A Sequence within the Cytoplasmic Tail of GpIIb Independently Activates Platelet Aggregation and Thromboxane Synthesis*

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All integrin α subunits contain a highly conserved KXGFFKR motif in their cytoplasmic domains that plays a crucial role in the regulation of integrin affinity for their ligands. We show that a lipid-modified peptide corresponding to the cytoplasmic region, 989–995, of the platelet integrin subunit glycoprotein GpIIb (αIIb), palmitoyl-KVGFFKR (Ppep; 10 μM), but not a similarly modified scrambled peptide (palmitoyl-FKFVRGK), can specifically induce platelet activation and aggregation equivalent to that of strong agonists such as thrombin. Ppep-induced aggregation is also associated with indices of platelet activation including thromboxane A2 (TXA2) synthesis (EC50 = 45 ± 5 μM), secretion of α-granules detected as enhanced surface expression of P-selectin (EC50 = 52 ± 8 μM), and conformational changes in GpIIb/IIIa measured by the monoclonal antibody, PAC-1 (EC50 = 7.7 ± 1 μM). The TXA2 receptor antagonist, SQ29548, PGE1, and the ADP scavenger, apyrase, differentially inhibit the aggregation response and TXA2 synthesis in response to Ppep. Similarly, GpIIb/IIIa antagonists (RO-449883 and integrin), which inhibit aggregation by greater than 90%, have little effect on peptide-induced TXA2 synthesis, suggesting that this event is independent of fibrinogen binding to GpIIb/IIIa. Alanine-stepping of the Ppep sequence identifies GFFK(991–994) as the critical residues in all peptide-mediated events. We conclude that this peptide can imitate the cytoplasmic domain of GpIIb and initiate parallel but independent signaling pathways, one leading to ligand binding and platelet aggregation and the other to intracellular signaling events such as TXA2 synthesis and secretion.

Integrins are a family of cell adhesion molecules composed of two subunits, α and β, which form a complex on the cell surface. Ligand recognition by integrins may be modulated by intracellular signals that interact with the cytoplasmic tails of the subunits. This has been demonstrated most clearly for the platelet glycoprotein (Gp)IIB/IIIa (αIIbβ3), the most abundant platelet integrin, which acts as a receptor for fibrinogen, fibrinectin, and other RGD-containing macromolecules (1). Under resting conditions, this receptor has a low affinity for its ligands (2). However, when platelets are stimulated by agonists such as thrombin or ADP, GpIIb/IIIa undergoes a conformational change (3) detected by the appearance of neoepitopes for monoclonal antibodies such as PAC-1 (4) and acquires a high affinity binding for its ligands, principally fibrinogen (5, 6). The binding of fibrinogen results in platelet aggregation, an early step in the generation of a thrombus. Deletion or mutation of the cytoplasmic domains of the integrin subunits can produce a constitutively active or inactive receptor (7–9), suggesting that signals resulting from cell activation interact with the intracellular components of GpIIb/IIIa to modify ligand recognition (outside-in signaling).

The cytoplasmic domains are also important for events occurring as a consequence of ligand-integrin interactions, so-called "outside-in" signaling. In the case of GpIIb/IIIa, ligand binding and receptor clustering is followed by an array of intracellular signals including thromboxane A2 (TXA2) generation and tyrosine phosphorylation events (10, 11). After ligand binding, GpIIb/IIIa becomes tightly associated with the membrane skeleton as focal contact structures form, composed of several cytoskeletal elements including talin, vinculin, and spectrin. Potential signaling molecules such as pp60c-src, pp62c-yes, phosphoinositide 3-kinase, and protein kinase C also associate with these focal contact structures (12, 13). Several sites within the cytoplasmic tail of the two integrin subunits have been implicated in this process (14–16). Phosphorylation of the cytoplasmic tail of GpIIb occurs in parallel with ligand occupancy, permitting the binding of signaling proteins SHC and GRB2 (17, 18). Naik and co-workers (19) showed that a 25-kDa calcium- and integrin-binding protein (CIB) interacts with the GpIIb cytoplasmic tail.

A highly conserved amino acid sequence exists immediately proximal to the transmembrane-spanning region in the α cytoplasmic domains of all integrins. This motif, KXGFFKR, has been shown to interact with calreticulin in an inducible manner in cells expressing the collagen receptor α2β1 (20). Furthermore, studies in which this sequence is deleted or mutated, have strongly suggested that it is involved in the regulation of the integrin affinity state (9, 16, 21–23). However, deletion or mutation of this region also modifies ligand recognition by the integrin and presumably, the conformation of the receptor, which will impact upon cellular events after ligand occupancy. Moreover, since modified integrins cannot be analyzed in native cells, it is necessary to study their functions in transfected cells that may not have a full complement of signaling molecules. As an alternative approach, we have examined the functional effects of a synthetic peptide sequence corresponding to the conserved amino acid motif on intact, human platelets. Our findings demonstrate that a lipid-soluble peptide, palmitoyl-KVGFFKR mediates platelet activation in a highly specific
manner and identifies a component of GPIIb that is involved in thromboxane formation independent of fibrinogen binding or platelet aggregation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Prostaglandin E₂ (PGE₂), apyrase, phorbol 12-myristate 13-acetate, and bovine thrombin were obtained from Sigma. SQ29548 (5-heptanoic acid, 7-[(3-[2-phenylamino]carbonyl)hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl) was obtained from Cayman Chemical (Ann Arbor, MI). PAC-I fluorogenic substrate was obtained from Becton Dickinson (San Jose, CA). Vectashield mounting medium for fluorescence was obtained from Vector Laboratories (Burlingame, CA). CD62P (anti-GMP-140) was obtained as a phosphatidylethanolamine conjugate from Cymbus Corporation (Hunts, UK). TXB₂ enzyme-linked immunosorbent assay kits were obtained from Assay Designs (Ann Arbor, MI). The GPIIb/IIIa antagonist Integrin tN was obtained from COR Therapeutics (San Francisco, CA). R044983 (1-[(N-p-amidinobenzoyl)-L-tyrosyl]-4-piperidinyl) oxy) acetic acid) was a gift from Dr. S. Roux of Hoffman LaRoche (Basle, Switzerland). All other materials were reagent grade or better.

Deionized water (Purite, grade 1) was used throughout all experiments.

**Peptide Synthesis**—A peptide corresponding to the minimal partial fragment of the GPIIb cytoplasmic tail containing the amino acid sequence (FKFVRGK) and a scrambled version of this sequence (FKFVVRKG) were synthesized on an Applied Biosystems automated peptide synthesizer (model 432A, Norwalk, CT) using a standard solid-phase Fmoc procedure. Corresponding peptides palmitoylated on the amino-terminal amino acid were also synthesized in an identical manner. Portions of both KVGGFKR and palmitoylated-KVGGFKR were subsequently labeled with carboxyfluorescein and used in immunofluorescence assays. Sequence L-alanine-substituted palmitoylated peptides (Palmitoyl-AVGFKR) [Pep K989A], Palmitoyl-KAGFKR [Pep V990A], Palmitoyl-KVAVFKR [Pep G991A], Palmitoyl-KYGFPR [Pep F992A], Palmitoyl-KVGAFK [Pep F993A], Palmitoyl-KVGFKFER [Pep R995A] and Palmitoyl-KVGFKFEA [Pep R995A] were also synthesized. All peptides were purified after synthesis using reverse phase liquid chromatography and confirmed using electrospray mass spectrometry.

**Platelet Aggregation**—Platelets obtained from volunteers free from medication were collected into 0.15 vol/vol acid-citrate-dextrose (ACD: 38 mM citric acid, 75 mM Na citrate, 124 mM dextrose) anticoagulant and washed using a modification of the method of Mendelsohn et al., 1991 (24). Briefly, blood was centrifuged at 180 g for 10 min. at room temperature. The supernatant was removed and the platelet pellet resuspended in 130 mM NaCl, 10 mM trisodium citrate, 9 mM NaHCO₃, 6 mM dextrose, 0.9 mM MgCl₂, 0.81 mM KH₂PO₄, 10 mM Tris pH 7.4, adjusted to 2 × 10⁶ cells/ml and supplemented with 1.8 mM CaCl₂. Platelet aggregations were performed at 37 °C in a BioData PAP-3 aggregometer (Hosham, PA). Peptide was dissolved in deionized water at a concentration of 1 mg/ml and used at the concentrations indicated to induce aggregation. Thrombin (0.2 units/ml) or thrombin receptor activating peptide (TRAP; 10 μM; SFLRNR) were used as positive controls in all experiments. Aliquots for TXB₂ analysis were snap frozen and stored at -80 °C until analysis.

**Fluorescent Microscopy**—Near-confluent human erythroleukemic (HEL) cells (25) were cultured overnight on Falcon culture Slides™ in RPMI 1640 (Gibco Laboratories) containing 10% fetal bovine serum and 10 μg/ml gentamycin. Cells were washed three times with phosphate-buffered saline and then mounted in vectashield-monomethacrylate. The slides were washed three times by immersion in phosphate-buffered saline and then mounted in vectashield-mounted mounting medium and analyzed on a Nikon diaphot microscope with epifluorescent attachment using 400X magnification.

**TXA₂ Analysis**—Washed platelets were diluted to 2 × 10⁵/ml and stimulated for 3 min with 20 μM arachidonic acid in the presence or absence of thrombin receptor activating peptide (TRAP) or thrombin at various concentrations. Platelet TXA₂ was measured as its stable metabolite TXB₂, by ELISA (26).

**FACS Analysis of P-selectin and PAC-1**—Washed platelets were placed in the aggregometer at 37 °C in the presence or absence of thrombin receptor activating peptide (TRAP) (10 μM) or the indicated concentrations of Ppep and stirred for 3 min. 50 μl aliquots were then placed on ice and incubated with CD62P-phosphatidylethanolamine as a marker for α-granule degranulation (27). Parallel samples were incubated with PAC-1 fluorescein isothiocyanate to determine the activation status of GPIIb/IIIa. Incubations were for 30 min. Samples were washed and resuspended in phosphate-buffered saline containing 0.1% bovine serum albumin and analyzed on a Becton Dickinson FACS-Scan (San Jose, CA) at 488/510 nm. Controls were included in all assays and were obtained by incubating platelets as above in the presence of 20 μM TRAP or 0.2 units/ml thrombin (positive control) or in the absence of any agonist (negative control). Nonspecific binding was determined in control and activated platelets using a fluorescein isothiocyanate-labeled γ-1 mouse IgG. Data are expressed as percent positive cells for P-selectin assays and as mean fluorescence for PAC-1 assays.

**RESULTS**

The membrane-proximal seven amino acids (Ly689-Arg995) of GPIIb were synthesized either as unmodified peptides or as palmitoylated isoforms and purified by high performance liquid chromatography. Peptide purity was ascertained by electrospray mass spectrometry and was routinely >99%.

Unmodified KVGGFKR had no effect on platelets. However, the addition of a palmitate group to the peptide increased its lipid permeability and permitted an interaction with intracellular components of the platelet. Palmitoylated peptide (Ppep45 μM), but not equal concentrations of palmitate alone, KVGGFKR alone, or palmitate plus unconjugated KVGGFKR caused aggregation in washed human platelets (Fig. 1A). There was a lag time of between 10 and 20 s before the initiation of aggregation. No obvious shape change response was observed in this time period. Palmitoylated scrambled peptide (P-FKFVRKGK), even at higher concentrations (120 μM), failed to cause significant platelet aggregation (Fig. 1B).

To establish if the peptide was gaining access to the platelet cytoplasmic milieu, carboxyfluorescein-labeled peptides were synthesized with and without the palmitate modification. In Fig. 2, we show that only the palmitoylated peptide can be observed in the cytoplasm of HEL cells, a platelet-like, human megakaryocyte cell line (25). No fluorescence was observed in cells incubated with carboxyfluorescein-labeled KVGGFKR that lacked the lipid modification. Similarly, flow cytometric analysis of either HEL cells or platelets shows that only the palmitoylated, fluorescein-labeled peptide had significant cellular association (Fig. 2, c and d). Access of labeled Ppep to cells is independent of the presence of GPIIb/IIIa, as excess of either Ppep or palmitoylated scrambled peptide compete equally for label uptake into cells. Furthermore, we have evidence of Ppep uptake into cells that do not express GPIIb/IIIa, including human umbilical vein endothelial cells (data not shown).

Pep-induced aggregation was acutely dose-dependent and was maximal at 10 μM (Fig. 3a). Platelet aggregation induced by Ppep, like that caused by thrombin, was accompanied by TXB₂ synthesis, whereas Ppep-induced thromboxane formation was paralleled by secretion, which can be measured by expression of the α-granular marker, CD62P or P-selectin (27), on the surface of the platelet (Fig. 3b). The maximum response determined by Michaelis-Menten kinetics was 645 ± 49.22 ng/ml TXB₂, with half-maximal effect (EC₅₀) at 45 μM peptide. Ppep-induced thromboxane formation was paralleled by secretion, which can be measured by expression of the α-granular marker, CD62P or P-selectin (27), on the surface of the platelet (Fig. 3b). The EC₅₀ for this effect was 52 μM, which corresponded to 41.3 ± 14% positive cells compared with 0.5 ± 9.1% positive cells in untreated, control platelets. The disparity in the EC₅₀ values for the signaling pathways (45–52 μM; thromboxane synthesis and secretion) compared with the dose of Ppep that produces a maximal aggregation response (10 μM) would suggest a different mechanism of activation for these respective events. Finally, Ppep induced PAC-1 expression (Fig. 3c) in a time- and dose-dependent manner.

This antibody recognizes an epitope on the GPIIb/IIIa complex that is exposed after platelet activation, permitting fibrinogen binding (4).

To define the membrane-proximal residues of the IIb subunit
important for the aggregating activity of this peptide, we sequentially substituted each residue in the peptide with alanine (Ala). The Ala-stepped peptides were then assayed for their ability to induce platelet aggregation, and samples were analyzed in parallel for TXA2 production (Fig. 4). Platelet aggregation activity was lost in Ppep G991A, Ppep F992A, Ppep F993A, and Ppep K994A. In contrast, activity was present but submaximal in Ppeps K989A, V990A, and R995A. A parallel profile of activity was observed for TXA2 synthesis activity with the Ala-stepped peptides. Thus, TXA2 synthesis and aggregation seem to be associated facets of Ppep stimulation.

To explore the mechanisms of Ppep-induced platelet activation, we examined the response to several inhibitors (Fig. 5). SQ29 548, a highly specific thromboxane receptor antagonist (10 μM), reduced platelet aggregation by 63% but did not abolish it. Similarly, apyrase (10 units/ml), an ADP scavenger, reduced platelet aggregation by 73%. Aggregation was also partially inhibited by PGE1 (5 μM), a concentration that abolished responses to thrombin (0.2 units/ml). These findings demonstrate that platelet aggregation was augmented by the formation of TXA2 and the release of ADP but was not initiated by these events. Similarly, Ppep-induced platelet activation is not mediated via the activation of a G-protein-dependent mechanism as these events are inhibited by PGE1. Thus it would appear that Ppep directly induces a high affinity state in GpIIb/IIIa and this is responsible for initiating the intracellular signaling events and platelet aggregation. Moreover, none of the inhibitors used altered TXA2 formation, suggesting a dissociation between aggregation and TXA2 synthesis. Consistent with this, two GpIIb/IIIa antagonists, RO 449883 (28) and integrin...
KVGFFKR Peptide Activates Platelet Signaling Pathways

The KVGFFKR sequence is common to α subunits of integrins and appears critical for receptor function. Deletion or mutagenesis analysis directed against this motif suggests that this sequence or a factor binding to it is critical for maintaining the integrin in its low affinity binding state (15, 16, 22). Thus, deletion of the KVGFFKR sequence in GpIIb/IIIa results in a constitutively active receptor capable of recognizing its ligand and binding it with high affinity. This cytoplasmic segment also recognizes intracellular proteins that may play a role in signal transduction such as calreticulin (20, 30, 31) and the calcium and integrin binding protein, CIB (19). We synthesized the peptide KVGFFKR and added a palmitoyl group to facilitate its access to the intraplatelet milieu, an approach that has been successful with other peptides (32–34). Fluorescence microscopy and flow cytometry demonstrate, using carboxyfluorescein-labeled peptides, that only peptides modified with palmitate gained access to intracellular regions of HEL cells and platelets. Staining in HEL cells was homogeneously distributed within the entire cytoplasmic area and was notably absent from the nuclear region.

The palmitoylated peptide, at concentrations as low as 10 μM, induced platelet activation and aggregation, whereas a scrambled peptide, similarly palmitoylated, had no effect even at concentrations as high as 120 μM. Unconjugated peptide, in the presence or absence of equimolar amounts of free palmitate, had no effect on platelet function, affording that the peptide must gain access to intracellular sites for biological effect. Neither palmitate alone or palmitoylated-scrambled peptide induced platelet aggregation, verifying that the effect on platelets was due to a specific Ppep-mediated response and not nonspecific effects such as to disruption of the cell membrane or cell lysis. Aggregation resulted from activation of GpIIb/IIIa as there was enhanced binding of the monoclonal antibody, PAC-1, which recognizes the active conformation of GpIIb/IIIa as there was enhanced binding of the monoclonal antibody, PAC-1, which recognizes the active conformation of GpIIb/IIIa. Platelet aggregation was inhibited by integrin and RO44 9883, highly specific GpIIb/IIIa antagonists, verifying that the aggregation response is dependent on activation of GpIIb/IIIa. The steep dose-response curve for Ppep-induced platelet aggregation reflects the inability of platelet aggregometry to detect small aggregates of platelets. Such aggregates can be observed by flow cytometry at doses as low as 2 μM Ppep as an increase in both forward and sideward scatter within the platelet population. The appearance of these small aggregates is accompanied by PAC-1 expression in a parallel manner. The EC_{50} for Ppep induction of PAC-1 binding was 3.7 ± 1.1 μM, and the maximum effect was achieved at 10 μM.

To address which residues of the peptide were responsible for the biological activity, we sequentially replaced each amino acid with an alanine residue. The results showed that the response was highly specific and that the activity resided predominantly in the region GFFKR corresponding to amino acids 990–994 of the GpIIb cytoplasmic tail. Mutation of the valine residue that varies most between different integrins, suggesting that cell activation and signaling via this mechanism may be common to all integrins. In studies with the alanine-stepped palmitoylated peptides, the peptides that gave a poor reaction in platelet aggregation assays were also poor inducers of TXA_{2} synthesis. These data are in agreement with the mutational studies by Hughes et al. in which similar alanine substitution...
revealed identical critical residues for constitutive expression of PAC-1 binding (16).

In addition to platelet aggregation, Ppep also induced platelet activation as measured by TXA₂ formation and secretion of granular contents. However, the products of these positive feedback pathways are not the mediators of Ppep-induced platelet activation. This finding is proven by two separate observations. First, the EC₅₀ values for Ppep-induced TXA₂ synthesis and P-selectin expression are approximately 50 μM, whereas maximal aggregation occurs at 10 μM Ppep. Second, inhibitor studies show that agents that scavenge ADP or that prevent changes in GpIIb/IIIa, exposing ligand-induced binding sites and, hence, to act as partial ligands. Integrelin, a cyclic heptapeptide RGD mimetic (35, 36), causes marked conformational changes in GpIIb/IIIa, exposing ligand-induced binding site epitopes (37), whereas RO 449883 does not (38). Both antagonists inhibited platelet aggregation but not TXA₂ synthesis. Identical results were also obtained using F(ab')₂ fragments of the inhibiting monoclonal antibody, 7E3 (39, 40) (data not shown). Thus, integrin occupancy by its ligand and integrin-initiated signaling may be distinguished as independent events. We conclude, therefore, that the interaction of Pal-KVGGFKR with GpIIb/IIIa represents a branch point in these two pathways such that both pathways require interaction with the peptide sequence and have the same intolerance to sequence mutation. Structure-function analysis pinpoints the region Gly-991–Lys-994 as the important region for these events. However, once the peptide has bound to its target, the processes of integrin ligation, clustering, and platelet aggregation proceed independently from the signaling pathways leading to TXA₂ synthesis. Our findings demonstrate that KVGGFKR can both activate GpIIb/IIIa and induce intracellular signals independently. We propose a model where the sequence KVGGFKR in the cytoplasmic region of GpIIb, adjacent to the transmembrane region, provides a site for tethering the integrin and stabilizing it in a low affinity state. This is consistent with the deletion and mutational studies of O'Toole et al. (9) and Hughes et al. (41) and may be mediated through an interaction with signaling protein(s). Competition by the peptide displaces the stabilizing signaling factor, simultaneously activating the receptor by releasing it from its tether and allowing signaling to proceed via the tethering protein. An alternative possibility is that the KVGGFKR sequentially promotes receptor clustering and li-
gand binding. The occurrence of integrin clustering may lead to cell signaling, whereas ligand binding is necessary for aggregation. Consistent with this possibility is the recent observation that integrin clustering can mediate cell signaling independent of ligand binding (23).

In conclusion, structure-function analysis of the intracellular segment of the GpIIb subunit (Lys-989–Arg-995) using the cell-permeable peptide, Palmitoyl-KVGFFKR, pinpoints this segment of the GpIIb subunit (Lys-989–Arg-995) at a critical segment having a critical role in integrin signaling. This peptide substitutes for the endogenous GpIIb cytoplasmic tail and independently stimulates integrin ligation, leading to aggregation and platelet signaling events such as TXA2 synthesis and secretion.

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