

## Molecular Identification of Aggrus/T1 $\alpha$ as a Platelet Aggregation-inducing Factor Expressed in Colorectal Tumors\*

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Platelets play an important role in hemostasis, thrombosis, and antimicrobial host defense and are also involved in the induction of inflammation, tissue repair, and tumor metastasis. We have previously characterized the platelet aggregation-inducing sialoglycoprotein (Aggrus/gp44) overexpressed on the surface of tumor cells. Because a platelet aggregation-neutralizing 8F11 monoclonal antibody that could specifically recognize Aggrus suppressed tumor-induced platelet aggregation, we have previously purified Aggrus by 8F11-affinity chromatography and found that purified Aggrus possessed the ability to induce aggregation of platelets. Here we show that Aggrus is identical to the T1 $\alpha$ /gp38P/OTS-8 antigen, the function of which in tumors is unknown. Expression of mouse Aggrus and its human homologue (also known as T1 $\alpha$ -2/gp36) induced platelet aggregation without requiring plasma components. Using the 8F11 antibody, we identified the highly conserved platelet aggregation-stimulating domain with putative *O*-glycosylated threonine residues as the critical determinant for exhibiting platelet aggregation-inducing capabilities. We compared the expression level of human aggrus mRNA using an array containing 160 cDNA pair samples derived from multiple human tumorigenic and corresponding normal tissues from individual patients. We found that expression level of aggrus was enhanced in most colorectal tumor patients. To confirm the protein expression, we generated anti-human Aggrus polyclonal antibodies. Immunohistochemical analysis revealed that Aggrus expression was frequently up-regulated in colorectal tumors. These results suggest that Aggrus/T1 $\alpha$  is a newly identified, platelet aggregation-inducing factor expressed in colorectal tumors.

Specific glycoproteins expressed on the surface of platelets enable the platelets to adhere to receptors exposed in areas of vascular damage (1). The process of adhesion activates platelet aggregation, leading to the formation of a platelet plug in the vessel wall. Activated platelets also induce the formation of a fibrin clot by carrying coagulation factors and providing a catalytic surface for the major interactions of the coagulation

cascade. Because there exists a clear link between atherosclerotic vascular disease, inflammation, tumor metastasis, and thrombosis (1–3), it is important to identify the mechanisms of platelet aggregation that have pathobiologic, prognostic, and treatment-related relevance. Studies on cancer metastasis have shown that some human and animal tumor cells possess platelet aggregation-inducing abilities that correlate with their metastatic potential (2, 3). Interactions between tumor cells and platelets have been considered to facilitate the arrest of tumor cell cluster in the microcirculation with the subsequent formation of experimental metastasis. However, the molecules associated with the tumor-induced platelet aggregation have not yet been identified.

We previously established several clones possessing different platelet aggregation-inducing capabilities from a mouse colon adenocarcinoma 26 cell line (4). By generating monoclonal antibodies, we revealed that the established 8F11 monoclonal antibody inhibited platelet aggregation *in vitro* (5, 6) and the pulmonary metastasis of highly metastatic clones of mouse colon adenocarcinoma 26 cell line *in vivo* (7). The 8F11 antibody recognized a cell-surface 44- or 41-kDa sialoglycoprotein (Aggrus) (5, 6). Using 8F11 affinity-column chromatography, we purified the 44-kDa mouse Aggrus and found that purified Aggrus itself could induce platelet aggregation with no requirement for plasma components (8). We tried to identify the protein; however, we could not obtain the peptide sequence from the purified Aggrus protein because of the abundant carbohydrate chains.

In this study, we identified the 8F11 antibody-reactive mouse Aggrus as mouse T1 $\alpha$  antigen (also known as mT1 $\alpha$ /gp38P/OTS-8) (9–11). Using the platelet aggregation-neutralizing 8F11 antibody, we identified the residue (PLAG<sup>1</sup> domain) critical for platelet aggregation-inducing capability of mouse Aggrus. Although mouse and human Aggrus (also known as T1 $\alpha$ -2/gp36) (9, 12) proteins have only 39% amino acid identity, the PLAG domains are highly conserved. Mutation of threonine residues in the PLAG domain abolished the platelet aggregation-inducing abilities of human and mouse Aggrus proteins. Using Cancer Profiling Array II, we found that the expression level of *aggrus* was enhanced in colorectal tumor patients. By generating polyclonal antibodies to human Aggrus/T1 $\alpha$ -2/gp36, we confirmed the increased human Aggrus/T1 $\alpha$ -2/gp36 protein expression in colorectal tumors. Therefore, Aggrus is a newly identified platelet aggregation-inducing factor that is overexpressed in colorectal tumors.

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<sup>1</sup> The abbreviations used are: PLAG, platelet aggregation-stimulating; AGR, Aggrus; siRNA, small interfering RNA oligonucleotide duplexes; PRP, platelet-rich plasma; CHO, Chinese hamster ovary.

## EXPERIMENTAL PROCEDURES

**Plasmids, siRNA, and Antibodies**—Mouse aggrus/mT1 $\alpha$ /gp38P cDNA (GenBank accession no. AJ297944 or AJ250246) (9, 10) was obtained by reverse transcription-PCR with NL-17 mRNA as the template. Human aggrus/hT1 $\alpha$ -2/gp36 cDNA (GenBank accession no. AF030428 or AJ225022) (9, 12) was obtained by PCR using a normal human lung cDNA library (CosmoBio, Tokyo, Japan) as the template. The amplified cDNAs were subcloned into a pcDNA3 vector, and a FLAG epitope tag was added at the COOH terminal. Substitution of the appropriate amino acid codons to alanine codons in aggrus cDNAs was accomplished using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) (13, 14). The mouse aggrus deleted cDNAs were also ligated into pET-21a vector (Novagen, Darmstadt, Germany), and the recombinant mouse Aggrus deletion mutant proteins were expressed in *Escherichia coli*. Five small interfering RNA oligonucleotide duplexes (siRNAs) were designed from the mouse aggrus/mT1 $\alpha$  sequence. In our experiments to suppress mouse Aggrus expression in NL-17 cells, we used the most effective siRNA from among them. The coding strands of the siRNA directed to residues 180–198 of mouse aggrus cDNA were 5'-UGAAUCUACUGGCAAGGCA-3' and 5'-UGCCUUGCCAGUAGAUUCA-3', respectively. Non-silencing control siRNA was purchased from QIAGEN (Valencia, CA). The oligonucleotides had 3' dTdT overhangs. The anti-human Aggrus polyclonal antibody (TT679) was obtained by immunizing rabbits with the synthetic peptide CEGGVAMPGEEDDVV, corresponding to amino acids 38–51 of human Aggrus plus N-terminal cysteine and purified using a column linked to the peptide. Rat monoclonal 8F11 antibody was purified from ascites fluid (5). Control rat IgG, anti-FLAG-M2 antibody, and anti- $\beta$ -actin antibody were obtained from Sigma.

**Cell Culture Conditions**—Chinese hamster ovary (CHO), mouse colon adenocarcinoma NL-14 and NL-17 (4), mouse lymph node stromal CA-12 (15), mouse osteoblastic MC3T3-E1 (11), and thioglycolate-elicited mouse peritoneal macrophages were cultured at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Biocell, Carson, CA), 2 mM L-glutamine (Invitrogen), and 100  $\mu$ g/ml of kanamycin.

**Transient Transfections and Establishment of Stable Transfectants**—Cells were transfected with appropriate amounts of plasmids using Superfect transfection reagent (QIAGEN) or LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturers' instructions (14). RNA interference was performed by transfecting control or mouse aggrus siRNA plus mock pcDNA3 vector into NL-17 cells (4) using LipofectAMINE 2000 reagent. Stable CHO transfectants or pooled transient CHO transfectants were selected by cultivating the transfectants in medium containing 1 mg/ml of G-418 (Geneticin), and the expression level of mouse or human Aggrus was confirmed by immunoblot analysis.

**Western Blotting, Immunoprecipitation, and Enzyme-linked Immunosorbent Assay**—Western blotting and immunoprecipitation were performed as described previously (14). The synthetic DGMVPPGIEC peptide (wild-type) corresponding to amino acids 39–47 of mouse Aggrus sequence plus C-terminal cysteine and its mutant peptides DGAVPPGIEC (M41A), DGMAPPGIEC (V42A), DGMVAPGIEC (P43A), and DGMVPAGIEC (P44A) were immobilized on a 96-well plate (mutated amino acids are shown in bold letters). The reactivity of 8F11 antibody and control rat IgG to the peptides was estimated by enzyme-linked immunosorbent assay (16).

**Flow Cytometry**—Parental CHO or CHO/mAGR cells were harvested by brief exposure to trypsin. After washing with phosphate-buffered saline, cells were treated with 8F11 antibody for 1 h at 4 °C after treatment with fluorescein isothiocyanate-conjugated anti-rat IgG (Cappel, Cochranville, PA). Fluorescence data were collected using a FACScalibur flow cytometer (BD Biosciences).

**Platelet Aggregation Assay**—Mouse platelet-rich plasma (PRP) was prepared from fresh, heparinized blood drawn from BALB/c mice (8). Human PRP was obtained from a healthy volunteer. Washed platelets were separated from PRP by centrifugation at 400  $\times$  g for 5 min, washed, and resuspended in phosphate-buffered saline (8). In some experiments, CHO/mAGR cells were incubated with 1 mg/ml of 8F11 antibody or normal rat IgG for 20 min on ice before incubation with platelets (5). The platelet aggregation was monitored using an NKK HEMA Tracer I (Niko Bioscientific Co., Tokyo, Japan) (5, 6).

**Reverse Transcription-PCR, PCR, and Cancer Profiling Array Analysis**—Reverse transcription-PCR and PCR were performed as described previously using the mouse aggrus sense (5'-CTCAAGCTTCAAGATGTGGACCGTGCCAGTG-3'), mouse aggrus antisense (5'-GAGGAATTCGGCGGAGAACCCTCCAGAAATC-3'), human aggrus sense (5'-TGTTGGAAGGTGTCAGCTCTG-3'), human aggrus antisense (5'-TTAGGGC-

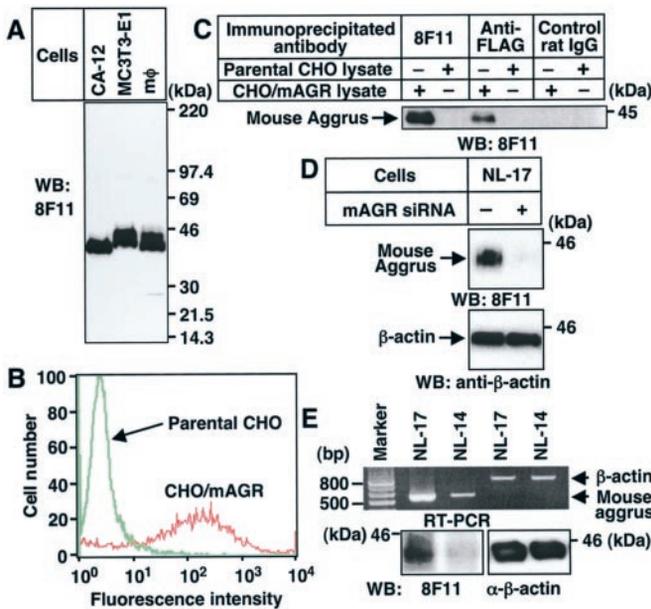
GAGTACCTTCCCC-3'), mouse  $\beta$ -actin sense (5'-GATATCGCTGCGC-TGGTCTGTCGAC-3'), mouse  $\beta$ -actin antisense (5'-CAAGAAGGAAGGCTGGAAAAGAGC-3'), human ribosomal protein S9 sense (5'-GATGAAGGACCCACGGCGTCTGTTCG-3'), and human ribosomal protein S9 antisense (5'-GAGACAATCCAGCAGCCAGGAGGGACA-3') oligonucleotides. We compared the expression level of human aggrus mRNA in the same patient using Cancer Profiling Array II according to the manufacturer's instructions (BD Biosciences). Cancer Profiling Array II consists of 160 cDNA pairs, synthesized from human tumorigenic and corresponding normal tissues. Each pair was independently normalized based on the expression of four housekeeping genes and immobilized in separate dots (17, 18). The full-length <sup>32</sup>P-labeled human aggrus cDNA was used for hybridization and radioactivity of each dot was visualized and quantified with a BAS1000 Bio-Imaging Analyzer (Fuji Film, Tokyo, Japan) (13). The expression level of human aggrus mRNA in the individual patients was normalized by measuring radioactivity of each dot after hybridization with <sup>32</sup>P-labeled ubiquitin cDNA.

**Immunohistochemistry**—Fixed and paraffin-embedded colorectal tissue specimens (LandMark Tissue Microarrays, lot 022P15A; Ambion, Austin, Texas) were deparaffinized, rehydrated, and treated to block endogenous peroxidase by 3% H<sub>2</sub>O<sub>2</sub> and nonspecific staining by casein-containing blocking reagent (DAKO Cytomation, Glostrup, Denmark). TT679 antibody was added (0.01  $\mu$ g/ml) for 18 h at 23 °C, followed by incubation with biotin-conjugated secondary anti-rabbit IgG antibody and peroxidase-conjugated streptavidin (LSAB2 kit; DAKO Cytomation). Color was developed with 3,3'-diaminobenzidine tetrahydrochloride tablet sets (DAKO Cytomation) used for 10 min.

## RESULTS

**Molecular Identification of A Platelet Aggregation-inducing Factor**—To identify the 8F11 antibody-reactive gp44/mouse Aggrus (mAGR) protein, we screened cells expressing the protein. We found that mouse osteoblastic MC3T3-E1 (11), lymph node stromal CA-12 (15), and macrophages expressed mouse Aggrus protein (Fig. 1A). Several reports suggested that mouse T1 $\alpha$  antigen (also known as mT1 $\alpha$ /gp38P/OTS-8) (9–11) and its human homologue (T1 $\alpha$ -2/gp36) (9, 12) were expressed in MC3T3-E1 cells (11) and lymphoid tissues (19). Because of the expression pattern, we hypothesized that mT1 $\alpha$ , which is required for normal lung cell proliferation and alveolus formation *in vivo* (20), is identical to an unidentified platelet aggregation-inducing factor (Aggrus). We generated pcDNA3 vector containing FLAG-tagged full-length mT1 $\alpha$  cDNA. When CHO cells were stably transfected with mT1 $\alpha$  cDNA (CHO/mAGR), transfected mT1 $\alpha$  antigen was expressed on the cell surface and was recognized by the 8F11 antibody (Fig. 1B). Immunoprecipitation followed by immunoblot analysis revealed that 8F11 antibody recognized the antigen itself (Fig. 1C). We designed five siRNAs (21) and found that the siRNA directed to residues 180–198 of mT1 $\alpha$  decreased the expression of endogenous mouse Aggrus in highly metastatic NL-17 and B16-F10 cells (Fig. 1D and data not shown). As we have reported previously (5), protein expression level of gp44/mouse Aggrus in highly metastatic NL-17 cells was higher than that in poorly metastatic NL-14 cells (Fig. 1E). Semiquantitative PCR analysis revealed the increased aggrus mRNA expression in NL-17 cells (Fig. 1E). These results indicate that the mT1 $\alpha$ /gp38P/OTS-8 antigen is identical to our previously characterized mouse Aggrus/gp44 protein.

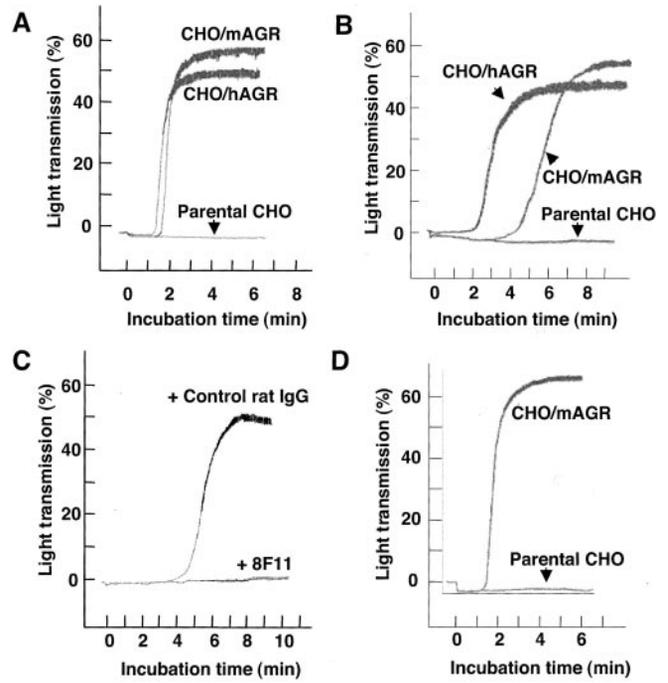
**Aggrus Promotes Aggregation of Human and Mouse Platelets without Requiring Plasma Components**—When CHO/mAGR cells were incubated with mouse PRP, CHO/mAGR cells, but not parental CHO cells, induced mouse and human platelet aggregation (Fig. 2, A and B), and the aggregation was suppressed by pretreatment of the cells with 8F11 antibody (Fig. 2C). Consistent with our previous report (8), plasma components were not required because washed platelets were also aggregated by incubation with CHO/mAGR (Fig. 2D). We cloned human aggrus cDNA (hAGR; also known as hT1 $\alpha$ -2 or gp36) (9, 12) and transiently transfected it into CHO cells (CHO/hAGR). Expression of human Aggrus on CHO cell sur-



**FIG. 1. Molecular identification of mouse Aggrus.** *A*, the cell lysate of lymph node stromal CA-12, osteoblastic MC3T3-E1, and thioglycolate-elicited peritoneal macrophages (*mφ*) were electrophoresed and immunoblotted with 8F11 antibody. *B*, flow cytometric analysis of 8F11 reactivity to parental CHO and a stable mouse Aggrus transfectant (CHO/mAGR). *C*, parental CHO and CHO/mAGR cell lysates were incubated with protein G agarose that had been conjugated with 8F11 antibody, anti-FLAG antibody, or control rat IgG. After washing, immunoprecipitated proteins were electrophoresed and immunoblotted with 8F11 antibody. *WB*, Western blot. *D*, NL-17 cells were transfected with pcDNA3 vector plus non-silencing control siRNA (–) or mouse aggrus (*mAGR*) siRNA (+). Cells were harvested, electrophoresed, and immunoblotted with 8F11 antibody or anti- $\beta$ -actin antibody. *E*, semi-quantitative reverse transcription-PCR analysis and immunoblot analysis of mouse Aggrus or  $\beta$ -actin mRNA and protein expression in NL-17 and NL-14 cells.

face induced mouse and human platelet aggregation (Fig. 2, *A* and *B*), regardless of poor homology (39% amino acid identity with mouse Aggrus) between mouse and human Aggrus proteins (9). These results indicate that human and mouse Aggrus proteins possessed the ability to induce platelet aggregation.

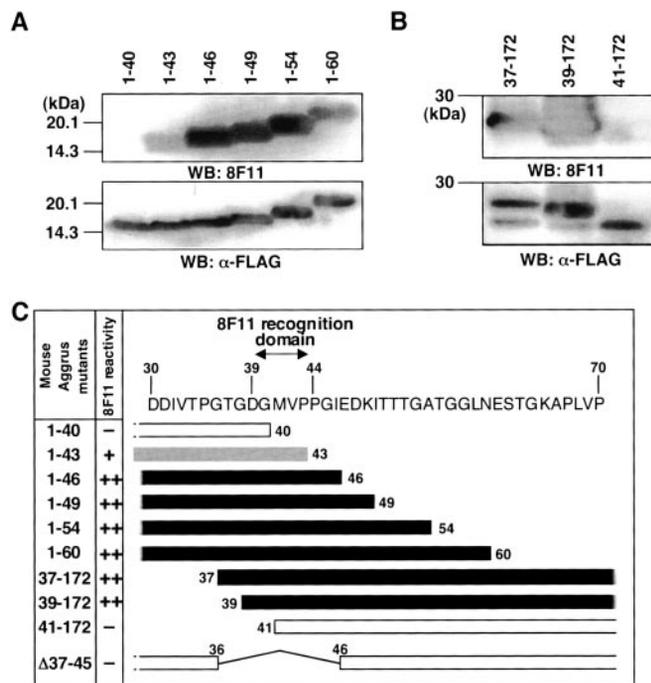
**Identification of the 8F11 Recognition Domain in Mouse Aggrus**—The mouse Aggrus-induced mouse platelet aggregation was perfectly suppressed by pretreatment of CHO/mAGR cells with 8F11 antibody (Fig. 2*C*). Thus, 8F11 may recognize the specific epitope of mouse Aggrus that interacts with platelets. To clarify the residues critical for platelet aggregation, we examined the reactivity of 8F11 antibody to recombinant mouse Aggrus protein expressed in *E. coli* (Fig. 3, *A–C*). The 39-DGMVPP-44 residue was identified as essential for 8F11 recognition of recombinant mouse Aggrus protein. To confirm the result, we transiently transfected the pcDNA3 vector containing mouse aggrus-point mutated cDNAs into CHO cells. The 8F11 antibody could recognize all point mutants except G40A, M41A, V42A, and P43A (Fig. 4*A*). Conversion of glycine-38 to alanine (G38A) decreased the 8F11-binding capability. This result was consistent with the result indicating that 8F11 recognized the synthetic 39-DGMVPPGIE-47 plus C-terminal cysteine but not its point-mutated peptides (Fig. 4*B*). Therefore, 39-DGMVPP-44 is the 8F11 recognition domain in mouse Aggrus. We established CHO cells transiently transfected with the pcDNA3 vector containing mouse aggrus-M41A cDNA (CHO/M41A). CHO/M41A had almost the same platelet aggregation-inducing ability that CHO/mAGR did (Fig. 4*C*). Moreover, the peptide coding 8F11-recognition domain could not induce platelet aggregation by itself (data not shown).



**FIG. 2. Mouse and human Aggrus proteins possessed the ability to induce aggregation of human and mouse platelets.** *A* and *B*, the platelet aggregation-inducing capability of parental CHO, a stable mouse Aggrus transfectant (CHO/mAGR), or a transient human Aggrus transfectant (CHO/hAGR) was estimated by incubating the cells with mouse PRP (*A*) or human PRP (*B*). *C*, CHO/mAGR cells were preincubated with 1 mg/ml of control rat IgG or 8F11 on ice. After incubation for 20 min, the platelet aggregation-inducing capability was estimated by incubating cell suspension with mouse PRP. *D*, the platelet aggregation-inducing capability of parental CHO or CHO/mAGR was estimated by incubating the cells with washed mouse platelets.

These results suggest that the identified 8F11 recognition domain may not be involved in direct binding to platelets.

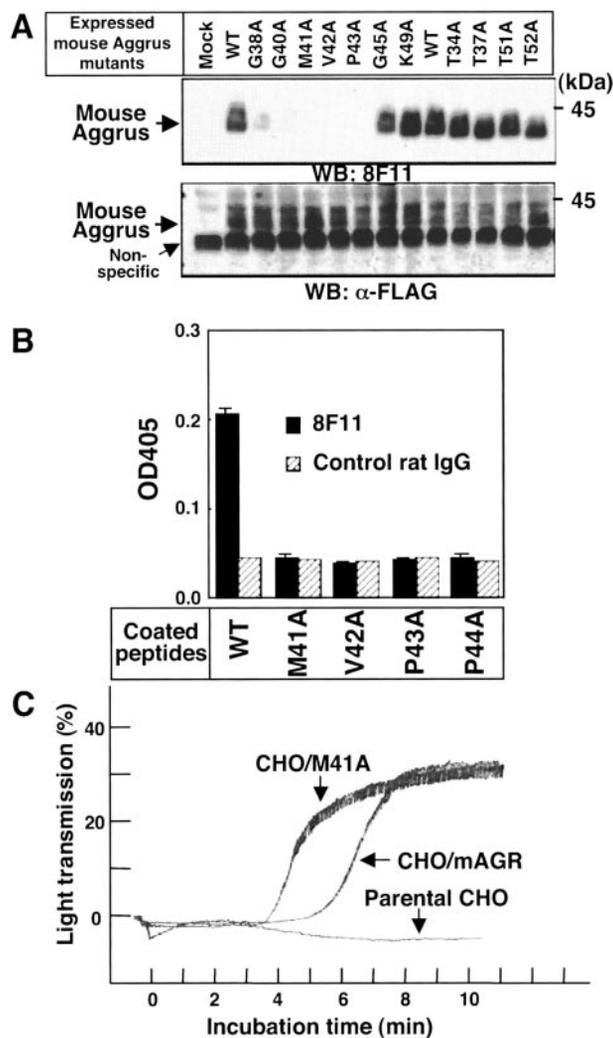
**Identification of the Platelet Aggregation-stimulating Domain in Aggrus As the Critical Determinant for Exhibiting Platelet Aggregation-inducing Capabilities**—As we have characterized previously (8), carbohydrate chains of mouse Aggrus/gp44 are involved in the platelet aggregation-inducing capability. We hypothesized that the 8F11 antibody might neutralize platelet the aggregation-inducing capability of mouse Aggrus by conformationally interfering with the carbohydrate chains near the 8F11 binding domain. We then generated several mouse aggrus point mutants in which putative *O*-glycosylated threonine residues near the 8F11-binding domain were converted to alanine residues. Mutation of these threonine residues affected neither 8F11 binding (Fig. 4*A*) nor cell surface expression (data not shown). However, conversion of threonine-34 to alanine (T34A) in mouse Aggrus eliminated the platelet aggregation-inducing capability (Fig. 5*A*). Because threonine followed by proline is likely to be *O*-glycosylated (22) and Edman microsequencing of the equivalent residues in the dog homologue of Aggrus revealed gaps at the threonine residues (23), threonine-34 in mouse Aggrus might be *O*-glycosylated, and the carbohydrate chain was involved in the interaction of the platelets and subsequent platelet aggregation. Sequence comparison of the residues around threonine-34 in mouse Aggrus and residues around threonine-52 in human, threonine-34 in rat (24), and threonine-41 or threonine-50 in dog (23) homologues showed mouse Aggrus to be highly conserved (Fig. 5*B*). We therefore termed the residues as PLAG (platelet aggregation-stimulating) domains. As expected, conversion of threonine-52 to alanine in human Aggrus (T52A-hAGR) specifically abolished its platelet aggregation-inducing



**FIG. 3. Identification of the 8F11 antibody-recognition domain in mouse Aggrus.** *A* and *B*, the indicated FLAG-tagged mouse aggrus deleted cDNAs were ligated into pET-21a vector, and the recombinant mouse Aggrus proteins were expressed in *E. coli*, electrophoresed, and immunoblotted with 8F11 antibody or anti-FLAG antibody. *WB*, Western blot. *C*, amino acid sequences of mouse Aggrus deletion mutants used in these experiments and their 8F11 reactivity are shown schematically (solid bars, strong binding; gray bar, weak binding; open bars, no binding).

capability (Fig. 5C). These results indicate that the PLAG domain (especially *O*-glycosylated threonine residues) is critical for platelet aggregation.

**Expression of Aggrus in Colorectal Tumors**—Mouse aggrus mRNA expression was observed in parental mouse colon adenocarcinoma 26 and its sublines, and the expression levels of aggrus mRNA in these cells were higher than that in normal mouse colon tissue (data not shown). Thus, we estimated the human aggrus expression in human tumors using Cancer Profiling Array II. In this array, normalized cDNA pairs from 160 primary tumor tissues and the corresponding normal tissues from individual patients were immobilized on nylon membrane (17, 18). Human aggrus expression increased in almost all intestinal tumors (colon, rectum, and small intestine) compared with corresponding normal tissues (Fig. 6A). After hybridization with <sup>32</sup>P-labeled ubiquitin cDNA, human aggrus expression in each patient was normalized. The average expression ratio of human aggrus in colon ( $n = 10$ ), rectum ( $n = 7$ ), and small intestine ( $n = 10$ ) tumor patients was about 2.8, 3.2, and 3.9, respectively (Fig. 6B). To confirm the result, we performed PCR with matched cDNA pairs from colon adenocarcinoma patient 5 and small intestine adenocarcinoma patient 2, whose cDNAs were immobilized in Cancer Profiling Array II (Fig. 6A, circled). Semiquantitative PCR analysis revealed that human aggrus expression was clearly enhanced in both tumor tissues (Fig. 6C). In contrast, human aggrus expression in lung, breast, and ovary varied from patient to patient (Fig. 6, A and B), and the average expression ratio in lung, breast, and ovary was about 1.6, 0.94, and 1.1, respectively. Human aggrus expression in adenocarcinoma of the intestine was higher than that in colon adenomas or small intestine carcinoid tumors (data not shown). Therefore, human aggrus expression might be specific for carcinoma. We obtained similar results using Cancer Profiling Array I (data not shown).

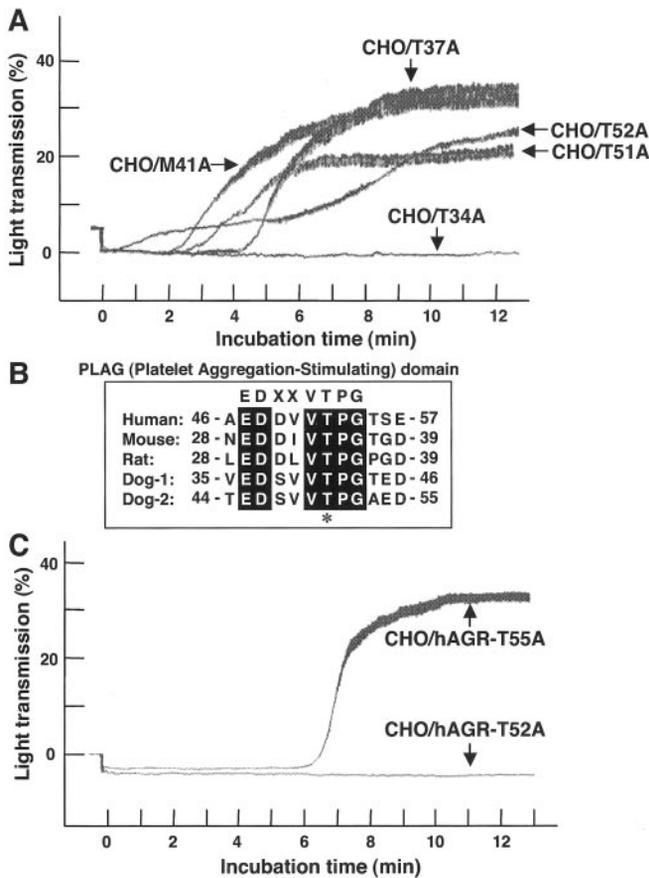


**FIG. 4. Confirmation of amino acid residues recognized by the platelet aggregation-neutralizing 8F11 antibody.** *A*, CHO cells were transiently transfected with a pcDNA3 vector containing none (*Mock*), wild-type (*WT*), or the indicated mutant mouse aggrus cDNAs. The cell lysates were immunoblotted with 8F11 or anti-FLAG antibody. *WB*, Western blot. *B*, the synthetic DGMVPPGIEC peptide (*WT*) corresponding to amino acids 39–47 of mouse Aggrus sequence plus C-terminal cysteine and its mutant peptides were immobilized on a 96-well plate, as described under “Experimental Procedures.” The reactivity of 8F11 antibody and control rat IgG was estimated by enzyme-linked immunosorbent assay. Each point represents a mean  $\pm$  S.D. of triplicate determinations. *C*, the platelet aggregation-inducing activity of parental CHO, a stable mouse Aggrus transfectant (*CHO/mAGR*), or a transient M41A-mouse Aggrus transfectant (*CHO/M41A*) was estimated by incubating the cells with mouse PRP.

To confirm the increased human Aggrus protein expression in colorectal tumors, we generated polyclonal antibodies to human Aggrus (TT679) because the 8F11 antibody cannot recognize human Aggrus (Fig. 7A). The generated TT679 antibody recognized the 36-kDa human Aggrus protein and did not cross-react with other protein species (data not shown). Strong staining was seen in human specimens derived from colon adenocarcinoma tissues (Fig. 7, B and C) but not in samples from normal colon tissues (Fig. 7, D and E). These results indicate that expression level of Aggrus is enhanced in most colorectal tumor patients.

#### DISCUSSION

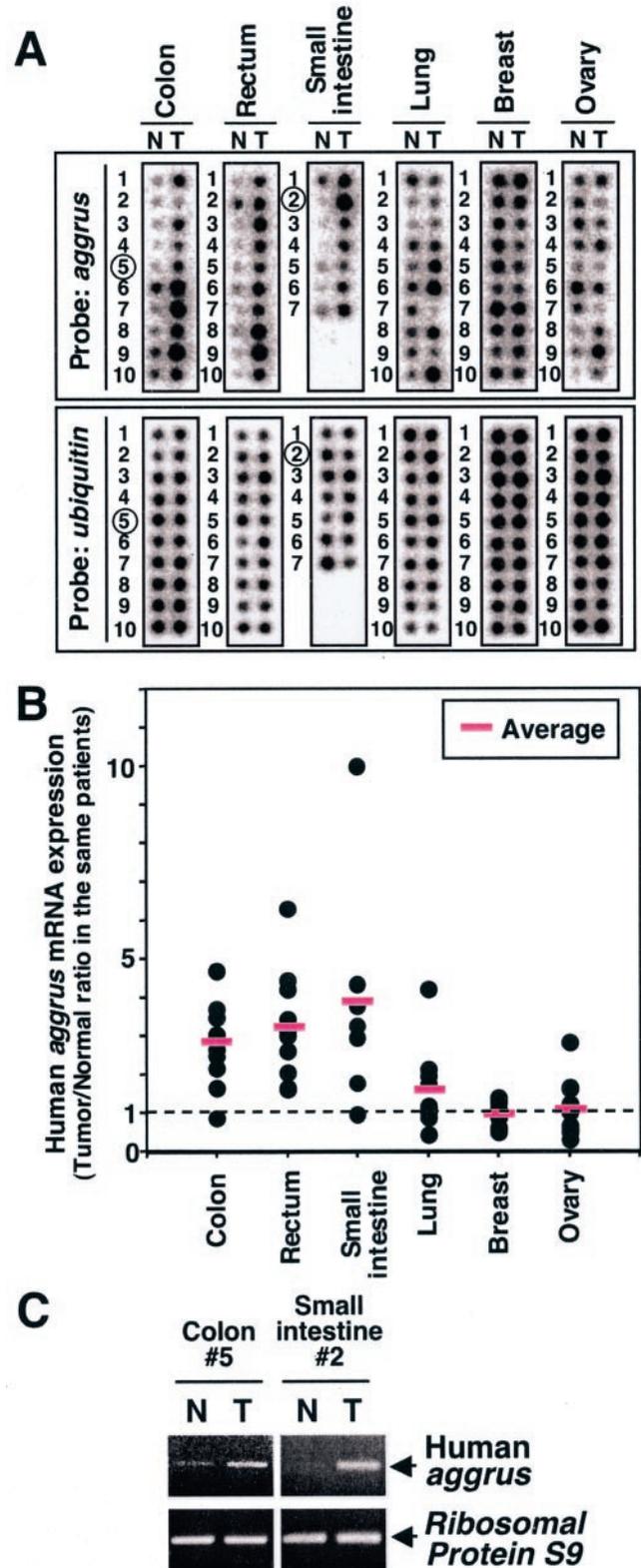
Several reports suggested that tumor-induced platelet aggregation is an early step in the development of a metastatic lesion (2, 3). Ultrastructural studies have also demonstrated that



**FIG. 5. Identification of PLAG domain in Aggrus protein.** *A*, CHO cells were transiently transfected with mouse aggrus cDNA carrying the indicated point mutations. Their platelet aggregation-inducing capabilities were estimated by incubating the cells with mouse PRP. *B*, alignment of the amino acid sequence of Aggrus homologues around the PLAG domain. Identical amino acids are denoted by white letters on black background. An asterisk indicates the possible O-glycosylation site. *C*, CHO cells were transiently transfected with T52A- or T55A-human aggrus cDNA (CHO/hAGR-T52A or CHO/hAGR-T55A, respectively). Their platelet-aggregation inducing capabilities were estimated by incubating the cells with mouse PRP.

platelets seem to enhance the development of arrested tumor emboli into a secondary metastatic colony (25, 26). Although several inhibitors of platelet aggregation have been reported to retard tumor metastasis in certain animal models (27–29), the tumor adhesive glycoproteins have not yet been identified.

We previously established several clones possessing different metastatic abilities from a mouse colon adenocarcinoma 26 cell line (4). Among these clones, a highly metastatic clone, NL-17, was found to exhibit a high platelet aggregation-inducing activity, although a poorly metastatic clone, NL-14, had marginal platelet aggregation-inducing capability (28, 30). Therefore, the ability to induce platelet aggregation was related to the metastatic potential. To identify the platelet aggregation-inducing molecule(s) associated with tumor metastasis, we generated monoclonal antibodies by immunizing rats with NL-17 cells. One of the established antibodies, 8F11, exhibited the platelet aggregation-neutralizing activity *in vitro* and the inhibitory activity of lung colonization *in vivo* (5, 7). The 8F11 antibody recognized a 44-kDa membrane sialoglycoprotein (mouse Aggrus/gp44) on NL-17 cells (5). Consistent with the platelet aggregation-inducing capabilities of NL-17 and NL-14 cells (28, 30), the expression level of 8F11 antibody-reactive mouse Aggrus in NL-17 cells was higher than that in NL-14 cells (Fig. 1E). We previously succeeded in purifying the mouse Aggrus from NL-17 cells (8) and found that the purified mouse



**FIG. 6. Human aggrus expression in colorectal, rectal, and small intestinal tumors.** *A* and *B*, human aggrus expression in tumor (T) and normal (N) tissues in the same patient was assessed by hybridizing <sup>32</sup>P-labeled human aggrus cDNA to Cancer Profiling Array II. The expression level of human aggrus in the individual patients was normalized by measuring radioactivity of each dot after hybridization with <sup>32</sup>P-labeled ubiquitin cDNA. The radioactivity of each dot was visualized (*A*) and quantified (*B*). Red bar, average of the human Aggrus expression ratio in each tissue. *C*, semiquantitative PCR analysis of human aggrus and ribosomal protein S9 expressions in colon adenocarcinoma patient 5 and small intestine adenocarcinoma patient 2 (indicated by circles in *A*).

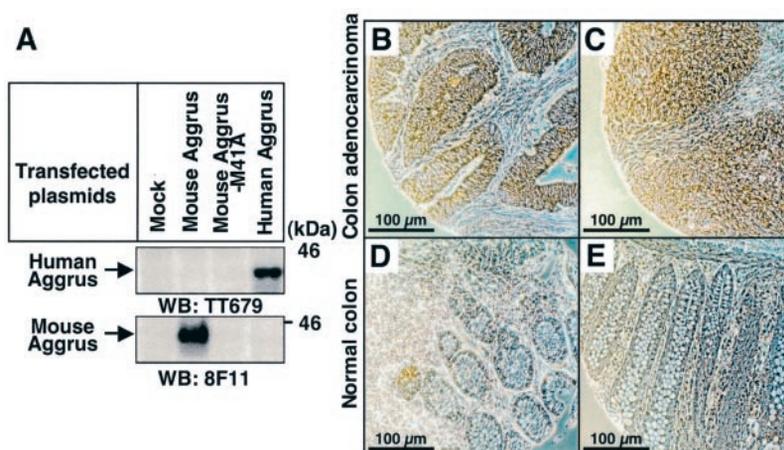


FIG. 7. **Human Aggrus protein expression in colon adenocarcinoma.** A, CHO cells were transiently transfected with a pcDNA3 vector containing the indicated mouse or human *aggrus* cDNAs. The cell lysates were immunoblotted with 8F11 antibody or anti-human Aggrus antibody (TT679). B–E, Immunohistochemical staining with anti-human Aggrus antibody (TT679) on human specimens derived from colon adenocarcinoma (B and C) compared with specimens derived from normal colon (D and E) tissues.

Aggrus itself possessed the ability to induce platelet aggregation. These data indicate that 8F11 antibody-reactive Aggrus is a platelet aggregation-inducing factor expressed on tumors.

We could not obtain the peptide sequence from the purified mouse Aggrus. Thus, we searched cells expressing mouse Aggrus. We found that mouse Aggrus was expressed on the surface of mouse osteoblastic MC3T3-E1 cells (Fig. 1A). Because the 38-kDa mouse T1 $\alpha$ /gp38P/OTS-8 antigen was originally isolated from MC3T3-E1 cells (11), we hypothesized that mouse Aggrus was identical to mouse T1 $\alpha$ /gp38P/OTS-8. Four lines of evidence support this assumption. First, the 8F11 antibody recognized CHO cells that had been transfected with mouse T1 $\alpha$ /gp38P/OTS-8 cDNA (Fig. 1A); second, the immunoprecipitated FLAG-tagged mouse T1 $\alpha$ /gp38P/OTS-8 protein was recognized by 8F11 antibody (Fig. 1C); third, siRNA directed to mouse T1 $\alpha$ /gp38P/OTS-8 decreased the 8F11 antibody-reactive endogenous mouse Aggrus expression in NL-17 (Fig. 1D); and fourth, expression of mouse T1 $\alpha$ /gp38P/OTS-8 on CHO cell surface induced platelet aggregation with no requirement for plasma components (Fig. 2). We observed that proteins recognized by 8F11 antibody in different mouse cell lines vary in their electrophoretic motility (Fig. 1A). We also previously reported that 8F11 antibody recognized a 44-kDa protein in colon adenocarcinoma sublines (5) and a 41-kDa protein in B16 melanoma sublines (6). The siRNA to *aggrus* decreased the expression level of both 44-kDa Aggrus in NL-17 cells (Fig. 1D) and 41-kDa Aggrus in B16-F10 cells (data not shown). Therefore, we think these variations are caused by differences in post-translational modification.

The identified mouse Aggrus was first identified as an early-response protein (OTS-8) that was induced by phorbol ester in osteoblastic MC3T3-E1 cells (11). Mouse Aggrus has also been reported as a cell-surface antigen (gp38/gp38P) that is expressed in type-I thymus epithelial cells and in peripheral lymphoid tissues (10, 19). Human, rat, and dog homologues (E11 antigen/podoplanin/RT140/PA2.26/gp36/gp40) have been already cloned (9, 12, 23, 24, 31) and have been reported to be molecules expressed by various epithelial and nonepithelial cell types. The molecules have putative extracellular and transmembrane domains and a short cytoplasmic tail with putative protein kinase C and cAMP phosphorylation sites. Sequence analysis indicates that Aggrus does not share common domains with other protein families of known function that predict its function. Thus, the physiological role of Aggrus has not yet been determined. Several reports suggested that Aggrus could be a regulator of fluid transport or a receptor for the influenza

C virus (9, 32). Our findings indicate that Aggrus functions as an adhesion molecule that promotes platelet aggregation in pathological conditions.

A striking feature of Aggrus proteins is their extraordinarily high content of serine and threonine residues that might be *O*-glycosylated. In dog Aggrus/gp40, 14 serine and threonine residues were identified by Edman degradation to be modified by *O*-glycosylation (23). Because our data (Fig. 4) suggested that 8F11 antibody might neutralize mouse Aggrus-induced platelet aggregation by conformationally interfering the carbohydrate chains near the 8F11-binding domain, we generated several mouse *aggrus* point mutants in which putative *O*-glycosylated threonine residues around the 8F11-binding domain. We revealed that putative *O*-glycosylated threonine-34 of mouse Aggrus is a critical residue for its platelet aggregation-inducing capability (Fig. 5). This result is consistent with our previous report (8) that sialylated carbohydrate chains of Aggrus/gp44 were involved in their platelet aggregation-inducing capabilities. Although human and mouse Aggrus proteins have only 39% amino acid identity (9), the residues around threonine-34 of mouse Aggrus are highly conserved (Fig. 5B). Mutation of the equivalent threonine-52 in human Aggrus abolished its platelet aggregation-inducing activity (Fig. 5C). We also cloned rat homologue of Aggrus/RT140/E11/podoplanin (33–35) and found that it could induce platelet aggregation (data not shown). Mutation of the threonine-34 to alanine in rat Aggrus abolished its platelet aggregation-inducing capability (data not shown). Thus, the PLAG domain is identified as critical residues for their platelet aggregation-inducing capability. Because heavily *O*-glycosylated proteins were identified as counter receptors for selectins (36, 37), it is possible that Aggrus binds to selectins for triggering platelet aggregation.

We also observed increased *aggrus* mRNA expression in most patients with intestinal tumors (Fig. 7, B–E). This result was consistent with the result that *aggrus* mRNA expression in colon adenocarcinoma 26 sublines was higher than that in normal mouse colons. Interestingly, the *aggrus* expression in adenocarcinoma of the intestine was higher than that in colon adenomas or small intestine carcinoid tumors (data not shown). Therefore, human *aggrus* expression might be specific for carcinoma and was strongly correlated with tumor progression.

Our results indicate that Aggrus is a newly identified platelet aggregation-inducing factor expressed on the surface of several tumors. Because platelet aggregation is associated with the development of arrested tumor emboli into a secondary

metastatic colony, therapies aimed at neutralizing Aggrus function could prove successful in inhibiting tumor metastasis.

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