

Integrin $\beta 3$ regions controlling binding of murine mAb 7E3: Implications for the mechanism of integrin $\alpha \text{IIb}\beta 3$ activation

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Abciximab, a derivative of the murine mAb 7E3, protects against ischemic complications of percutaneous coronary interventions by inhibiting ligand binding to the $\alpha \text{IIb}\beta 3$ receptor. In this study we identified regions on integrin $\beta 3$ that control 7E3 binding. Murine/human amino acid substitutions were created in two regions of the βA domain that previous studies found to influence 7E3 binding: the C177–C184 loop and K125–N133. The T182N substitution and a K125Q mutation reduced 7E3 binding to human $\beta 3$ in complex with αIIb . The introduction of both the human C177–C184 region and human W129 into murine $\beta 3$ was necessary and sufficient to permit 7E3 binding to the human $\alpha \text{IIb}/\text{murine } \beta 3$ complex. Although we cannot exclude allosteric effects, we propose that 7E3 binds between C177–C184 and W129, which are within 15 Å of each other in the crystal structure and close to the $\beta 3$ metal ion-dependent adhesion site. We previously demonstrated that 7E3 binds more rapidly to activated than unactivated platelets. Because it has been proposed that $\alpha \text{IIb}\beta 3$ changes from a bent to an extended conformation upon activation, we hypothesized that 7E3 binds less well to the bent than the extended conformation. In support of this hypothesis we found that 7E3 bound less well to an $\alpha \text{IIb}\beta 3$ construct locked in a bent conformation, and unlocking the conformation restored 7E3 binding. Thus, our data are consistent with $\alpha \text{IIb}\beta 3$ existing in variably bent conformations in equilibrium with each other on unactivated platelets, and activation resulting in $\alpha \text{IIb}\beta 3$ adopting a more extended conformation.

The integrin $\alpha \text{IIb}\beta 3$ and $\alpha \text{V}\beta 3$ receptors are important in a number of physiologic and pathologic phenomena, including hemostasis, thrombosis, tumor angiogenesis, and bone resorption (1, 2). The murine mAb 7E3 (3) blocks ligand binding to both $\alpha \text{IIb}\beta 3$ and $\alpha \text{V}\beta 3$ receptors (4, 5). Abciximab is a mouse/human chimeric Fab fragment of the mAb 7E3 that inhibits $\alpha \text{IIb}\beta 3$ -mediated platelet aggregation and is approved for human use to prevent the ischemic complications associated with percutaneous coronary interventions (6).

Previous studies of 7E3 binding to cells expressing recombinant $\alpha \text{IIb}\beta 3$ receptors demonstrated that: (i) swapping select murine for human αIIb sequences does not decrease 7E3 binding (7), (ii) removing the specificity determining loop (SDL) (K156–G189 sequence) from $\beta 3$ results in loss of 7E3 binding (8), (iii) swapping the murine for human C177–C184 sequence within the SDL region in $\beta 3$ results in loss of 7E3 binding (7), and (iv) swapping the murine S129–T133 sequence for the human W129–N133 sequence results in partial loss of 7E3 binding (7).

The above human/mouse swapping studies identified regions within $\beta 3$ that affect 7E3 binding, but the biochemical and functional properties of these chimeric receptors were not characterized. In addition, the W129–N133 region contains two amino acid differences between human $\beta 3$ ($\beta 3\text{Hu}$) and mouse $\beta 3$ ($\beta 3\text{M}$) and the C177–C184 region contains three amino acid differences (Table 1). To define further the regions on $\alpha \text{IIb}\beta 3$

that control 7E3 binding, we assessed the effects of individual amino acid substitutions on 7E3 binding to cells expressing $\alpha \text{IIb}\beta 3$, as well as the effects of these substitutions on receptor biochemistry and function. In addition, we studied the effect of mutating a highly conserved lysine residue (K125) that appears to link the C177–C184 loop to the α -helix containing W129–N133.

Finally, in view of our localization of the region involved in 7E3 binding to the head region of $\beta 3$ adjacent to the arginine-glycine-aspartic acid (RGD) binding site, our previous studies demonstrating that 7E3 IgG (but not 7E3 Fab) binds much more rapidly to activated than unactivated platelets, (3, 9), and our recently proposed model of $\alpha \text{IIb}\beta 3$ undergoing a change from a bent to an extended conformation upon activation, (10, 11), we also assessed the binding of 7E3 IgG and 7E3 Fab to an $\alpha \text{IIb}\beta 3$ receptor reversibly locked in a bent conformation (10).

Materials and Methods

mAbs. The mAbs used in this study were: 7E3 IgG and Fab (anti- $\alpha \text{V}\beta 3 + \alpha \text{IIb}\beta 3$) (4), 10E5 ($\alpha \text{IIb}\beta 3$ -specific) (5), LM609 ($\alpha \text{V}\beta 3$ -specific) (kindly provided by David Cheresch, The Scripps Research Institute, La Jolla, CA), AP3 ($\beta 3$ -specific) (kindly provided by Peter Newman, Blood Center of Southeastern Wisconsin, Milwaukee) (12), FITC-labeled goat anti-mouse IgG F(ab')₂ (The Jackson Laboratory), and purified mouse IgG (The Jackson Laboratory).

Generation of Constructs. $\beta 3\text{Hu}$ and human αIIb cDNAs were generously provided by Peter Newman; $\beta 3\text{M}$ cDNA was a generous gift from Patrick Ross (Washington University School of Medicine, St. Louis). Table 1 lists the constructs used for this study. Mutations in $\beta 3\text{Hu}$ and $\beta 3\text{M}/\text{pcDNA}3.1$ cDNA constructs were generated by using either the splice by overlap extension PCR method as described (13) or the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) as per the manufacturer's instructions. The generation of pEF- $\alpha \text{IIbR}320\text{C}$ and pCDNA3.1- $\beta 3\text{R}563\text{C}$ has been described (10).

Cell Transfections. Normal or mutant $\alpha \text{IIb}\beta 3$ receptors were expressed in Chinese hamster ovary (CHO), human 293T, and

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Abbreviations: RGD, arginine-glycine-aspartic acid; CHO, Chinese hamster ovary; $\beta 3\text{Hu}$, human $\beta 3$; $\beta 3\text{M}$, mouse $\beta 3$; MFI, mean fluorescent intensity; MIDAS, metal ion-dependent adhesion site.

See accompanying Biography on page 13111.

[§]B.S.C. is an inventor of abciximab, and in accord with federal law and the policies of the Research Foundation of the State University of New York, shares in royalties paid to the foundation for the sale of abciximab.

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Table 1. Constructs for mammalian cell expression

Construct	Name	129–133	177–184
Human $\beta 3$	$\beta 3$ Hu	¹²⁹ WSIQN ¹³³	¹⁷⁷ CYDMKTT ¹⁸⁴
Human $\beta 3$ mouse 177-184	$\beta 3$ Hu–M177-184	¹²⁹ ----- ¹³³	¹⁷⁷ --N--NA ¹⁸⁴
Human $\beta 3$ D179N	D179N	¹²⁹ ----- ¹³³	¹⁷⁷ --N----- ¹⁸⁴
Human $\beta 3$ T182N	T182N	¹²⁹ ----- ¹³³	¹⁷⁷ -----N ¹⁸⁴
Human $\beta 3$ T183A	T183A	¹²⁹ ----- ¹³³	¹⁷⁷ -----A ¹⁸⁴
Human $\beta 3$ C177A + C184A	C177A-C184A	¹²⁹ ----- ¹³³	¹⁷⁷ A-----A ¹⁸⁴
Human $\beta 3$ C177A-M-C184A		¹²⁹ ----- ¹³³	¹⁷⁷ A-N--NAA ¹⁸⁴
Human $\beta 3$ W129S	W129S	¹²⁹ S----- ¹³³	¹⁷⁷ ----- ¹⁸⁴
Human $\beta 3$ N133T	N133T	¹²⁹ -----T ¹³³	¹⁷⁷ ----- ¹⁸⁴
Human $\beta 3$ K125A/R/Q	K125A/R/Q	¹²⁹ ----- ¹³³	¹⁷⁷ ----- ¹⁸⁴
Human α Ib-R320C $\beta 3$ -R563C	α IbR320C β 3R563C	¹²⁹ ----- ¹³³	¹⁷⁷ ----- ¹⁸⁴
Mouse $\beta 3$	$\beta 3$ M	¹²⁹ S---T ¹³³	¹⁷⁷ --N--NA ¹⁸⁴
Mouse $\beta 3$ human 177-184	$\beta 3$ M–Hu177-184	¹²⁹ S---T ¹³³	¹⁷⁷ ----- ¹⁸⁴
Mouse $\beta 3$ human 177-184 + S129W	$\beta 3$ M–Hu177-184+W129	¹²⁹ S---T ¹³³	¹⁷⁷ ----- ¹⁸⁴

human 293 cells. CHO cells express relatively small amounts of endogenous hamster αV , which can combine with transfected $\beta 3$ to form $\alpha V\beta 3$. The 293 and 293T cells express relatively small amounts of endogenous human αV , which can combine with transfected $\beta 3$ to form $\alpha V\beta 3$. The expression of these small amounts of $\alpha V\beta 3$ was considered in designing the experiments and interpreting the results. Transfections were performed with Lipofectamine (GIBCO/BRL), PerFectin (Gene Therapy System, San Diego), or CaCl_2 /Hepes methods (14). Transfections using Lipofectamine and PerFectin were performed according to the manufacturer's instructions. For transfections using the CaCl_2 /Hepes method, cells were plated to $\approx 20\%$ confluency in 100-mm tissue culture dishes and incubated overnight at 37°C . A DNA transfection solution [DNA (6–10 μg) and 2 M CaCl_2 (62 μl) in 500 μl of double distilled H_2O and 500 μl of Hepes-buffered saline] was added to the cells and incubated for an additional 48 h; the cells were then washed with PBS and harvested. For stable transfections, cells were selected in media containing 800 $\mu\text{g}/\text{ml}$ G418 for 2–4 weeks. To obtain a population of cells expressing higher levels of human and chimeric receptors, cells were labeled with the mAb 10E5 (α Ib $\beta 3$ -specific) and sorted with a FACSCalibur cell sorter (Becton Dickinson).

Flow Cytometry. Cells ($4\text{--}6 \times 10^5$ cells per ml) were incubated with primary antibody (5–10 $\mu\text{g}/\text{ml}$) for 30 min at room temperature followed by FITC-labeled goat anti-mouse IgG F(ab')₂ (10 μg per sample) for 30 min on ice. For some experiments 7E3 and 10E5 were labeled with Alexa Fluor 488 (Molecular Probes) as per the manufacturer's protocol and then used to directly label the cells. Cells were washed, resuspended in PBS containing 2% FBS, and analyzed with a FACSCalibur flow cytometer. Background controls were transfected cells incubated with secondary FITC-labeled antibody alone or cells transfected with pCDNA3.1 and then incubated with FITC-labeled 10E5 and/or 7E3; background levels were set such that the control cells had $\leq 5\%$ positive cells. In some experiments surface $\beta 3$ receptor expression was normalized for total $\beta 3$ integrin expression by using the anti- $\beta 3$ Hu-specific mAb VIPL2. In some experiments cells were pretreated with 2 mM DTT for 15 min at room temperature before adding the mAbs.

Fibrinogen Adhesion Assay. To minimize any contribution of $\alpha V\beta 3$ receptors to fibrinogen adhesion of transfected cells, the assay was conducted in the presence of Ca^{2+} , which preferentially supports α Ib $\beta 3$ -mediated ligand binding rather than $\alpha V\beta 3$ -mediated ligand binding (15). Wells of polystyrene (Nunc) flat-bottom 96-well plates were precoated with Tris-saline buffer

(50 mM Tris·HCl/100 mM NaCl, pH 7.4) (100 μl per well) containing 10 $\mu\text{g}/\text{ml}$ human fibrinogen (Enzyme Research Laboratories, South Bend, IN) for 1 h at room temperature. The wells were blocked with DMEM (GIBCO/BRL) (100 μl per well) containing 1% BSA for 1 h at room temperature. Cells (10^6 cells per ml) were preincubated with mAbs 7E3 or 10E5, or purified mouse IgG (10 $\mu\text{g}/\text{ml}$), and 2 mM CaCl_2 for 20 min at room temperature, and then added to the wells (100 μl per well) and incubated at 37°C for 1 h. Nonadherent cells were removed by gently rinsing the wells three times with Tris-saline buffer (200 μl per well). Adherent cells were fixed with 4% formaldehyde for 20 min, and then stained with a solution containing 0.5% crystal violet and 20% methanol for 30 min at room temperature. After washing, a 10% acetic acid solution was added (100 μl per well) to solubilize the dye, and the OD₅₅₀ of the solution was measured on a microplate reader (Thermo Max, Biotronic Technologies, Stoughton, MA). The OD₅₅₀ using untransfected cells was considered background and subtracted from the OD₅₅₀ using transfected cells.

Biosynthetic Labeling and Immunoprecipitation. Cells (5×10^5 per 60-mm tissue culture well; two wells per group) were incubated at 37°C in medium containing [³⁵S]methionine/cysteine (300 μCi per well) for 2–4 h. Cells were then incubated for 30 min on ice with lysis buffer (150 mM NaCl/50 mM Tris·HCl, pH 7.5) containing 1% Triton X-100 and PMSF (2 mM), leupeptin (10 μM), calpeptin (20 μM), and *N*-ethylmaleimide (10 mM). Lysates were centrifuged at 4°C at $12,000 \times g$ for 30 min, and supernatants were precleared with protein A or protein G Sepharose (Amersham Pharmacia) at 4°C for 30 min. Using equivalent amounts of trichloroacetic acid-precipitable radioactivity ($\approx 5\text{--}6 \times 10^6$ counts per sample), immunoprecipitations were performed with 10E5 or 7E3 (8 $\mu\text{g}/\text{ml}$). Protein G Sepharose (5%) was added and incubated for 1 h at 4°C . The beads were washed twice with lysis buffer containing 500 mM NaCl, and then the bound material was eluted with SDS/PAGE sample buffer at 100°C . Samples (reduced and nonreduced) were separated by SDS/PAGE (7.5% gel) and the dried gels were exposed to film (Blue Sensitive, Denville Scientific, Metuchen, NJ). Preliminary studies demonstrated that antibody LM609 immunoprecipitated only trace amounts of $\alpha V\beta 3$ from stable 293 cell lines expressing either α Ib $\beta 3$ or just $\beta 3$, indicating that only trace amounts of $\alpha V\beta 3$ are produced by these cells (data not shown).

Immunoblotting. Preparation of cell lysates and immunoprecipitation with 10E5 or 7E3 were performed as above. For some experiments cells were labeled for 30 min with a sulfo-*N*-

Table 2. Percentage (\pm standard deviation) of 293T cells expressing α Ib and either normal or mutated β 3 that binds 10E5 or 7E3 as judged by flow cytometry

Construct	10E5	7E3	7E3/10E5	P value (10E5 vs. 7E3)
β 3Hu	42 \pm 12	46 \pm 17	1.1	n.s.
β 3Hu-M177-184	16 \pm 10	0.1 \pm 0.2	0.01	<0.01
β 3M	36 \pm 18	2 \pm 2	0.06	<0.01
β 3M-Hu177-184	32 \pm 8	24 \pm 6	0.75	<0.01
β 3M-Hu177-184+W129	32 \pm 8	32 \pm 8	1	n.s.

n.s., Not significant.

hydroxysuccinimide-biotin (1 mg/ml) (Pierce) on ice; the reaction was stopped by adding glycine (5 mM final concentration). Samples (reduced and nonreduced) were separated by SDS/PAGE (7.5% gel) and electro-transferred to poly(vinylidene difluoride) membranes. Membranes were blocked in 5% nonfat dry milk for 1 h at room temperature, washed, and hybridized with a primary antibody (10 μ g/ml) for 2 h at room temperature or overnight at 4°C. Membranes were washed twice in 0.1% Tween-TBS and incubated with horseradish peroxidase (HRP)-conjugated AP3 (anti- β 3) or HRP-avidin for 1 h at room temperature. Membranes were washed and developed by using chemiluminescence as per the manufacturer's instructions (Amersham Pharmacia).

Molecular Modeling. The structural effects of the substitutions were analyzed by using a molecular modeling approach using the CHARMM molecular program as described (16). Briefly, the individual amino acids were substituted into the β -A domain of β 3 and then energy minimization was performed with the Adopted Basis Newton-Raphson method (17).

Results

7E3 Binding to Receptors Containing β 3M C177–C184 Amino Acid Residues. Antibodies 10E5 and 7E3 reacted with similar percentages of 293T cells transfected with normal human α Ib and normal β 3Hu, 42 \pm 12% and 46 \pm 17%, respectively (Table 2). In sharp contrast, when 293T cells were transfected with human α Ib in conjunction with β 3Hu containing the murine C177–C184 sequence (β 3Hu–M177-184), 16 \pm 10% were positive with 10E5, but only 0.1 \pm 0.02% were positive with 7E3 (P < 0.01). Similar results were obtained with CHO cells expressing endogenous hamster α V with either β 3Hu or β 3Hu–M177-184; both sets of cells reacted with antibody LM609, which is specific for α V β 3 (data not shown). Thus, the binding of 7E3 to both α Ib β 3 and the hamster/human hybrid α V β 3 were affected by substituting the murine C177–C184 sequence.

To ascertain whether the flow cytometry data correlate with antibody-induced inhibition of ligand binding, transfected CHO cells were tested for their ability to adhere to fibrinogen (Fig. 1A). Cells transfected with human α Ib and either β 3Hu or β 3Hu–M177-184 adhered to immobilized fibrinogen, and adhesion of both transfected cell lines was markedly inhibited by mAb 10E5 (Fig. 1A). In contrast, although the adhesion of cells transfected with β 3Hu was markedly inhibited by mAb 7E3, 7E3 did not inhibit the adhesion of cells transfected with β 3Hu–M177-184.

To assess the biochemical characteristics of the receptors, transfected cells were grown in the presence of [35 S]methionine/cysteine for 3 h and lysed; the resulting lysates were then immunoprecipitated with either mAb 10E5 or mAb 7E3 (Fig. 1B). 10E5 immunoprecipitated radiolabeled receptors from the lysates of the cells transfected with human α Ib in conjunction with either β 3Hu or β 3Hu–M177-184. When samples were

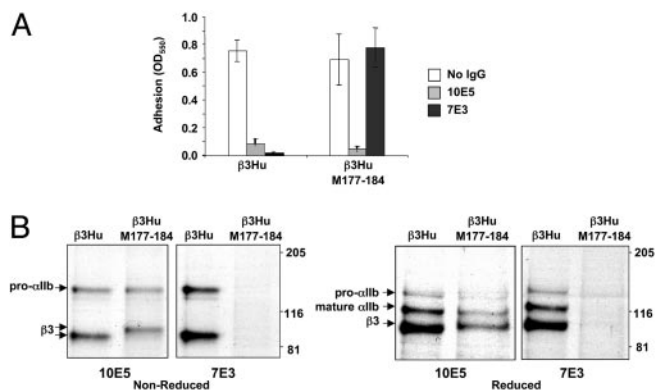


Fig. 1. Effects of swapping of murine sequence in β 3Hu on 7E3 binding. (A) Adhesion to immobilized fibrinogen of CHO cells transiently expressing β 3Hu or β 3Hu–M177-184 in association with human α Ib. Cells were preincubated with 2 mM Ca^{2+} and different antibodies. Adhesion is expressed as OD₅₅₀ of solubilized crystal violet-stained cells. Data presented are the mean \pm standard deviation of three experiments. (B) Radiograph of immunoprecipitates under nonreduced and reduced conditions of 35 S-labeled cells using 10E5 or 7E3. Cells were transiently transfected with β 3Hu or β 3Hu–M177-184 in association with human α Ib.

analyzed under nonreduced conditions, β 3Hu–M177-184 migrated slower than did β 3Hu. Under reduced conditions, however, the migration of β 3Hu and β 3Hu–M177-184 were similar or identical, suggesting that the difference in migration patterns under nonreduced conditions was caused by loss of, or altered, disulfide bond formation in β 3Hu–M177-184. Trace amounts of pro- α Ib were also observed in reduced samples of 10E5 immunoprecipitates of both constructs. As expected, β 3Hu–M177-184 was not immunoprecipitated by 7E3, confirming the data obtained in the flow cytometry and adhesion studies.

Biochemical, Functional, and Immunologic Characterization of α Ib β 3 Receptors with Single Murine/Human Amino Acid Substitutions in the β 3 C177–C184 Sequence. To identify the amino acid(s) within the murine C177–C184 sequence that affect the binding of 7E3, we prepared β 3 constructs containing the three single amino acid substitutions that distinguish the murine from the human sequence (D179N, T182N, T183A) (Fig. 2A). Cells were cotransfected with human α Ib and either normal or mutant β 3. Receptors reactive with 10E5 were expressed at comparable levels as demonstrated by similar patterns of α Ib β 3 immunoprecipitation of biosynthetically labeled α Ib β 3 by 10E5 (Fig. 2A). In contrast, although 7E3 and 10E5 immunoprecipitated comparable amounts of α Ib β 3 from β 3Hu, D179, and T183 cells, it precipitated only a small amount of α Ib β 3 from T182N, and nearly none of the α Ib β 3 from β 3Hu–M177-184. The results of the adhesion studies were consistent with the immunoprecipitation data (Fig. 2C). Thus, 10E5 inhibited adhesion with all of the constructs, indicating the dominant role of α Ib β 3 in mediating the adhesion. In sharp contrast, 7E3 did not inhibit the adhesion of the β 3Hu–M177-184 cells and only slightly reduced the adhesion of the T182N cells. Under nonreduced conditions, the β 3 containing the T182N substitution immunoprecipitated by 10E5 migrated as a doublet, with the less intense lower band migrating at the same M_r as β 3Hu and the more intense upper band migrating at the same M_r as β 3Hu–M177-184 (Fig. 2A and B). In contrast 7E3 appeared to only immunoprecipitate the minor form that migrated at the same M_r as β 3Hu (Fig. 2A and B). These data suggest that 7E3 can bind to a subpopulation of T182N receptors that adopts the proper conformation, but we cannot rigorously exclude the possibility that these observations are caused by differences in affinity. The

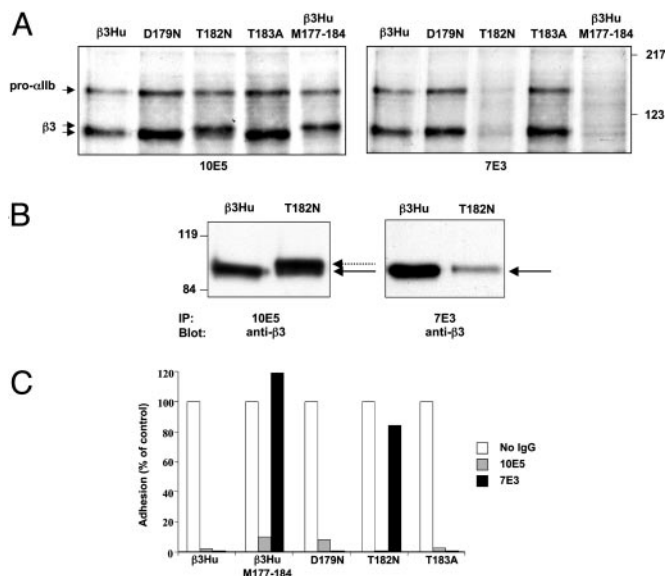


Fig. 2. Effects of introducing single murine substitutions in β 3Hu C177–C184. (A) Radioautographs of ^{35}S -labeled cells immunoprecipitated with 10E5 or 7E3 and electrophoresed under nonreduced conditions in SDS/PAGE. (B) Immunoblot analysis using mAb AP3 (anti- β 3) of 7E3 or 10E5 immunoprecipitates of lysates of cells transfected with β 3Hu or T182N. T182N was immunoprecipitated by 10E5 and migrated as a closely spaced doublet. Only the minor band migrating with the same M_r as β 3Hu was immunoprecipitated by 7E3. (C) Adhesion to immobilized fibrinogen of 293T cells expressing native and mutated β 3. Cells were preincubated with 2 mM Ca^{2+} . Data are the mean of two experiments and are normalized for adhesion of cells in the absence of antibodies.

β 3Hu–T182N doublet disappeared when the samples were reduced (data not shown).

Effect of Modifying the C177–C184 Disulfide Bond on 7E3 Binding. To determine whether 7E3 binding to β 3 receptors has an absolute requirement for disulfide bond formation between residues C177 and C184, constructs were generated in which alanines were substituted for the two cysteines. Cell lysates were immunoprecipitated with 10E5 or 7E3 and immunoblotted with mAb AP3. 10E5 immunoprecipitated receptors containing C177A+C184A; under nonreduced conditions this β 3 mutant migrated as did β 3Hu–M177–184, that is, more slowly than β 3Hu (Fig. 3). Because the C177A+C184A mutant cannot form the normal C177–C184 disulfide bond, these data support the hypothesis that both the β 3Hu–M177–184 and T182N substitutions also disrupt the C177–C184 disulfide bond. At least a portion of

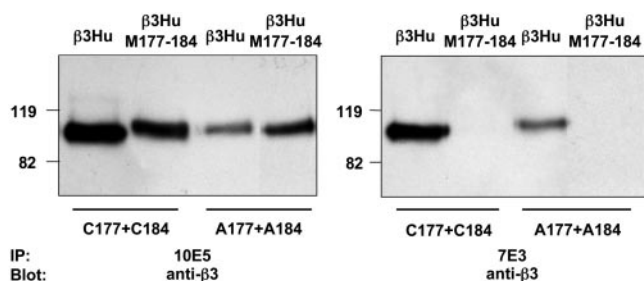


Fig. 3. Effect of substituting alanines for C177 and C184 on 10E5 and 7E3 binding. Lysates of constructs containing β 3Hu or β 3Hu–M178–183 and/or C177A+C184A residues were immunoprecipitated by 10E5 (Left) or 7E3 (Right), electrophoresed under nonreduced conditions in SDS/PAGE, and immunoblotted with anti- β 3 mAb AP-3.

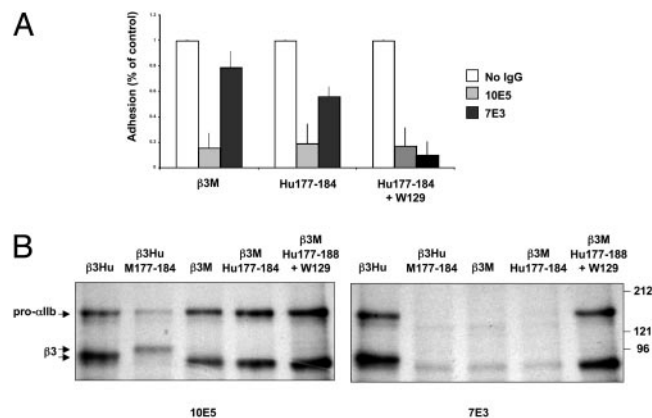


Fig. 4. Engineering of 7E3 epitope into β 3M. (A) Adhesion to immobilized fibrinogen of cells stably expressing native and mutated β 3. Cells were preincubated with 2 mM Ca^{2+} and purified murine IgG, 10E5, or 7E3. Data are the mean of three experiments \pm standard deviation and are normalized for adhesion of cells in the presence of purified murine mAb. (B) Radioautographs of immunoprecipitates using 10E5 or 7E3 of lysates of ^{35}S -labeled cells transfected with β 3Hu, β 3M, or chimeric β 3 and electrophoresed under nonreduced conditions in SDS/PAGE.

the C177A+C184A-containing receptors could also be immunoprecipitated with 7E3, suggesting that at least some of the molecules adopt a conformation(s) recognized by 7E3 even in the absence of a disulfide bond between residues 177 and C184 in β 3Hu (Fig. 3). The substitution of the three murine residues (M177–184) into the C177A+C184A construct (A177–A184), however, eliminated 7E3 binding. Thus, both the conformation and the amino acid composition of the C177–C184 sequence can profoundly affect 7E3 binding.

Effect of β 3 W129S and T133N Substitutions on 7E3 Binding to α IIb β 3 Receptors. Puzon-McLaughlin *et al.* (7) previously showed that substituting both β 3 W129S and N133T partially decreased 7E3 binding, so we assessed the effects of making the two individual single amino acid substitutions on 7E3 binding. By flow cytometry and immunoprecipitation, neither single amino acid substitutions affected 7E3 binding (data not shown), suggesting that both substitutions are required in combination to partially decrease 7E3 binding.

Studies to Engineer the 7E3 Epitope into Receptors Containing β 3M and Human α IIb. β 3M constructs were generated containing either β 3Hu amino acids C177–C184 (β 3M–Hu177–184) or β 3Hu C177–C184 plus the β 3Hu tryptophane at position 129 (β 3M–Hu177–184+W129). Cells transiently or stably expressing human α IIb in combination with WT β 3M or the chimeric β 3 subunits were analyzed for 7E3 binding by flow cytometry, SDS/PAGE, and cell adhesion. By flow cytometry, 7E3 binding to cells expressing β 3M–Hu177–184 was greater than 7E3 binding to cells expressing β 3M (Table 2), but significantly less than 10E5 binding to the same cells ($P < 0.01$). In sharp contrast, 7E3 binding to cells expressing β 3M–Hu177–184+W129 was virtually identical to 10E5 binding ($P = \text{not significant}$) (Table 2). Cell adhesion to immobilized fibrinogen in the absence of inhibitory antibodies was similar for cells expressing β 3M, β 3M–Hu177–184, or β 3M–Hu177–184+W129 (Fig. 4A). 10E5 inhibited adhesion of cells expressing each of these chimeric receptors by 80% or more. In contrast, and consistent with the flow cytometry data, 7E3 did not significantly inhibit adhesion to immobilized fibrinogen of cells expressing β 3M, only partially inhibited adhesion of cells expressing β 3M–Hu177–184, and nearly completely inhibited adhesion of cells expressing β 3M–Hu177–

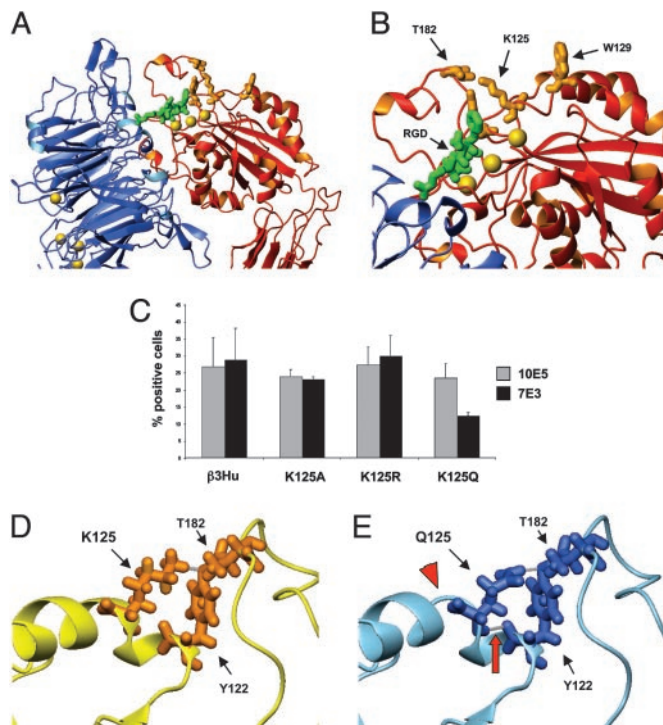


Fig. 5. The role of K125 in 7E3 binding. (A) Crystal structure of selected regions of the external domain of integrin α V β 3 in complex with the peptide RGDW as per Xiong *et al.* (31). The α V propeller is in blue, and the β 3- β A domain is in red. The peptide is in green, and Mn^{2+} ions are depicted as yellow spheres. (B) Higher-power view of the top surface of the β 3- β A domain in association with the peptide. The three yellow spheres represent the three Mn^{2+} ions in the ligand-associated metal binding site, MIDAS, and adjacent to MIDAS positions, respectively. The amino acids depicted in orange are the ones described in the text as important in 7E3 binding. (C) Effect of K125 substitutions on 10E5 and 7E3 binding. Data are the mean \pm standard deviation of three independent experiments. (D and E) Molecular modeling of the effect of the Q125 substitution on C177-C184 and α -helix 1. Models are rotated 180° in comparison with A and B. Residues at positions 122, 125, and 182 are shown for β 3Hu (D) and K125Q (E). Selected hydrogen bonds are depicted in gray and the new hydrogen bond between Y122 and K125Q is identified by a red arrow. Notice the reorientation of K125 as a result of the additional hydrogen bond, along with the loss of a portion of the α -helical structure of α -helix 1 (red arrowhead).

184+W129. These findings were supported by immunoprecipitation analyses of ^{35}S -labeled cell lysates in which 7E3 did not immunoprecipitate the receptors from cells expressing β 3M or β 3M-Hu177-184, but did immunoprecipitate the receptors from cells expressing β 3M-Hu177-184+W129 (Fig. 4B).

Effect of K125 Substitutions on 7E3 Binding to α IIB β 3 Receptors. We further investigated the effects of substitutions in K125, which is present in both β 3Hu and β 3M. K125 is on α -helix 1 in close proximity to W129; it forms hydrogen bonds with Y122, and the latter forms a hydrogen bond with T182, thus linking the α -helix 1 to the C177-C184 loop (Fig. 5 A and B) (18). Neither the K125A nor the K125R substitution altered 7E3 binding. However, when K125 was mutated to a glutamine, the binding of 7E3 was reduced by \approx 50% relative to 10E5 (Fig. 5C). The structural effects of these substitutions were analyzed by using a molecular modeling approach. The alanine and arginine substitutions at K125 did not affect the structure or the hydrogen-bonding pattern in the region. In contrast, the substitution of the smaller glutamine for K125 resulted in the development of a new hydrogen bond between Q125 and Y122, as well as a 1-Å shift in T182 toward Y122 and a 1-Å shift in Y122 toward Q125 (Fig.

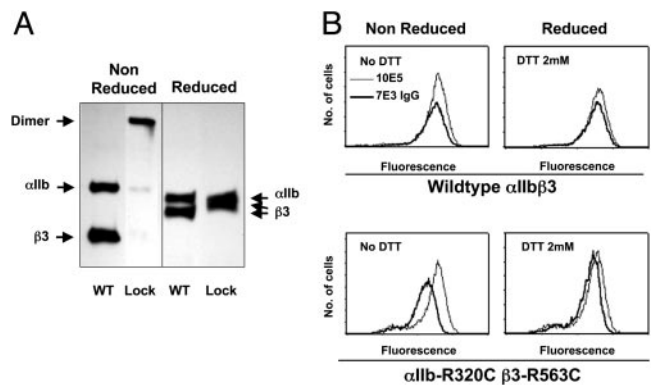


Fig. 6. 7E3 binding to α IIB β 3 and α IIB-R320C β 3-R563C. (A) Immunoprecipitation with 10E5 of lysates of surface biotin-labeled 293 cells expressing α IIB β 3 or α IIB-R320C β 3-R563C. Samples were electrophoresed under nonreduced and reduced conditions. (B) Fluorescent intensity of cells expressing α IIB β 3 or α IIB-R320C β 3-R563C after incubation with Alexa⁶⁴⁷-7E3 IgG or Alexa⁶⁴⁷-10E5, with or without treatment with 2 mM DTT.

5 D and E, arrowhead). In addition, there was partial loss of α -helix 1 (from amino acids 125-127), increasing the flexibility of this region (Fig. 5 D and E, arrow).

Effects of Locking α IIB β 3 in a Bent Conformation on 7E3 and 10E5 Binding.

Some 293 cells were generated that stably expressed α IIB β 3 or the mutant α IIB-R320C β 3-R563C receptor, which locks α IIB β 3 into a bent position by introducing a disulfide bond between the α IIB propeller and the EGF3 domain of β 3 (10). In accord with our previous report, almost all of the mutant receptors formed disulfide-bonded bent-locked heterodimers (Fig. 6A). As judged by flow cytometry, the mean fluorescent intensity (MFI) binding ratio of 7E3 IgG/10E5 to α IIB β 3-expressing cells was 0.78 ± 0.04 . In sharp contrast, the 7E3 IgG/10E5 MFI ratio was only 0.56 ± 0.05 with cells expressing α IIB-R320C β 3-R563C ($P < 0.008$; Fig. 6B and Table 3). When the smaller 7E3 Fab fragment was tested, the 7E3 Fab/10E5 MFI ratios were nearly identical in cells expressing the normal and the mutated receptors (0.58 ± 0.04 and 0.56 ± 0.04). Mild reduction with DTT (2 mM for 15 min) did not affect 10E5 binding to either α IIB β 3 or mutant-expressing cells (Table 3 and Fig. 6B). DTT increased 7E3 IgG binding to normal α IIB β 3 by only 12%, but increased the 7E3 IgG binding to the cells expressing the mutant receptor such that the 7E3 IgG/10E5 MFI ratio increased by 32%. The 7E3 Fab binding increased by only 5% and 9% with DTT reduction of α IIB β 3 and mutant receptors, respectively.

Table 3. MFI of cells expressing α IIB β 3 or α IIB-R320C β 3-R563C with presence or absence of 2 mM DTT

	10E5	7E3 IgG	7E3 Fab
α IIB β 3	435 \pm 85	336 \pm 49	255 \pm 64
α IIB β 3 + DTT	440 \pm 96 (1%)	379 \pm 55 (12%)	267 \pm 77 (5%)
α IIB-R320C β 3-R563C	389 \pm 63	218 \pm 43	218 \pm 53
α IIB-R320C β 3-R563C + DTT	387 \pm 64 (-1%)	290 \pm 53 (32%)*	237 \pm 78 (9%)

Data are the mean \pm SD of four separate experiments. In parentheses is shown the change in MFI after DTT treatment.

* $P < 0.008$ with DTT treatment.

Discussion

In the present study we provide immunologic, biochemical, and functional data on the binding of 7E3 and the mechanism of integrin activation. Because the binding properties of 7E3 IgG and related forms of 7E3 have been well characterized (3, 5, 9), these data have implications for 7E3's mechanism of inhibition of ligand binding as well as the structural changes in $\beta 3$ integrins that accompany platelet activation. In accord with the study of Puzon-McLaughlin *et al.* (7) we found that swapping the murine for the $\beta 3$ Hu C177–C184 sequence ($\beta 3$ Hu–M177-184) greatly reduced 7E3 binding. This chimeric $\beta 3$ subunit migrated slower than $\beta 3$ Hu on SDS/PAGE under nonreduced conditions, suggesting that the insertion of the murine residues in the human backbone reduces the likelihood of forming the C177–C184 bond. This hypothesis is strengthened by the observation that $\beta 3$ Hu–M177-184 migrated at the same M_r as a construct in which the C177–C184 bond could not be formed because alanines were substituted for C177 and C184. However, it is not the loss of the disulfide bond itself that causes the loss of the 7E3 epitope, because at least some of the receptors with the C177A and C184A substitutions could still be immunoprecipitated by 7E3. Only when the $\beta 3$ M 179–183 sequence was swapped into the C177A+C184A construct was 7E3 binding lost.

Of the three differences in amino acids in the human and murine C177–C184 sequences, only the T182N substitution reduced the binding of 7E3 as judged by immunoprecipitation. In accord with these binding data, adhesion to immobilized fibrinogen of cells expressing this mutated receptor was not inhibited by 7E3. Remarkably, the $\beta 3$ subunit containing the T182N substitution migrated in SDS/PAGE gels as a closely spaced doublet. The minor band migrated as did $\beta 3$ Hu and was immunoprecipitated by 7E3. The major band migrated more slowly, like the $\beta 3$ subunit containing alanines in place of cysteines at positions 177 and 184, and could not be immunoprecipitated by 7E3. Our hypothesis is that the T182N substitution decreases the likelihood of the formation of the C177–C184 disulfide loop, thus resulting in the presence of two different conformations, one in which the C177–C184 disulfide bond forms (faster migrating) and the other in which it does not (slower migrating). 7E3 can react with the former, but not the latter, indicating that 7E3 binding does not absolutely require the threonine at position 182. These data were confirmed with functional studies of 7E3-induced inhibition of adhesion to immobilized fibrinogen.

Puzon-McLaughlin *et al.* (7) also reported that swapping the murine W129–N133 sequence into $\beta 3$ Hu resulted in partial loss of 7E3 binding. We did not observe a loss of 7E3 binding when the two individual substitutions in this region (W129S and N133T) were made, suggesting that both substitutions are required to affect 7E3 binding.

Because the loss of 7E3 binding upon altering one or more amino acids in $\beta 3$ provides only presumptive evidence for a direct role of the amino acid in 7E3 binding, we sought more convincing evidence by attempting to re-engineer $\beta 3$ M so that 7E3 would bind to it when complexed with human α IIB. We demonstrated that insertion of the human C177–C184 sequence into $\beta 3$ M supported partial binding of 7E3 as judged by flow cytometry, but the binding appeared to be of low affinity because 7E3 did not immunoprecipitate the receptor, and 7E3 only partially inhibited the adhesion to immobilized fibrinogen of cells expressing this construct. When the W129S substitution was added to $\beta 3$ M containing the human C177–C184 loop, however, 7E3 not only reacted with the receptor by flow cytometry and immunoprecipitation, but also eliminated cell adhesion. Thus, substitutions in both regions of $\beta 3$ M are required for 7E3 binding to cells coexpressing human α IIB.

Table 4. 7E3 binding in different species and amino acid sequences of Site1 and Site2 on $\beta 3$

Species	7E3 activity	129–133	177–184
Human	+++++	¹²⁹ WSIQN ¹³³	¹⁷⁷ CYDMKTTTC ¹⁸⁴
Dog	+++	¹²⁹ S---- ¹³³	¹⁷⁷ ----- ¹⁸⁴
Rat	+	¹²⁹ S---T ¹³³	¹⁷⁷ --T--ST-- ¹⁸⁴
Pig	–	¹²⁹ E---- ¹³³	¹⁷⁷ ----- ¹⁸⁴
Mouse	–	¹²⁹ S---T ¹³³	¹⁷⁷ --N--NA-- ¹⁸⁴

Analysis of species differences in the ability of 7E3 to inhibit platelet α IIB $\beta 3$ is also consistent with the importance of both human C177–C184 and W129 in 7E3 binding (19–21). As shown in Table 4, a decreasing order of binding affinity of 7E3 for human (5), dog (22), and rat (23) platelets is observed, whereas 7E3 does not bind at all to platelets from pig (B.S.C., unpublished observation) and mouse. Dog $\beta 3$ shares the same 177–184 sequence as human, but has the W129S substitution found in $\beta 3$ M. Rat $\beta 3$ has the same W129S and N133T substitutions as mouse, but it does not have the T182N substitution found to be so important in the loss of 7E3 binding to $\beta 3$ M; instead it has a conservative T182S substitution. Pig $\beta 3$ is particularly instructive because the pig and human 177–184 sequences are identical, but pig has a W129E substitution that introduces a charged amino acid in place of a hydrophobic residue.

Although W129 is not continuous with C177–C184 in the primary sequence of $\beta 3$ Hu, the crystal structure of α V $\beta 3$ shows these regions to be contiguous in the folded protein (18). These data provide very strong complementary support for the biochemical data indicating that both regions participate in 7E3 binding. Moreover, this region is very close to the $\beta 3$ metal ion-dependent adhesion site (MIDAS), which participates in ligand binding. Thus, although we cannot exclude the possibility of allosteric effects, we propose that the 7E3 epitope is on or near the ≈ 15 -Å ridge that extends between the C177–C184 loop and

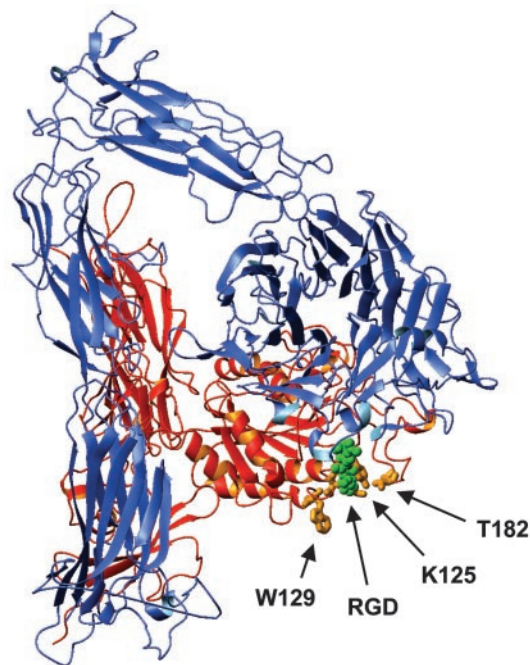


Fig. 7. Crystal structure of entire extracellular domain of α V $\beta 3$ with amino acids Y122, K125, W129, and K181, which are implicated in the 7E3 epitope, shown in orange. The RGD peptide is shown in green. Figure was prepared with MOL MOL (32).

W129 (Fig. 5 A and B). This proposed location will, however, require direct confirmation by crystallographic analysis. This region contains the conserved residues Y122 and K125. Y122 may also be important to the 7E3 epitope because Y122 appears to form hydrogen bonds with both K125 and T182. Thus, we found that the K125Q substitution, which alters the hydrogen-bonding pattern that links the C177–C184 loop to α -helix 1, decreased 7E3 binding by $\approx 50\%$, strengthening the hypothesis that α -helix 1, which contains W129, is important in maintaining the 7E3 epitope.

The proximity of the proposed 7E3 epitope to the MIDAS provides an explanation for the ability of 7E3 to inhibit ligand binding and suggests that 7E3 inhibits ligand binding by steric hindrance, an allosteric effect on the RGD binding region, or both effects. Our previous studies demonstrating that RGD peptides in solution do not inhibit 7E3 binding to α IIb β 3 (24), but that 7E3 inhibits the binding to α IIb β 3 of RGD peptides immobilized on beads (24), are also consistent with 7E3 operating through steric hindrance, allosteric changes in the RGD binding region, or both effects. The data in this study also provide an explanation for our previous observation that 7E3 blocks the binding of antibody LM609 to α V β 3 because previous studies by Puzon-McLaughlin *et al.* (7) demonstrated that LM609 did not bind to human α V β 3 when the region immediately adjacent to the C177–C184 loop was swapped with the murine sequence.

Based on the crystallographic data of Xiong *et al.* (18) and our NMR and electron microscopic data (10, 25) we previously proposed that the α V β 3 receptor assumes variably bent conformations. Thus, if α IIb β 3 is similarly bent, the 7E3 epitope may be only partially accessible on the most highly bent forms of the receptor as a result of steric hindrance from the legs of α IIb and/or β 3. If the legs of the receptor enter the plasma membrane with a perpendicular orientation, the plasma membrane may also limit access of 7E3 to its epitope (Fig. 7) (25); a model based on electron cryomicroscopy, however, suggests that the legs do not enter the membrane with a perpendicular orientation, (26) and thus this issue remains open.

We previously observed that 7E3 IgG binds more slowly to unactivated platelets than the smaller 7E3 Fab fragment and that multimers of 7E3 (dimers, trimers, and tetramers) bind more slowly than 7E3 IgG (5). Moreover, we observed that the on rate of 7E3 IgG increases by >2 -fold with platelet activation, but the on rate of 7E3 Fab increases much less with activation (9). These

observations are consistent with our model in which the extent of receptor bending is variable and there is a dynamic equilibrium between the different bent conformations (10, 25). They are also consistent with our data that activation results in the receptor assuming a less bent or perhaps fully extended conformation (10). These data do not, however, exclude the possibility that additional changes occur in the head and tail regions of the molecule (2, 27–30). Our previous studies with RGD peptides tethered to small beads by variably sized glycine tethers also are consistent with this working model because beads containing peptides with long, but not short, tethers could bind to both unactivated and activated platelets, whereas beads with peptides with short tethers bound much better to activated platelets (24).

To test the effect of locking the receptor in a bent position on 7E3 binding, we analyzed 7E3 IgG binding to cells expressing α IIb-R320C β 3-R563C, in which a disulfide bond constrains the receptor in a bent conformation, and found that the binding was reduced as compared to 7E3 IgG binding to cells expressing normal α IIb β 3. Consistent with the reduction in binding being a result of the disulfide bond locking the receptor in a bent position, mild reduction of the receptor with DTT markedly reduced the difference in 7E3 IgG binding to mutant and native α IIb β 3. Further support for a size-dependent access mechanism comes from our findings that the binding of the much smaller 7E3 Fab to cells expressing α IIb-R320C β 3-R563C or native α IIb β 3 was nearly equivalent and that DTT treatment only minimally increased 7E3 Fab binding to the mutant receptor.

In conclusion, we provide data on 7E3 binding and propose a location for the 7E3 epitope in the 3D structures of α IIb β 3 and α V β 3. The strategic localization of this region relative to the β 3 ligand binding site provides a structural explanation for the inhibitory effects of 7E3 on ligand binding. Moreover, combining the data on the size and the activation-dependent nature of 7E3 binding to platelets with structural data on α V β 3 provides insights into the molecular dynamics of the receptor under basal conditions and the conformational changes it undergoes with activation.

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