted Dami cell multimerin contained a large component of N-linked carbohydrate, evidenced by large mobility shifts of the p-196 and p-165 subunits after treatment with endoglycosidase F/*N*-glycosidase F (Fig. 5, lane *16*). Additionally, the secreted Dami cell multimerin was resistant to endoglycosidase F (Fig. 5, lane *12*) and to endoglycosidase H (Fig. 5, lane *10*), indicating that, like platelet multimerin, all of the N-linked carbohydrate had been processed to complex forms. The Dami cell p-196 subunit migrated with an apparent reduced molecular mass of 145 kD (5% polyacrylamide gels; Fig. 5, lane *16*) after removal of N-linked carbohydrate. As observed with the deglycosylated subunits of platelet multimerin, the smaller p-165 subunit of Dami cell multimerin contained a smaller polypeptide component than the larger p-196 subunit (Fig. 5, lane *16*). Because the deglycosylated p-155 platelet multimerin subunit was smaller (Fig. 5, lane *15*) than the deglycosylated subunits of multimerin from Dami cells (Fig. 5, lane *16*), we postulate that there is more complete proteolytic processing of the protein derived from platelets.

The 196-kD Dami cell multimerin subunit contained sialic acid that was not completely removed by treatment with *N*-glycanase F (Fig. 5, lanes *16* and *18*), suggesting the presence of O-linked carbohydrate. *O*-glycanase digestion resulted in a 9-kD mobility shift in the neuraminidase-treated 196-kD subunit (Fig. 5, lanes *20* and *22*), confirming the presence of O-linked sugars on the p-196 subunit of multimerin. However, complex, N-linked carbohydrate is the predominant type.

Processing of multimerin during biosynthesis. Pulse-chase studies were undertaken to follow the processing of multimerin during its biosynthesis. Dami cells were metabolically labeled (20 min) and biosynthesis was followed in the culture supernatants and cell lysates at varying chase intervals after pulse labeling (Fig. 6). This study demonstrated that the first multimerin subunit synthesized was 170 kD. Subsequently the 196-kD protein became detectable in the cell lysate with the amount progressively increasing over the next hour. Secretion of the protein was first detected at 1 h and almost all of the synthesized multimerin was secreted within 3 h of biosynthesis. The secreted multimerin was primarily the 196-kD protein with smaller amounts of the 165-kD subunit.

We postulated that the increase in the molecular weight of multimerin subunit during biosynthesis was due to the addition of carbohydrate. Additionally, because the pulse-chase studies indicated rapid secretion, we postulated that the intracellular multimerin from 18-h labeling experiments was mainly the recently synthesized, precursor form of the protein. To address these issues, the multimerin prepared from 18-h metabolic labeled Dami cell lysate was compared to the precursor protein synthesized during a 0.5-h metabolic labeling (Fig. 7 *a*). Both lysates contained a 170-kD multimerin subunit that was susceptible to endoglycosidase H digestion, indicating the

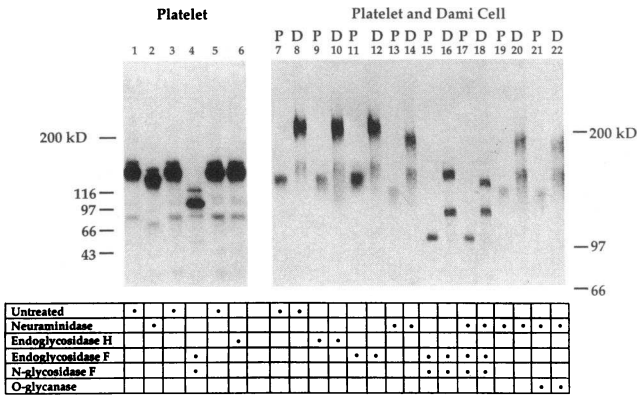

Figure 5. The carbohydrate composition of multimerin. The carbohydrate composition of platelet and Dami cell multimerin was assessed by digestion with endo and exoglycosidases. Platelet multimerin subunits were analyzed by 4–8% SDS-PAGE (lanes *1–6*) and 5% SDS-PAGE was used to compare the Dami cell (*D*) and platelet (*P*) multimerin subunits (lanes *7–22*). Both platelet (lanes *4* and *15*) and Dami cell (lane *16*) multimerin subunits are highly glycosylated with mainly complex, N-linked carbohydrate. The Dami cell p-196 multimerin subunit (lane *22*) contains O-linked carbohydrate, evidenced by a mobility shift after treatment with *O*-glycanase. Comparison of the deglycosylated individual subunits demonstrates that there are differences in their polypeptide components (lanes *4* and *15–22*).

presence of high mannose N-linked carbohydrate that had not been converted to complex forms. These results indicate that most of the multimerin found within Dami cells is the newly synthesized precursor that undergoes further processing of N-linked carbohydrate before secretion of the mature subunits.

Prevention of N-glycosylation in Dami cells with tunicamycin resulted in the biosynthesis of a smaller precursor protein that migrated at 132 kD (Fig. 7 *b*, lane *3*) in contrast to the 170-kD protein synthesized in the absence of tunicamycin (Fig. 7 *b*, lane *2*). The endoglycosidase H treatment of the early precursor protein resulted in an equivalent reduction in apparent M_r (Fig. 7 *a*), suggesting that the 170-kD multimerin precursor contained only high mannose forms of N-linked carbohydrate.

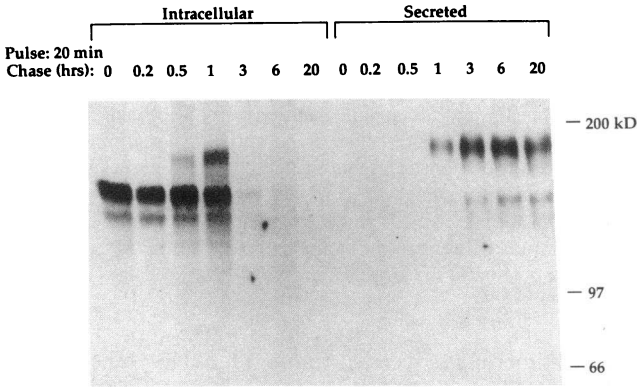

Figure 6. Pulse-chase study of multimerin biosynthesis by Dami cells. Day 2, PMA-stimulated Dami cells were pulse labeled for 20 min with [^{35}S]methionine. Multimerin immunoprecipitates were prepared from cell lysates and supernatants collected at 0, 0.2, 0.5, 1, 3, 6, and 20 h after pulse and analyzed by SDS-PAGE. This study demonstrates that multimerin is synthesized as a 170-kD subunit, followed by further processing to generate the secreted 196- and 165-kD subunits. The larger 196-kD subunit first appears in the cell lysate at 0.5 h and its appearance is rapidly followed by secretion of 196- and 165-kD subunits. Almost all of the synthesized protein was secreted by 3 h. The band at 140 kD in the cell lysate was not consistently seen in other pulse chase experiments and may represent a coprecipitated protein.

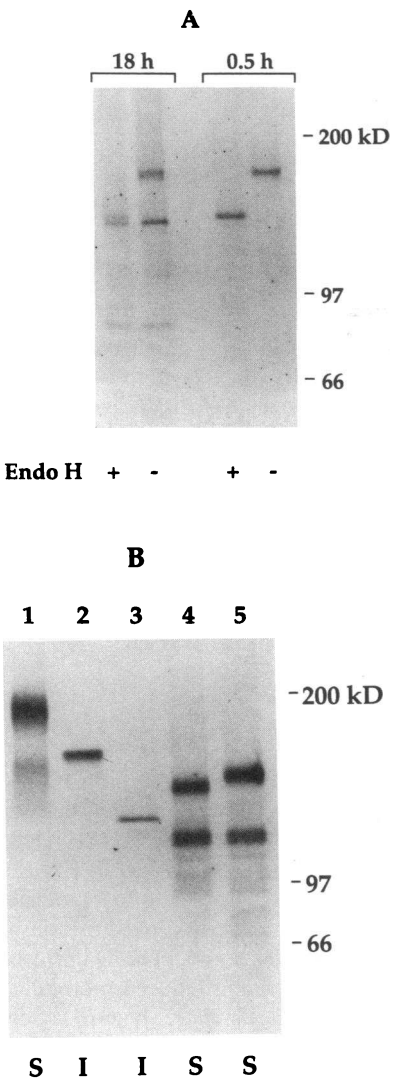

Figure 7. Comparison of intracellular and secreted Dami cell multimerin. The carbohydrate processing of multimerin was investigated using cell lysates and supernatants from 0.5 h (with and without tunicamycin) and 18-h [^{35}S]-methionine metabolically labeled, day 2 PMA-stimulated Dami cells. Immunoprecipitates were incubated with glycosidases or buffer, followed by reduced 5% SDS-PAGE. (*A*) The 170-kD intracellular multimerin subunit from an 18-h labeling study comigrated with the multimerin precursor synthesized during a 0.5-h labeling. Both proteins contained endoglycosidase H sensitive, high mannose N-linked carbohydrate. These findings indicate that the multimerin contained in the 18-h cell lysate is the recently synthesized, precursor form of multimerin. The multimerin immunoprecipitate from the 18-h lysate contained an additional endoglycosidase H resistant 130-kD protein, not seen in previous 18-h lysates (Fig. 3). This may represent a coprecipitated protein. (*B*) The carbohydrate composition of the intracellular (*I*) and secreted (*S*) multimerin subunits were compared. The mature secreted protein consists of 196 and 165 kD subunits (lane *1*, 18-h labeling) in contrast to the intracellular 170-kD subunit (lane *2*, 0.5-h labeling). Prevention of N-glycosylation with tunicamycin resulted in the synthesis of a 132-kD protein (lane *3*, 0.5-h labeling) that did not comigrate with either of the *N*-glycosidase F–treated, secreted subunits of multimerin (lane *5*, 18-h labeling). Neuraminidase treatment further reduced the M_r of the N-deglycosylated, secreted multimerin (lane *4*, 18-h labeling), indicating the presence of non–N-linked carbohydrate on the secreted 196-kD subunit.

This study demonstrates that formation of the mature, secreted protein involves conversion of high mannose N-linked carbohydrate to complex forms, the addition of O-linked carbohydrate and also indicates that proteolysis is required to generate the 165-kD subunit.

Further studies were performed to investigate O-glycosylation during the biosynthesis of multimerin. The presence of O-linked carbohydrate was suggested by the higher M_r of the N-deglycosylated, 196-kD secreted multimerin subunit (Fig. 7 *b*, lane *5*), compared with the 132-kD protein synthesized in the presence of tunicamycin (Fig. 7 *b*, lane *3*). The presence of O-linked carbohydrate was confirmed by demonstrating that neuraminidase reduced the M_r of the N-deglycosylated p-196 (Fig. 7 *b*, lane *4*; Fig. 5, lane *18*) and *O*-glycanase further reduced the M_r of p-196 (Fig. 5, lane *22*). The N-deglycosylated, secreted p-165 was smaller than the precursor protein synthesized in the presence of tunicamycin (Fig. 7 *b*, lanes *5* and *3*), indicating that production of the smaller subunit of the secreted protein requires proteolysis. The observed differences in migration of the Dami cell p-165 and platelet p-170 multimerin subunits may reflect differences in either glycosylation or proteolysis.

Because the megakaryocytic cell line synthesized a larger multimerin subunit than found in platelets, we looked for evidence that the multimerin contained within platelets had originated from a larger precursor protein. A 200-kD band was occasionally observed in reduced multimerin immunoprecipitates from surface-labeled thrombin-activated platelets. To determine if this protein was a component of multimerin, two-dimensional nonreduced/reduced electrophoresis was performed. The 200-kD protein was present within the multimerin multimers, covalently linked to the p-155 and p-170 subunits by interchain disulfide bonds (Fig. 8).

Comparison of the multimeric composition of platelet multimerin and the multimerin secreted by Dami cells. Agarose/acrylamide gels were used to investigate the nonreduced structure of multimerin synthesized by Dami cells. Multimerin immunoprecipitates were prepared from Dami cell culture supernatants (18-h metabolic labelings) and from surface radiolabeled, thrombin-activated platelets. In comparison to the multimers from platelets, the constitutively secreted multimers from Dami cells were comprised mainly of the smallest multimers of the protein (Fig. 9). Two-dimensional, nonreduced/reduced electrophoresis demonstrated that the different sized subunits of Dami cell multimerin were linked by interchain disulfide bonds.

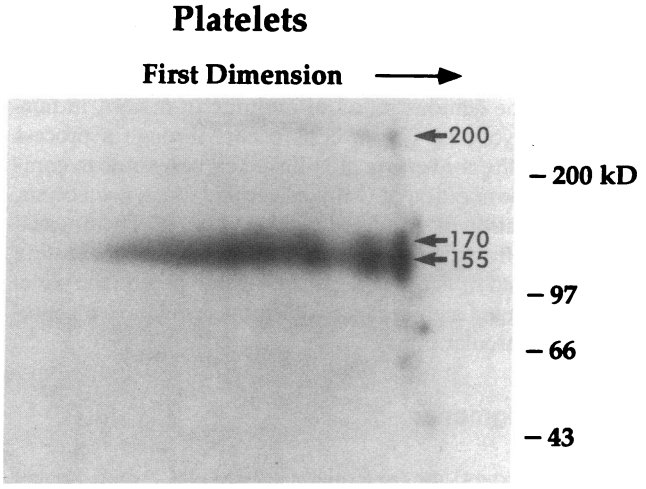

Figure 8. The composition of platelet multimerin multimers. The nature of the association of a 200-kD protein with platelet multimerin was investigated by two-dimensional nonreduced/reduced electrophoresis. Multimerin immunoprecipitates containing the 200-kD protein were prepared using ^{125}I surface radiolabeled activated platelets. Immunoprecipitates were separated by nonreduced agarose/acrylamide gel electrophoresis followed by reduced SDS-PAGE. The individual multimers of platelet multimerin contain mainly p-155 and p-170 subunits. However, the 200-kD protein also was identified as a minor component of the disulfide-linked multimers. The M_r of the 200-kD subunit was determined using 5% SDS-PAGE; anomalous migration was observed on 5–20% gradient gel shown in this figure.

Discussion

Multimerin is an unusually large, disulfide-linked, multimeric protein that exhibits variability in its native multimeric size, ranging from less than 450 kD to many million Daltons (2). In our previous studies, we demonstrated that it was a unique, soluble platelet protein and demonstrated the activation-dependent nature of multimerin expression on the platelet surface (1). Further investigation demonstrated that multimerin resembles von Willebrand factor in its complex multimeric composition and that multimerin and von Willebrand factor are the two largest proteins in platelets (2). The purpose of the studies described in this report was threefold. The first studies were designed to determine the intracellular location of multimerin in platelets. The second series of investigations focused on identifying if the protein was present in megakaryocytes and whether it could be synthesized by Dami cells, a malignant megakaryocytic cell line. The third series of studies investigated the similarities and differences between the platelet and Dami cell forms of multimerin.

Previously, we have shown that multimerin is a soluble protein contained within platelets (1). Platelet activation results in a dramatic increase in the expression of multimerin on the platelet surface (1). In the current studies, we used immunoelectron microscopy to demonstrate that multimerin was contained within the α granules of resting platelets. Because the eccentric location of multimerin within the α granule resembled that of von Willebrand factor (12), dual labeling experi-

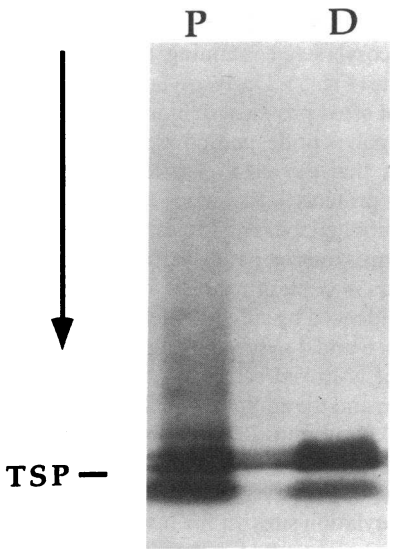

Figure 9. Comparison of the multimeric composition of platelet and Dami cell multimerin. Multimerin immunoprecipitates were prepared using ^{125}I surface radiolabeled activated platelets (*P*), and culture supernatant from 18-h [^{35}S]methionine metabolically labeled day 2 PMA-stimulated Dami cells (*D*). Immunoprecipitates were analyzed by agarose/acrylamide gel electrophoresis. The arrow indicates the direction of migration. The position of thrombospondin (*TSP*, M_r 450 kD) is shown for reference. Compared to platelet multimerin, PMA-stimulated Dami cells secrete mainly the smaller multimers of multimerin.

ments with different sized protein A–gold were used to compare the distribution of these proteins. These studies demonstrated colocalization of multimerin and von Willebrand factor to the same region of the α granule (Fig. 1). In agreement with the measurable increase in multimerin on the platelet surface after platelet activation (1), multimerin was shown to redistribute to the open cannallicular system and to the external plasma membrane after activation, as well as being secreted to the extracellular medium.

The soluble nature of multimerin suggested one of two different pathways of synthesis: either endogenous biosynthesis by megakaryocytes or endocytosis of an exogenously synthesized protein. Usually, proteins that are endocytosed by megakaryocytes are present in a greater concentration in plasma than in platelets (3). In contrast, endogenously synthesized platelet proteins typically are present in greater concentration within platelets compared to plasma (3). Our previous studies failed to identify multimerin as a constituent of normal plasma (1). As a result, we postulated that multimerin was synthesized by megakaryocytes, then stored within granules.

Confirmation of endogenous biosynthesis by megakaryocytes was obtained using Dami cells, a megakaryocytic cell line. These cells synthesize a variety of platelet glycoproteins including von Willebrand factor (4). Immunohistochemistry demonstrated the presence of multimerin in normal megakaryocytes and in Dami cells (Fig. 2). Furthermore, using metabolic labeling, we demonstrated that PMA-activated Dami cells were able to synthesize multimerin (Fig. 3). The protein synthesized by the Dami cells was confirmed to be multimerin in two ways. First, two different antibodies against platelet multimerin (polyclonal and monoclonal) each immunoprecipitated the same protein. Second, Cleveland mapping demonstrated extensive peptide homology between platelet multimerin and the protein synthesized by Dami cells (Fig. 4). The Dami cell protein further resembled platelet multimerin in its soluble nature and disulfide-linked multimeric structure.

We have shown that platelet multimerin is primarily comprised of a 155-kD subunit with a smaller amount of a 170-kD subunit. Using protease digestion, peptide homology was demonstrated between these two proteins (Fig. 4). Glycosidase digestion of the p-155 and p-170 proteins demonstrated that both proteins were heavily glycosylated, containing mainly complex, N-linked carbohydrate (Fig. 5). Deglycosylation of p-155 and p-170 yielded different sized proteins, demonstrating that p-170 contains a larger polypeptide component. Together, these observations suggest that the p-155 protein is derived from the p-170 protein by proteolytic cleavage.

Next, pulse-chase metabolic labeling studies were used to follow the biosynthesis of multimerin. Multimerin was synthesized as a 170-kD precursor containing high mannose, N-linked carbohydrate, as evidenced by susceptibility to endoglycosidase H digestion (Figs. 6 and 7). Subsequent modification included the processing of N-linked carbohydrate from high mannose to complex forms and the addition of O-linked carbohydrate to generate a 196-kD subunit. Removal of all N-linked carbohydrate from the mature, 196-kD subunit produced a 51-kD shift in apparent molecular mass, which would indicate approximately 17 N-glycosylation sites on the multimerin precursor protein (11). Proteolysis resulted in the production of a smaller, 165-kD, secreted multimerin subunit.

In contrast to platelet multimerin, the protein synthesized and secreted by Dami cells differed in its subunit sizes. The rapid secretion of multimerin synthesized in vitro by the PMA-activated Dami cells may account for less complete proteolytic processing compared to platelet multimerin synthesized in vivo. Similar incomplete proteolysis of von Willebrand factor secreted by cultured endothelial cells has been observed (13). Additionally, the malignant Dami cells may differ from normal megakaryocytes in their expression of the glycosyltransferases involved in N-glycosylation. This may account for the small differences in M_r observed between the Dami cell p-165 and the platelet p-170 multimerin subunits.

In contrast to platelet multimerin, which ranges in size up to many million Daltons (2), the multimerin synthesized and secreted by Dami cells contained less of the high molecular mass multimers (Fig. 9). The von Willebrand factor constitutively secreted by cultured endothelial cells also has been demonstrated to contain a predominance of the smaller multimers (13).

Our studies suggest that the platelet multimerin subunits originate from a larger precursor protein. We have occasionally observed that reduced platelet multimerin immunoprecipitates contain small quantities of a 200-kD protein that resembles the Dami cell p-196 (2). Using two-dimensional, nonreduced/reduced electrophoresis, we demonstrated that the 200-kD protein was a component of the disulfide-linked multimers of platelet multimerin (Fig. 8). Collectively, the evidence suggests that multimerin is synthesized both in vivo and in vitro as a larger precursor protein and that the mature, platelet multimerin undergoes proteolysis during biosynthesis and storage.

These studies indicate that multimerin is located within the α-granules of resting platelets and redistributes to the plasma membrane and surface-connected cannalicular system following activation. Multimerin is a heavily glycosylated protein that exists as variably sized, disulfide-linked multimers of related subunits. The synthesis of multimerin by the megakaryocytic cell line supports endogenous biosynthesis by megakaryocytes. Further confirmation of a megakaryocytic origin in vivo will require the demonstration of multimerin mRNA in normal megakaryocytes. Biosynthesis occurs through a process that involves the conversion of N-linked carbohydrate to complex forms, the addition of O-linked carbohydrate, proteolysis, and the formation of disulfide-linked multimers. The unusual repeating multimeric structure of multimerin, its localization to platelets, and its activation-induced expression on the platelet surface suggest a physiologic role in the cellular events associated with vascular injury.

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