Gln-589 is highlighted in cyan. Note that Ser-462 is located above the α-*genu*, while Ser-528 is located on the side of the thigh domain distal to the α-genu.

Fig. 5. Effect of anti-αVβ3 mAbs on fibrinogen binding. A. FITC-fibrinogen binding to cells expressing αVβ3 in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+} (open column) or in the presence of 2 mM Mg^{2+} and 5 μM EGTA (hatched column) is shown. The ratio of the MFI (FL1) to the MFI (FL2) in the gated cell population was used to normalize the binding with the expression of αVβ3 on the cell surface. B. FITC-fibrinogen binding to cells expressing wild-type αVβ3 in the presence of 2 mM Mg^{2+} and 5 μM EGTA was examined. Binding in the presence of 100 μg/mL of the indicated mAb is shown in the hatched column. An equivalent volume of PBS, instead of the mAb solution, was included to obtain the control binding. The asterisks indicate statistically different binding abilities, compared with the control (*P<0.01, **P<0.05).

Fig. 6. Effect of integrin extension on the function of the anti-αVβ3 mAbs. A. SDS-PAGE analysis of αVβ3 expressed on CHO cells. Cell lysates from biotin-labeled cells expressing αVβ3 were immunoprecipitated with anti-αV mAb P2W7. The precipitates were subjected to a 7.5% non-reducing gel and visualized using chemiluminescence. The positions of the molecular weight markers are shown on the left side of panel. Lane 1, parent CHO; lane 2, wild-type αVβ3; lane 3, Q589NAT. Note that the αV carrying the Q589NAT mutation (arrowhead) migrated more slowly than the wild type. B. FITC-fibrinogen binding to cells expressing αVβ3 carrying the Q589NAT mutation was examined as described in Fig. 4A. Binding in the presence of 1 mM Ca^{2+} and 1 mM Mg^{2+} (open column) or in the presence of 2

mM Mg^{2+} and 5 μM EGTA (solid column) is shown. C. The effect of the anti-αVβ3 mAbs on fibrinogen binding to cells expressing αVβ3 carrying the Q589NAT mutation was examined, as described in Fig. 5B. Among the mAbs, only 7E3 significantly inhibited binding.

Fig. 7. Effect of integrin extension on the binding of the anti-αVβ3 mAbs. Wild-type αV or αV carrying the Q589NAT mutation was transiently expressed together with wild-type β3 in CHO cells. The binding of function-blocking mAbs to these cells was examined by FACS. The MFI obtained from the whole cell population with each mAb was normalized by the MFI obtained with an anti-αV mAb 17E6 that represents the αV expression. There was no significant difference in the binding of leg-binding mAbs in cells expressing Q589NAT (solid column) as compared with cells expressing wild-type αV (hatched column).

	mIgG	10C4	23C6	LM609	17E6	69-6-5	AMF-7	M9	P2W7	P3G8	SZ21
CHO	5.26	4.77	4.34	4.48	4.94	5.23	3.57	3.15	18.99	4.25	3.63
β3	2.72	55.83	70.09	61.93	3.64	4.66	2.92	3.93	4.08	5.84	49.89
αVβ3	3.47	61.09	77.73	68.92	67.32	64.4	64.17	71.05	57.59	63.15	54.52
αIIbβ3	3.92	57.69	58.25	65.47	3.66	4.25	3.07	4.08	4.61	5.56	51.73
V/B	4.18	58.73	69.11	61.9	53.13	45.19	3.08	3.78	6.00	6.36	52.16
B/V	4.24	51.6	63.63	57.3	7.11	6.44	58.39	63.47	49.97	55.63	49.16

Table 1. **MAb binding to cells expressing tail-swapping chimeras.** MAb binding to cells expressing wild-type human β3 (β3), wild-type human αVβ3 (αVβ3), wild-type human αIIbβ3 (αIIbβ3), tail-swapping mutants (V/B, B/V), and to parent CHO cells (CHO) was examined. The numbers represent the percentage of the cell population stained with each mAb.

	mIgG	AMF-7	M9	P2W7	P3G8	17E6	SZ21
CHO	7.83	5.86	5.72	15.51	5.78	5.91	4.8
αVβ3	5.6	68.11	78.41	63.75	67.97	76.32	59.23
VT	4.72	3.37	4.09	6.91	6.44	74.37	68.6
VC1	10.88	82.45	90.34	71.17	76.44	90.39	83.9
VC2	4.7	64.82	81.14	69.67	69.52	78.53	74.97
T	6.4	49.93	62.12	45.45	44.62	4.45	62.75
C1	5.87	3.8	4.03	5.82	6.37	4.05	61.79
C2	5.36	5.53	5.16	6.57	8.25	6.8	71.52

Table 2. **MAb binding to cells expressing domain-swapping chimeras.** The numbers represent the percentage of the cell population stained with each mAb.

	mIgG	AMF-7	M9	P2W7	P3G8	17E6	SZ21
CHO	3.62	2.56	2.95	13.3	3.2	4.07	1.16
WT	6.8	74.11	85.85	78.21	83.47	87.07	71.41
I441V	4.15	76.76	70.66	72.64	77.61	79.97	70.78
T460ICP	5.15	5.84	3.26	7.01	70.72	77.52	65.15
T460I	5.88	62.11	63.27	54.8	56.43	70.81	64.01
S462P	5.52	14.66	3.75	17.17	66.41	76.18	66.88
V486T	11.93	72.65	78.35	71.75	64.14	82.23	71.1
N492H	10.04	70.78	79	70.14	72.01	82.49	73.56
E496DV	14.9	73.25	76.95	67.31	75.08	75.01	58.72
Y515HN	8.47	59.79	67.31	59.04	65.69	66.81	50.75
S520V	8.33	65.13	72.76	61.29	73.55	71.38	60.68
N524T	12.98	60.75	69.31	58.6	65.03	66.37	57.98
I527VF	9.55	74.93	78.94	59.25	9.26	84.31	81.13
I527V	3.12	75.57	78.3	64.88	77.78	83.6	67.47
S528F	5.32	80.94	82.19	76.5	10.59	85.83	78.1
L532Q	26.68	62.2	77.22	58.2	62.38	76.84	71.26
I539V	13.13	73.98	67.01	57.12	68.84	76.24	64.92
Y565Q	11.64	70.1	77.22	64.52	71.32	80.13	72.63
T571A	27.84	68.76	69.91	54.89	59.55	75.22	75.28
I586V	9.99	70.96	78.21	61.16	70.96	68.54	76.34

Table 3. **MAb binding to cells expressing human-to-mouse αV mutants.** The numbers represent the percentage of the cell population stained with each mAb. Bindings significantly lower than those for 17E6 or SZ21 are marked in red.

REFERENCES

Adair BD, Xiong JP, Maddock C, Goodman SL, Arnaout MA et al. (2005) Three-dimensional EM structure of the ectodomain of integrin {alpha}V{beta}3 in a complex with fibronectin. The Journal of cell biology 168(7): 1109-1118.

Blue R, Li J, Steinberger J, Murcia M, Filizola M et al. (2010) Effects of limiting extension at the alphaIIb genu on ligand binding to integrin alphaIIbbeta3. The Journal of biological chemistry 285(23): 17604-17613.

Chen W, Lou J, Hsin J, Schulten K, Harvey SC et al. (2011) Molecular dynamics simulations of forced unbending of integrin alpha(v)beta(3). PLoS computational biology 7(2): e1001086.

Chen X, Xie C, Nishida N, Li Z, Walz T et al. (2010) Requirement of open headpiece conformation for activation of leukocyte integrin alphaXbeta2. Proceedings of the National Academy of Sciences of the United States of America 107(33): 14727-14732.

Cheresh DA (1987) Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. Proceedings of the National Academy of Sciences of the United States of America 84(18): 6471-6475.

Chuntharapai A, Bodary S, Horton M, Kim KJ (1993) Blocking monoclonal antibodies to alpha V beta 3 integrin: a unique epitope of alpha V beta 3 integrin is present on human osteoclasts. Experimental cell research 205(2): 345-352.

Coller BS (1985) A new murine monoclonal antibody reports an activation-dependent change in the conformation and/or microenvironment of the platelet glycoprotein IIb/IIIa complex. The Journal of clinical investigation 76(1): 101-108.

de Vries JE, Keizer GD, te Velde AA, Voordouw A, Ruiter D et al. (1986) Characterization of melanoma-associated surface antigens involved in the adhesion and motility of human melanoma cells. International journal of cancer 38(4): 465-473.

Friedlander DR, Zagzag D, Shiff B, Cohen H, Allen JC et al. (1996) Migration of brain tumor cells on extracellular matrix proteins in vitro correlates with tumor type and grade and involves alphaV and beta1 integrins. Cancer Res 56(8): 1939-1947.

Horton MA, Lewis D, McNulty K, Pringle JA, Chambers TJ (1985) Monoclonal antibodies to osteoclastomas (giant cell bone tumors): definition of osteoclast-specific cellular antigens. Cancer Res 45(11 Pt 2): 5663-5669.

Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. Cell 110(6): 673-687.

Kamata T, Irie A, Tokuhira M, Takada Y (1996) Critical residues of integrin alphaIIb subunit for binding of alphaIIbbeta3 (glycoprotein IIb-IIIa) to fibrinogen and ligand-mimetic antibodies (PAC-1, OP-G2, and LJ-CP3). The Journal of biological chemistry 271(31): 18610-18615.

Kamata T, Handa M, Sato Y, Ikeda Y, Aiso S (2005) Membrane-proximal {alpha}/{beta} stalk interactions differentially regulate integrin activation. The Journal of biological chemistry 280(26): 24775-24783.

Kamata T, Handa M, Ito S, Sato Y, Ohtani T et al. (2010) Structural requirements for activation in alphaIIb beta3 integrin. The Journal of biological chemistry 285(49): 38428-38437.

Lehmann M, Rabenandrasana C, Tamura R, Lissitzky JC, Quaranta V et al. (1994) A monoclonal antibody inhibits adhesion to fibronectin and vitronectin of a colon carcinoma cell line and recognizes the integrins alpha v beta 3, alpha v beta 5, and alpha v beta 6. Cancer Res 54(8): 2102-2107.

Litvinov RI, Mekler A, Shuman H, Bennett JS, Barsegov V et al. Resolving two-dimensional kinetics of the integrin alphaIIbbeta3-fibrinogen interactions using Binding-Unbinding Correlation Spectroscopy. The Journal of biological chemistry.

Luo BH, Takagi J, Springer TA (2004a) Locking the beta3 integrin I-like domain into high and low affinity conformations with disulfides. The Journal of biological chemistry 279(11): 10215-10221.

Luo BH, Strokovich K, Walz T, Springer TA, Takagi J (2004b) Allosteric beta1 integrin antibodies that stabilize the low affinity state by preventing the swing-out of the hybrid domain. The Journal of biological chemistry 279(26): 27466-27471.

Mitjans F, Sander D, Adan J, Sutter A, Martinez JM et al. (1995) An anti-alpha v-integrin antibody that blocks integrin function inhibits the development of a human melanoma in nude mice. Journal of cell science 108 (Pt 8): 2825-2838.

Puzon-McLaughlin W, Kamata T, Takada Y (2000) Multiple discontinuous

ligand-mimetic antibody binding sites define a ligand binding pocket in integrin alpha(IIb)beta(3). The Journal of biological chemistry 275(11): 7795-7802.

Springer TA, Zhu J, Xiao T (2008) Structural basis for distinctive recognition of fibrinogen gammaC peptide by the platelet integrin alphaIIbbeta3. The Journal of cell biology 182(4): 791-800.

Takagi J, Petre BM, Walz T, Springer TA (2002) Global conformational rearrangements in integrin extracellular domains in outside-in and inside-out signaling. Cell 110(5): 599-511.

Takagi J, Strokovich K, Springer TA, Walz T (2003) Structure of integrin alpha5beta1 in complex with fibronectin. The EMBO journal 22(18): 4607-4615.

Tang XY, Li YF, Tan SM (2008) Intercellular adhesion molecule-3 binding of integrin alphaL beta2 requires both extension and opening of the integrin headpiece. J Immunol 180(7): 4793-4804.

Tokuhira M, Handa M, Kamata T, Oda A, Katayama M et al. (1996) A novel regulatory epitope defined by a murine monoclonal antibody to the platelet GPIIb-IIIa complex (alpha IIb beta 3 integrin). Thromb Haemost 76(6): 1038-1046.

von Schlippe M, Marshall JF, Perry P, Stone M, Zhu AJ et al. (2000) Functional interaction between E-cadherin and alphav-containing integrins in carcinoma cells. Journal of cell science 113 (Pt 3): 425-437.

Wayner EA, Orlando RA, Cheresh DA (1991) Integrins alpha v beta 3 and alpha v beta 5 contribute to cell attachment to vitronectin but differentially distribute on the cell surface. The Journal of cell biology 113(4): 919-929.

Weisel JW, Nagaswami C, Vilaire G, Bennett JS (1992) Examination of the platelet membrane glycoprotein IIb-IIIa complex and its interaction with fibrinogen and other ligands by electron microscopy. The Journal of biological chemistry 267(23): 16637-16643.

Xiao T, Takagi J, Coller BS, Wang JH, Springer TA (2004) Structural basis for allostery in integrins and binding to fibrinogen-mimetic therapeutics. Nature 432(7013): 59-67.

Xie C, Shimaoka M, Xiao T, Schwab P, Klickstein LB et al. (2004) The integrin alpha-subunit leg extends at a Ca2+-dependent epitope in the thigh/genu interface upon activation. Proceedings of the National Academy of Sciences of the United States of America 101(43): 15422-15427.

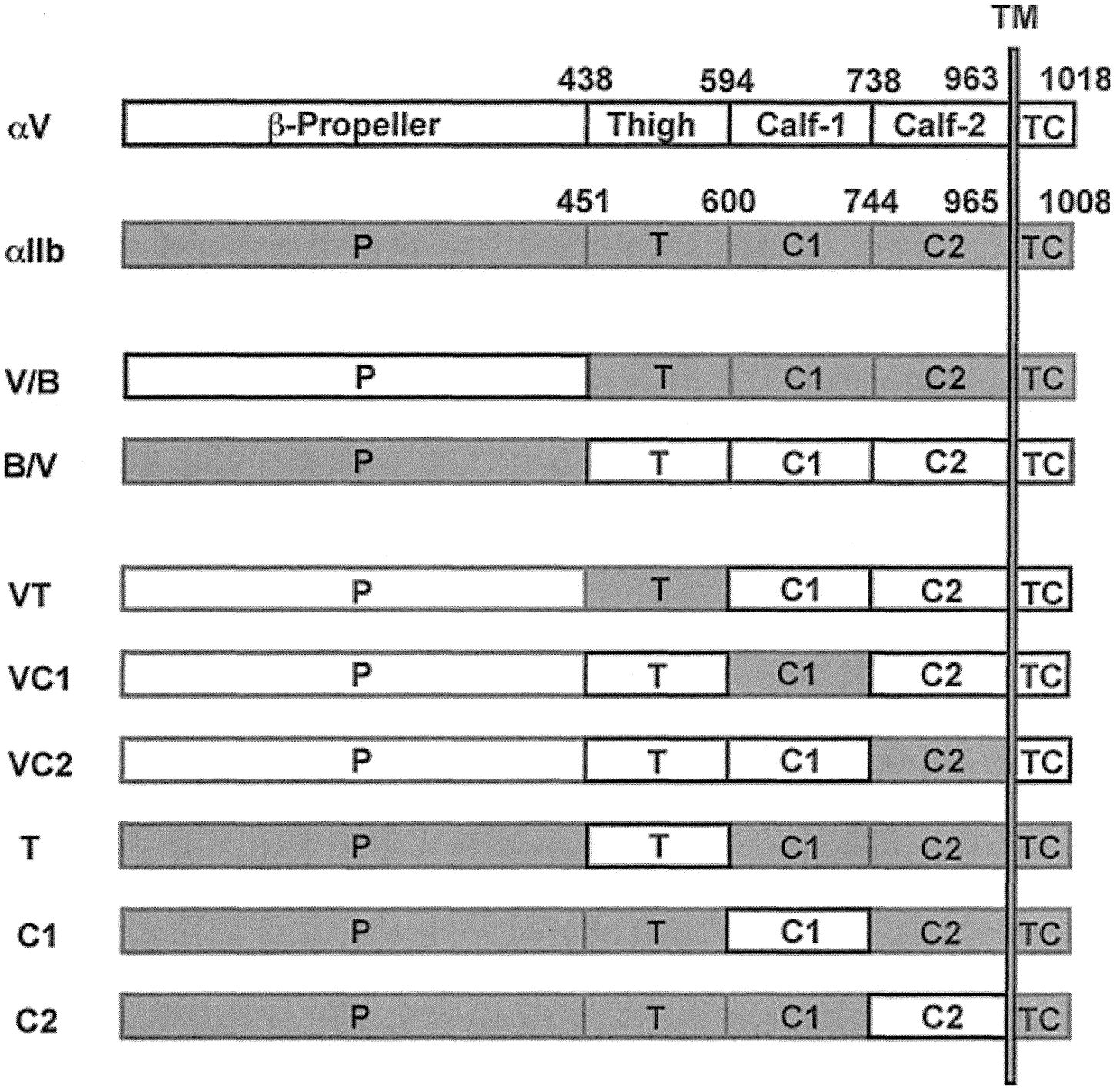
Xiong JP, Stehle T, Zhang R, Joachimiak A, Frech M et al. (2002) Crystal structure of the extracellular segment of integrin alpha Vbeta3 in complex with an Arg-Gly-Asp ligand. Science (New York, NY 296(5565): 151-155.

Xiong JP, Stehle T, Diefenbach B, Zhang R, Dunker R et al. (2001) Crystal structure of the extracellular segment of integrin alpha Vbeta3. Science (New York, NY 294(5541): 339-345.

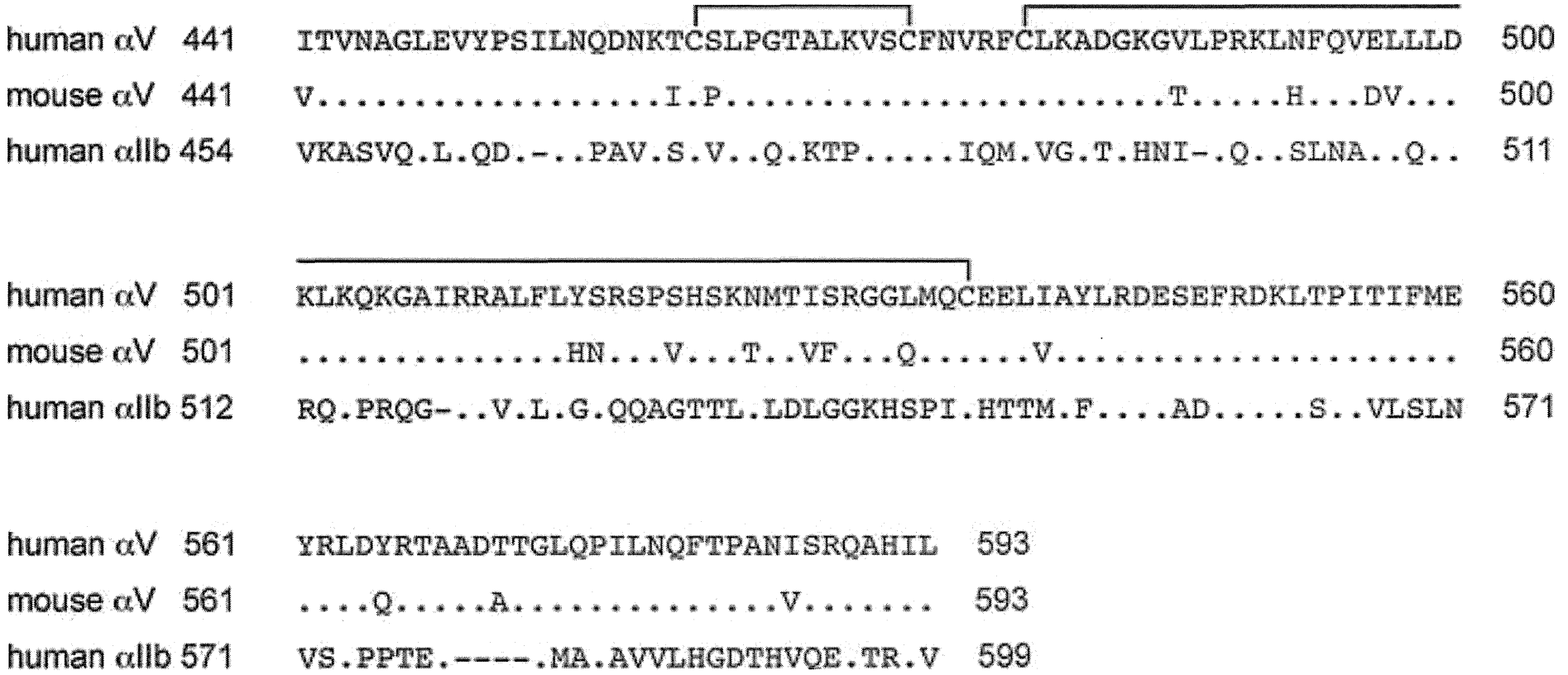
Xiong JP, Mahalingham B, Alonso JL, Borrelli LA, Rui X et al. (2009) Crystal structure of the complete integrin alphaVbeta3 ectodomain plus an alpha/beta transmembrane fragment. The Journal of cell biology 186(4): 589-600.

Ye F, Liu J, Winkler H, Taylor KA (2008) Integrin alpha IIb beta 3 in a membrane environment remains the same height after Mn2+ activation when observed by cryoelectron tomography. Journal of molecular biology 378(5): 976-986.

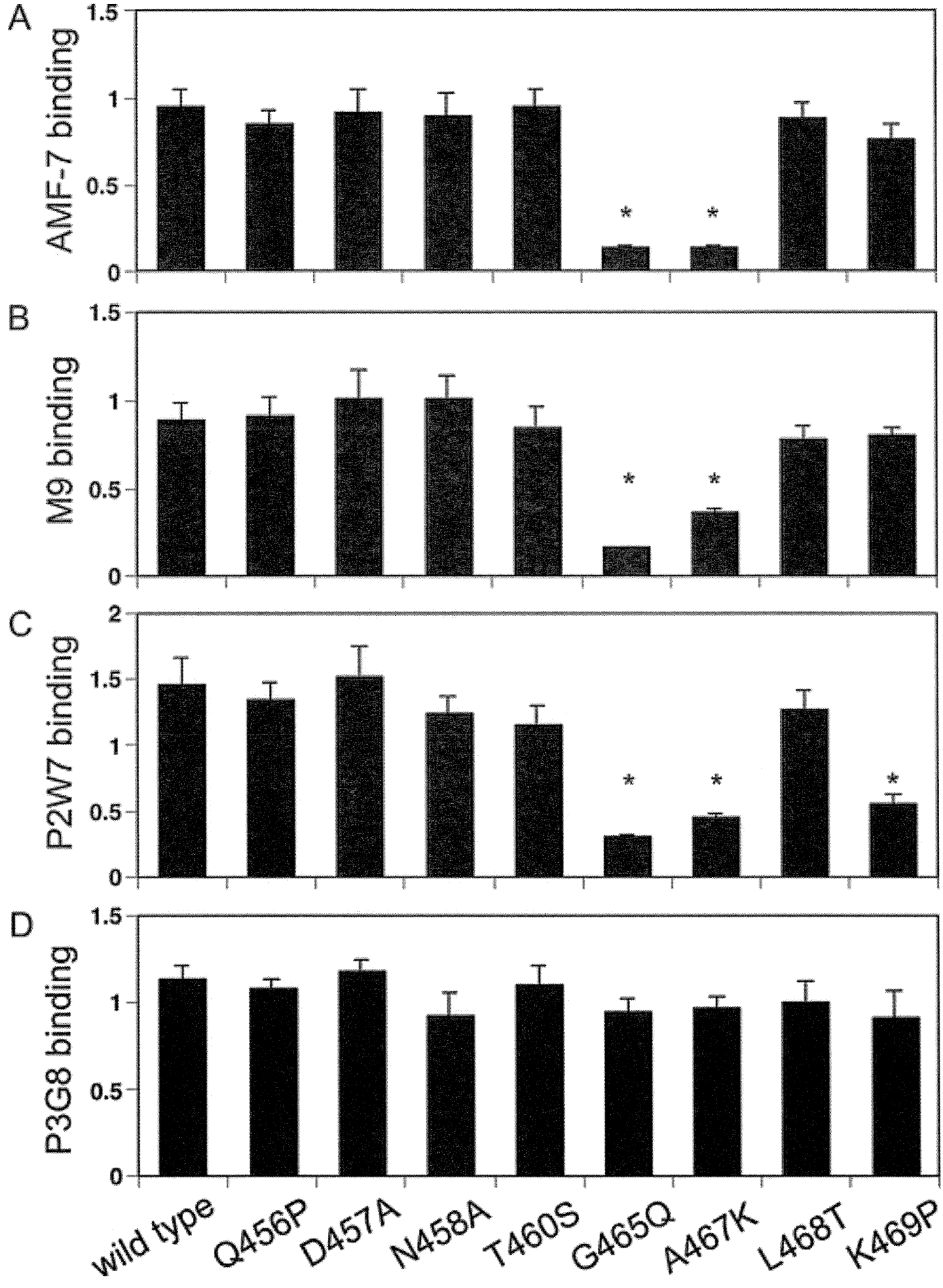
Ye F, Hu G, Taylor D, Ratnikov B, Bobkov AA et al. (2010) Recreation of the terminal events in physiological integrin activation. The Journal of cell biology 188(1): 157-173.



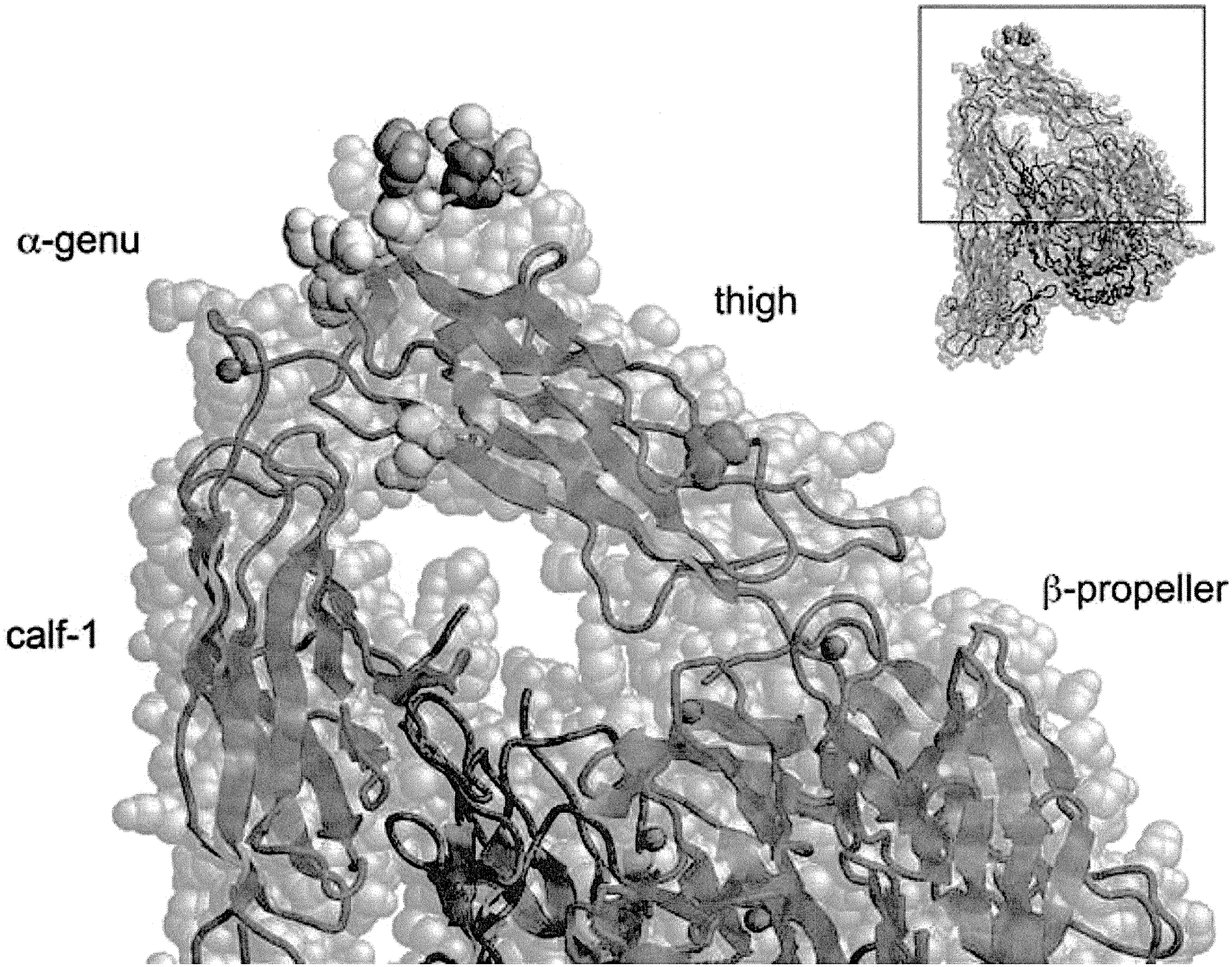
Click here to download high resolution image

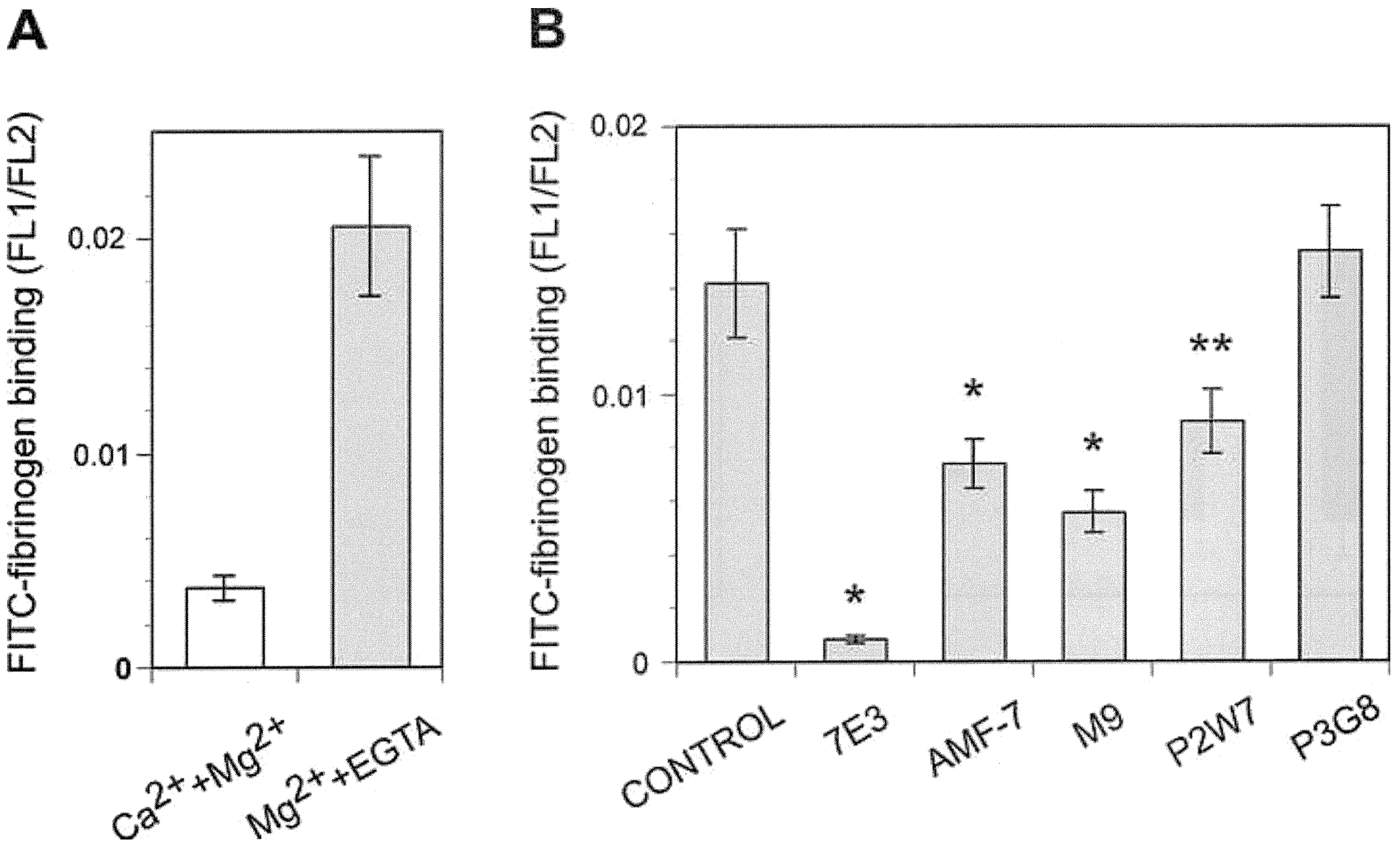


ıre 3
k here to download high resolution image



Click here to download high resolution image





Click here to download high resolution image