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Identification and Characterisation of a Platelet GPIb/V/IX-like Complex on Human Breast Cancers: Implications for the Metastatic Process

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The glycoprotein (GP) Ib/V/IX receptor complex is an important adhesion molecule, originally thought to be unique to the megakaryocytic lineage. Recent evidence now indicates that GPIb/V/IX may be more widely expressed. In this study we report the presence of all subunits of the complex on four breast cancer cell lines, and 51/80 primary breast tumours. The surface expression of GPIb/V/IX was confirmed by flow cytometry, and by immunoprecipitation of biotin surface-labelled tumour cells. Western blotting of cell lysates under reducing conditions revealed that tumour cell-GPIbα had a relative molecular weight of 95 kDa as compared to 135 kDa on platelets. Despite the discrepant protein size, molecular analyses on the tumour cell-GPIbα subunit using RT-PCR and DNA sequencing revealed 100% sequence homology to platelet GPIbα. Tumour cell-GPIb/V/IX was capable of binding human von Willebrand factor (vWf), and this binding caused aggregation of tumour cells in suspension. Tumour cells bound to immobilised vWf in the presence of EDTA and demonstrated prominent filapodial extensions indicative of cytoskeletal reorganisation. Furthermore, in a modified Boyden chamber assay, prior exposure to vWf or a GPIbα monoclonal antibody, AK2, enhanced cell migration. The presence of a functional GPIb/V/IX-like complex in tumour cells suggests that this complex may participate in the process of haematogenous breast cancer metastasis.

Key words: Platelet glycoproteins — von Willebrand factor — Metastasis — Breast cancer

In order for a tumour cell to metastasize it must be able to travel through the circulation avoiding host immune cells and then adhere to, and pass through, the vascular endothelium. Although it has long been known that platelets are an indispensable component of the metastatic process,[1, 2] only recently has it become evident that tumour cells themselves express immunologically-related platelet proteins that participate in metastasis.[3–5]

The glycoprotein (GP) Ib/V/IX complex is an adhesive protein and an integral membrane component of circulating platelets. The complex is made up of four transmembrane polypeptide subunits: GPIbα, GPIbβ, GPV and GPIX. GPIbα is a 135-kDa glycosylated protein disulfide-linked to the 30-kDa GPIbβ chain. The 25-kDa GPIX chain is non-covalently, but tightly, associated with GPIbβ. The role of the latter two subunits in platelet adhesion is poorly understood; however, both GPIbβ and GPIX are required for efficient plasma membrane association of the complex.[6] The 82-kDa GPV subunit is more loosely associated with GPIb/IX in a molar ratio of 0.5:1. GPIb/V/IX complex plays a pivotal role in thrombosis and haemostasis through its interaction with the adhesive glycoprotein von Willebrand factor (vWf) (reviewed extensively in ref. 7). The GPIbα subunit of the complex contains the ligand-binding sites for vWf in the NH_2-terminal domain (reviewed in refs. 7, 8), and also interacts with actin-binding protein (ABP),[9] linking actin filaments to the cytoskeleton. Functional operation of the complex through the GPIbα-vWf axis requires the associated surface expression of both the GPIbβ and GPIX subunits,[6, 10] as well as the cytoplasmic interaction with ABP.[11, 12]

GPIb/V/IX complex expression was originally thought to be restricted to cells of the megakaryocytic lineage. Several authors have demonstrated that this is not the case, and have shown that a functional GPIb/V/IX complex resides on the surface of human vascular endothelial cells.[13–15] Several other reports describe the presence of the GPIbα subunit on a variety of other cells including human erythroleukaemia (HEL) cells,[16] aortic smooth muscle cells,[17] synovial cells[18] and dermal dendrocytes.[19] Recent publications by Oleksowicz *et al.* demonstrated the presence of an immunorelated GPIbα subunit on a breast carcinoma cell line MCF-7 and several fresh human breast carcinomas.[20, 21] The immunologically related GPIbα identified in these studies was found to be 100 kDa in size, and was significantly upregulated by treatment of the cells with PMA. However, the presence of other members of the complex was not investigated in these studies. In this

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study, we report, for the first time, the surface expression of all subunits of the GPIb/V/IX complex on four breast cancer cell lines. In addition to demonstrating expression, we also provide evidence that the complex is capable of mediating tumour-cell aggregation, as well as morphological and behavioural changes in response to human vWf.

MATERIALS AND METHODS

Cell lines Cancer cell lines HT29, MCF-7, T47D, SKBR3, MDA-MB-231 (ATCC, Manassas, VA) were cultured in RPMI 1640, supplemented with 10% fetal calf serum (FCS) and 1.25 U/ml human insulin. The myoepithelial cell line 184, originally isolated by Dr. M. Stampfer,[22] was maintained in MCBD Medium170 supplemented with 5% bovine pituitary extract (Gibco BRL, NY). CHO cells transfected with cDNA encoding GPIbα (CHO-α) (gift from Dr. Jose Lopez) were cultured in DMEM, supplemented with 10% FCS. Cells were harvested using Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) containing EDTA (0.6 m*M*) and EGTA (5 m*M*).

Antibodies The monoclonal antibodies SZ1 (anti-GPIX; Immunotech, Marseilles, France), AK2 and WM23 (anti-GPIbα; gift from Dr. Michael Berndt), SZ2 (anti-GPIbα; Immunotech), ALMA12 (anti-GPIbα; gift from Dr. Shaun Jackson), SW16 (anti-GPV; Research Diagnostics, Inc., Flanders, NJ), 5D2 (anti-vWf; gift from Dr. Shaun Jackson), and CD42c polyclonal antibody to GPIbβ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were obtained as indicated. Rabbit-anti-mouse-biotin, streptavidin-horseradish peroxidase (streptavidin-HRP), and goat-anti-mouse-HRP were all from DAKO (Carpinteria, CA). Goat-anti-mouse-FITC and donkey-anti-goat-FITC were both from Silenus (Chemicon, CA).

Immunostaining Cell lines used for immunostaining were grown onto glass slides (LabTek, Nalge Nunc, NY), fixed with ice-cold acetone for 20 min then washed with Tris-buffered saline (TBS) prior to immersion in 3% H_2O_2 for 5 min to block endogenous peroxidase. After washing, slides were blocked with non-immune rabbit serum (DAKO) diluted 1:5 in TBS containing 2% bovine serum albumin (TBS/BSA). Slides were then incubated with 20 μg/ml isotype control murine IgG (mIgG) or the monoclonal antibody (mAb) SZ2 for 60 min at room temperature. After washing, the slides were incubated for 30 min with a biotinylated rabbit-anti-mouse secondary antibody diluted 1:100 in TBS/BSA. Slides were washed again and then incubated with streptavidin-HRP diluted 1:100 in TBS. Finally the slides were washed once more before application of 3,3′-diaminobenzidine substrate (Sigma "FAST" DAB, St. Louis, MI). After development, sections were rinsed in distilled water, dehydrated and mounted with Eukitt (O.Kindler GmbH & Co., Freiburg, Germany). Paraffin sections (5 μm) of human breast cancers were dewaxed, rehydrated and subject to immunostaining as described above, with the addition of counterstaining with Mayer's haematoxylin prior to dehydration and mounting.

Flow cytometry Directly after harvesting, cells were washed twice in PBS and resuspended at 2×10^6 per ml in PBS containing 1% BSA. Cells were incubated with either control IgG or 10 μg/ml of the following antibodies: SZ1, AK2, WM23, SZ2, ALMA12, SW16, and CD42c. Cells were fixed with 1% paraformaldehyde and permeabilised with Triton X-100 (0.1%) prior to treatment with CD42c. Cells were washed twice in PBS before incubation with the appropriate FITC-labeled secondary and washed three times prior to analysis. Flow cytometry was performed using a FACStar Plus cytometer (Becton Dickinson, San Jose, CA) with argon laser excitation at 488 nm. Emission spectra were collected using a 530±30 nm band pass filter for both FITC and Alexa-488. Ten thousand cells were assessed in each experiment.

Immunoprecipitation and western blotting For immunoprecipitation, washed platelets, MDA-MB-231 and 184 cells, were surface labelled with NHS-LC-biotin according to the manufacturer's recommendations (Pierce, Rockford, IL). Unbound biotin was removed by three washes with PBS and the cells were then lysed in Tris buffer containing 1% Triton X-100 and the protease inhibitors leupeptin, PMSF, aprotinin, benzamidine and sodium orthovanadate. Lysed cells were incubated on ice for 30 min, then centrifuged at 10 000*g* for 15 min, and the equivalent of 2×10^7 cells were used for immunoprecipitation with 2 μg of either AK2 or isotype control mIgG. Following incubation for 60 min at room temperature, 50 μl of packed goat-anti-mouse Sepharose beads (Zymed, San Francisco, CA) were added for 2 h at 4°C. The beads were washed with lysis buffer, resuspended in SDS-Laemmli buffer and boiled under reducing conditions for 3 min. Samples were resolved on 4–20% SDS-PAGE according to Laemmli[23] and transferred onto PVDF membrane. The membrane was blocked for 1 h with 5% BSA in PBS, probed with SA-HRP, and washed extensively, and the resulting bands were visualized using ECL chemiluminescence (NEN Life Science Products, Boston, MA).

For western blot, total protein lysates of platelets or MDA-MB-231 were prepared as above without biotinylation. Lysates corresponding to 1 μg and 200 μg of platelets, and MDA-MB-231 total protein, respectively, were run on 8% SDS-PAGE and transferred onto PVDF membrane. The membrane was blocked for 1 h with 5% BSA in PBS, and then probed with the mAb WM23. After washing, the membrane was probed with goat-anti-mouse-HRP for 1 h, washed extensively and visualised using chemiluminescence as described above.

RT-PCR and sequencing Total RNA was extracted from MDA-MB-231 and CHO-α cells as previously described.[24] Total RNA (10 μg) was reverse transcribed to produce

Table I. Primer Sequences Used in RT-PCR

Primer	Sequence 5′-3′	PCR amplicon of GPIbα
P1	CTGTGACAAGAGGAATCT	137–686
P2	CCCGTGGAGAAAAGCAA	
P3	CTGGACGTCTCCTTCAACCGGCTGACCT	430–1063
P4	AGTGGATTCTTGTGTTGGATGCAAGGA	
P5	CCTGGGGTCTATTCTAC	1023–1560
P6	GACCCAAGACATAGAAG	
P7	GAACTTGATCAGCCACCA	1470–1920
GPIbRT	TCAGAGGCTGTGGCCAGAGTACCTAA	

cDNA using the primer GPIbRT (Table I) and the Perkin Elmer GeneAmp RNA PCR kit according to the manufacturer's recommendations (Perkin Elmer, Ringwood, NJ). The resultant cDNA was used immediately in PCR with the primer pairs shown in Table I. For PCR, 3 μl of the cDNA reaction was used as a template in 100 μl reactions using 40 pmol of each primer and *Tth* Plus DNA Polymerase under the conditions prescribed by the manufacturer (Biotech International, Belmont, WA, Australia). Amplification using primer sets P1/P2 and P5/P6 was performed under the following conditions; 94°C for 3 min, 35 cycles of 94°C for 30 s; 56°C for 30 s; 72°C for 60 s, followed by a final extension step of 72°C for 10 min. Amplification using primer sets P3/P4 and P7/GPIbRT was performed as above except the annealing temperature was increased to 58°C. Controls for each PCR amplification included RNA not subject to an RT step, and a no template control. Amplicons generated from MDA-MB-231 were gel purified using Qiagen Gel Extraction Kit (QIAGEN Pty, Ltd., Victoria, Australia), sequenced using cycle sequencing (ABI BigDyes, Perkin Elmer) and analysed with the Perkin Elmer ABIPrism 377 automated DNA sequencer.

Tumour cell aggregation Washed MDA-MB-231 cells (1×10^6/ml) were resuspended in modified Tyrode's buffer containing EDTA (2 m*M*) and EGTA (5 m*M*). Cells were then incubated with 20 μg/ml human vWf (gift of Dr. Shaun Jackson) in the presence of 1 mg/ml ristocetin (Sigma) in a 1.5 ml tube, with gentle rocking for 10 min. Cells rocked in the absence of vWf were used as a control. Cell suspensions were wet-mounted and visualised using Nomarsky optics on a Zeiss microscope and images were captured using Microcomputer Imaging Device ("MCID," Imaging Research, Inc., Ontario, Canada). In some experiments, cells were incubated with the mAbs AK2, 5D2 or with 10 μg/ml of the snake venom metalloprotease Mocarrhagin (gift from Dr. Michael Berndt).

vWf binding Purified vWf was labeled with the fluorochrome, Alexa-488, according to the manufacturer's instructions (Molecular Probes, Eugene, OR) modified as described previously.[25] The concentration of the vWf-Alexa conjugate was determined by protein assay (BCA Protein Assay, Pierce). Unlabelled vWf and Alexa-488-labelled vWf were resolved on 1% agarose gel electrophoresis according to Ruggeri and Zimmerman.[26] There was no apparent difference in the multimer distribution of unlabelled versus Alexa-488-labelled vWf. Tumour cells were harvested as described above and resuspended at 2×10^6 per ml in PBS containing 1% BSA, 10 m*M* EDTA and RGDS peptide (Sigma). Tumour cells were incubated with 20 μg per ml of either isotype control mIgG or the anti-GPIbα monoclonal antibody SZ2. The blocked tumour cells were incubated with 0.1 to 10 μg per ml vWf-Alexa and 1 mg per ml ristocetin for 10 min at room temperature. The cells were washed three times with PBS containing 1% BSA to remove unbound vWf, and the bound vWf was measured by flow cytometry.

Static adhesion to vWf Glass coverslips were coated with either human vWf (10 μg/ml) or fibrinogen (10 μg/ml) (gift of Dr. Shaun Jackson), overnight at 4°C. Coated coverslips were washed 3 times with PBS before MDA-MB-231 cells (1×10^6/ml) were applied, and allowed to adhere for 60 min at 37°C. Non-adherent cells were removed by three gentle washes with PBS and the adherent cells fixed to the coverslips with 3.7% formaldehyde for 10 min. Images of cells remaining bound to the coverslips were captured using "MCID" software and Nomarsky optics on a Zeiss microscope.

Migration assay MDA-MB-231 cells in culture were serum starved at 80% confluence for 24 h and treated for a further 1 or 24 h with either human vWf (10 μg/ml), AK2 (10 μg/ml or Tris-saline buffer alone as a control. Cell migration was determined using a 48-well microchemotaxis chamber assay (Neuroprobe, Cabin John, MD) as previously described.[27] Migration was quantified in terms of the number of cells traversing a collagen IV-coated (40 μg/ml) 8 μm-pore polycarbonate membrane (Poretics, Livermore, CA). Briefly, MDA-MB-231 cells, after stimulation, were harvested and resuspended in serum-free RPMI 1640 containing 0.1% BSA to a concentration of 1×10^6/ml. Cells were loaded into the upper chambers with either RPMI/0.1%BSA or RPMI/10%FCS as the

chemoattractant in the lower chamber. Chambers were incubated at 37°C and 5% CO_2 in a humidified incubator for 4 h. After incubation the membranes were removed, fixed and stained with Diff-Quik (Baxter Scientific, IL) and mounted onto glass slides. Non-migrating cells were removed by gentle wiping with a moist cotton swab. Cells having traversed the membrane were counted (at least 5 high-power fields) and each assay was performed in triplicate. Statistical analysis of the results was performed with Prism software (GraphPad, Inc., San Diego, CA) using a one-way analysis of variance (ANOVA) and a Bonferroni post-test. *P* values of <0.05 were considered significant.

RESULTS

Immunocytochemistry and flow cytometry The first of several lines of experimental evidence to demonstrate the expression of a GPIb/V/IX-like complex by malignant breast epithelium is presented in Fig. 1. Immunoperoxidase staining reveals moderate immunoreactivity for GPIbα in 4/4 breast cancer cell lines tested (Fig. 1A). Staining in all cell lines was mainly cytoplasmic and had a slightly punctate distribution. The normal myoepithelial breast cell line 184 demonstrated no staining for GPIbα (data not shown). Immunostaining of 80 primary breast cancers confirmed that the expression of GPIbα was not just an *in vitro* phenomenon, with 51/80 (64%) of specimens showing GPIbα immunoreactivity. Staining was clearly restricted to the malignant epithelium, with the surrounding myoepithelial cells and stroma clearly negative (Fig. 1B). Flow cytometry on unfixed, unpermeabilised tumour cells confirmed that GPIbα is expressed on the surface of the cells (Fig. 2A). When compared with platelets, the monoclonal antibody (mAb) AK2 gave a much

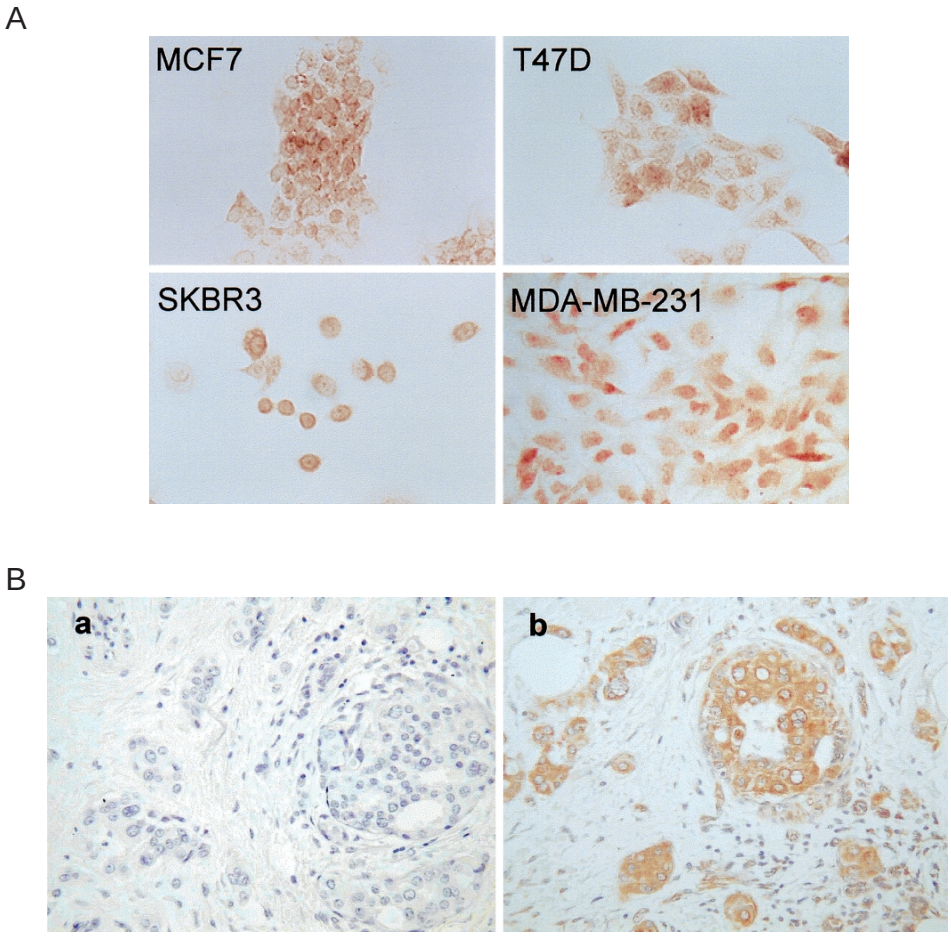

Fig. 1. Immunocytochemical identification of GPIbα in breast cancer. (A) Cultured MCF-7, T47D, SKBR3 and MDA-MB-231 cells all show immunoreactivity when probed with the GPIbα mAb, SZ2, as outlined in "Materials and Methods." (B) Example of immunostaining of an invasive ductal carcinoma of the breast with (a) isotype control mIgG and (b) SZ2, demonstrating GPIbα immunoreactivity in the malignant epithelium.

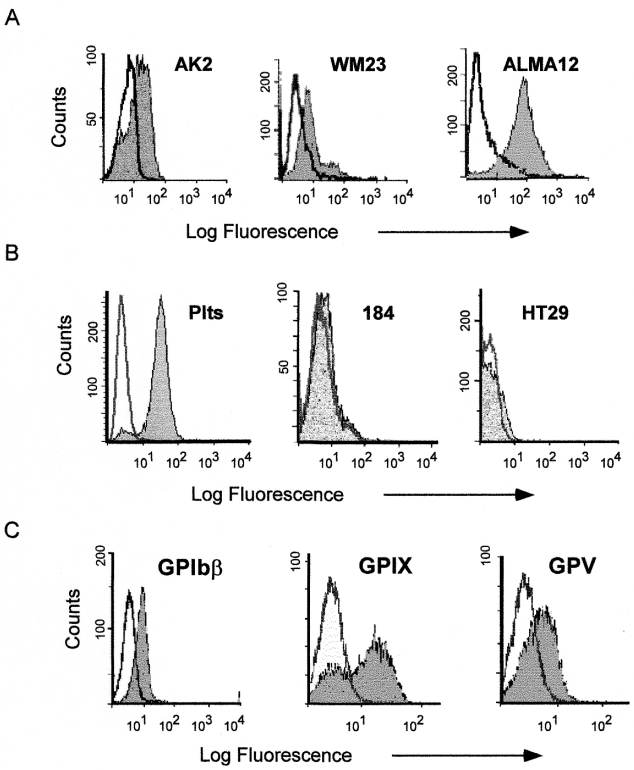

Fig. 2. Human breast cancer cell lines express all subunits of the GPIb/V/IX complex. (A) Flow cytometry histograms of MDA-MB-231 cells, shown as representative of all four breast cancer cell lines tested, demonstrating surface expression of GPIbα. Note the different shifts with the various GPIbα mAb as indicated. AK2 binds at the GPIbα NH_2 terminus within the first leucine-rich repeat at amino acid residues 36–59. WM23 binds within the highly glycosylated macrogylcopeptide domain of GPIbα. ALMA12 also binds at the NH_2 terminus of GPIbα between amino acid residues 1–282. (B) Histograms of controls: platelets, 184 cell line and HT29 colorectal cell line probed with the GPIbα mAb AK2. Platelets showed identical histograms when probed with WM23 and ALMA12 (data not shown). (C) Histograms demonstrating immunoreactivity for GPIbβ (pAb CD42c), GPIX (mAb SZ1) and GPV (mAb SW16) on MDA-MB-231 cells shown as representative of all four breast cancer cell lines. The negative control for each histogram is isotype control immunoglobulin and is represented by the unshaded plot.

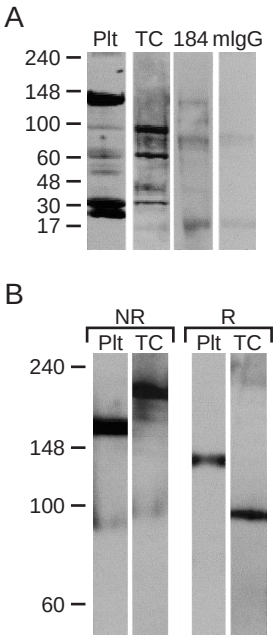

Fig. 3. Characterisation of the tumour-cell GPIb/V/IX by immunoprecipitation and western blotting. (A) Immunoprecipitation of biotin-labeled platelets (Plt), MDA-MB-231 tumour cells (TC) and 184 myoepithelial cell line (184) by the mAb AK2. The molecular weight markers are indicated on the extreme left, and the far right panel shows MDA-MB-231 cells immunoprecipitated with isotype control mIgG (mIgG). (B) Western blot of platelets and MDA-MB-231 lysates corresponding to 1 μg and 200 μg of total protein, respectively. Lysates were run on 8% SDS-PAGE, blotted to PVDF and probed with the mAb WM23. Molecular weight markers are indicated on the extreme left. NR, non-reduced; R, reduced.

reduced reactivity with tumour cells (Fig. 2B). In tumour cells, there was a distinct difference in the fluorescence intensity depending on the epitope specificity of the GPIbα mAb used for staining. In platelets, mean fluorescence intensity was identical irrespective of the GPIbα antibody used (AK2, WM23 or ALMA12; data not shown). This suggests there may be some conformational difference between breast epithelial GPIb/V/IX-like complex and its platelet form. No immunoreactivity for GPIbα was detected on a normal myoepithelial cell line, 184 or a colon cancer cell line, HT29 (Fig. 2B). Flow cytometry performed with mAbs against the GPV and GPIX subunits of the complex (Fig. 2C) confirmed expression of these molecules on the surface of the breast cancer cells. Flow cytometry for the GPIbβ subunit was performed using a polyclonal antibody raised against the intracellular portion of the complex. Surface expression of GPIbβ can be inferred, however, by the binding of the conformation-sensitive mAb SZ1, which only recognises the GPIX subunit when it is correctly complexed with GPIbβ.[28] The data shown in Fig. 2 are representative of results seen with other breast tumour cell lines MCF-7, T47D and SKBR3, which all showed comparable levels of GPIb/V/IX. Although the magnitude of variation between GPIbα expression was small, the cell line MDA-MB-231 demonstrated the highest levels of expression, and therefore more detailed characterisation of the immunorelated GPIb/V/IX was performed on this cell line.

Immunoprecipitation and western blotting To identify

the immunoreactive surface protein, immunoprecipitation was performed with biotin surface-labeled platelets, MDA-MB-231 cells and a normal breast myoepithelial cell line, 184 (Fig. 3A). Under reducing conditions the mAb AK2 immunoprecipitates platelet proteins of apparent molecular weights 135, 30 and 25 kDa, which correspond to the GPIbα, GPIbβ and GPIX subunits, respectively. Minor reactive bands at 95, 60 and 50 were also observed in the platelet immunoprecipitates and represent proteolytic degradation products of GPIbα. In the MDA-MB-231 cells, major reactive bands were detected at approximately 95, 60 and 30 kDa with a minor reactive band at 45 kDa. No specific immunoreactive bands were detected in the 184 cell line, or when the MDA-MB-231 cells were immunoprecipitated with isotype control mIgG. Western blotting of total protein lysates from platelets and MDA-MB-231 with the mAb WM23 provides evidence that the tumour-cell immunoreactive band at 95 kDa is a form of GPIbα (Fig. 3B). In order to visualise this immunoreactive band, considerably more protein had to be loaded from MDA-MB-231, indicating a lower level of expression than in platelets, in agreement with the lesser shift seen with this mAb in flow cytometry. Interestingly, the western blot under non-reducing conditions shows a band of approximately 200 kDa for MDA-MB-231 as compared to 170 kDa for platelets. The difference in protein size under both non-reducing and reducing conditions suggests that members of the complex, other than GPIbα, may have different sizes to those observed in platelets. The larger immunoreactive bands seen in the immunoprecipitation of MDA-MB-231 would support this hypothesis.

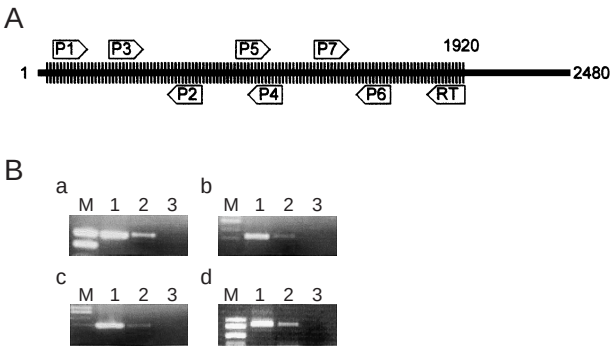

Fig. 4. Molecular characterisation of tumour-cell GPIb/V/IX. (A) Schematic diagram of the gene coding for GPIbα, showing the coding region (shaded) and the relative positions of primer pairs. (B) Amplicons generated by PCR using primer pairs P1/P2 (a), P3/P4 (b), P5/P6 (c) and P7/GPIbRT (d). In all gels: M, molecular size standard; 1, CHO-α; 2, MDA-MB-231 cDNA; and 3, MDA-MB-231 RNA with no RT.

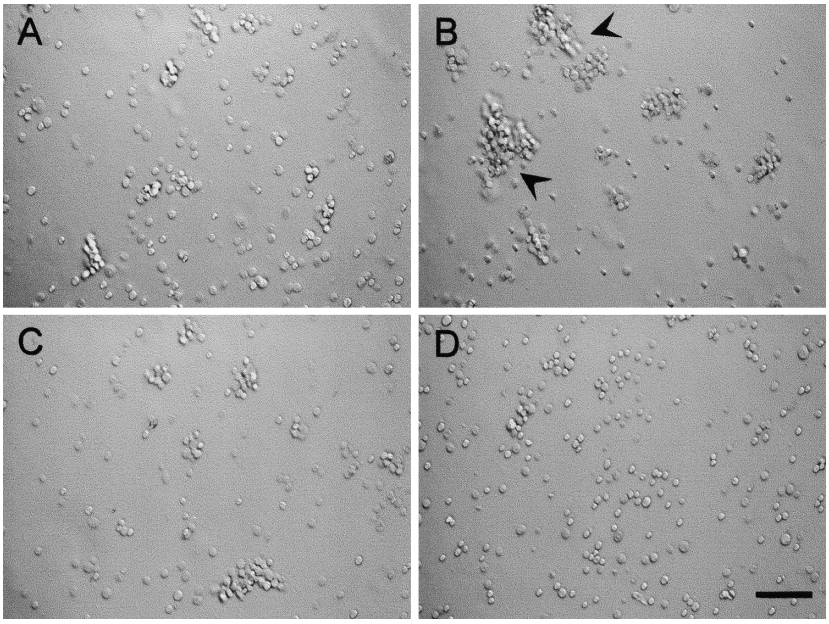

Fig. 5. vWf induced MDA-MB-231 aggregation. MDA-MB-231 cells were treated with 20 μg/ml vWf in the presence of 1 mg/ml ristocetin as outlined in "Materials and Methods" and suspensions photographed after 10 min. (A) Control untreated cells incubated in the absence of vWf. (B) Cells incubated with vWf and ristocetin showing larger aggregates (arrows) and fewer single cells than the control. (C) As in (B) with the addition of the anti-vWf A1 domain mAb, 5D2. (D) As in (B) except cells were preincubated with the GPIbα cleaving protease, Mocarhaggin. Note the absence of aggregates in (D). Scale bar in D=100 μm (A–D).

RT-PCR and DNA sequencing Consistent with the relatively low levels of protein expression, GPIbα mRNA could not be detected in this study by northern blot (data not shown), but was detected by RT-PCR, as shown in Fig. 4. The entire mRNA sequence for GPIbα is 2480 bp and is shown schematically in Fig. 4A. The sequence that corresponds to the mature GPIbα polypeptide is contained within this 2480 bp from 90–1920. We designed oligonucleotide primer pairs to overlap, and span this coding region (Fig. 4A). Using total RNA extracted from MDA-MB-231, all primer pairs amplified a specific product of the expected size, as did the positive control (CHO-α cells). The amplicons were confirmed to be derived from specific mRNA, rather than contaminating genomic DNA, as no amplification was observed without the RT step. All PCR amplicons were gel purified and sequenced directly using the automated ABI prism sequencer. We found that the coding region (between 137–1920) of the mRNA of tumour cell GPIbα was 100% identical to the published sequence for platelet GPIbα (GenBank Accession # J02940).

Tumour-cell GPIb/V/IX complex functionality To examine the functionality of the tumour cell GPIb/V/IX-like complex as a receptor for vWf we performed vWf-induced aggregation studies as outlined in "Materials and Methods." Unless otherwise indicated, all aggregation and binding studies were conducted in the presence of EDTA and EGTA to abolish the divalent-cation dependent vWf-binding RGD motif of the vitronectin receptor (integrin αvβ3). MDA-MB-231 cells formed aggregates when treated with physiological concentrations of purified human vWf in 1 mg/ml ristocetin when compared to untreated cells (Fig. 5, A and B). No aggregation was observed with ristocetin alone (data not shown). Pretreatment of the cells with mAb AK2 against the vWf binding domain of GPIbα did not inhibit this aggregation (data not shown), but moderate inhibition was achieved by addition of the mAb 5D2 (Fig. 5C). This antibody is directed against the GPIbα binding site in the A1 domain of human vWf. Complete inhibition of aggregation was achieved by

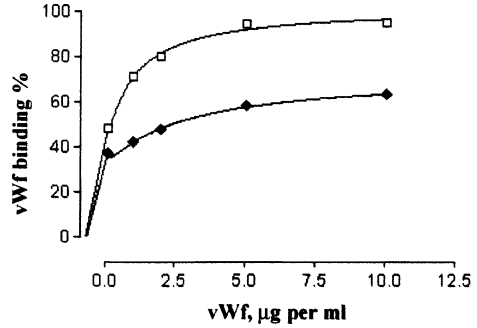

Fig. 6. Inhibition of binding of vWf to tumour cells by anti-GPIbα monoclonal antibody SZ2. Washed MDA-MB-231 cells were incubated with 20 μg/ml SZ2 or isotype control mIgG. SZ2 maps to the anionic sulfated region bounded by amino acid residues 276–282. Blocked tumour cells were then incubated with 0.1–10 μg/ml human vWf-Alexa and 1 mg/ml ristocetin, in the presence of EGTA and EDTA, as outlined in "Materials and Methods." Bound vWf-Alexa was measured by flow cytometry. The curve represents the best fit of the data to the rectangular hyperbolic binding equation by nonlinear regression. The apparent dissociation constant and maximal binding of vWf were 0.9±0.1 μg/ml and 85±6% in the presence of control IgG (□) and 2.7±1.1 μg/ml and 53±5% in the presence of 20 μg/ml SZ2 (◆).

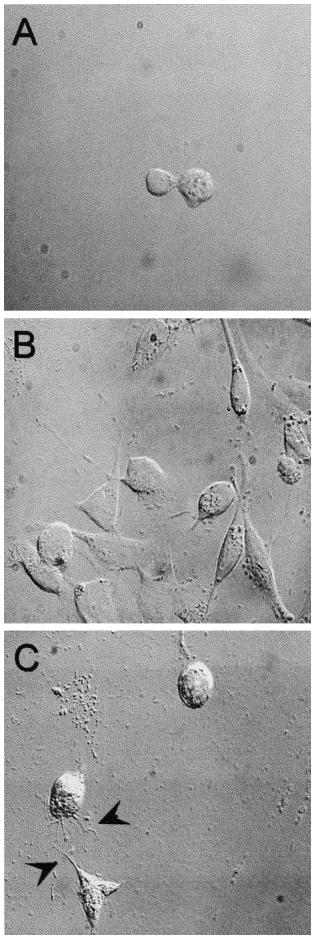

Fig. 7. MDA-MB-231 adhesion to immobilised vWf. Washed MDA-MB-231 cells were allowed to adhere to coverslips coated with either fibrinogen or vWf, in the presence of calcium or EDTA/EGTA, for 60 min at 37°C. (A) Very few cells remain adherent to fibrinogen in the presence of EDTA/EGTA and they present a very round morphology. (B) Cells spread readily on vWf in the presence of calcium. (C) Cells that remain attached to vWf in the presence of EDTA/EGTA demonstrate prominent filapodial extensions indicative of actin reorganisation.

pretreatment of the cells with the GPIbα-specific protease[29] Mocarhaggin (Fig. 5D).

GPIbα-specific vWf binding to MDA-MB-231 was also assessed using purified vWf labelled with the fluorochrome Alexa. Washed MDA-MB-231 cells were preincubated with either isotype control mIgG or mAb SZ2. SZ2 binds to the sulfated tyrosine residues of GPIbα, identical to the cleavage point of Mocarhaggin. Cells were then incubated with 0.1 to 10 μg/ml of Alexa-vWf in the presence of EDTA, EGTA and 1 mg/ml ristocetin, and the resultant binding was measured by flow cytometry. Slight aggregation was noted in both mIgG control and SZ2 reactions, but this was easily disrupted with gentle pipetting. MDA-MB-231 tumour cells bound human vWf in a dose-dependent, saturating manner (Fig. 6) and binding could be inhibited by up to 36% by the GPIbα mAb SZ2. The apparent dissociation constant of vWf in the absence of GPIbα inhibition was 0.9±0.1 μg/ml. This corresponds to a molar dissociation constant of approximately 0.45 n*M*, which is in good agreement with the estimates for vWf binding to platelet GPIbα (ref. 30) and references cited therein).

MDA-MB-231 cells were also examined for their adherence to immobilised vWf (Fig. 7). On vWf in the absence of EDTA and EGTA, many tumour cells were noted to have attached and spread, presumably a reaction mediated by calcium-dependent integrins. When the cells were placed on vWf in the presence of EDTA and EGTA fewer cells remained bound than in the presence of calcium; however, those that did remain adherent demonstrated prominent filapodial extensions, reminiscent of activated platelet morphology and demonstrative of cytoskeletal reorganisation.

Tumour cell migration assay The mAb AK2 binds to the N-terminus of platelet GPIbα within the first leucine-rich repeat domain, and is a potent inhibitor of vWf binding to platelet GPIbα. However, AK2 was not able to block vWf induced aggregation and adhesion of MDA-MB-231 cells. In the standard Boyden chamber assay, the inclusion of vWf in the matrix resulted in increased numbers of MDA-MB-231 migrating through the matrix, but this could not be inhibited by AK2 (data not shown). Considering these findings, we hypothesised that AK2 may be activating the cells to a more motile, migratory phenotype, acting on tumour cell GPIbα in a manner analogous to a vWf ligand. MDA-MB-231 cells that had been treated with human vWf or AK2 were assessed for their migratory potential in a standard 4-h chemomigration assay. Cells that had traversed a collagen-coated membrane were stained and counted (Fig. 8). Exposure to either vWf or AK2 led to a significant increase in the number of cells migrating across the membrane, when compared to control conditions, suggesting that AK2 was acting on the cells to upregulate motility in the same way as vWf.

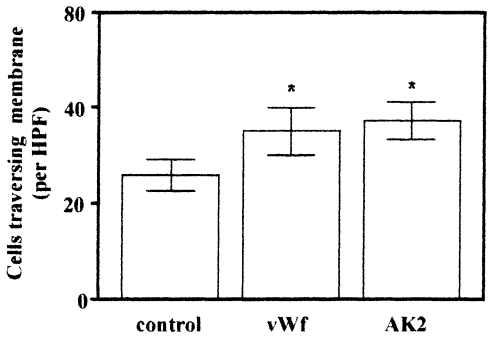

Fig. 8. vWf stimulation causes upregulation of MDA-MB-231 migration. Analysis of the migratory response of MDA-MB-231 to stimulation with vWf was performed in a 48-well microchemotaxis chamber as outlined in "Materials and Methods." Cells were stimulated with human vWf or the mAb AK2 1 or 24 h prior to the migration assay. Cells stimulated with Tris buffer only were used as a control. The data presented are expressed as the mean of cell counts taken from 15 high-powered fields (five fields from each replicate well). The results are composite of both time points, and are representative of two experiments performed in triplicate. The asterisk denotes statistical significance when compared to the control ($P<0.001$; one-way ANOVA, Bonferroni's method).

DISCUSSION

The data presented in this study clearly indicate that malignant human breast epithelial cells express a GP complex closely related to the GPIb/V/IX complex of platelets. Using a panel of monoclonal and polyclonal antibodies and flow cytometry, we have shown proteins immunologically related to the four subunits of GPIb/V/IX on four different breast cancer cell lines. We found no evidence of GPIbα on the colon carcinoma line HT29, or on the normal breast myoepithelial cell line 184. Immunohistochemistry performed by us (manuscript in preparation), and another investigator,[20] on human breast carcinoma tissue confirms that the expression of a GPIb/V/IX-like complex on malignant breast epithelium is also an *in vivo* phenomenon, and thus it may have important implications for the metastatic process. The clinical significance of GPIb/V/IX-like complex expression by human breast cancers in terms of disease progression and outcome is presently being investigated in our laboratory (manuscript in preparation).

Although all 4 individual subunits of GPIb/V/IX are present on the surface of breast cancer cells, the immunologically related GPIb is of discrepant molecular weight. Platelet GPIb(α+β) has an apparent molecular weight of 170 kDa, with approximate molecular weights for the individual disulfide-bonded subunits of 135 and 25 kDa for α and β, respectively. Under non-reducing conditions, the tumour cell-related GPIb appears to be 200 kDa in size,

and the GPIbα constitutes 95 kDa of this molecular weight as assessed by western blot under reducing conditions. The other bands immunoprecipitated with GPIbα mAbs were 60 and 30 kDa, which we believe may represent immunorelated GPIbβ and GPIX, respectively. This is not an unreasonable assumption as the GPIbβ subunit on endothelial cells has recently been shown to be larger than the platelet form (48 kDa vs. 25 kDa) in the absence of a size discrepancy with GPIX.[15] Considering that in platelets the GPIbα and GPIbβ subunits are disulfide-bonded, the additional molecular weight contributed by a 60 kDa GPIbβ would not quite account for the 200 kDa band seen in the western blot under non-reducing conditions. Considering that the protein is approximately half the molecular weight under reducing as compared with non-reducing conditions raises the possibility that tumour cell GPIbα may be forming a disulfide-linked homodimer. This may better explain the size discrepancy on SDS-PAGE.

An immunologically related GPIbα molecule with an apparent molecule weight of 60 kDa has been isolated from HEL cells.[31] Subsequent sequencing of this protein[32] revealed that it corresponded to an unglycosylated form of GPIbα. One other group has reported an immunorelated GPIbα on a breast cancer cell line, MCF-7, with a molecular weight similar to that reported here.[21, 33] In their studies on MCF-7 breast cancer cells, Oleksowicz *et al.* report the finding of two specific GPIbα transcripts on northern blot analysis,[21] which is unusual considering the entire coding sequence for GPIbα is encoded by a single exon. The authors proposed that the additional larger transcript they observed could account for the discrepancy in GPIbα molecular weight. We were unable to detect any specific mRNA transcripts by northern blot, supportive of our findings of lower levels of protein observed in tumour cells, compared with platelets. We were, however, able to amplify the coding region of GPIbα mRNA using RT-PCR. Sequencing of the PCR products demonstrated that the tumour cell GPIbα was identical to the reported platelet GPIbα sequence, a finding which is inconsistent with the notion that the protein size difference has a genetic origin.[21] We suggest that the 95-kDa band we consistently observed in our immunoprecipitations and western blots represents different post-translational processing of tumour cell GPIbα versus the platelet molecule.

Platelet GPIbα is quite sensitive to proteolysis, therefore the possibility that the 95-kDa tumour cell protein is a degradation product cannot be dismissed. In the absence of protease inhibitors, platelet GPIbα readily hydrolyses to produce a protein with this molecular weight, termed macroglycopeptide.[34] It is possible that the tumour cell GPIbα is more proteolytically sensitive than its platelet counterpart, and readily yields the macroglycopeptide fragment, even in the presence of standard concentrations of protease inhibitors.

As is the case with GPIb/V/IX on endothelial cells,[14] we have found the difference in size, and potential difference in conformation, does not alter its ability to bind vWf. Indeed recent evidence demonstrates that GPIb/V/IX on endothelial cells is in part responsible for cell migration.[35] This study clearly demonstrates that the tumour cell GPIb/V/IX-like complex is capable of binding vWf under static conditions and this binding mediates tumour cell aggregation. It is not unreasonable to speculate that tumour cell GPIb/V/IX-like complex could facilitate metastasis by allowing circulating cancer cells to bind plasma vWf and enhance their interaction with platelets, resulting in an arrested tumour cell-platelet emboli.

In addition, it may be possible that tumour cell GPIb/V/IX-like complex is involved in direct interaction with subendothelial vWf once a tumour cell embolus has lodged in the vasculature. In platelets, GPIb/V/IX is structurally and functionally linked to the actin cytoskeleton through direct linkage to ABP and actin tubules.[9] Recent studies have shown that this association is also present in CHO cells transfected only with the GPIb/V/IX complex.[36] The filapodial extension seen in static vWf adhesion assays presented here is definitive evidence of cytoskeletal reorganisation. This would suggest that the tumour cell GPIb/V/IX-like complex shares a similar association with the cytoskeleton to that of platelet GPIb/V/IX. The close association of GPIb/V/IX with actin and ABP would make it a good candidate for a tumour cell receptor involved in the migration of cells. Migration assays performed in this study, and those on vascular endothelial cells expressing GPIb/V/IX[35] support this hypothesis. Using a standard chemomigration assay we have shown that prior exposure to vWf induces a more migratory phenotype. This suggests that circulating tumour cells' motility is upregulated in response to the initial binding of vWf. It can be argued that other receptors, primarily the vitronectin receptor (αvβ3 integrin), could be responsible for these observations. Unfortunately, the chemomigration assay could not be performed in the presence of calcium chelators so the involvement of αvβ3 cannot be ruled out. However, surprising results were found when AK2, a monoclonal antibody against the vWf binding domain of GPIbα, was used in the same assay. Because AK2 could not inhibit vWf stimulated invasion, and in fact appeared to augment it (data not shown), we used it as a prior stimulator and found that AK2 alone could increase the migratory potential of the tumour cells. We believe these results suggest AK2 binds to GPIbα on tumour cells and mimics vWf as a ligand to induce the same signaling pathway, resulting in increased cell motility. One mAb directed against the vWf binding domain of GPIbα has been reported to activate platelets and cause small aggregate formation, and intracellular signaling[37] similar to that seen with vWf-stimulated platelets.[38] It may be that an altered

conformation of the complex due to aberrant glycosylation is allowing AK2 to elicit the same response in tumour cells via GPIbα.

GPIb/V/IX expression has always been thought to be restricted to megakaryocytes and platelets. Recently it has become accepted that this molecule also resides on vascular endothelial cells, although its function here remains an enigma. Even more recently, the complex has been described on dermal dendrocytes where again its function is unknown.[19] In this study we present evidence that a GPIb/V/IX-like complex is also expressed by breast carcinomas, and functions as a receptor for vWf, capable of inducing behavioural and morphological changes of the cell.

Although the process of metastasis is complicated and multifactorial, our studies lead us to hypothesize that tumour-associated GPIb/V/IX-like complex may be involved in several steps of the metastatic cascade, and is clearly deserving of further study.

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