The platelet antigens CD9, CD42 and integrin α1Ibβ3 can be topographically associated and transduce functionally similar signals

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Investigation of the specific effects of different mAb known to stimulate platelets (agonist mAb) is complicated by interaction of the Fc portion of these mAb with the platelet FcγRII. This has led to the conclusion that nearly all agonist-mAb-induced activation of platelets is mediated by this receptor. However, the target antigen-mediated signal can be analysed provided that the effects of FcγRII engagement can either be reduced or eliminated. We have therefore blocked platelet FcγRII with IV.3 Fab fragments (an anti-FcγRII mAb), and stimulated the platelets by cross-linking intact agonist mAb with F(ab')2, fragments of an Fc-specific anti-mouse antibody. By analysing functional platelet responses and protein-tyrosine phosphorylation, we found that such non-FcγRII-mediated cross-linking of CD9, CD42 and glycoprotein (gp) Ibα/IIa generates closely similar signals. Since this may indicate molecular associations, we analyzed the surface topography of platelets using the chemical cross-linking agent dithiobis(sulfosuccinimidyl propionate). We found that a proportion of CD9, gpIbα/IIa and CD42 molecules associate with each other on the platelet surface membrane. Thus, our results suggest that these antigens are able to form a larger molecular complex and induce similar signals. Furthermore, cross-linking of CD9 and CD42 stimulated thrombosthenic platelets completely lacking gpIbα/IIa. These data therefore indicate that CD9 and CD42 can signal independently of gpIbα/IIa, and that signals generated by all these molecules may converge on a common pathway.

Keywords: glycoprotein Ib; integrin; CD9; transmembrane-4 superfamily; signal transduction.

Many cell receptors are now known to be multicomponent complexes involving ligand-binding and signalling proteins. Members of the transmembrane-4 superfamily (or tetraspan superfamily) of membrane proteins, which includes CD9 (Boucheix et al., 1991), are frequently found within such complexes, where they are thought to participate in receptor signalling (reviewed by Wright and Tomlinson, 1994). The B-cell and T-cell antigen receptors are particularly good examples of such multicomponent structures (Matsumoto et al., 1993; Imai et al., 1995).

Integrin heterodimers can form complexes with other membrane proteins, including a transmembrane-4 protein, CD9 (Slupsky et al., 1989; Rubinstein et al., 1994; Nakamura et al., 1995). Thus, in stimulated platelets, the principal integrin, glycoprotein (gp) Ibα/IIa (also known as α1Ibβ3, CD41/CD61), has been shown by chemical cross-linking to be associated with CD9 (Slupsky et al., 1989). In addition, there is evidence that gpIbα/IIa and gpIbα/IX [CD42b/CD42a; a receptor for von Willebrand factor and thrombin (Roth, 1991; Granli et al., 1994)] may also be associated (Fox, 1985; Davies and Palek, 1982; Jung and Moroi, 1983).

These observations raise the possibility that gpIbα/IIa, gpIbα/IX and CD9 can all associate into multicomponent adhesion/signalling receptor complexes. In the present paper, we provide evidence from mAb-probing and chemical-cross-linking studies that CD9, gpIbα/IX and gpIbα/IIa can form such complexes. Furthermore, by using thrombosthenic platelets, we show that signals generated by antibody-induced cross-linking of CD9 and gpIbα/IX are independent of the presence of gpIbα/IIa.

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MATERIALS AND METHODS

Materials. BSA (Cohn Fraction V), prostaglandin E1, Hepes, fibrinogen, thrombin, phosphotyrosine agarose (PT66-agarose), Staphylococcus aureus V8 protease, and papain were from Sigma. Dithiobis(sulfosuccinimidyl propionate) (DTSSP) cross-linking agent was from Pierce Chemicals. Protein-G-Sepharose was from either Pharmacia or Zymed. Anti-glycocalcin mAb (AN-51) was from DAKO. LeuA1 [IgG, against the 67-kDa platelet surface antigen platelet and T-cell antigen 1 (PTA1); Kd = 1.2 nM, 1200 binding sites/platelet (Scott et al., 1989)], NEWEI [IgG, against denatured PTA1] and IA7 (IgG, against CD36) were kind gifts from Dr G. Burns (Newcastle, Australia). IV.3 [IgG2a, against FcγRII (CD32); Kd = 0.6 nM (McCrue et al., 1990)] was purchased from Medarex, or purified from the supernatants of cultured hybridoma cells #ATCC-HB
217 (American Type Culture Collection, USA). SYB-1 (IgG, against CD9) was a kind gift from Dr C. Boucheix (Institut National de la Santé et de la Recherche Médicale, Paris). PL2-49 (IgG, against gp11b) was a kind gift from C. Kapian (Paris). SZ-2 (IgG, against gpIIb, CD42) was purchased from Immunotech S. A. 5-Hydroxy[14C]tryptamine was purchased from Amersham. F(ab)2), fragments of goat anti-mouse IgG (Fc specific) antibodies were purchased from Jackson Immunoresearch. Horse-saradish peroxidase-conjugated anti-phosphotyrosine mAb PY-20 was purchased from ICN biomedicals.

Preparation of IV.3 Fab fragments. Fab fragments of IV.3 were prepared by papain digestion of whole IgG (Roussseau et al., 1986) as follows. Cysteine (0.01 M) was added to a 1 mg/ml solution of mAb (1 ml) and incubated for 30 min at 37°C. 10 U of papain-agarose (Sigma) were added and the mixture incubated for 4 h at 37°C with constant agitation. The mixture was centrifuged at 12000 × g for 30 s, and the supernatant containing digested mAb was drawn off and dialysed overnight against 137 mM NaCl, 2 mM KCl, 1.5 mM KH2PO4, 6.5 mM Na2HPO4, pH 7.4 (Na-Cu/P), mAb Fab fragments were separated from undigested mAb and mAb Fe fragments by means of protein-A-Sepharose. The purity of the Fab-fraction preparation was assessed by SDS/PAGE (data not shown). Antibody binding was checked by the ability of the IV.3 Fab to block platelet aggregation induced by cross-linked-intact IV.3 (data not shown).

Preparation of washed platelets. Whole venous blood was obtained by voluntary donation from healthy laboratory personalities. The blood was anticoagulated by the addition of a 0.1 vol. 3.8% sodium citrate and centrifuged at 150 × g for 10 min to obtain platelet-rich plasma. 2 mM EGTA was added to platelet-rich plasma, which was carefully layered on top of a discontinuous albumin-density gradient composed of 25% and 34% BSA dissolved in 138 mM NaCl, 2.9 mM KCl, 1 mM MgCl2, 1 mM glucose, 0.5 mM Na2HPO4, 20 mM Heps, pH 7.4 (buffer A) and centrifuged for 30 min at 750 × g. The platelet band at the interface between the two albumin layers was removed and transferred into buffer A containing 0.3% BSA by column chromatography on a Sepharose 2B column (1 cm × 20 cm). After elution, the concentration of the washed platelets was assessed turbidimetrically and adjusted to 3 × 10^9 platelets/ml (Mustard et al., 1989; Timmons and Hawiger, 1989).

Analysis of the platelet response to antigen-specific cross-linking. Washed platelets were equilibrated at 37°C and incubated with 20 μg/ml IV.3 Fab for 5 min. Intact mAb (either 2 μg/ml LEO-A1 (anti-PTA1), 5 μg/ml SYB-1 (CD9), 2 μg/ml SZ-2 (gpIIb, CD42), 2 μg/ml IA7 (CD36), or 5 μg/ml PL2-49 (gpIIb/IIIa complex)) was added, followed by addition of 30 μg/ml (Fc-specific) anti-mouse IgG antibody F(ab)2, fragments 2 min later. Platelet aggregation was monitored, and/or platelets were used for the analysis of either dense-granule release or protein-tyrosine phosphorylation.

Platelet aggregation was measured in an aggregation module (Racettech) at 37°C and a constant spin rate of 900 rpm. The platelet suspension was allowed to equilibrate to the conditions of the aggregometer for 3 min before the addition of agonist. Aggregation was recorded as increases in light transmission against time.

Measurement of platelet-dense-granule release. Platelets in plasma were incubated with 5-hydroxy[14C]tryptamine (specific activity = 50 μCi/mmol; 1 nmol/10^9 platelets) for 30 min at 37°C. For the secretion assay, the platelets (3 × 10^9 platelets/ml) were incubated in the aggregometer at 37°C in the presence of agonist under stirred conditions. After 2 min of activation, the platelets were fixed by addition of an equal volume of ice-cold 2% paraformaldehyde in NaCUP, and kept on ice until the end of the experiment. These fixed platelets were centrifuged for 2 min at 12000 × g in a microcentrifuge, and a 400-μl aliquot of the supernatant was taken for scintillation counting (Holmsen and Dangelmaier, 1989).

Measurement of platelet-α-granule release. Platelet-α-granule release was measured by fluorescence-activated cell sorting (FACS) analysis of GMP-140 (CD62, P-selectin) expression with a fluorescein-isothiocyanate (FITC)-conjugated anti-GMP-140 mAb (Immunotech). Prior to the analysis, activated or resting platelets were fixed in 1% paraformaldehyde for at least 1 h on ice. The cells were washed three times with NaCl/P, then incubated with 50-fold-diluted FITC-conjugated anti-GMP-140 Ig at 4°C for at least 30 min. The GMP-140 expression on platelets was determined by means of a Becton Dickinson FACSort.

Analysis of tyrosine phosphorylation. Washed platelets were incubated for 4 min at 37°C under stirred conditions in the presence or absence of agonist, then lysed with an equal volume of cold 2% Triton X-100, 2% sodium deoxycholate, 0.2% SDS, 300 mM NaCl, 50 mM Tris, pH 7.4, 10 mM EDTA, 60 mM sodium pyrophosphate, 100 mM NaF, 200 μM Na3VO4, and 1 μg/ml each of antipain, leupeptin, chymostatin and pepstatin (buffer B) (Huang et al., 1991). Lysis was allowed to proceed on ice for at least 30 min and the samples were cleared by centrifugation at 16000 × g for 30 min. The supernatants were immunoprecipitated with anti-phosphotyrosine—agarose at 4°C for at least 2 h.

The agarose beads were washed once with 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 25 mM Tris pH 7.4, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na3VO4, and boiled in 2× SDS/PAGE sample buffer for 5 min. The immunoprecipitated platelet proteins were separated by SDS/PAGE and electrophoreted onto Immobilon (Millipore) membrane using 192 μM glycine, 25 mM Tris, pH 8.3 (Huang et al., 1991). The residual protein-binding capacity of the Immobilon membrane was saturated with a solution of 3% gelatin in 100 mM NaCl, 20 mM Tris, pH 7.2, 0.05% Tween-20, 0.05% Nonidet P-40 (buffer C) at 37°C for 1 h. The blocked membrane was washed twice with buffer C and incubated at room temperature for 2 h with horse-saradish-peroxidase-conjugated anti-phosphotyrosine mAb PY-20 (0.1 μg/ml) in buffer C containing 0.5% gelatin. The membrane blot was washed five times with buffer C, developed with enhanced chemiluminescence reagents (Amersham) and recorded on Hyperfilm.

SDS/PAGE analysis. SDS/PAGE analysis of proteins was performed according to the procedure of Laemmli (1970) with single-concentration or gradient polyacrylamide gels.

Radioiodination of platelet membrane proteins. Platelet-rich plasma was prepared from fresh citrated venous blood as described, mixed with prostaglandin E1 (to 1 μg/ml) and EGTA (to 5 mM), and centrifuged at 750 × g for 10 min. The platelets were suspended in 2 ml buffer A and radioiodinated by the lactoperoxidase technique (Slupska et al., 1993) as follows. Lactoperoxidase (to 0.2 mg/ml) was added to the platelet suspension, followed by 1 μCi Na125I. The reaction was initiated by the addition of 40 μl 0.12% (by vol.) H2O2. The iodination was allowed to proceed at room temperature for 15 min, followed by the addition of 40 μl 0.12% H2O2, and incubation for 15 min. EGTA and prostaglandin E1 were added, and the platelets were separated from unbound 125I by means of a discontinuous BSA-density gradient, followed by gel filtration on Sepharose 2B. The final platelet suspension was in buffer A.

Chemical cross-linking of platelet membrane proteins. Platelets (4×10^9/ml) suspended in buffer A were incubated at 37°C for 15 min before the treatment with either cross-linking agents (0.5 mg/ml DTSSP) or dimethyl sulfide (Me2SO) (as
carrier) at 37°C for 15 min. With respect to the usage of Me₃SO in these experiments, the final concentration never exceeded 1% of the final volume of the platelet suspension. This was followed by brief centrifugation (3000 x g for 60 s) and suspension of the platelet pellet in buffer A. Platelets were subsequently lysed with an equal volume of buffer B for 30 min on ice (see above). The platelet lysates were centrifuged (12,000 x g) at 4°C for 30 min, the supernatants were cleared with protein-G-Sepharose, followed by immunoprecipitation of platelet proteins with mAb which had been adsorbed onto protein-G-Sepharose. The immunoprecipitated proteins were separated by SDS/PAGE. Gels containing proteins that had been radioiodinated were autoradiographed.

**FACS analysis of platelet proteins after chemical cross-linking.** Platelets that had been treated with either dithiobisis(succinimidyl propionate) (DSP) or DTSSP were washed once, then incubated with selected mAb for 1 h at 22°C without agitation. The platelets were fixed with 1% paraformaldehyde on ice for at least 30 min, followed by washing three times with NaCl/P. The platelets were incubated with FITC-conjugated goat anti-mouse IgG (Becton Dickinson; 1:1000 diluted in NaCl/P) for 30 min at 4°C, and analyzed by means of a FACSsort.

**Limited proteolytic digestion of proteins.** The regions of the dried gel corresponding to CD9, gpIib, and those proteins that had the same mobility as CD9 on SDS/PAGE were rehydrated for 30 min in 0.1% SDS, 1 mM EDTA, 12.5 mM Tris, pH 6.8. The rehydrated gel slices were transferred to a 15% polyacrylamide/SDS gel, and digested with 200 μg/ml protease (either S. aureus V8 protease for CD9, or papain for gpIib) during electrophoresis (Slupsky et al., 1993).

**RESULTS**

CD9, CD41 and CD42 mAb induce platelet aggregation independently of FcγRII binding. Platelet stimulation by mAb generally requires Fc-FcγRII interaction (Morel et al., 1989; Aiken et al., 1990; Worthington et al., 1990; Rubinstein et al., 1991a; Zuzel et al., 1991). Therefore, when mAb are used as probes of target antigens, FcγRII signalling has to be taken into account. However, previous work by us and others has demonstrated that the target antigens of several mAb themselves have active signalling roles (Morel et al., 1989; Slupsky et al., 1992; Griffith et al., 1991; Alessio et al., 1993), and that in the generation of an antigenic signal, FcγRII has an anchorage or cross-linker function (Slupsky et al., 1992; Anderson et al., 1991; Rubinstein et al., 1991b; Horsewood et al., 1991). Therefore, to avoid signalling associated with FcγRII engagement, it is necessary to block this receptor and to provide an alternative cross-linking of antigen-bound mAb. In this study, we have blocked FcγRII by IV3 Fab fragments and cross-linked antigen-bound mAb with F(ab')₂, fragments of Fc-specific goat anti-mouse Ig.

Such FcγRII-independent cross-linking of either SYB-1 (CD9), PL2-49 (gpIib/IIa), or SZ-2 (gpIib) on washed platelets resulted in variable degrees of partial platelet aggregation (Fig. 1A). In contrast, non-FcγRII-mediated cross-linking of either of two other platelet mAb (L0eA1 against PTA1 and IA7 against CD36) did not induce platelet aggregation, although with L0eA1 the changes in light transmission indicated that the platelets changed their shape.

The aggregation responses to cross-linked CD9 or CD42 mAb observed under the above experimental conditions were independent of gpIib/IIa because similar responses were observed with platelets from a patient with Glanzmann’s thrombasthenia (Fig. 1B), who completely lacked this principal platelet integrin. Moreover, the platelet response to cross-linked CD9 (Fig. 2A) or CD42 (data not shown) mAb was not inhibited by RGDS peptides confirming that the induced aggregation was not integrin mediated.

This apparent independence of gpIib/IIa raises the possibility that the observed aggregation was passive, antibody-mediated platelet agglutination. However, although raising intracellular cAMP by prostaglandin E₁ had no effect on CD9-induced aggregation (Fig. 2A), this aggregation was inhibited by cyto-
Fig. 2. Effects of various pharmacological agents on platelet aggregation in response to FcγRII-independent cross-linking of CD9 and CD42. Washed platelets (3 × 10⁷/ml) from normal donors were incubated with (A) 10 µg/ml prostaglandin E₁ (PGE₁) (2 min) or 0.5 mM RGDS peptide (2 min), or (B) 20 µg/ml cytochalasin B (10 min) prior to the addition of IV.3 Fab (a), mAb, and cross-linking Ab (b) as described. These results are representative of three separate experiments with different donors.

Fig. 3. CD62 expression of platelets in response to FcγRII-independent cross-linking of signalling antigens. Washed platelets from (A) normal donors, or (B) a thrombasthenic donor were stimulated for 2 min with the indicated mAb, fixed with paraformaldehyde, and probed for CD62 expression by FACS analysis with an FITC-conjugated CD62 mAb as described in Materials and Methods. Results are presented as relative cell number versus fluorescence intensity.

The aggregation response of platelets to cross-linked CD9 and CD42 mAb is accompanied by α-granule but not dense-granule release. Since platelet aggregation is often preceded or accompanied by secretion, the responses of Fc-receptor-blocked platelets to cross-linked mAb were examined for evidence of granule release. None of mAb employed in the present study (against CD9, CD41, CD42, PTA1 and CD36) induced dense-granule release from 5-hydroxy[¹⁴C]tryptamine-labelled platelets (data not shown). In contrast, when platelet expression of CD62 was examined to assess α-granule release, it was observed that the cross-linking of mAb against CD9, CD42 or CD41 caused an increase in CD62 expression, whereas cross-linking of anti-PTA1 or anti-CD36 mAb did not (Fig. 3A). Furthermore, with the exception of cross-linked CD41 (gpIIb/IIIa) mAb, similar results were observed with thrombosthenic platelets (Fig. 3B).

These results indicate that specific α-granule release accompanies the platelet aggregation induced by cross-linking mAb against CD9, CD41 and CD42. Moreover, like the induction of platelet aggregation, this process for CD9 and CD42 does not require gpIIb/IIIa.

To understand the nature of the signalling process leading to the α-granule release and platelet aggregation induced by non-FcγRII-mediated cross-linking of mAb, the effects of such cross-linking on protein-tyrosine phosphorylation were investigated next.

Platelet stimulation by FcγRII-independent cross-linking of mAb generates signals for intracellular protein-tyrosine phosphorylation. Normal platelets. In FcγRII-blocked platelets, non-cross-linked CD9, CD41 and CD42 had no effect on protein-tyrosine phosphorylation (Fig. 4A). However, cross-linking of all these antibodies induced strong increases in the tyrosine phosphorylation of proteins of 53 kDa (band e), 56 kDa (band d) and 64 kDa (band c), as well as the phosphorylation of a protein of 129 kDa (band a). Moreover, cross-linked mAb against CD9, CD41 and CD42 also induced the phosphorylation of a protein of 74 kDa (band b).

In contrast, cross-linked anti-PTA1 only induced a clear increase in tyrosine phosphorylation of a single protein of 64 kDa (band c), while cross-linked CD36 mAb (IA7) had no effect.

When the intensities of tyrosine-phosphorylated protein bands were compared, the strongest induction of protein-tyrosine phosphorylation was seen to take place in platelets stimulated by cross-linked CD9, CD41 and CD42 mAb. This indicates that protein-tyrosine phosphorylation induced by these antibodies is related to the above described ability of CD9, CD41 and CD42 to induce platelet-α-granule release and aggregation.

Thrombasthenic platelets. When thrombosthenic platelets were used in similar experiments (Fig. 4B), proteins of 53, 56, 64 and 74 kDa (bands e, d, c and b) became phosphorylated on tyrosine in response to platelet stimulation by cross-linked CD9 or CD42 mAb (Fig. 4B). Therefore, responses of thrombas-
In normal and thrombathenic platelets, cross-linked IV.3 induced the phosphorylation of a protein of 43 kDa (band f). This protein is probably FcγRII itself, since it is known that the receptor becomes phosphorylated on tyrosine in response to FcγRII-mediated platelet stimulation (Huang et al., 1992; Hunter et al., 1993). Since a 43-kDa protein was not observed among the proteins phosphorylated on tyrosine in response to other cross-linked antibodies, this confirms that the platelet response to antibodies other than IV.3 did not include a residual FcγRII-mediated component. Furthermore, since platelet stimulations by cross-linked CD9, CD41, CD42 and IV.3 antibodies resulted in the reduction of α-granule release, these results suggest that the tyrosine phosphorylation of the 53, 56, 64 and 74-kDa proteins may represent reactions downstream of the point at which different activation pathways converge.

Taken together, our results demonstrate that CD9 and CD42 mAb, when cross-linked, are able to induce gpIb/IIIa-independent stimulation signals, and that cross-linked CD9, CD42, CD41 and IV.3 (but not CD36 or LeOa1) induce similar protein-tyrosine phosphorylation. Since the similarity of the signals induced by these mAb could indicate spatial proximity of their target antigens, we investigated the topographical association of these proteins on the platelet membrane.

Chemical cross-linking of platelet membrane proteins indicates topographical associations between CD9, CD41 and CD42 antigens. To assess whether these molecules form macromolecular complexes within the platelet membrane, the membrane-impermeable chemical cross-linking agent DTSSP was employed. This agent is homobifunctional and thiol cleavable, and will cross-link primary amino groups over 1.2 nm. After treatment of platelets with DTSSP, cell lysates were immunoprecipitated with antibodies against the following proteins.

**CD9.** gpIb/IIIa immunoprecipitated with CD9 (SYB-1) from lysates of platelets treated with DTSSP (Fig. 5A). This finding is comparable to results obtained using DSP (a membrane-permeable cross-linking agent)-treated platelets (Slupsy et al., 1989; data not shown). This is not surprising, since DSP and DTSSP are structurally similar compounds. However, CD9 precipitation from lysates of DTSSP-treated platelets showed an additional associated protein whose mobility on SDS/PAGE matched that of gpIbα. This protein was identified as gpIbβ on the basis of peptide maps, generated by limited proteolytic digestion with pepsin, of the protein precipitating with CD9 and specifically immunoprecipitated gpIbβ (data not shown).

**CD42.** When an anti-gpIbα mAb was used (AN-51), CD9 and gpIb/IIIa immunoprecipitated with CD42 (gpIbα) from lysates of DTSSP-treated platelets (Fig. 5A). The identity of CD9 was confirmed by comparison of peptide maps, generated by limited proteolytic digestion with *S. aureus* V8 protease, of the protein precipitating with gpIbα and of specifically immunoprecipitated CD9 (data not shown). These findings were reproducible using another CD42 mAb (SZ-2) against a different epitope of gpIbα (data not shown). Thus, taken together with the above results with anti-CD9 mAb, these results suggest that some CD9, CD42 and gpIb/IIIa molecules on the platelet membrane associate to form a macromolecular complex.

**CD32 and PTAI.** When other platelet membrane antigens were examined by immunoprecipitation after platelet treatment with DTSSP, it was found that PTAI and CD32 were greatly reduced or lost from the soluble part of platelet lysates, and that mAb to these antigens failed to coprecipitate other membrane proteins (Fig. 5A). Further analysis by FACS showed that respective mAb binding to PTAI or CD32 was diminished in DTSSP-treated platelets (data not shown). This indicates that the reduced immunoprecipitability of these proteins after DTSSP...
Fig. 5. Chemical cross-linking studies of CD9, CD36, CD42, CD32 and PTA1. Radiiodinated platelets were treated with cross-linking agent (DTSSP) or Me$_2$SO (0), lysed, and immunoprecipitated with the indicated antibodies. This was followed by separation of the immunoprecipitated proteins by SDS/PAGE and autoradiography. (A) Platelets from normal donors. (B) Platelets from a patient with thrombocytopenia. IA7/IV.3, sample was immunoprecipitated simultaneously with two mAb respectively against CD36 and FcγRII, whereas the last lane shows the profile of iodinated proteins in the whole platelet lysate. The identities of the indicated proteins are a = gpIIb (CD42b), b = gpIIb (CD41), c = gpIlla (CD61), d = gpIV (CD36), e = PTA1 (LeoA1 antigen), f = FcγRII (CD32), g = CD9, and h = gpIX (CD42a).

treatment is probably caused by the loss of antibody-reactive epitopes, rather than through the cross-linking of these respective antigens to other molecules.

CD36. Immunoprecipitation of CD36, like that of CD9 and CD42, and in contrast to that of CD32 and PTA1, appeared to be unaffected by platelet treatment with DTSSP (Fig. 5A). Furthermore, CD36 appeared to precipitate with trace amounts of gpIIb/IIa (Fig. 5A), indicating that CD36 may also form a complex with this integrin.

Chemical cross-linking of membrane proteins of thrombocytopenic platelets. When identical experiments were carried out with thrombocytopenic platelets, similar results were obtained, with the exception of the expected absence of gpIIb/IIa in the CD9 and CD42 precipitates (Fig. 5B). Despite this absence, some CD9 precipitated with CD42 and vice versa, suggesting that the CD9 and CD42 association does not necessarily require the presence of gpIIb/IIa. PTA1 and CD32 were lost to immunoprecipitation after thrombocytopenic platelets were treated with DTSSP.

DISCUSSION

In the present study, by using mAb as functional probes and by chemical cross-linking, we show a close functional and topo-

graphical association between platelet CD9, CD41 and CD42 surface antigens. In contrast, similar studies of the target antigens of two other mAb (against CD36 and PTA1) provided no evidence of such associations.

To demonstrate antigen-specific effects of the mAb, we blocked reagent–Fc-receptor binding with Fab fragments of an anti-FcγRII mAb. In such Fc-receptor-blocked platelets, secondary antibody cross-linking of CD9, CD41 and CD42 between bound to their respective target antigens caused similar platelet aggregation, α-granule secretion, and similar patterns of protein-tyrosine phosphorylation. This clearly indicates a convergence of signals generated by CD9, CD41 and CD42 on a common pathway linked to the platelet-aggregation/secretion response. Moreover, the data demonstrate the need for the physical cross-linking provided by second-layer antibodies because the above effects were not observed if platelets were not cross-linked. Thus, it would appear that such physical cross-linking (of mAb-bound antigen on adjacent cell membranes) resembles the rigid cross-linking achieved through the engagement of multiple receptors by large multimeric ligands.

In contrast, Fc-receptor-independent cross-linking of anti-CD36 or anti-PTA1 mAb failed to induce aggregation, although some shape change occurred in platelet suspensions treated with anti-PTA1 mAb. Likewise, such cross-linking of both mAb failed to cause α-granule secretion. mAb-induced signalling via CD9, CD41 and CD42 involved tyrosine phosphorylation of a similar group of unidentified proteins. In contrast, anti-PTA1 produced phosphorylation of a single 64-kDa band, while an anti-CD36 mAb had no effect on protein-tyrosine phosphorylation in Fc-receptor-blocked platelets. These data suggest that the group of phosphorylated proteins (with the exception of the 64-kDa band) are specifically involved in signalling leading to aggregation/secretion. Phosphorylation of the 64 kDa protein may be specifically related to the induction of platelet shape change, since in anti-PTA1-stimulated cells this response was not followed by aggregation/secretion. The absence of tyrosine phosphorylation and functional responses to the anti-CD36 mAb suggests that the reported agonist effects of this particular mAb (Aiken et al., 1990; Alessio et al., 1993) entirely depend on FcγRII signalling.

It is unlikely that the number of surface molecules accounts for the failure of cross-linked anti-CD36 and anti-PTA1 mAb to induce aggregation. FcγRII is represented on the platelet membrane in much lower numbers [approximately 2000 molecules/platelet (McCrae et al., 1990)] than is CD36 [approximately 12000 (Aiken et al., 1990; Alessio et al., 1993)], yet the cross-linking of FcγRII on normal and thrombocytopenic platelets induced aggregation as well as α-granule and dense-granule release. Moreover, it is unlikely that differences in the cytoskeletal associations of CD9, CD42, CD36, gpIIb/IIa and PTA1 account for the differences in the ability of the respective mAb (to these antigens) to induce aggregation, gpIIb, gpIIb/IIa and CD36 have been demonstrated to associate with platelet cytoskeleton (Fox, 1985), and, while such associations have not been shown for either CD9 or PTA1 (Wright and Tomlinson, 1994; Scott et al., 1998; Zazal et al., 1991), it is not inconceivable that they exist. This is because the aggregation response mediated by CD9 was dependent on cytoskeletal organisation since it was inhibited by cytochalasin B (this study), and because anti-PTA1–mAb-induced tyrosine phosphorylation in degranulated platelets is inhibited by C3-exo-enzyme-catalyzed ADP ribosylation of rhoA (Slupsky et al., 1996), a molecule associated with cytoskeleton (Furman et al., 1993).

Similar studies with thrombocytopenic platelets indicated that cell responses to anti-CD9 and anti-CD42 are largely independent of the presence of CD41 (gpIIb/IIa). Although aggregation
was reduced, there was a substantial residual response that was not inhibited by RGDS peptide. Such aggregation is presumably analogous to the homotypic aggregation induced in B cells by CD9 mAb (Masellis-Smith et al., 1990; Letarte et al., 1993). Increasing intracellular cAMP concentration with prostaglandin E1 did not affect this gpIIb/IIIa-independent aggregation.

Although signalling via the cross-linking of mAb against CD9, CD41 and CD42 involved tyrosine phosphorylation of a similar group of proteins, some individual differences were apparent. In particular, cross-linked CD9 and CD42 induced the additional phosphorylation of a protein at 74 kDa (band b in Fig. 4A), whereas this band was not present in cross-linked CD41-stimulated platelets. This indicates that the phosphorylation of the 74-kDa band may be specific for the signalling pathways employed by CD9 and CD42, and, taken together with the overall similarity in induced protein-tyrosine phosphorylation, adds further support to the notion that all three antigens, CD9, CD41, and CD42, have convergent signalling pathways.

Although tyrosine phosphorylation in normal and thrombocytic platelets was broadly similar, the phosphorylation of the 129-kDa protein observed in normal platelets did not take place upon mAb stimulation of thrombocytic cells. This suggests that the 129-kDa protein is gpIIb/IIIa, the phosphorylation of which is known to be dependent on ligand-induced cross-linking of gpIIb/IIIa (Lipfert et al., 1992; Huang et al., 1993). The phosphorylation of the 64-kDa protein was not observed in anti-PTA1-stimulated thrombocytic platelets, but was present in thrombocytic cells stimulated with mAb against CD9 and CD42. This suggests that the 64-kDa-protein phosphorylation induced by anti-PTA1 antibody, but not by mAb against CD9 and CD42, is gpIIb/IIIa dependent.

All the results discussed above involved FcγRII-blocked platelets, but we also studied signalling induced by cross-linking of FcγRII itself. Such stimulation closely resembled that induced by mAb against CD9, CD41 and CD42 except that the Fc-receptor itself became tyrosine phosphorylated, and that the platelet response included dense-granule and α-granule release. Since neither dense-granule release nor FcγRII phosphorylation were seen after platelet stimulation with the other antibodies, these findings exclude any residual Fc-receptor signalling in IV.3-Fab-blocked platelets.

In thrombocytic platelets, Fc-receptor stimulation, like PTA1 cross-linking, had no effect on 64-kDa-protein phosphorylation. This suggests that signals induced by these two antigens are similar with regard to the dependence of 64-kDa-protein phosphorylation on the presence of gpIIb/IIIa.

Having demonstrated close similarities in the stimulation signals and platelet functional responses to mAb against CD9, CD41 and CD42, we postulated that the target antigens of these mAb may participate in the formation of larger molecular complexes within which the platelet activation signal is generated. Such association of diverse molecules into multicomponent cell-activation receptors is well known for T-cell and B-cell receptors (Matsumoto et al., 1993; Imai et al., 1995; Julius et al., 1993). To investigate this possibility, we used the homobifunctional cross-linking agent DTSSP. This agent is membrane impermeable, thiol cleavable, and will cross-link primary amino groups over 1.2 nm. Platelet treatment with this reagent demonstrated that CD9 and CD42 are physically associated with gpIIb/IIIa and, in relatively small amounts, with each other. The present study therefore indicates that a subpopulation of CD9, CD42 and gpIIb/IIIa are topographically associated. This is supported by reports that demonstrate CD42 and gpIIb/IIIa association under similar chemical cross-linking conditions (Davies and Palek, 1982; Jung and Mutoi, 1983), and by a report that showed that a trace amount of a CD9-like molecule precipitated with CD42 from lysates of 'H-labelled normal platelets (Miller et al., 1986). It is well established that CD9 and other proteins of the transmembrane-4 family participate in the formation of multicomponent receptors such as major-histocompatibility-complex class-II, T-cell and B-cell antigen receptors (Matsumoto et al., 1993; Imai et al., 1995; Angelisova et al., 1994). We have previously demonstrated that CD9 may be physically associated with the gpIIb/IIIa integrin, and others have reported a similar direct association with αβ3 integrins (Rubinstein et al., 1994; Nakamura et al., 1995; Berdichevsky et al., 1996; Mannion et al., 1996). The present study shows that CD9 can be part of a larger molecular complex consisting of CD9, gpIIb/IIIa and CD42. Thus, we demonstrate that integrins can directly associate with other adhesion receptors (CD42), as well as with transmembrane-4 (e.g. CD9) proteins. The formation of such multicomponent adhesion-receptor complexes can potentially influence the overall ligand-binding affinity, specificity, and signalling of the individual components.

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