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The Macrophage $\alpha_M\beta_2$ Integrin α_M Lectin Domain Mediates the Phagocytosis of Chilled Platelets*

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$\alpha_M\beta_2$ integrin receptors on myeloid cells mediate the adhesion or uptake of diverse ligands. Ligand binding occurs in the α_M chain, which is composed of an I domain and a lectin domain. The α_M I domain binds iC3b, fibrinogen, intercellular adhesion molecule-1, and other ligands and mediates the adhesion of neutrophils to platelet glycoprotein Ibα (GPIbα). $\alpha_M\beta_2$ also recognizes β-GlcNAc residues on GPIbα that are clustered on platelets after cooling. The phagocytosis of chilled platelets could be reconstituted when Chinese hamster ovary cells were transfected with $\alpha_M\beta_2$. Replacement of the I domain or the lectin domain of the α_M chain with the corresponding domain from the α_X chain (p150) revealed that the activity of the $\alpha_M\beta_2$ integrin toward chilled platelets resides within the lectin domain and does not require the I domain. Additional evidences for this conclusion are: 1) Sf9 cells expressing solely the α_M lectin domain bound chilled platelets, and 2) soluble recombinant α_M lectin domain inhibited the phagocytosis of chilled platelets by $\alpha_M\beta_2$-expressing THP-1 cells, whereas I domain substrates showed no inhibitory effect. Therefore chilled platelets are removed from blood by an interaction between β-GlcNAc residues on clustered GPIbα and the lectin domain of α_M chain of the $\alpha_M\beta_2$ integrin, distinguishing this interaction from those mediated by the α_M I domain.

The demand for platelet transfusions continues to increase due to the intensive treatment of hematologic and oncologic diseases. The estimated total number of platelet units collected in 2003 in the United States was over 5.9 million, and the fact that refrigerated platelets do not circulate after transfusion, therefore requiring storage at room temperature, complicates this potentially life-saving procedure. Platelets stored at room temperature (RT)[1] lose hemostatic function. Furthermore the risk of bacterial infections transmitted through RT platelet concentrate transfusion is 50 times higher than through transfusion of refrigerated red blood cell products. Thus, regulatory agencies limit platelet storage to 5 days, a limitation that severely compromises platelet inventories and creates chronic shortages (1, 2).

We recently defined the clearance mechanism of refrigerated platelets. Cooled platelets irreversibly reorganize von Willebrand factor receptors (the (GPIb$\alpha\beta$IX)$_2$V complex) into clusters on the platelet surface. The hepatic macrophage integrin receptors, $\alpha_M\beta_2$ (complement receptor type 3, Mac-1, or CD11b/CD18), recognize clustered GPIbα, leading to ingestion of cooled platelets (3). Previously we provided indirect evidence that $\alpha_M\beta_2$ recognizes GPIbα on cooled platelets through exposed β-GlcNAc residues on *N*-linked oligosaccharides and proposed that this interaction is mediated by the α_M lectin domain (4). Other investigators have also shown that the C terminus of platelet GPIbα, via its flanking and leucine-rich repeat region, binds to the neutrophil α_M I domain of $\alpha_M\beta_2$ (5).

$\alpha_M\beta_2$ was initially described as an opsonic receptor that binds to the iC3b fragment and induces the phagocytosis of pathogens (6–8). The α_M subunit consists of three subdomains: an I domain, a divalent cation binding domain, and a lectin domain. Ligands such as iC3b, intercellular adhesion molecule-1, or fibrinogen bind in a cation-dependent manner within the α_M I domain (9–12). The cation-independent lectin domain, located C-terminally to the I domain and proximal to the membrane (13), is unique because of its broad sugar specificity. Typically C3-opsonized microorganisms display iC3b in combination with their cell wall polysaccharides, stimulating phagocytosis and/or cytotoxic degranulation resulting in the elimination of the microorganism (14). $\alpha_M\beta_2$ is also capable of phagocytosis of non-opsonized pathogens that is mediated by the binding of the pathogen cell wall to the α_M lectin (sugar binding) domain (15).

The fact that specific sugar residues on GPIbα on chilled platelets elicit phagocytic recognition by $\alpha_M\beta_2$ receptors impli-

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[1] The abbreviations used are: RT, room temperature; $\alpha_M\beta_2$, Mac-1, CR3, CD11b/CD18; iC3b, opsonic fragment of C3 that binds to $\alpha_M\beta_2$ and $\alpha_X\beta_2$; GPIbα, glycoprotein Ibα; β-D-GlcNAc-1-Me, methylated β-*N*-acetylglucosamine; CHO, Chinese hamster ovary; Sf9 cells, *Spodoptera frugiperda* cells; THP-1 cells, human monocytic cell line; $\alpha_X\beta_2$, p150,95, CR4, CD11c/CD18; CMFDA, 5-chloromethylfluorescein diacetate; CM-Green, CellTracker Green CMFDA fluorescent dye; CMTMR, (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine); CM-Orange, CellTracker Orange CMTMR fluorescent dye; GST, glutathione *S*-transferase; FLNa, filamin A; MOC, mocarhagin; BSA, bovine serum albumin; HBSS, Hanks' balanced salt solution; s-lectin, soluble α_M lectin domain; sWGA, succinyl wheat germ agglutinin; FITC, fluorescein isothiocyanate; Ab, antibody; mAb, monoclonal Ab.

cates the α_M lectin domain in this recognition. However, platelet GPIbα also mediates adhesion to neutrophil $\alpha_M\beta_2$ through the α_M I domain (5). In this report, we investigate the components of the α_M subunit required for the recognition and ingestion of chilled platelets. Chilled platelets not opsonized by serum (3) are ingested by the $\alpha_M\beta_2$ integrin. We show here that chilled platelet phagocytosis by differentiated THP-1 cells could be recapitulated in Chinese Hamster Ovary (CHO) cells expressing $\alpha_M\beta_2$ on their surface (16). CHO cells stably expressing the α_M lectin domain in which segments of the $\alpha_M\beta_2$ α-subunit (α_M) were replaced with homologous segments of $\alpha_X\beta_2$ (p150,95, CR4, CD11c/CD18) α-subunit (α_X) recognized and ingested chilled platelets. Moreover Sf9 cells expressing the α_M subunit adhered to chilled platelets 3–4 times more avidly than to room temperature platelets. Furthermore a soluble, functional α_M lectin domain significantly inhibited chilled platelet phagocytosis by differentiated THP-1 cells. In contrast α_M I domain, fibrinogen, a small I domain peptide (M2) (17), or an anti-α_M I domain antibody did not significantly diminish chilled platelet ingestion. Our data demonstrate that the α_M lectin domain mediates the $\alpha_M\beta_2$-dependent phagocytosis of chilled platelets by myeloid cells.

EXPERIMENTAL PROCEDURES

Materials—We obtained FITC-conjugated anti-human CD61 mAb and LPM19c mouse anti-human α_M mAb from Accurate Chemical & Scientific Corp. (Westbury, NY). Phycoerythrin-conjugated anti-human α_M mAb (clone 44), phycoerythrin-conjugated anti-human β_2 mAb, FITC-conjugated mouse IgG$_1$, κ were from Pharmingen. Phycoerythrin-conjugated mouse anti-human α_X Ab (clone 3.9) was from Chemicon International (Temecula, CA). Mouse anti-His Ab was from Amersham Biosciences. Peroxidase-conjugated goat anti-mouse Ab was from Jackson Immunoresearch Laboratories (West Grove, PA). Anti-human GPIbα mAb (SZ2) was from Immunotech (Marseille, France), FITC-anti-His Ab was from Novus Biologicals, (Littleton, CO). FITC-anti-human α_M mAb (clone 44), prostaglandin E$_1$, phorbol 12-myristate 13-acetate, methylated β-GlcNAc (β-D-GlcNAc-1-Me; synonymous with methyl 2-acetamido-2-deoxy-β-D-glucopyranoside) were from Sigma. FITC-conjugated zymosan A bioparticles, CellTracker™ Green CM-FDA (CM-Green), and CellTracker Orange CMTMR (CM-Orange) were from Molecular Probes, Inc. (Eugene, OR). Takara LA *Taq* polymerase was from Takara Biomedicals (Otsu, Shiga, Japan). Transforming growth factor-β1 was from Oncogene Research Products (Cambridge, MA). 1,25-(OH)$_2$ vitamin D$_3$ was from Calbiochem. pCDM1 vector containing α_M cDNA (18) subcloned into pCDM8 expression vector and α_M C-terminal binding mouse-anti human mAb CBRM1/25 were provided by Dr. T. A. Springer (The Center for Blood Research, Harvard Medical School, Boston, MA). CHO cells expressing β_2 and α_M/α_X chimeras (16) were provided by Dr. G. D. Ross (James Graham Brown Cancer Center, University of Louisville, Louisville, KY). The pgext-2T vector encoding the human α_M I domain as a soluble GST-α_M fusion construct (19) was provided by Dr. M. A. Arnaout (Renal Unit, Mass General Hospital, Harvard Medical School, Boston, MA). The peptide M2 (Pro^{201}–Lys^{217} of the α_M I domain), scM2 (17), and mAb LPM19c specific for the α_M I domain were obtained from Dr. D. I. Simon (Cardiovascular Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA). Recombinant baculovirus to express soluble GalNAc-T2 in Sf9 cells (20) was obtained from Dr. H. H. Wandall (School of Dentistry, University of Copenhagen, Copenhagen, Denmark), and recombinant baculovirus to express filamin A (FLNa) in Sf9 cells (21) was a gift from Dr. F. Nakamura (Hematology Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA). Mocarhagin (MOC) and mouse anti-human GPIbα mAb (WM23) were provided by Dr. M. Berndt (Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria, Australia).

Platelet Preparation—Venous blood was obtained from healthy human volunteers by venipuncture into 0.1 volume of Aster Jandl citrate-based anticoagulant (85 mM sodium citrate, 69 mM citric acid, 111 mM glucose, pH 4.6). Approval was obtained from the institutional review boards of both Brigham and Women's Hospital and Harvard Medical School, and informed consent was approved according to the Declaration of Helsinki. Platelet-rich plasma was prepared by centrifugation at $100 \times g$ for 20 min, and platelets were separated from platelet-rich plasma by gel filtration through a Sepharose 2B column (Amersham Biosciences) (22) or by a metrizamide gradient (23). Platelets prepared by gel filtration were allowed to rest at 37 °C for 30 min in 145 mM NaCl, 10 mM HEPES, 3.5 mM NaH_2PO_4, 5 mM KCl, 2 mM $MgCl_2$, 3 mg/ml bovine serum albumin (BSA), and 10 mM glucose, pH 7.4. Gel-filtered human platelets were used in all experiments except for immunoprecipitation experiments.

For the *in vitro* phagocytic assays, isolated platelets were stained with 1.8 μM CM-Orange (fluorescent dye), washed by centrifugation, and resuspended in 10 mM HEPES, 140 mM NaCl, 3 mM KCl, 0.5 mM $MgCl_2 \cdot 6H_2O$, 10 mM glucose, and 0.5 mM $NaHCO_3$, pH 7.4, as described previously (3). For the platelet binding assay to Sf9 cells expressing α_M surface proteins, isolated platelets were stained with 2.5 μM CM-Green for 10 min at 37 °C. The platelets were washed and resuspended as described for CM-Orange labeling above. Cleavage of GPIbα with MOC was performed as described previously (3).

Platelet Temperature Protocol—To study the effects of chilling on platelet function, human platelets were incubated at RT (~22 °C) or at ice bath temperatures for 2 h and then rewarmed for 15 min at 37 °C before *in vitro* analysis.

Cell Lines and Culture Conditions—Monocytic THP-1 cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Differentiation of THP-1 cells, which is accompanied by increased expression of $\alpha_M\beta_2$, was induced by treatment of the cells with 1 ng/ml transforming growth factor-β and 50 nM 1,25-(OH)$_2$ vitamin D$_3$ for 24 h (24). $\alpha_M\beta_2$ expression was analyzed by flow cytometry (FACSCalibur flow cytometer, BD Biosciences) using a phycoerythrin-conjugated anti-human α_M antibody. 10,000 events were acquired and analyzed using CELLQuest software. CHO cells expressing β_2 and α_M/α_X chimeras were maintained in minimum essential α medium with L-glutamine and deoxyribonucleotides (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 μM methotrexate (Sigma). Sf9 insect cells (Invitrogen) were cultured at 27 °C in Sf900 II SFM (Invitrogen) supplemented with 50 units/ml penicillin and 50 μg/ml streptomycin. Sf9 cells were allowed to adhere during the expression of recombinant protein constructs or otherwise maintained in suspension under constant stirring at 135 rpm.

In Vitro Platelet Phagocytic Assay Using Stimulated THP-1 Cells—Differentiated THP-1 phagocytic cells (1×10^6 cells/ml) were activated by the addition of 150 pg/ml phorbol 12-myristate 13-acetate for 15 min at 37 °C and plated onto human albumin (1 mg/ml)-coated 24-well plates (1×10^6 cells/well) and allowed to adhere for 45 min at 37 °C in RPMI 1640 medium (3). The cells were washed and maintained in Hanks' balanced salt solution (HBSS) (Cellgro, Mediatech) containing Ca^{2+}/Mg^{2+} or HBSS without Ca^{2+}/Mg^{2+} containing 2 mM EGTA and 2 mM EDTA to determine the effects of divalent cations on platelet phagocytosis. To determine the effects of the various α_M-specific inhibitors, the adherent phagocytes were incubated with 20 μg/ml anti-human α_M I domain mAb LPM19c or 250 μM fibrinogen for 30 min under gentle agitation. CM-Orange-labeled RT or chilled platelets (5×10^8 cells/ml) were incubated for 30 min at 37 °C under gentle agitation with 0.1–10 μg/ml soluble I domain, 0.1–10 μg/ml soluble lectin domain, 80 μM I domain-mimicking peptide M2 or scrambled scM2 peptide. Supernatants from Sf9 cells expressing intracellular FLNa (21) or secreted GalNAc-T2 (20) were used as controls for Sf9 medium containing the soluble α_M lectin domain. CM-Orange-stained chilled/rewarmed or RT platelets (5×10^8 cells/ml) were added to each well containing 10^6 differentiated THP-1 cells and allowed to incubate for 30 min at 37 °C under gentle agitation. Surface-associated platelets were removed through digestion with 0.05% trypsin-EDTA (Invitrogen) followed by the addition of trypsin inhibitors for 5 min. THP-1 cells were detached from the wells and incubated with FITC-anti-CD61 mAb, which recognizes the platelet-specific β_3 integrin. Platelet ingestion was determined and quantified by flow cytometry on a FACSCalibur flow cytometer (BD Biosciences) (3). Data were acquired in $\log_{10}$ fluorescence. The percentage of phagocytes positive for CM-Orange fluorescence when incubated with RT platelets was set to one to calculate the ratio of the phagocytic increase for the chilled platelet population.

In Vitro Platelet Phagocytic Assay Using CHO Cells Expressing $\alpha_M\beta_2$, $\alpha_X\beta_2$, or $\alpha_M/\alpha_X\beta_2$ Chimeras—The phagocytic assay for platelets by differentiated THP-1 cells was adapted for CHO cells stably expressing α-chain chimeras of α_M and α_X subunit (16). The following constructs were used. 1) α_M I domain was replaced by the homologous α_X domain in the α_M chain and designated as α_M lectin domain (originally designated MeXbM (16). 2) The α_M lectin domain and the N-terminal domain were replaced by the homologous α_X domains and designated as α_M I

domain (originally designated XeMbX (16)). 24-well plates (Falcon™, BD Biosciences) were coated with 1% human serum albumin (Sigma). 5×10^5 CHO cells were plated per well and were allowed to adhere at 37 °C for 24 h. The CHO cells were stimulated with 150 pg/ml phorbol 12-myristate 13-acetate for 15 min at 37 °C, and the cell culture medium was washed off and replaced with an equal volume of HBSS containing Ca^{2+}/Mg^{2+} or 1 mM β-D-GlcNAc-1-Me. Ingestion of cold platelets by CHO cells was determined (3). Briefly chilled/rewarmed or RT platelets were loaded with CM-Orange and added to the CHO cells. Phagocytosis was quantified by measuring the incorporation of CM-Orange fluorescence (CM-Orange-labeled platelets) into CHO cells by flow cytometry. Bound (*versus* ingested) platelets were identified by labeling the cells after the phagocytic period with FITC-labeled anti-integrin-β_3 (CD61) Abs specific for platelets, *i.e.* bound platelets are FITC-positive, ingested platelets are negative. 10,000 events were acquired in each sample and analyzed using CellQuest software. Data were acquired in $\log_{10}$ fluorescence. Fluorescently labeled anti-α_M, anti-α_X, or anti β_2-integrin mAbs were used to confirm that all chimeric molecules were expressed on the surface of CHO cells and that the expression levels of the α and β chains were equivalent. The percentage of CHO cells expressing $\alpha_M\beta_2$, $\alpha_X\beta_2$, or $\alpha_M/\alpha_X\beta_2$ chimeras positive for CM-Orange fluorescence when incubated with RT platelets was set to one to calculate the ratio of the phagocytic increase for the chilled platelet population by the chimera-expressing CHO cells.

Immunoblot Analysis and Immunoprecipitation—For immunoblotting, platelets or proteins were denatured by the addition of one-fourth volume of 4× SDS-PAGE loading buffer containing 20% β-mercaptoethanol (25). All samples were boiled for 5 min. For immunoprecipitation experiments, isolated platelets were lysed by the addition of 0.5 volume of 3× lysis buffer (3% Nonidet P-40, 150 mM Tris/HCl, pH 7.4, 450 mM NaCl, 3 mM EGTA, 3 mM phenylmethylsulfonyl fluoride, 3 mM Na_3VO_4, 30 μg/ml leupeptin, and 30 μg/ml aprotinin) (26). Insoluble material was removed by centrifugation at 1400 × g for 10 min, and the soluble fraction was immunoprecipitated with 50 μg/ml GST-I domain bound to glutathione-Sepharose 4B beads (Amersham Biosciences). Soluble lectin was incubated with CBRM1/25 and protein G-Sepharose 4 fast flow beads (Amersham Biosciences). As control, the antibody was incubated with beads without soluble lectin. Platelet lysates were incubated with the soluble lectin-bead conjugate. Immune complexes were collected and solubilized in SDS-PAGE buffer containing β-mercaptoethanol. Proteins were separated by SDS-PAGE on 8% or 4–20% polyacrylamide gels (Cambrex, Rockland, ME) and stained with Coomassie Blue or transferred onto Immobilon-P membrane (Millipore). Membranes were blocked with 1% BSA in 100 mM NaCl, 20 mM Tris/HCl, pH 7.4, and probed with 5 μg/ml SZ2 mouse anti-human GPIbα mAb (GPIbα bound to GST-I domain on beads), mouse anti-human GPIbα mAb WM23 (GPIbα bound to soluble lectin on beads) with LPM19c/α_M (purified I domain), and with anti-His (His fusion proteins expressed in Sf9 cells) followed by a peroxidase-conjugated goat anti-mouse Ab. Detection was performed with an enhanced chemiluminescence system (Pierce).

Microscopy—12-mm round coverslips (Fisher Scientific) were coated with 1% BSA (Sigma) in 24-well plates onto which 8×10^4 CHO cells expressing the various α_M/α_X and β_2 constructs were plated. The cells were allowed to adhere for 2 h, stimulated with phorbol 12-myristate 13-acetate for 15 min at 37 °C, and washed briefly using HBSS containing Ca^{2+}/Mg^{2+}. 10^7 RT-maintained or chilled and rewarmed CM-Orange-labeled platelets were added to the CHO cells into HBSS containing Ca^{2+}/Mg^{2+} and incubated with gentle shaking for 1 h at 37 °C. The CHO cells were washed once, fixed in 4% formaldehyde, HBSS (EM Science, Gibbstown, NJ) for 20 min at RT, and then washed three times for 5 min at RT. Ingestion of cold platelets by CHO cells was determined. Ingested cells are visualized by CM-Orange fluorescence. Adherent platelets were identified by labeling the cells after the phagocytic period with FITC-labeled anti-integrin-β_3 (CD61). The cells were washed three times for 5 min, and the glass coverslips were mounted on microscope slides (Fisher Scientific) with Aqua Poly/Mount (Polysciences, Inc., Warrington, PA). All washes were carried out at RT using HBSS containing Mg^{2+}/Ca^{2+}. Differential interference contrast objective images were obtained on a Zeiss Axiovert 200 microscope equipped with a 100× objective using an ORCA-II (charge-coupled device) camera and captured using Metamorph software (Universal Imaging Corp., Downingtown, PA). When both green and red fluorescences were overlaid, the images were imported to Adobe Photoshop.

Expression of α_M Recombinant Cell Surface His-α_M (Ala^{136}–Ser^{1098}) and His-α_M Lectin Domain (Val^{400}–Ser^{1098}) in a Baculovirus Expression System—Primers IDF1 (5′-GGG AAG CTT GCC TTC TTG ATT GAT GGC TC-3′, sense), TMF1 (5′-GGG AAG CTT GTG CAA AGC CTG GTT CTG-3′, sense), and TMR1 (5′-GGG AAG CTT TCA AGA GCT GCC CAC GAT GAG-3′, antisense) (27) were used to amplify the 3000-bp His-α_M and 2200-bp His-α_M lectin domain constructs using human α_M cDNA from the pCDM1 vector (18) as a template. The PCR protocol was as follows: 1) 94 °C for 1 min; 2) 98 °C for 20 min; 3) 60 °C for 3 min; and 4) 68 °C for 8 min. Steps 2 to 4 were repeated for 32 times, and end with 72 °C for 8 min. The fragments were inserted into the pCR 2.1-TOPO vector (Invitrogen) and subcloned into the NotI (New England Biolabs) and BamHI sites of the pFastBacHTa vector (Invitrogen). Correct α_M fragment insertion was verified using restriction enzymes and sequencing. DH10Bac competent cells (Invitrogen) were used to transform the recombinant pFastBacHTa DNA into a Bacmid with the (BAC-TO-BAC baculovirus system, Invitrogen). Sf9 cells were transfected with Bacmid DNA, and harvested virus was amplified. Sf9 cells were infected with a multiplicity of infection of 0.1–0.5 of virus and cultured for 3–5 days. The two membrane-bound (His_6) tagged proteins encoding amino acid residues His-α_M (Ala^{136}–Ser^{1098}) and His-α_M lectin domain (Val^{400}–Ser^{1098}) contained 20 vector-derived amino acids at the N terminus. Surface protein expression was verified by flow cytometry using anti-His FITC-conjugated or anti-α_M (clone 44) FITC-conjugated Abs. Sf9 cells were washed and resuspended in HBSS containing Ca^{2+}/Mg^{2+} and 1% BSA. 5×10^6 cells were incubated with 5 μg/ml anti-His-FITC, the anti-α_M-FITC mAb, or the appropriate FITC-conjugated IgG control at 4 °C for 1 h and then analyzed by flow cytometry. The functionality of the surface-expressed α_M lectin domain was determined by binding FITC-conjugated zymosan A bioparticles. Washed Sf9 cells were resuspended in HBSS containing Ca^{2+}/Mg^{2+}, 3% BSA and incubated with 0.1 mg/ml FITC-conjugated zymosan A bioparticles for 30 min at 4 °C. Following incubation, the cells were washed three times with HBSS at RT and analyzed by flow cytometry. The fluorescence acquisition and analysis of fluorescently labeled Sf9 cells were performed in $\log_{10}$ fluorescence. The number of dead *versus* alive virus-transfected Sf9 cells was determined prior to each functional assay using trypan blue staining. FLNa-transfected Sf9 cells (21) were used as control cells in all functional assays described.

Expression of α_M Soluble Recombinant Lectin Domain (Val^{400}–Asn^{1089}) in Sf9 Cells—An expression construct designed to encode amino acid residues of the α_M lectin domain (designated as s-lectin domain) was amplified using the primer pair TMF1 (sense) and SECR (antisense) (5′-AGC AAG CTT TTA GTT GGG GAC CTC GAA CGG-3′) and the pCDM1 vector containing α_M cDNA as a template and then cloned into the pCR2.1-TOPO vector as described above. The fragment was cut out and inserted directionally between the BamHI and NotI restriction sites of the pAcGP67A vector (Pharmingen). This vector contains a signal peptide that mediates forced secretion of the recombinant protein into the Sf9 cell culture medium. Correct insertion was verified by restriction enzymes. The plasmid pAcGP67A-s-lectin was co-transfected with Baculo-Gold™ DNA (Pharmingen), and recombinant baculovirus was obtained after three successive amplifications into Sf9 cells. The α_M soluble lectin domain (Val^{400}–Asn^{1089}), contains 18 extra amino acids at the N terminus.

Expression of Soluble GST-I Domain in Escherichia coli—Pgext-2T vector, encoding the human α_M I domain as a GST-α_M fusion construct, was used to express and purify GST-I domain as described previously (19). Briefly the protein was expressed in DH5-α *E. coli*, and the obtained GST-I domain was purified using glutathione-Sepharose 4B beads (Amersham Biosciences). GST-I domain beads were equilibrated using 50 mM Tris/HCl, pH 8, and the GST-I domain was eluted using 50 mM Tris/HCl, 10 mM glutathione, pH 8. In some experiments the GST-I domain bound to beads was first treated with 0.5 μg/ml thrombin (Sigma) (19) to remove the GST tag. The thrombin was inactivated by the addition of benzamidine-Sepharose 4 fast flow beads (Amersham Biosciences) for 30 min at RT. The molecular size of the eluted protein was determined by SDS-PAGE after Coomassie Blue staining and by immunoblotting using the anti-I domain-specific mAb LPM19c. The protein concentration was determined using the BCA protein assay reagent kit (Pierce) or densitometry of the Coomassie-stained SDS-polyacrylamide gel using BSA as a standard.

Platelet Binding Assay to Sf9 Cells Expressing Recombinant α_M Surface Proteins—Sf9 cells were harvested 4–5 days postinfection by centrifugation at 1000 rpm for 5 min and washed with cold HBSS Ca^{2+}/Mg^{2+}. Dead virus-infected Sf9 cells may nonspecifically take up fluorescently labeled platelets. To access the number of dead cells *versus* alive virus-infected cells, samples of cells were stained with trypan blue. Numbers of dead cells/live cells were comparable between virus-infected control cells expressing intracellular FLNa (21) (negative control) and cells infected with recombinant virus to express α_M surface proteins. Surface protein expression was apparent after day 3, was

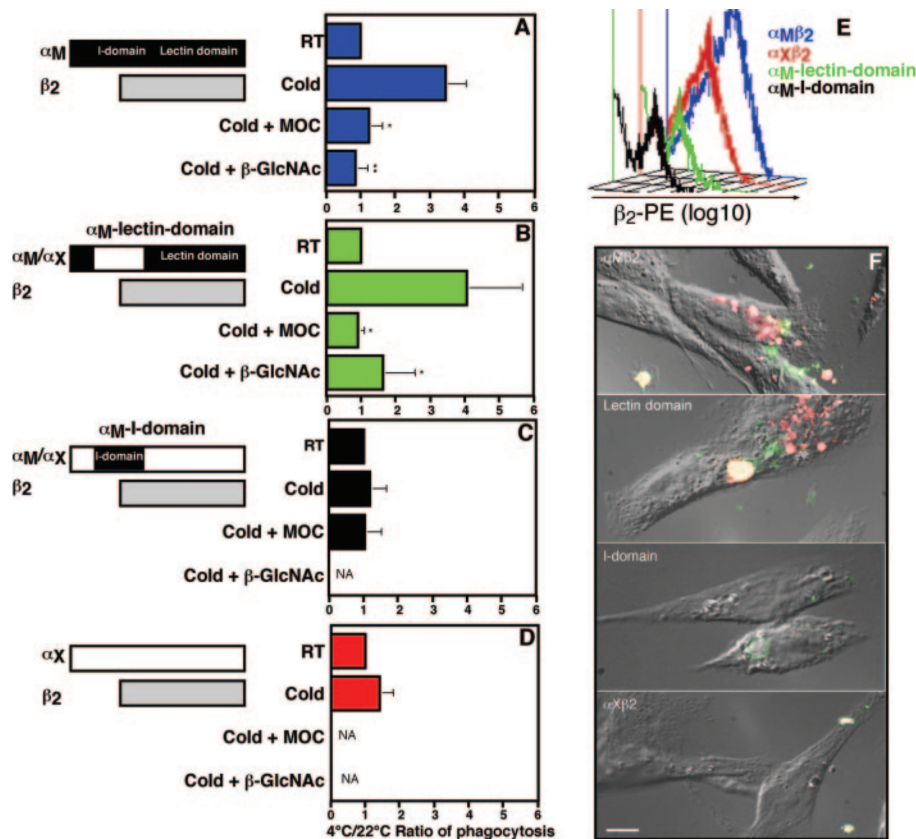

FIG. 1. **CHO cells expressing $\alpha_M\beta_2$ and specifically the α_M lectin domain phagocytize chilled platelets.** *A* and *B*, chilled platelets, but not RT platelets, are phagocytized by CHO cells expressing $\alpha_M\beta_2$ and by CHO cells expressing α_M lectin domain. The GPIbα N terminus contains exposed β-D-GlcNAc residues. The phagocytosis of chilled platelets can be inhibited by MOC, a snake venom that cleaves off the N terminus of GPIbα or by β-D-GlcNAc-1-Me (*β-GlcNAc*) at 1 mM. *C* and *D*, in contrast, CHO cells expressing $\alpha_X\beta_2$ or the α_M I domain chimera do not ingest chilled platelets. The data are the mean ± S.D. of three independent experiments in triplicate. The α_M/α_X chimeras expressed in CHO cells used in a particular experiment are schematically indicated. The wild type α_M subunit is in *black*, the α_X subunit is in *white*, and the β_2 subunit is in *gray*. *E*, flow cytometric analysis of the expression levels of the β_2 subunit in CHO cells. $\alpha_M\beta_2$ (*blue*; 91.24 ± 0.84 fluorescence-positive cells) and $\alpha_X\beta_2$ (*red*; 77.17 ± 11.39 fluorescence-positive cells) have similar expression levels. The α_M I domain (*black*; 55.13 ± 1.14 fluorescence-positive cells) and α_M lectin domain (*green*; 64.41 ± 12.05 fluorescence-positive cells) have slightly lower but comparable expression levels. *F*, CHO cells expressing wild type $\alpha_M\beta_2$ or α_M/α_X chimera containing the α_M lectin domain together with the β_2 subunit (*Lectin domain*) phagocytize chilled CM-Orange-stained platelets (*red*) as determined by light microscopy. Non-ingested, adherent platelets appear in *green/orange* because adherent CM-Orange-labeled platelets also stain with the anti-CD61 FITC-conjugated mAb. The *scale bar* is 5 μm. **, $p < 0.01$; *, $p < 0.05$.

maximal 4–5 days after virus infection, and decreased after 6 days postinfection. Cells at 4 or 5 days postinfection were used for all flow cytometry experiments. The fluorescence acquisition and analysis of fluorescently labeled Sf9 cells were performed in $\log_{10}$ fluorescence. Expression levels of His-α_M (Ala^{136}–Ser^{1098}) and His-α_M lectin domain (Val^{400}–Ser^{1098}) were compared in each experiment using anti-His FITC-conjugated Ab. 1×10^8 CM-Green-stained platelets were incubated together with 5×10^6 washed Sf9 cells in HBSS Ca^{2+}/Mg^{2+} in the presence or absence of 1 mM β-D-GlcNAc-1-Me for 30 min with gentle shaking at 37 °C. Sf9 cells were washed two times with HBSS and then analyzed by flow cytometry.

Statistics—In experiments using differentiated THP-1 cells, CHO cells, or Sf9 cells, the percentage of phagocytes positive for CM-Orange or CM-Green fluorescence when incubated with 22 °C platelets was set to one to calculate the ratio of the phagocytic increase for the chilled platelet population. Values are expressed as average ± S.D. or S.E. Groups were compared using the non-paired *t* test. *p* values < 0.05 were considered significant. **, $p < 0.01$; *, $p < 0.05$. Calculations were performed using Microsoft Excel software.

RESULTS

CHO Cells Expressing the α_M Lectin Domain Phagocytize Chilled Platelets—Experiments using differentiated THP-1 cells have provided evidence that the $\alpha_M\beta_2$ receptor mediates chilled platelet phagocytosis (3). As shown in Fig. 1, CHO cells stably expressing $\alpha_M\beta_2$ (16) ingested chilled platelets stained with CM-Orange 3–4 times more avidly than they ingested RT CM-Orange-stained platelets. As reported for THP-1 cells, platelet ingestion was inhibited by MOC (a snake venom that cleaves off the N terminus of GPIbα) as well as by 1 mM β-D-GlcNAc-1-Me (3, 4) (Fig. 1*A*). CHO cells expressing $\alpha_X\beta_2$ did not ingest chilled platelets (Fig. 1*D*). CHO cells expressing β_2 and chimeras of different α_M and α_X (p150) domains where the α_M I domain or α_M lectin domain was replaced by the corresponding α_X domains were used to define which portion of α_M is required to mediate chilled platelet phagocytosis. The phagocytic capacity of CHO cells expressing integrin chimeras containing either the α_M I domain or the α_M lectin domain (missing the I domain) were compared (Fig. 1, *B* and *C*). CHO cells expressing an α_M chain containing the lectin domain ingested chilled human platelets (Fig. 1*B*) to a degree comparable to that of the ingestion mediated by the intact α_M subunit (Fig. 1*A*). However, CHO cells expressing an α_M chain chimera having only the I domain (Fig. 1*C*) did not phagocytize chilled platelets. Selective uptake of chilled platelets mediated by the lectin domain of α_M is shown by fluorescence microscopy in Fig. 1*F*. CHO cells expressing $\alpha_M\beta_2$ or the α_M lectin domain in combination with β_2 in a chimera of α_M/α_X contained CM-Orange-stained chilled platelets (Fig. 1*F*, *red*). Also shown are surface-adherent platelets (*green/orange*) that stained with anti-CD61-FITC (*green*) if exposed on the surface of CHO cells. CHO cells expressing $\alpha_X\beta_2$ or α_M I domain/β_2 did not ingest chilled platelets and had few, if any, platelets bound to their apical surfaces. As reported for the intact $\alpha_M\beta_2$ receptors ex-

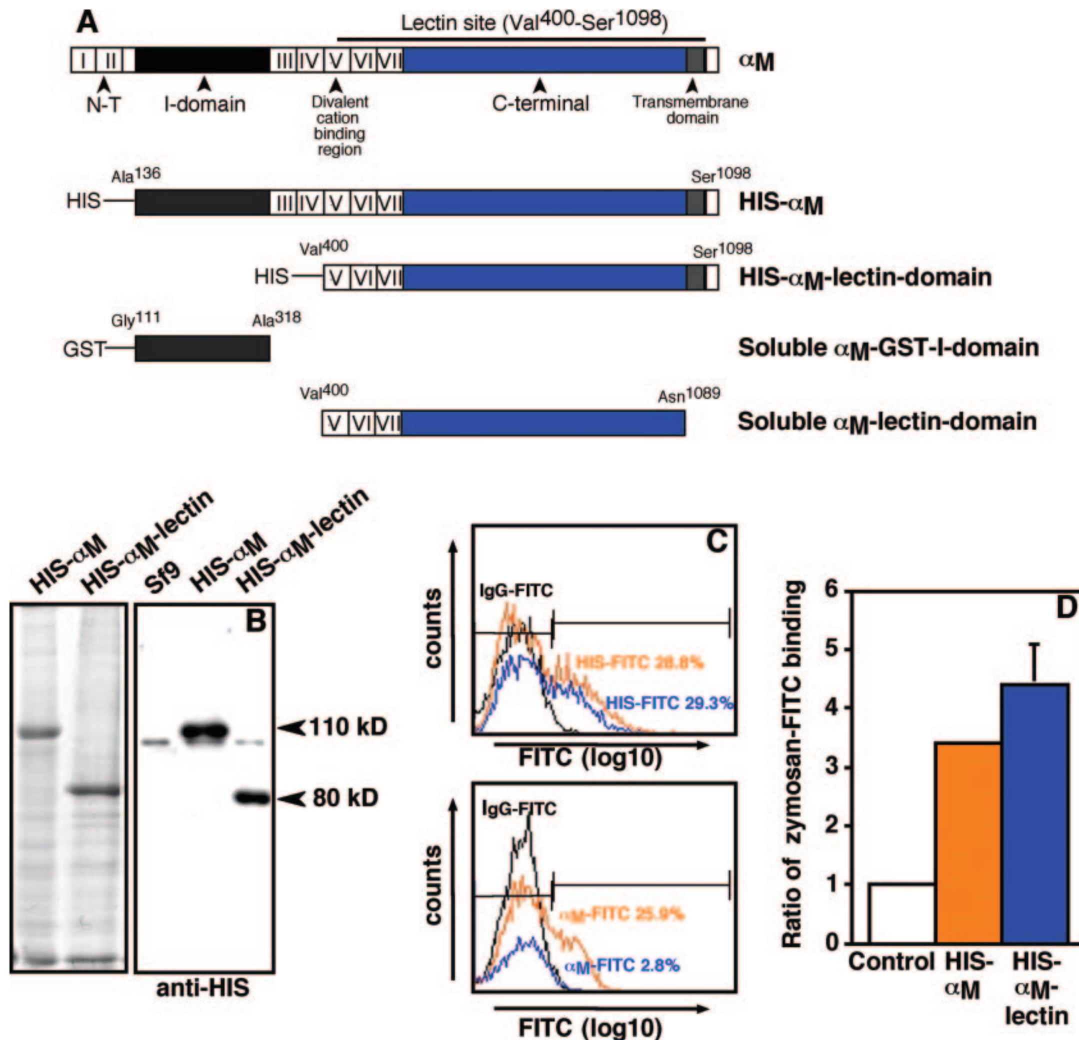

FIG. 2. **Sf9 cells expressing different domains of α_M.** *A*, primary structure of α_M and recombinant proteins expressed in Sf9 insect cells or *E. coli*. His-α_M (Ala^{136}–Ser^{1098}), His-α_M lectin domain (Val^{400}–Ser^{1098}), and soluble α_M lectin domain (Val^{400}–Asn^{1089}) were expressed in Sf9 insect cells. The soluble α_M GST-I domain (Gly^{111}–Ala^{318}) was expressed as a GST fusion protein in *E. coli*. *N-T*, N terminus. *B*, Sf9 cells expressing the recombinant His-α_M or His-α_M lectin constructs were subjected to SDS-PAGE under reducing conditions and stained with Coomassie Brilliant Blue (*left panel*). The *right panel* shows the corresponding immunoblot using the anti-His Ab including the uninfected control Sf9 cells sample (*Sf9*). The apparent molecular mass of the His-α_M is 110 kDa and of the His-α_M lectin is 80 kDa. *C*, surface expression of His-α_M (*orange*) and His-α_M lectin domain (*blue*) was determined by flow cytometry using the FITC-conjugated anti-His Ab (*top panel*). Approximately 30% of the transfected Sf9 cells expressed His-α_M or His-α_M lectin domain on their surfaces. The FITC-conjugated anti-α_M I domain (clone 44) mAb (*bottom panel*) stained the His-α_M construct (~25.9%) but failed to stain the His-α_M lectin domain (~2.8%) as expected. The nonspecific FITC-conjugated IgG control is shown in *black*. *D*, FITC-conjugated zymosan A bioparticles bind to the His-α_M lectin domain or to the His-α_M expressed on Sf9 cells as measured by flow cytometry. Virus-infected Sf9 cells expressing recombinant intracellular FLNa were used as control cells (*Control*). The mean ± S.D. of three individual experiments is shown (n = 3).

pressed in CHO cells, 1 mM β-D-GlcNAc-1-Me or cleavage of the GPIbα N terminus with the snake venom MOC inhibited chilled human platelet phagocytosis mediated by the α_M lectin domain (Fig. 1*B*). Flow cytometric analyses confirmed that all chimeric proteins were expressed on the surface of CHO cells and that the expression levels of the α and β chains were equivalent. The expression level of the β_2 subunit (Fig. 1*E*) was used to evaluate the expression levels of the different recombinant receptors. $\alpha_M\beta_2$ and $\alpha_X\beta_2$ had similar expression levels and α_M I domain and α_M lectin domain had slightly lower but comparable expression levels.

Chilled Platelets Bind to Sf9 Cells Expressing the α_M Lectin Domain—I domain-deficient (truncated) recombinant α_M fragments expressed on insect cells without β_2 exhibit affinity for ^{125}I-β-glucan that is comparable to that of the affinity of neutrophil $\alpha_M\beta_2$ (27). We used a similar approach to construct and express recombinant α_M domains on insect cells. Fig. 2*A* shows the expression of recombinant α_M subunit proteins. Membrane-spanning recombinant α_M proteins were expressed in Sf9 insect cells containing the α_M (Ala^{136}–Ser^{1098}) I domain, lectin domain, and C terminus designated His-α_M or α_M (Val^{400}–Ser^{1098}) lacking the I domain but having a portion of the divalent cation-binding region, the lectin domain, and the C terminus, designated His-α_M lectin domain. Fig. 2*B* shows that the Sf9 cells express the two truncated receptor forms abundantly at the expected sizes of 110 kDa (His-α_M) and 80 kDa (His-α_M lectin domain), respectively. Immunoblotting with an anti-His antibody confirmed the identity of the α_M-His recombinant molecules (Fig. 2*B*, *anti-His*). Flow cytometry, using anti-His-FITC or anti-α_M-FITC (clone 44) antibodies, demonstrated that the recombinant proteins were expressed on the surface of Sf9 cells. Both recombinant α_M proteins stain with the anti-His antibody (Fig. 2*C*, *top panel*), and ~30% of Sf9 cells expressed the His-α_M (*orange*) or the His-α_M lectin domain (*blue*). The expression levels of the two α_M recombinant proteins were similar. His-α_M-infected Sf9 cells (*orange*) stained with an an-

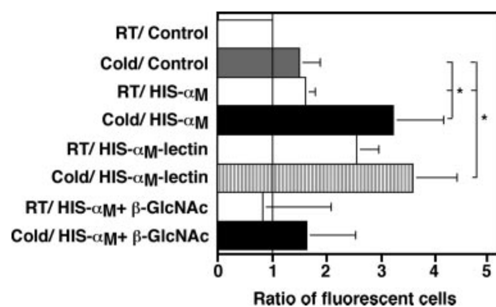

FIG. 3. **Sf9 cells expressing α_M subunit bind 3–4 times more chilled platelets than control cells; soluble β-GlcNAc inhibits this binding.** Binding of chilled (*Cold*) and RT CM-Green-stained platelets to Sf9 cells expressing His-α_M (*RT/His-α_M* and *Cold/His-α_M*) or His-α_M lectin domain (*RT/His-α_M-lectin* and *Cold/His-α_M-lectin*) and to virus-infected cells expressing intracellular FLNa (*RT/Control* and *Cold/Control*) was determined by flow cytometry. Addition of 1 mM β-D-GlcNAc-1-Me (β-*GlcNAc*) to His-α_M-expressing cells inhibited the binding of chilled platelets. The mean ± S.D. of three experiments in triplicate is shown. *, $p < 0.05$.

ti-α_M I domain antibody as expected and the His-α_M lectin domain-infected cells (*blue*) did not (Fig. 2*C*, *bottom panel*). The lectin domain of His-α_M and the His-α_M lectin domain constructs appear to be functional because FITC-labeled zymosan A bioparticles bound to Sf9 insect cells expressing them (Fig. 2*D*) as detected by flow cytometry.

We incubated chilled or RT platelets with Sf9 insect cells expressing either the His-α_M or the His-α_M lectin domain. 3–4-fold more CM-Green-stained, chilled human platelets bound to His-α_M- or His-α_M lectin domain-expressing Sf9 cells than did CM-Green-stained platelets maintained at room temperature and incubated with control or His-α_M-expressing cells (Fig. 3). Cells expressing His-α_M lectin domain showed increased binding to RT platelets as well compared with control cells; however, binding of the His-α_M lectin domain to chilled platelets was higher than to RT-maintained platelets. Binding of chilled platelets to His-α_M was inhibited by the addition of 1 mM β-D-GlcNAc-1-Me (Fig. 3), again indicating that binding is mediated by β-GlcNAc carbohydrate moieties. Control virus-infected Sf9 cells incubated with chilled or RT platelets did not show differential platelet binding.

Soluble α_M Lectin Domain, but Not Soluble α_M I Domain, Inhibits Chilled Platelet Phagocytosis by Differentiated THP-1 Cells—The α_M lectin and I domains were expressed as soluble proteins in Sf9 cells and *E. coli*, respectively (Fig. 2*A*). Soluble α_M I domain, expressed as a GST fusion protein and purified from *E. coli* (19), bound weakly to GPIbα in platelet lysates as shown in Fig. 4*C*. The soluble α_M I domain also bound to fibrinogen, a known ligand to the I domain, as determined by co-immunoprecipitation.[2] Adherence of the soluble I domain to glycocalicin and inhibition of neutrophil binding to platelet monolayers by small α_M I domain peptides was shown earlier (5, 17). The soluble α_M I domain, however, did not inhibit the phagocytosis of chilled CM-Orange-stained human platelets *in vitro* at doses of 0.1–10 μg/ml (Fig. 4*E*) or 100 μg/ml.[2] The soluble α_M lectin domain had an apparent size of 72 kDa, slightly smaller than the expected 78 kDa, and also immunoprecipitated GPIbα from platelet lysates, pointing to its functionality (Fig. 4*D*). In contrast to the soluble I domain, addition of >0.5 μg/ml of soluble α_M lectin domain inhibited, in a dose-dependent fashion, the ingestion of chilled platelets by differentiated THP-1 cells (Fig. 4*E*), and 1 μg/ml was maximally effective.

[2] E. C. Josefsson, H. H. Gebhard, T. P. Stossel, J. H. Hartwig, and K. M. Hoffmeister, unpublished data.

Other assays were performed to rule out I domain participation in chilled platelet phagocytosis. A synthetic peptide (M2) duplicating Pro^{201}–Lys^{217} of the α_M I domain, but not its scrambled version (scM2), binds directly to glycocalicin and inhibits $\alpha_M\beta_2$-dependent neutrophil adhesion to glycocalicin or to adherent platelets (17). However, neither M2 nor scM2 affected chilled platelet phagocytosis by differentiated THP-1 cells at concentrations shown to inhibit neutrophil binding of platelets (Fig. 5). Chilled platelet phagocytosis was also independent of the presence or absence of divalent cations (Fig. 5), and it was not greatly affected by a specific α_M I domain antibody, LPM19c, or by fibrinogen (Fig. 5). Based on these results, we conclude that the α_M I domain does not play a significant role in the phagocytosis of chilled human platelets in our *in vitro* phagocytic assay.

DISCUSSION

Platelet GPIbα is known to bind to the α_M I domain, which has homology to the von Willebrand factor A1 domain, and to mediate platelet adhesion to neutrophils (5, 17). Chilling causes GPIbα clustering on platelets that results in their recognition by the $\alpha_M\beta_2$ receptor leading to chilled platelet phagocytosis and rapid clearance from the circulation by macrophages (3). We now report that chilled platelet phagocytosis can be reconstituted by expressing $\alpha_M\beta_2$ and provide evidence that chilled platelet phagocytosis is mediated by the α_M lectin domain, not the α_M I domain.

We have previously shown that GPIbα contains exposed β-GlcNAc residues on *N*-linked glycans based on the following evidence. 1) GPIbα is the principal binding target of peroxidase-labeled succinyl wheat germ agglutinin (sWGA), a lectin that specifically binds to exposed β-GlcNAc residues. 2) Cooling of platelets increases sWGA binding. 3) Coverage of β-GlcNAc residues with galactose prevents the increase in sWGA binding to cooled human platelets and their phagocytosis (4). We speculated that sWGA mimics the α_M lectin domain and binds to exposed and clustered β-GlcNAc residues to mediate chilled platelet phagocytosis.

To determine whether $\alpha_M\beta_2$ is the only receptor mediating chilled platelet ingestion and to determine the specific α_M domains involved in cooled platelet phagocytosis, we used CHO cells expressing $\alpha_M\beta_2/\alpha_X\beta_2$ chimeras containing either a functional α_M I domain (XeMbX) or an α_M lectin domain (MeXbM). These chimeras (XeMbX and MeXbM) have been used previously to restrict the iC3b, intercellular adhesion molecule-1, or fibrinogen binding sites to the α_M I domain (16) as well as to determine the location and sugar specificity of the α_M lectin domain (13). Furthermore CHO cells expressing the $\alpha_M\beta_2$ receptor are capable of phagocytizing both opsonized and non-opsonized zymosan, recapitulating the non-opsonic, lectin-mediated, and opsonic I domain-mediated functions of $\alpha_M\beta_2$ when expressed on myeloid cells (28). CHO cells expressing wild type $\alpha_M\beta_2$ or α_X chain chimera having the α_M lectin domain ingested chilled platelets that were not opsonized by serum, whereas CHO cells expressing α-chain chimera containing the α_M I domain did not. Chilled platelet phagocytosis by the $\alpha_M\beta_2$ and the α_M lectin domain chimera was inhibited following the removal of the GPIbα N terminus, the site of *N*-linked carbohydrate residues, or by the addition of soluble methylated β-GlcNAc proving that chilled platelets interact with the α_M lectin domain through exposed β-GlcNAc residues. β-GlcNAc exposure on mammalian cells is an unexpected finding, and it is even more surprising that a macrophage lectin site recognized and initiated the ingestion of chilled platelets, whereas in contrast, it did not initiate phagocytosis of RT-maintained platelets. We have previously speculated that clustering of the exposed β-GlcNAc residues provides the required density to bind

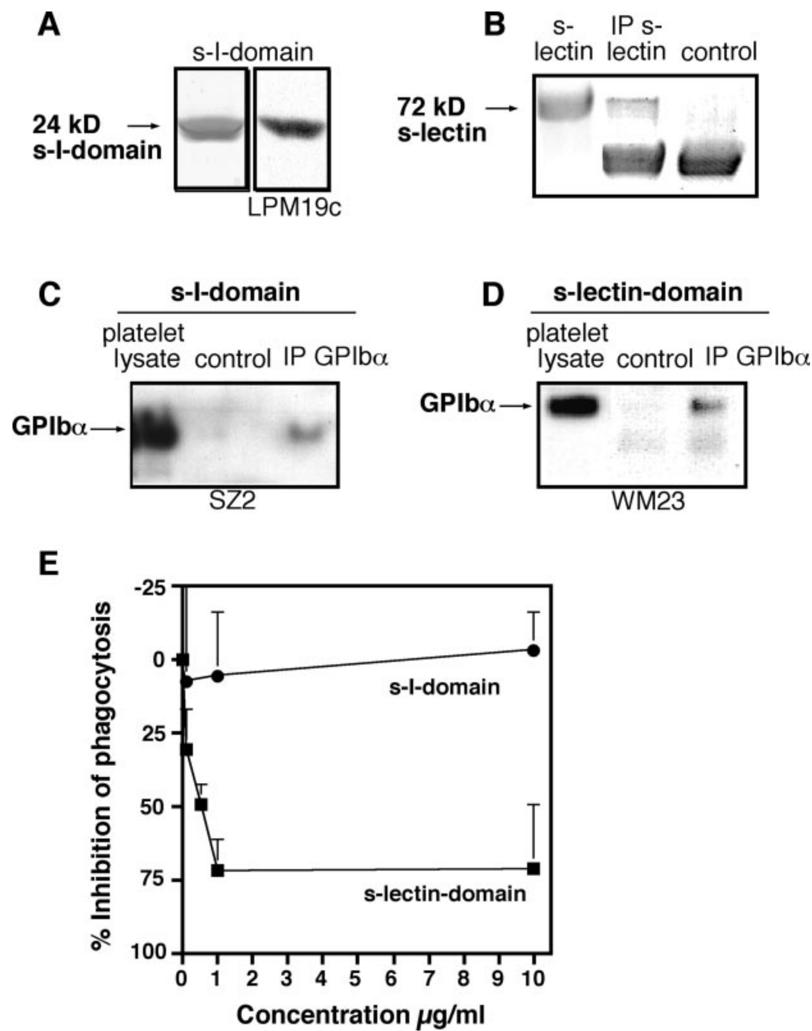

FIG. 4. **Soluble α_M lectin domain, but not soluble α_M I domain, inhibits chilled platelet phagocytosis by differentiated human monocytic THP-1 cells.** *A*, the molecular size of the soluble α_M I domain (*s-I-domain*), following the removal of the GST portion, was determined by SDS-PAGE and Coomassie Brilliant Blue staining. The soluble α_M I domain has an apparent size of 24 kDa (*left panel*) as expected. The corresponding immunoblot using the specific anti-α_M-I domain mAb LPM19c is shown (*right panel*). *B*, the molecular size and specificity of the s-lectin domain expressed in Sf9 insect cells was determined by SDS-PAGE and Coomassie Brilliant Blue staining. The apparent molecular size of the s-lectin domain is ~72 kDa (*left lane*). The specificity of the s-lectin domain was verified by immunoprecipitation (*IP*) using the anti-lectin domain monoclonal antibody CBRM1/25 (*IP s-lectin*) because the CBRM1/25 antibody failed to recognize the construct in immunoblots. The immunoprecipitation using a control Ab is shown in the *right lane* (*control*). *C* and *D*, the soluble α_M I domain and the s-lectin domain bound to GPIbα from platelet lysates, showing the functionality of both domains. *C*, the GST-conjugated soluble α_M I domain, but not the control, bound to GPIbα (*IP GPIbα*) as shown by immunoblot analysis using the specific anti-GPIbα antibody clone (*SZ2*). The platelet lysate is shown in the *left lane*. *D*, the s-lectin domain (*IP GPIbα*), but not the control, bound to GPIbα as determined by immunoblotting using the specific anti-GPIbα antibody clone (*WM23*). The platelet lysate is shown in the *left lane*. *E*, the s-lectin, but not the soluble α_M I domain, inhibited chilled platelet phagocytosis by differentiated human monocytic THP-1 cells. CM-Orange-stained RT or chilled platelets were incubated with 0.1–10 μg/ml s-lectin or soluble α_M I domain prior to the addition of the platelets to THP-1 cells. The analysis of THP-1 cells that ingested CM-Orange-stained platelets and appear as single CM-Orange-positive cells is shown. The results show the mean ± S.E. of three to four experiments.

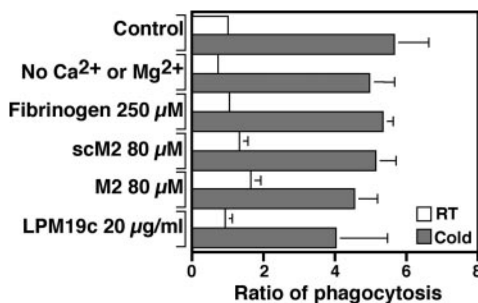

FIG. 5. **The I domain ligands (fibrinogen and α_M I domain mAb LPM19c) and the I domain peptide (M2, Pro^{201}–Lys^{217} of the α_M I domain) that binds GPIbα as defined by Ehlers *et al.* (17) or the omission of divalent cations do not significantly ($p > 0.05$) inhibit the ingestion of chilled platelets by differentiated human monocytic THP-1 cells.** The results show the mean ± S.D. of three to four individual experiments in triplicate.

to the $\alpha_M\beta_2$ and induce phagocytosis, an event that does not require opsonization by serum (3). The α_M lectin carbohydrate-mediated, cation-independent recognition of pathogens such as *Mycobacterium tuberculosis* and yeast (6, 13) plays a major role in the non-opsonic phagocytosis of pathogens (15). It is possible that chilled platelets bind to the α_M lectin domain and induce clustering of the $\alpha_M\beta_2$ integrins and initiate phagocytosis. Another hypothesis is that possibly the mechanism described for chilled platelet clearance also applies to normal platelet clearance. As platelets age in the circulation they may lose sialic acid and galactose residues, exposing β-GlcNAc leading to their elimination by phagocytes expressing $\alpha_M\beta_2$ receptors. Hence the chilled platelet clearance mechanism may not have evolved solely to remove platelets damaged by cold temperatures, and rather it may serve to eliminate senile platelets. It is tempting to speculate that von Willebrand factor receptors containing

GPIbα chains become clustered upon deglycosylation, a mechanism described for the T-cell receptor in β1,6-*N*-acetylglucosaminyltransferase *V*-deficient mice (29). Senile platelets with greater levels of exposed β-GlcNAc would be more likely to cluster GPIbα and therefore more readily cleared from the circulation.

The ability of Sf9 cells expressing His-α_M or the His-α_M lectin domain to bind chilled or RT platelets was also studied. Sf9 cells expressing the His-α_M bound to chilled platelets better than to room temperature platelets, an interaction again inhibited by the competition with soluble methylated β-GlcNAc. In contrast, Sf9 cells expressing His-α_M lectin domain bound chilled platelets but only slightly better than RT platelets. Zymosan particles also exhibited increased binding to Sf9 cells expressing the His-α_M lectin domain indicating that the truncated form has a higher affinity for its ligands. Hence truncation of the α_M subunit in the B-propeller region might interfere with the protein folding and therefore confer conformational changes to the α_M lectin domain that enhance its binding to unclustered β-GlcNAc residues on the surface of RT platelets. Surface expression of the His-α_M lectin domain on insect cells without the β-subunit may also lead to clustering of the α_M lectin domain, facilitating increased binding to exposed ligands on RT platelets or zymosan particles. Furthermore expression of the β domain may help mask the α_M lectin domain and partially prevent binding of the lectin domain ligands such as β-GlcNAc. Proteins expressed by Sf9 cells, however, are poorly glycosylated compared with vertebrate cells. The diminished glycosylation capacity of Sf9 cells may also alter the binding/affinity of the lectin domain as this domain is highly glycosylated in mammalian cells.

The quantification of exposed galactose or β-GlcNAc residues on platelets using commercially available lectins, such as sWGA or *Ricinus communis* agglutinin I, is technically challenging. We constructed and expressed a soluble form of the α_M lectin domain, speculating that this domain may recognize more specifically the changes in GPIbα structure and its *N*-linked glycans induced by chilling, making it a useful reagent to probe for changes on platelets induced by chilling. Such an assay would prove to be valuable in a clinical transfusion setting if the galactosylation method for chilled platelets proves to be feasible in humans. Here we demonstrate that the α_M lectin domain bound specifically to GPIbα and that low concentrations of the soluble domain inhibited, in a dose-dependent fashion, chilled platelet ingestion by differentiated THP-1 cells (3). In contrast, soluble α_M I domain, the small I domain peptide M2, scM2, or fibrinogen did not inhibit the phagocytosis of chilled human platelets *in vitro* by differentiated THP-1 cells. Chilled platelet phagocytosis was also independent of the presence of divalent cations, pointing to a minor contribution of the α_M I domain to chilled platelet phagocytosis and to the specificity and potency of the α_M lectin domain. Although some mAbs such as MN-41 specific for α_M I domain epitopes block polysaccharide binding to the C-terminal lectin domain (13), the α_M I domain-specific antibody LPM19c had a slight but not significant inhibitory effect ($p > 0.05$) on chilled platelet phagocytosis by differentiated THP-1 cells. Taken together, these results point to the high specificity of the α_M lectin domain and demonstrate that, independently of the presence or absence of the I domain, it can mediate chilled platelet phagocytosis. Binding of the α_M lectin domain to platelets may therefore be used in the future to probe for the changes induced by chilling and repair by galactose transfer.

In summary, we have previously demonstrated that transfused chilled platelets remain in the circulation of α_M-deficient mice (3) despite GPIbα clusters on their surfaces. Since cooled platelets exhibit normal hemostatic functions and cooling does not detectably alter von Willebrand factor-platelet interactions (3), hemostatic, adhesive, and clearance functions mediated by GPIbα may be separable. Here we demonstrate that chilled platelet phagocytosis depended solely on the α_M lectin domain and was independent of the α_M cation-binding domain and α_M I domain. These findings point to the versatility of GPIbα: it mediates platelet adhesion to the α_M I domain of neutrophils in flowing arterial blood and chilled platelet ingestion through the α_M lectin domain by sinusoidal macrophages. Finally the clearance mechanism for chilled platelets may be applicable to "senile" platelets as well, suggesting its relevance in the clearance mechanism for platelets in mammals.

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