New Synthesis of a Platelet-specific Protein:
Platelet Factor 4 Synthesis in a Megakaryocyte-enriched
Rabbit Bone Marrow Culture System

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ABSTRACT The site of synthesis of platelet-specific proteins remains to be established. With
the use of short-term megakaryocyte-enriched cultures, direct evidence was obtained to show
that megakaryocytes synthesize the platelet-specific protein, platelet factor 4. A megakaryocyte-
enriched fraction of rabbit bone marrow for culture was obtained by centrifugal elutriation
and cultured with [3H]leucine. Newly synthesized [3H]-platelet factor 4 was sought by copuri-
fication with added carrier rabbit platelet factor 4, using heparin agarose affinity chromatog-
raphy and immunoprecipitation with specific goat anti-rabbit platelet factor 4 antisera. SDS
PAGE of the washed immunoprecipitates demonstrated a [3H]-leucine-containing peak which
migrated identically with purified homogeneous rabbit platelet factor 4. A second, slightly
larger molecular-weight protein was identified in the gels also, suggesting that rabbit platelet
factor 4 may be synthesized as a larger molecular-weight precursor in rabbit megakaryocytes.
These results provide direct evidence that the platelet-specific protein, platelet factor 4, is
synthesized in rabbit megakaryocytes before it is packaged into α-granules for release in
circulating platelets.

Several proteins found in platelet α-granules are unique to
platelets and presumably unique to megakaryocytes, the bone
marrow precursor cell of platelets. The best known of these
proteins are platelet factor 4, β-thromboglobulin, and the
platelet-derived growth factor (1–9). During normal blood
coagulation, the platelet undergoes the “release reaction”, se-
creting α-granule constituents to the extracellular milieu.
While the precise role of the released proteins is unknown,
these platelet release proteins have been shown to have the
capacity to profoundly influence inflammation, wound healing,
DNA synthesis, and cell growth. They may be important also
in the development of atherosclerosis in humans (10–17).
Platelet factor 4 and β-thromboglobulin are the most fully
characterized of the secreted proteins. The complete amino
acid sequence of each protein has been published (18–21). No
information is available on the synthesis of these proteins and
little is known on how they are packaged into α-granules.
Platelets synthesize little if any protein; it is assumed that
megakaryocytes synthesize platelet-specific proteins. Alterna-
tively, these proteins may be synthesized elsewhere and actively
taken up by platelets for storage and subsequent release. Sup-
port for megakaryocytic origin of platelet factor 4 was recently
provided by the demonstration in human bone marrow prep-
arations that only megakaryocytes (7) and potential megakary-
ocyte progenitors (8) showed positive immunofluorescence
after incubation with purified fluorescein-labeled rabbit anti-
human platelet factor 4; these results do not preclude the
uptake of platelet factor 4 by megakaryocytes after its synthesis
elsewhere.

The present experiments use short-term rabbit marrow cul-
tures enriched for megakaryocytes by centrifugal elutriation
(22, 23) to provide direct evidence that platelet factor 4 is
iodinated by the chloramine T technique (28) as described in detail previously (27).

Anti-rabbit platelet factor 4 serum was obtained from goats after immunization with purified rabbit platelet factor 4. The antisera is monospecific for platelet factor 4, on the basis of a single precipitin line of sera Against Rabbit Platelet Factor 4:

Harvesting of Megakaryocytes: Megakaryocytes were harvested from bone marrow from New Zealand white rabbits by methods adapted from those described previously (21, 22). Briefly, 8–10 rabbits were anesthetized with Nembutal (Abbott Laboratories, North Chicago, IL) and were sacrificed by cardiac puncture. The femora, tibiae, and humeri were removed aseptically, cleaned of adherent tissue, and kept in cold Hank's balanced salt solution (HBSS; Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), containing 50 U/ml penicillin and 50 U/ml streptomycin. After the bones were opened with a rongeur, the marrow was scooped free with a spatula and the bone marrow tissues were immersed in 10 ml of cold phosphate-buffered saline (PBS) (pH 6.7), supplemented with 1.5% bovine serum albumin (BSA), containing 50 U/ml penicillin and 50 U/ml streptomycin, in a 9-cm plastic petri dish (Costar Packaging, Cambridge, MA). The tissues were cut with small scissors into 2-mm pieces and transferred to a 50-ml plastic tube (Falcon Labware, Oxford, CA). Marrow suspensions were vigorously pipetted 18–20 times with wide-bore pasteur pipettes to break the cell clumps.

The marrow cells were filtered through a stainless steel sieve (100 mesh) and poured into 50-ml plastic tubes. The bone marrow suspensions were centrifuged at 150 g for 5 min at 4°C, and the cells washed twice with PBS supplemented with 1.5% BSA (pH 6.7). The marrow cells were suspended and introduced into the Beckman elutriator (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 1 x 10^7 cells/ml. The elutriator buffer was PBS supplemented with 1.5% BSA (pH 6.7) and precooled to 4°C. The cell elutriator was washed with 70% ethanol to prevent cell loss before starting the separation and maintained at 4°C. Megakaryocytes were harvested with a buffer flow of 30 ml/min and a centrifuge speed of either 2.793 rpm (channel fractions >10 mm/h) or 1.975 rpm (>20 mm/h). The megakaryocytes from the elutriator chamber were recovered by resuspending the pellet with a 5-ml syringe fitted with an 18-gauge needle. Cells from other fractions of the elutriator run were also collected and used in control cultures. The harvested megakaryocytes were washed two times with calcium- and magnesium-free Hank's Buffered Salts Solution (HBSS) containing 1.5% BSA (pH 7.2) by centrifugation at 200 g for 5 min at 4°C. The total nucleated cell count was electronically determined with a Coulter Counter Model B. Megakaryocytes in cell suspensions were stained with hematoxylin and counted microscopically as described previously (7). 1.2-3.0 x 10^7/ml cells were recovered from 4 ml of calcium- and magnesium-free HBSS; megakaryocytes comprised 1–4% of the isolated total cell population. Control cultures contained 0.1% or fewer megakaryocytes. Fig. 1 shows a low power micrograph of the harvested megakaryocytes stained with hematoxylin.

Morphological Observations of Megakaryocytes: The isolated megakaryocytes were cultured in HBSS containing 2.3% BSA, pH 7.4, in siliconized glass tubes (19 x 140 mm) covered with caps. Cultured cells were incubated under 5% CO_2 at 37°C for 3 h with 2.5 x 10^5 cells in 2.0–2.5 ml of medium. Solutions and culture media were sterilized by passage through 0.22-µm filters (Millipore Corp., Bedford, MA). The cell culture systems were incubated for up to 48 h at 37°C. The morphological appearance of the cell systems was largely unchanged. Trypan blue exclusion was used to assess the bone marrow cell viability. As judged by morphological appearance, up to 50% of the megakaryocytic cells excluded Trypan blue. The percentage of cells excluding Trypan blue did not decrease over 48 h of culture, which supported previous results on liquid cultures of guinea pig megakaryocytes (24). The morphological appearance of the cells remained constant, suggesting that megakaryocytes maintained their viability in culture.

Synthesis of Platelet Factor 4 in the Culture of Megakaryocytes: The cultured megakaryocytes were incubated at 37°C with HBSS containing 2.3% BSA, pH 7.4, and 5 µCi/ml or 10 µCi/ml [3H]leucine (New England Nuclear, Boston, MA). After an appropriate labeling period, the reaction was stopped by addition of one-tenth volume of 10 mM leucine in HBSS and by cooling to 0°C. The radiolabeled megakaryocytes were washed three times by centrifuging at 200 g for 5 min at 4°C with cold PBS containing 1.5% BSA (pH 6.7). The megakaryocytes were resuspended in 0.15 M NaCl, 10 mM phosphate (pH 7.5), containing 0.5% Triton X-100 and 100 U/ml Trasylol (FBA Pharmaceuticals, New York) and disrupted for 10 s with sonication. The final volume of labeled megakaryocyte-enriched cell suspensions was adjusted to 2-6 x 10^7 cells/ml. After 15-min extraction at 0°C, the extract was centrifuged at 10,000 g for 30 min at 4°C, and the small pellet of Triton-X-100-insoluble material was discarded.

Purification of Newly Synthesized Heparin Neutralizing Protein with Heparin Sepharose Column: The Triton X-100 treated soluble supernatant was diluted 1:10 with 0.15 M NaCl, 10 mM phosphate (pH 7.4). The diluted cell extract was applied to a 1 x 5 cm column of heparin-Sepharose equilibrated with 0.15 M NaCl, 10 mM Tris, HCl pH 8.6. The column was washed with 200 ml of 0.5 M NaCl, 10 mM Tris HCl (pH 8.7), and eluted in a linear gradient between 10 ml of 0.5 M NaCl, 10 mM Tris HCl (pH 8.7), and 10 ml of 3 M NaCl, 10 mM Tris HCl (pH 8.7). The eluates were collected in 1-ml aliquots in plastic tubes containing 2 µg/ml heparin. Conductivity was measured with a conductivity meter. The radioactivity in the eluates was counted with Dimilume-30 (Packard Instrument Co., Inc. Dowsers Grove, IL) in a Searle Mark III liquid scintillation spectrometer (Amer sham Corp., Arlington Heights, IL).
anti-goat γ-globulin (Cappel Laboratories Inc., Cochranville, PA) was then added to reaction mixtures and the reaction mixture was incubated for 20 h at 4°C. Immunoprecipitates were recovered by centrifugation at 8,000 g for 5 min in a Beckman microfuge (Beckman Instruments, Inc.) and were washed three times with ice-cold 0.15 M NaCl, 0.5% Triton X-100, 10 mM phosphate (pH 7.4), and once with 0.15 M NaCl, 10 mM phosphate (pH 7.4). The washed precipitates were dissolved with 0.2 ml of NCS (New England Nuclear, Boston, MA), and the radioactivity was counted with neutralizer (Research Products International Corp., Mt. Prospect, IL) in a liquid scintillation spectrometer. A correction for nonspecific precipitation was made by subtracting the radioactivity in precipitates derived from the normal serum control. The radioactivity in nonspecific precipitates did not exceed 10% of the total precipitate radioactivity in any analysis.

Analysis of Labeled Immunoprecipitates on SDS Gel: The washed immunoprecipitate was subjected to electrophoresis in a 10% SDS polyacrylamide gel according to the modification of Weber and Osborn (30). The washed pellet was resuspended in 20 μl of 0.15 M NaCl, 10 mM phosphate and dissolved in 20 μl of 20 mM Tris, 2 mM EDTA, 2% SDS, 15% sucrose, 80 μg/ml Pyramin and 80 mM dithiothreitol. It was then boiled for 3 min and applied to the tops of gels (0.5 x 7 cm). The gels contained 10% acrylamide and were equilibrated with a gel buffer composed of 1% SDS and 100 mM Tris, pH 7.4. Protein bands were stained with Coomassie Blue, and 2-mm slices of gels were cut for analysis. Slices were transferred to scintillation vials and solubilized as described by Ames (31). Radioactivity of the sliced gels were measured in a liquid scintillation spectrometer.

RESULTS
Morphological Observations on Harvested Megakaryocytes

Preliminary experiments were done to seek α-granule-like particles in cultured megakaryocytes. Electron micrographs show that the megakaryocytes harvested after 48 h of culture had abundant cytoplasmic granules (Fig. 2A). Higher magnifications showed greater detail of these granules (Fig. 2B). The granules observed appear in electron micrographs to be identical to granules found in platelets; isolation and biochemical characterization of the megakaryocytic granules are required to definitively establish the precise relationship of megakaryocyte and platelet granules.

Immunofluorescence Studies

Experiments were done to detect platelet factor 4 antigen in the megakaryocyte preparations. When the megakaryocyte-enriched suspensions and cultured megakaryocytes were incubated with goat anti-rabbit platelet factor 4 antisera and fluorescein-conjugated rabbit anti-goat γ-globulin, prominent immunofluorescent staining was observed in all megakaryocytes, similar to that reported previously using identical staining techniques on human bone marrow smears (7). No megakaryocytes were stained when normal goat serum was substituted for goat anti-platelet factor 4 serum. The positive staining of megakaryocytes was sharply reduced by prior adsorption of goat anti-platelet factor 4 serum with purified platelet factor 4. The platelet factor 4 antigen in megakaryocytes appears roughly equal in fluorescent staining to that in platelets.

Incorporation of [3H]leucine into Platelet Factor 4 Antigen

Platelet factor 4 antigen in disrupted megakaryocytes was precipitated by double antibody immunoprecipitation as de-
were assumed to be nonspecific contaminants, on the basis of
in different preparations. These minor peaks (~10% of total)
of this peak has not been definitively established. A very small
radioactivity was applied, ~14,000 Mwt) was found consistently in gels of
immunoprecipitates from megakaryocyte cultures. The identity
radioactivity into this peak by 77%. Boiled megakaryocytes
and megakaryocyte poor elutriator fractions had <10% of the
radioactive protein peak illustrated in Fig. 4.

Elution Pattern of Newly Synthesized Product
on a Heparin Affinity Column

Megakaryocytes (6 x 10^5/ml) were cultured as noted above
with [3H]leucine (10 MCl/ml) for 2 h. The megakaryocytes were
then washed three times with cold PBS containing 1.5% BSA
at 4°C, and disrupted by sonication. This extract (6 x 10^6
megakaryocytes/ml, 2 ml) was applied to 1.5-m1 heparin affinity
column as described in Materials and Methods. A linear
salt gradient was used to elute labeled protein. Fig. 4 shows a
single peak of radioactivity which appeared at a molarity of
1.0-1.5 M NaCl. Recovery of [3H]leucine in this fraction was
0.6%. Cycloheximide (100 Mg/ml) inhibited incorporation of
radioactivity into this peak by 77%. Boiled megakaryocytes
and megakaryocyte poor elutriator fractions had <10% of the
radioactive protein peak illustrated in Fig. 4.

SDS Gel Electrophoretic Profile of an
Immunoprecipitate of Newly Synthesized
Antigen by Cultured Megakaryocyte

Platelet factor 4 containing fractions from heparin agarose
chromatography were immunoprecipitated as noted above.
[3H]leucine recovered in the washed immunoprecipitate of
material recovered from the heparin agarose column averaged
~10-15%. The radioactive immunoprecipitable platelet factor
4 antigen (120-min incubation) was dissolved in buffer (see
Materials and Methods) and boiled for 3 min at 100°C and
analyzed by SDS polyacrylamide gels as described also in
Materials and Methods. This analysis consistently showed two
peaks of radioactivity (Fig. 5). The most rapidly migrating
peak (~44% of 3H applied, ~10,000 MOL wt) co-migrated
precisely with purified rabbit platelet factor 4 shown in the gel
before slices were prepared for measurement of [3H]labeled
protein (Fig. 5). This material co-eluted with carrier platelet
factor 4 from heparin agarose and coprecipitated with carrier
platelet factor 4 with specific antiplatelet factor 4 antisera.

A slightly less rapidly migrating protein peak (~46% of 3H
applied, ~14,000 MOL wt) was found consistently in gels of
immunoprecipitates from megakaryocyte cultures. The identity
of this peak has not been definitively established. A very small
peak of radioactivity (~28-30 min, Fig. 5) were variably found
in different preparations. These minor peaks (~10% of total)
to be nonspecific contaminants, on the basis of

DISCUSSION

While it is generally assumed that platelet-specific proteins are
synthesized in megakaryocytes for packaging and subsequent
release in circulating platelets, the direct demonstration of the
synthesis of platelet-specific proteins has not been reported.
These studies were designed to determine whether megakary-
cocytes synthesize the platelet-specific protein, platelet factor 4.
The purification of a newly synthesized protein which copuri-
fied with homogeneous rabbit platelet factor 4 using heparin
affinity chromatography and which coprecipitated with ho-
mogeneous rabbit platelet factor 4 using specific goat anti-
rabbit platelet factor 4 provides strong direct evidence that
megakaryocytes have synthesized platelet factor 4 and that
megakaryocytes are the site of synthesis of the platelet-specific

![Figure 3](image-url)  
**Figure 3** Demonstration of the incorporation of [3H]leucine into platelet factor 4 antigen. Linear incorporation is found for 90 min and, in other experi-
ments, for up to 120 min.

![Figure 4](image-url)  
**Figure 4** The elution pattern of newly synthesized protein from the megakaryocyte-enriched culture system from a heparin-agarose column. The column was washed extensively with 500 mM NaCl
and eluted with a linear 0.5-1.5 M NaCl gradient. A large peak of radiolabeled material elutes in the range of 1.3 M NaCl and co-elutes with carrier rabbit platelet factor 4. The elution pattern of newly synthesized protein was the same as that of the 125I-labeled purified rabbit platelet factor 4.

![Figure 5](image-url)  
**Figure 5** SDS gel electrophoresis of the material eluting from the heparin agarose column which has been subsequently precipitated by a double antibody technique, using goat anti-rabbit platelet factor 4 and rabbit anti-goat IgG. DPM in slices of the gel is compared with a photograph of the gel containing carrier purified rabbit platelet factor 4 stained with Coomassie Blue before slicing of the gel for measurement of 3H-labeled protein. A peak of radioac-
tivity is found in the SDS gel which co-migrates with purified
platelet factor 4. The migration of marker proteins is shown for
reference. Bovine serum albumin (68,000), carbonic anhydrase (30,000), lysozyme (14,300), and PF4.
proteins. Non-megakaryocyte-enriched rabbit bone marrow cultures did not synthesize this protein, providing additional evidence that megakaryocytes alone synthesize platelet factor 4. This view is strengthened by our previous finding that fluorescein-labeled specific antplatelet factor 4 antisera reacted with megakaryocytes but not with other cells in rabbit bone marrow (7).

Criteria used to establish that the newly synthesized, purified protein is platelet factor 4 are: the protein co-elutes from heparin agarose at the same ionic strength as purified platelet factor 4; the protein is precipitated by a specific goat anti-rabbit platelet factor 4 antisera; and the newly synthesized protein migrates in polyacrylamide gels identically with rabbit platelet factor 4 purified to apparent homogeneity. Cycloheximide, an inhibitor of protein synthesis, blocks the synthesis of this platelet factor 4-like protein when added to the megakaryocyte-enriched culture systems.

A slower migrating (SDS gel electrophoresis) [3H]leucine-labeled protein copurifies with platelet factor 4. This protein binds to heparin agarose, is eluted from heparin agarose with carrier platelet factor 4, and is precipitated together with platelet factor 4 by specific anti-rabbit platelet factor 4 antisera, and seems likely to be the precursor of platelet factor 4 as synthesized in the megakaryocyte. Such a precursor would be important to identify and would provide important information on the mechanism of synthesis of platelet a-granule proteins. We have not established that the larger-molecular-weight peak is the precursor of the smaller-molecular-weight peak. Data from preliminary pulse-chase experiments over an extended incubation period did not support a precursor/product relationship between these two peaks of radioactivity. It is possible that the antisera to rabbit platelet factor 4 reacts with a second, newly synthesized protein in cell extracts, such as the PSPz protein, or charged proteins.

A slower migrating (SDS gel electrophoresis) [3H]leucine-labeled protein purified from rabbit platelets by Muggli et al. (32). Further studies are required to elucidate the identity of this higher-molecular-weight protein precipitated by our anti-PF4 antisera. Testing of the anti-rabbit platelet factor 4 antisera by immunodiffusion did not demonstrate cross-reacting protein species.

As a result of the abnormally low frequency of megakaryocytes in marrow (<0.1% of nucleated cells), biochemical studies of megakaryocytes have been possible only following isolation and purification on the basis of cell size (22, 23, 33–36), density (37), or both (1, 9, 38–41). Megakaryocytes isolated by these procedures have been shown to contain factor VIII antigen (1), fibrinogen (39), 5-hydroxytryptamine (40), and a platelet-derived growth factor in cell extracts, such as the PSPz protein, or charged proteins.

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