A Molecular Mechanism of Integrin Crosstalk: $\alpha_v\beta_3$ Suppression of Calcium/Calmodulin-dependent Protein Kinase II Regulates $\alpha_5\beta_1$ Function

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Abstract. Many cells express more than one integrin receptor for extracellular matrix, and in vivo these receptors may be simultaneously engaged. Ligation of one integrin may influence the behavior of others on the cell, a phenomenon we have called integrin crosstalk. Ligation of the integrin $\alpha_v\beta_3$ inhibits both phagocytosis and migration mediated by $\alpha_5\beta_1$ on the same cell, and the β_3 cytoplasmic tail is necessary and sufficient for this regulation of $\alpha_5\beta_1$. Ligation of $\alpha_5\beta_1$ activates the calcium- and calmodulin-dependent protein kinase II (CamKII). This activation is required for $\alpha_5\beta_1$-mediated phagocytosis and migration. Simultaneous ligation of $\alpha_v\beta_3$ or expression of a chimeric molecule with a free β_3 cytoplasmic tail prevents $\alpha_5\beta_1$-mediated activation of CamKII. Expression of a constitutively active CamKII restores $\alpha_5\beta_1$ functions blocked by $\alpha_v\beta_3$-initiated integrin crosstalk. Thus, $\alpha_v\beta_3$ inhibition of $\alpha_5\beta_1$ activation of CamKII is required for its role in integrin crosstalk. Structure-function analysis of the β_3 cytoplasmic tail demonstrates a requirement for Ser752 in β_3-mediated suppression of CamKII activation, while crosstalk is independent of Tyr747 and Tyr759, implicating Ser752, but not β_3 tyrosine phosphorylation in initiation of the $\alpha_v\beta_3$ signal for integrin crosstalk.

Key words: integrin • vitronectin • kinase • crosstalk • signaling

DYNAMIC interaction of cells with the complex protein mixtures found in the extracellular matrix occurs during many biologic and pathologic processes including development, wound healing, hemostasis, metastasis, inflammation, and thrombosis (13, 18). Most cells express multiple integrin receptors capable of interaction with the numerous ligands found in complex tissues. The simultaneous ligation of multiple integrins mandates coordination of the resulting signals. The coordination of integrin signaling into a hierarchy with a net effect on cell behavior has been called integrin crosstalk (2, 3, 17).

Numerous examples of integrin crosstalk have been reported. The common theme of these reports lies in the regulation of the function of one integrin (the target) as a result of coligation of a second integrin (the transducer) on the same cell. Examples of integrin crosstalk have been demonstrated in numerous primary cell types and cell lines including macrophages (2), T cells (17, 23), smooth muscle cells (1), neutrophils (10), monocytes (15), umbilical vein endothelial cells (20), malignant astrocytomas (16), CHO cells (7, 9), K562 cells (2, 3), and embryonic kidney 293 cells (20). Crosstalk may be initiated by transducing integrins belonging to the β_1 (11, 15, 16, 22), β_2 (17, 23), or β_3 (1–3, 7, 9, 10, 20) family with targets in any of these families as well. Integrin functions affected by crosstalk include phagocytosis (2, 3, 10), soluble ligand binding (7, 15), adhesion (9, 17, 23), migration (1, 17, 20), gene expression (11), and receptor-mediated endocytosis (16).

It is important to note that all reported cases of integrin crosstalk are unidirectional, that is, ligation of the target integrin does not affect the transducer integrin. In many cases, the transducing integrin is much less highly expressed than the target integrin (2, 3). This suggests a hypothesis that the receptor pairs involved in crosstalk are not simply competing for interaction with a signaling molecule, but rather that ligation of the transducing integrin initiates a unidirectional signaling cascade which affects

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the function of the target integrin. The molecular mechanisms of integrin crosstalk remain undetermined. With a single exception (20), crosstalk signals from the transducing integrin require the cytoplasmic tail of the β-subunit, and where it has been examined, the β-subunit cytoplasmic tail has been sufficient for initiation of signaling (3).

We have previously described integrin crosstalk between $\alpha_v\beta_3$ and $\alpha_5\beta_1$ in macrophages and in a K562 cell transfection model of macrophage integrins (2, 3). We have shown that ligation of $\alpha_v\beta_3$ inhibits $\alpha_5\beta_1$-mediated phagocytosis, which requires the high affinity state of the integrin, without affecting $\alpha_5\beta_1$-mediated adhesion, which is independent of the high affinity state of the integrin. The cytoplasmic tail of β_3 is necessary and sufficient for this crosstalk. $\alpha_v\beta_3$-mediated inhibition of $\alpha_5\beta_1$ phagocytosis occurs at a step subsequent to $\alpha_5\beta_1$ binding of ligand and is reversed by H7, a pharmacologic inhibitor of serine/threonine kinases.

In this report, we define a molecular mechanism required for $\alpha_v\beta_3$-to-$\alpha_5\beta_1$ crosstalk. Ligation of $\alpha_5\beta_1$ enhances the activity of the calcium/calmodulin-dependent protein kinase II (CamKII)[1]. This increase in CamKII activity is required for $\alpha_5\beta_1$-dependent migration as well as $\alpha_5\beta_1$-dependent phagocytosis. Simultaneous ligation of $\alpha_v\beta_3$ inhibits $\alpha_5\beta_1$ activation of CamKII activity, thus blocking $\alpha_5\beta_1$ migration and phagocytosis. Mutational analysis of the β_3 cytoplasmic tail demonstrates that Ser752 is required for both $\alpha_v\beta_3$-initiated inhibitory crosstalk to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ suppression of CamKII activity, while tyrosine phosphorylation of the β_3 cytoplasmic tail has no effect on this activity. These results describe a potential molecular pathway for integrin crosstalk that involves integrin regulation of CamKII activity.

Materials and Methods

Cells and β_3 cDNA Mutation

Human peripheral blood monocyte-derived macrophages were prepared as previously described (2). The human erythroleukemic cell line K562 transfected with cDNA encoding $\alpha_v\beta_3$ ($K\alpha_v\beta_3$), $\alpha_v\beta_3$ in which the tyrosine residue at position 747 or 759 was mutated to phenylalanine ($K\alpha_v\beta_3$Y747F and $K\alpha_v\beta_3$Y759F, respectively) and $Tac\beta_3$ (chimera of the extracellular and transmembrane domain of the IL2 receptor α-chain [Tac subunit] and the cytoplasmic tail of β_3, $KTac\beta_3$) were derived and maintained as described (2, 3). In addition K562 cells were similarly transfected with cDNA encoding $Tac\beta_3$ in which the serine residue at position 752 was replaced with proline ($KTac\beta_3$S-P), cysteine ($KTac\beta_3$S-C), alanine ($KTac\beta_3$S-A), or glutamic acid ($KTac\beta_3$S-E). Expression of all $Tac\beta_3$S-X chimeras was equivalent to $Tac\beta_3$ (3) as determined by flow cytometry as described (2; see Table I). For construction of $Tac\beta_3$S-X, the HindIII and XhoI fragment of $pTac\beta_3$ encoding the CT of β_3 (3) was ligated into HindIII-XhoI–digested pBluescript (Stratagene), creating pBSKSPB3-TAIL. This contruct was subjected to PCR using a 5′ T7 oligonucleotide (Stratagene) with the 3′ oligonucleotide (5′-CCCCCCTCGAGTTA-AGTGCCCCGGTACGTGATATTGGTGAAGGT-XXX-CGTGGC-3′) where XXX is AGG for S^{752}P, ACA for S^{752}C, GCT for S^{752}A, and TTC for S^{752}E. The resulting products were digested with HindIII-XhoI and ligated into $pTac\beta_3$ digested with HindIII-XhoI, creating $pTac\beta_3$S-P, $pTac\beta_3$S-C, $pTac\beta_3$S-A, and $pTac\beta_3$S-E, respectively.

1. *Abbreviations used in this paper:* CamKII, calcium/calmodulin-dependent protein kinase II; FN, fibronectin; IMDM, Iscove's Modified Eagle's Medium; LIBS, ligand-induced binding site; MLCK, myosin light chain kinase; VN, vitronectin.

Table I. Integrin Expression in Transfected K562 Cells

	P5D2 (β_1)	AP3 (β_3)	4E3 (Tac)	mAb16 (α_5)	IC12 (α_v)
K562	9.83	0.37	0.42	13.2	0.22
$K\alpha_v\beta_3$	10.1	29.2	0.44	13.0	31.4
$K\alpha_v\beta_3$Y747F	9.99	29.7	0.44	12.8	30.9
$K\alpha_v\beta_3$Y759F	9.87	30.0	0.37	13.1	29.7
$K\alpha_v\beta_3$S752A	10.7	29.8	0.44	12.6	29.9
$KTac\beta_3$	11.1	0.45	8.77	13.0	0.33
$KTac\beta_3$S-P	10.5	0.57	9.32	13.3	0.34
$KTac\beta_3$S-A	9.89	0.49	8.91	12.5	0.26
$KTac\beta_3$S-E	11.0	0.57	9.74	12.8	0.29
$KTac\beta_3$S-C	9.87	0.46	11.2	13.2	0.41
$KTac\beta_5$	10.3	0.67	9.88	13.1	0.31

K562 cells also were transfected with full-length $\alpha_v\beta_3$ in which the serine at position 752 of β_3 was mutated to alanine as described for the mutation of β_3 tyrosine residues (4). In brief, nested PCR was performed on pBLY100 using the overlapping oligonucleotides 5′-GAGGCCAC-GCCTACCTTCACCAATATCACG-3′ and 5′-CTCCGGTGCGGATG-GAAGTGGTTATAGTGC-3′ encoding the S-A mutation with oligonucleotides in the mutation cassette (4). After the nested PCR reaction, the wild-type β_3 CT was replaced with the S-A mutant CT by NdeI-NheI restriction. Transfection, selection, and fluorescent cell sorting for expression levels of $\alpha_v\beta_3$S752A equivalent to wild-type $\alpha_v\beta_3$ was as described previously, resulting in $K\alpha_v\beta_3$S752A (Table I). Modified cDNAs were verified by dideoxy nucleotide sequencing.

Phagocytosis, Adhesion, and Migration

Phagocytosis assays were performed as described (2) by flow cytometry using either FITC-FN– or FITC-mAb16 (anti-α_5)–coated 3.0-μm beads. Data are presented as a Phagocytic Index, the number of beads internalized per 100 cells.

Chemotaxis assays were performed in modified Boyden chambers (Neuroprobe) using 14.0-μM polycarbonate filters as described (19). Vitronectin (VN), fibronectin (FN), and BSA were added to basal chambers at 5 μg/ml and mAb at 10 μg/ml were added to apical chambers coincident with cells. Cells in Iscove's Modified Eagle's Medium (IMDM) adjusted to 1 mM Ca^{2+} and 1 mM Mg^{2+} with 0.5% human serum albumin and 2.0 mM Mn^{2+}Cl were incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere for migration. Migration was quantitated by counting the number of cells per high power field on the underside of the filter after Giemsa staining.

Adhesion assays were performed as described in FN-coated (10 μg/ml) microtiter wells (2). Data are presented as the percent of added cells adherent after 1 h at 37°C.

CamKII Activity Assay

Transfected K562 cells or monocyte-derived macrophages were stimulated as described in the text, washed once by centrifugation in ice-cold IMDM and suspended in ice-cold homgenization buffer containing Hepes (50 mM), EDTA (4 mM), EGTA (2 mM), sucrose (0.25 M), dithiothreitol (1 mM), phenylmethylsulfonyl fluoride (0.2 mM), Na_3VO_4 (2.0 mM), NaF (5.0 mM), phenyl-arsine-oxide (10.0 mM), and leupeptin (10 μg/ml), pH 7.5. Suspended cells were sonicated on ice and assayed for CamKII activity against the synthetic substrate autocamtide II (KKALRRQETVDAL) (21). An aliquot of cell extracts was used for protein determination by BCA. Parallel aliquots were assayed for CamKII activity in a 25-μl reaction mixture containing Hepes (50 mM), magnesium acetate (10 mM), Na_3VO_4 (2.0 mM), NaF (5.0 mM), phenyl-arsine-oxide (10.0 mM), $CaCl_2$ (1 mM), calmodulin (0.1 μM; Sigma Chemical Co.), autocamtide II (20 μM), and γ-[^{32}P]ATP (0.1 mM, 3,000 cpm/pmol). The reaction was initiated by ATP addition and terminated by addition of trichloroacetic acid to a final concentration of 10%. The reaction mixture was centrifuged through phosphocellulose separation units (Pierce) and washed as described (26). CamKII activation results from phosphorylation events that result in kinase activity which is no longer dependent upon exogenous calcium or calmodulin. CamKII activity in cellular extracts was measured by quantitating the incorporation of radioactive phosphate into a synthetic CamKII

substrate (autocamtide-2) in the presence (calcium/calmodulin-independent + calcium/calmodulin-dependent activity) or absence (calcium/calmodulin-independent activity) of calcium and calmodulin. The activation of CamKII (autonomous activity) is expressed as a direct percentage of the total cellular CamKII activity (1) in which:

$$\% \text{ CamKII activity} = \frac{\text{Autonomous activity}}{\text{Total activity}} \times 100$$

where autonomous activity equals the CamKII activity without calcium or calmodulin and total activity equals CamKII activity with calcium or calmodulin.

CamKII expression levels in transfected cell lines was assessed by immunoprecipitation as previously described, followed by Western blot analysis (1).

Infection of K562 Cells with Adenovirus Encoding Constitutively Active CamKII

$K\alpha_v\beta_3$, $KTac\beta_3$, and $KTac\beta_5$ were infected with a replication defective adenovirus in which the E1 region was replaced with the CMV early promoter and the cDNA for a constitutively active CamKII (Ad-CMV.CKIID3) or β-galactosidase (AdCMV.gal) and viral stocks propagated and titered as described (1). Transfected K562 cells at 5×10^6/ml in IMDM were infected with recombinant adenovirus at a multiplicity of infection of 100 for 1 h followed by the addition of normal growth medium to dilute cells to a concentration of 0.5×10^6/ml. After 4–6 h, cells were harvested for analysis of CamKII activity or functional assay as described in Results. Viability of all infected cell types exceeded 85% at the initiation, and 70% at the conclusion of experimental time courses.

Proteins and Antibodies

FN was purified by gelatin affinity and VN by heparin affinity as previously described (2, 3). Monoclonal antibodies 7G2 (anti–human β_3), W6/32 (anti–human HLA), IC12 (anti–human α_v), AP3 (anti–human β_3), P1F6 (anti–human β_5), 4E3 (anti-IL2Rα, gp55, TAC), and mAb16 (anti–human α_5) have been previously described and were used in excess at 5.0 μg/ml unless otherwise indicated (2, 3).

Reagents

The kinase inhibitors H7 (50 nM), KN62 (2.5 μM), KN04 (5.0 μM), KT5926 (20 nM), and ML-9 (2 μM) were included in some assays where indicated and all were from LC Laboratories (Woburn, MA). All other reagents were from Sigma Chemical Co. unless otherwise indicated.

Data Presentation

Data are presented as the mean ± SEM from at least three replicates for all studies. Significance was determined by analysis of variance followed by Duncan's comparison testing. A minimum confidence interval of 95% was employed for all studies.

Results

$\alpha_v\beta_3$ Crosstalk Regulates $\alpha_5\beta_1$-mediated Migration

We have previously described a phenomenon, termed integrin crosstalk, in which ligation of $\alpha_v\beta_3$ prevents $\alpha_5\beta_1$-mediated phagocytosis in macrophages and in K562 cells expressing transfected $\alpha_v\beta_3$. To determine if integrin crosstalk regulated $\alpha_5\beta_1$ functions other than phagocytosis, we evaluated the effects of $\alpha_v\beta_3$ ligation on the migration of K562 cells on the $\alpha_5\beta_1$ ligand FN. K562 cells did not migrate specifically to FN in IMDM containing 1 mM Ca^{2+} and 1 mM Mg^{2+}. However, addition of 2 mM Mn^{2+} or the $\alpha_5\beta_1$ conformation-stabilizing mAbs 8A2 or A1A5 at 5.0 μg/ml greatly enhanced the FN-specific migration of these cells, consistent with a requirement for high affinity $\alpha_5\beta_1$ in migration (data not shown). As shown in Fig. 1 A, the migration of untransfected K562 to FN in the presence of

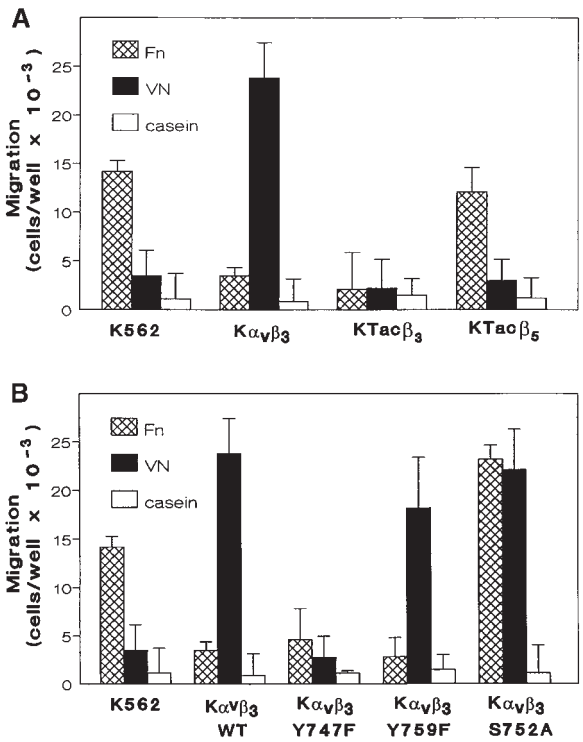

Figure 1. $\alpha_v\beta_3$ crosstalk regulates $\alpha_5\beta_1$-mediated migration. Untransfected K562 cells, $K\alpha_v\beta_3$, $KTac\beta_3$, and $KTac\beta_5$ were permitted to migrate to vitronectin (VN), fibronectin (Fn) or casein through 14.0-μM pores in a modified Boyden chamber apparatus as described in Materials and Methods (A). For structure-function analysis of integrin crosstalk, untransfected K562 cells and K562 cells expressing transfected wild-type $\alpha_v\beta_3$ ($K\alpha_v\beta_3$) or $\alpha_v\beta_3$ bearing single amino acid mutations Tyr747-Phe ($K\alpha_v\beta_3$Y747F), Tyr759-Phe ($K\alpha_v\beta_3$Y759F) or Ser752-Ala ($K\alpha_v\beta_3$S752A) were assayed for migration to Fn, VN, or casein as described in Materials and Methods (B). Migration was quantitated manually by counting cells adherent to the basal side of the filter and reported as the number of cells per well. Shown are mean ± SEM of four determinations with no fewer than eight replicates.

2 mM Mn^{2+} was enhanced sixfold over migration to the nonspecific protein casein; this migration was completely inhibited by mAb to $\alpha_5\beta_1$ (data not shown). We also examined $\alpha_v\beta_3$-mediated migration in K562 expressing this transfected integrin in addition to the endogenous $\alpha_5\beta_1$. $K\alpha_v\beta_3$ migrated in response to VN (Fig. 1 A); migration response to VN was inhibited by mAb to α_v or β_3 (data not shown). However, migration of $K\alpha_v\beta_3$ to FN was severely impaired compared with untransfected or mock transfected K562 (Fig. 1 A). Migration of $K\alpha_v\beta_3$ to FN was restored by the addition of the ser/thr kinase inhibitor H7 (50 nM), while addition of H7 had no effect on $K\alpha_v\beta_3$ migration to VN (data not shown). Restored migration of $K\alpha_v\beta_3$ to FN in the presence of H7 was completely inhibited by mAb to β_1 (data not shown). These results completely parallel the previously described $\alpha_v\beta_3$-mediated crosstalk which inhibits $\alpha_5\beta_1$-mediated phagocytosis (3) and support the hypothesis that the coligation of $\alpha_v\beta_3$ by FN regulates $\alpha_5\beta_1$-mediated K562 cell migration to FN be-

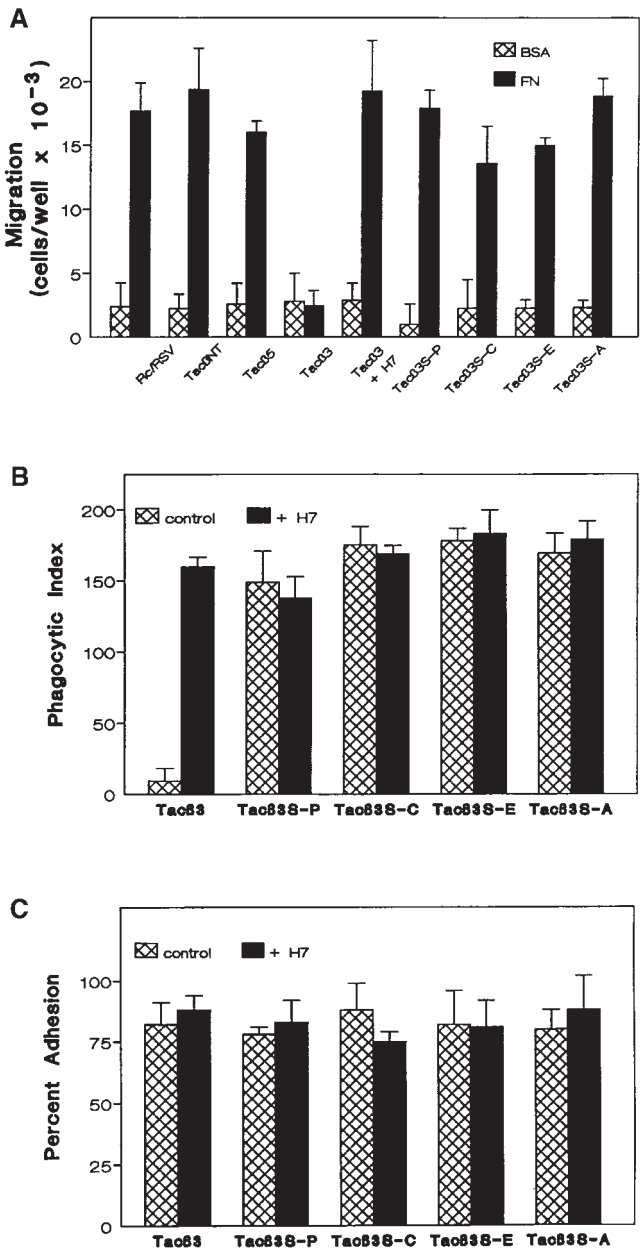

Figure 2. Role of β_3 Ser752 in $\alpha_v\beta_3$ crosstalk. K562 cells transfected with vector alone (Rc/RSV) or a chimeric molecule composed of the extracellular and transmembrane domains of the IL2 receptor (Tac) and the cytoplasmic tail of integrin subunits; no tail (TacNT), β_3 (Tacβ_3), β_5 (Tacβ_5), or β_3 in which Ser752 had been mutated to Pro (Tacβ_3S-P), Cys (Tacβ_3S-C), Ala (Tacβ_3S-A), or Glu (Tacβ_3S-E) were evaluated for their ability to migrate to FN (A), phagocytose FN opsonized beads (B), and adhere to FN-coated tissue culture plastic (C) as described in Materials and Methods. Parallel samples were treated with 50 nM H7 as indicated. Shown are mean ± SEM of no fewer than three determinations performed in triplicate.

cause this function, like phagocytosis, requires a high affinity form of $\alpha_5\beta_1$.

To demonstrate definitively that $\alpha_v\beta_3$ regulation of $\alpha_5\beta_1$-mediated migration was another example of integrin crosstalk, we examined migration to FN in KTacβ_3 and KTacβ_5, K562 cells expressing chimeric molecules comprised of the extracellular domain of the IL2 receptor and the cytoplasmic tail domain of the β_3 or β_5 integrin, respectively. Expression of Tacβ_3, but not Tacβ_5, leads to constitutive inhibition of $\alpha_5\beta_1$-mediated phagocytosis in K562 cells (3; see Fig. 7 B). Expression of Tacβ_3, but not Tacβ_5 (Fig. 1 A) or Tac lacking a cytoplasmic tail (KTacNT, Fig. 2 A), completely inhibited $\alpha_5\beta_1$-mediated migration to FN. The constitutive inhibition of migration to FN in KTacβ_3 was reversed by the addition of 50 nM H7 (Fig. 2 A). These studies demonstrate that $\alpha_5\beta_1$-mediated migration and $\alpha_5\beta_1$-mediated phagocytosis are similarly regulated by $\alpha_v\beta_3$ or the isolated β_3 CT and that this regulation is dependent upon a ser/thr kinase regulated by H7. These data suggest that both $\alpha_5\beta_1$-mediated migration and $\alpha_5\beta_1$-mediated phagocytosis are regulated by $\alpha_v\beta_3$-initiated crosstalk.

β_3 Ser752 Is Required for β_3 Crosstalk

We have previously demonstrated that expression of the isolated β_3 cytoplasmic tail is sufficient for initiation of $\alpha_v\beta_3$ crosstalk (Fig. 1 A and reference 12). To further delineate the required sequence elements of this unique regulatory pathway, we introduced point mutations in the β_3 cytoplasmic tail and analyzed their effects upon $\alpha_v\beta_3$-initiated crosstalk to $\alpha_5\beta_1$-mediated migration.

In a spontaneously occurring Glanzmann's Thrombasthenia mutation, the serine residue at position 752 of the β_3 CT is mutated to proline (6). This mutation results in loss of platelet β_3 function and a severe bleeding disorder. In vitro study has shown that Ser752 of the β_3 CT is required for the conformational change associated with elevated affinity of β_3 for ligand (8). To test whether Ser752 also is required for integrin crosstalk, we expressed an $\alpha_v\beta_3$ receptor in K562 cells in which Ser752 of β_3 was mutated to Ala (Fig. 1 B). While the ligation of wild-type $\alpha_v\beta_3$ blocked $\alpha_5\beta_1$-mediated migration on FN (Fig. 1 B), the S752A mutant migrated as well as the untransfected cells. In addition, the S752A mutant migrated as well as wild-type $\alpha_v\beta_3$ on VN (Fig. 1 B), consistent with reports that this mutation does not affect ligand binding by β_3 integrins (8). This demonstrates that failure of the S752A mutant to initiate crosstalk did not result from an inability to recognize ligand.

Recently, a tyrosine in the β_3 cytoplasmic tail, Tyr747, has been implicated in activation-dependent $\alpha_v\beta_3$ adhesion to VN (4). In contrast to the S752A mutation, Y747F had no effect on $\alpha_v\beta_3$-initiated integrin crosstalk (Fig. 1 B). Consistent with the previous report of a requirement for this tyrosine in firm adhesion, the Y747F mutation did abolish migration of K$\alpha_v\beta_3$Y747F to VN (Fig. 1 B). Mutation of Tyr759 to Phe (Y759F) did not affect either crosstalk or the migration function of $\alpha_v\beta_3$. These data demonstrate that the crosstalk signaling and adhesive functions of $\alpha_v\beta_3$ have distinct and independent sequence requirements in the β_3 cytoplasmic tail.

To evaluate further the requirement for β_3 S752 in integrin crosstalk, additional mutations at that position were made in the consititutively inhibitory Tacβ_3 construct. Mutation of Ser752 to Glu, Pro, or Cys as well as Ala abolished the inhibitory activity of Tacβ_3 on $\alpha_5\beta_1$-dependent migration (Fig. 2 A) and $\alpha_5\beta_1$-dependent phagocytosis (Fig. 2 B). Like the wild-type β_3 cytoplasmic tail, none of

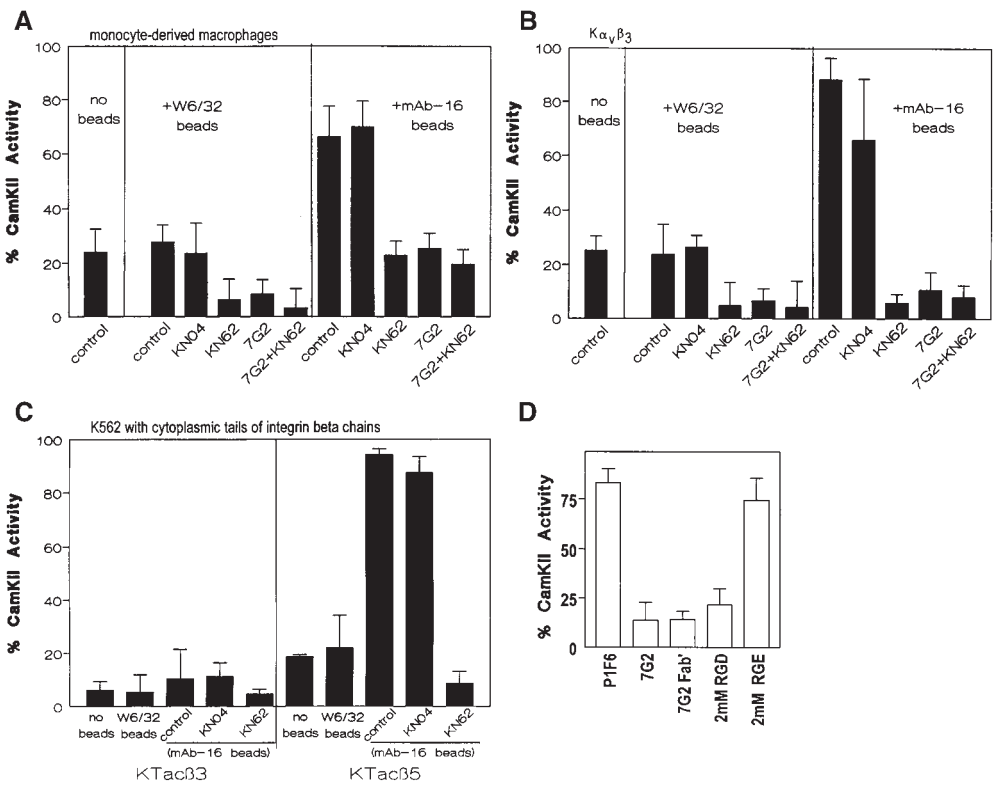

Figure 3. Regulation of CamKII activity by $\alpha_5\beta_1$ and $\alpha_v\beta_3$. Human monocyte-derived macrophages (A) or K562 cells expressing transfected $\alpha_v\beta_3$ ($K\alpha_v\beta_3$, B) or integrin β_5 ($KTac\beta_5$) or β_3 ($KTac\beta_3$) cytoplasmic tail chimeras (C) were assayed for activity of the calcium- and calmodulin-dependent protein kinase II against a synthetic substrate as described in Materials and Methods. Cells were either left unstimulated (no beads) or presented with control phagocytosis targets (W6/32 beads) or with an $\alpha_5\beta_1$-specific phagocytosis target (mAb-16 beads) in the presence and absence of KN62 (2.5 μM), KN04 (5.0 μM) or 5.0 μg/ml mAb 7G2 either individually or in combinations as indicated. D shows the inhibition of CamKII activity in $K\alpha_v\beta_3$ after $\alpha_5\beta_1$ ligation with mAb-16 beads by soluble mAb 7G2 (5.0 μg/ml), 7G2 Fab′ (3.8 μg/ml), and 2.0 mM GRGDSP peptide and the lack of inhibition by mAb P1F6 or 2 mM GRGESP. Regulated CamKII activity is presented as the percent of total activity as described in Materials and Methods. Shown are mean ± SEM for no fewer than three determinations in each group of all panels.

the mutants affected K562 binding to FN-coated surfaces, a function that does not require the high affinity state of $\alpha_5\beta_1$ (Fig. 2 C). The addition of H7 reversed the $Tac\beta_3$ inhibition of $\alpha_5\beta_1$-mediated migration and phagocytosis (Fig. 2, A and B).

$\alpha_5\beta_1$ and $\alpha_v\beta_3$ Differentially Regulate CamKII

$\alpha_v\beta_3$ ligation inhibits the $\alpha_5\beta_1$ high affinity functions of phagocytosis and migration, without effect upon $\alpha_5\beta_1$-mediated adhesion. Alterations in $\alpha_5\beta_1$ affinity can be regulated by calcineurin, a calcium/calmodulin-dependent phosphatase and CamKII (calcium/calmodulin-dependent protein kinase II; reference 1). Recently, inhibition of CamKII activity by $\alpha_v\beta_3$ ligation in smooth muscle cells was reported (1). Therefore, we evaluated $\alpha_v\beta_3$ regulation of CamKII as a potential mediator of $\alpha_v\beta_3$-initiated crosstalk.

CamKII activity was measured in human monocyte-derived macrophages in the presence and absence of an $\alpha_5\beta_1$-specific phagocytosis target (mAb-16–coated latex beads) (3). Ligation of macrophage $\alpha_5\beta_1$ with mAb-16 beads enhanced CamKII activity twofold, while ligation with a control target (W6/32 beads) had no effect (Fig. 3 A). Both basal and stimulated CamKII activities were decreased by the CamKII inhibitor KN62, but not the structurally related, but non-inhibitory KN04. Ligation of $\alpha_v\beta_3$ with soluble mAb 7G2 prevented the rise in CamKII activity induced by mAb-16 beads (Fig. 3 A). No additional decrease in CamKII activity was detected when KN62 and 7G2 were combined. Thus, β_3 ligation prevented the $\alpha_5\beta_1$-induced rise in CamKII activity.

To explore further the hypothesis that CamKII mediates $\alpha_v\beta_3$ regulation of $\alpha_5\beta_1$, we evaluated the regulation of CamKII in $K\alpha_v\beta_3$. Binding of mAb-16 beads to $K\alpha_v\beta_3$, and to vector-transfected K562 (data not shown), resulted in an increase in CamKII activity (Fig. 3 B) that was not seen when $K\alpha_v\beta_3$ were incubated with W6/32 beads that bound to the cells equivalently. As in macrophages, the $\alpha_5\beta_1$-mediated rise in CamKII activity was prevented by ligation of $\alpha_v\beta_3$ with soluble mAb 7G2 (Fig. 3 B) and by 7G2 Fab fragments or Arg-Gly-Asp peptide (Fig. 3 D). As seen in macrophages, inhibition of the $\alpha_5\beta_1$-induced increase in CamKII activity by $\alpha_v\beta_3$ ligation was blocked by KN62, but not KN04.

Previously we have demonstrated that the cytoplasmic tail of β_3 is both necessary and sufficient for $\alpha_v\beta_3$ inhibitory crosstalk to $\alpha_5\beta_1$ (reference 3 and Fig. 2, A and B). In the presence of mAb-16 beads, expression of $Tac\beta_3$, but not $Tac\beta_5$, prevented the $\alpha_5\beta_1$-mediated rise in CamKII activity (Fig. 3 C). These results indicate that $\alpha_5\beta_1$ and $\alpha_v\beta_3$ differentially regulate CamKII activity in macrophages and K562 cells.

To determine if the failure of the $K\alpha_v\beta_3S752A$ to initiate crosstalk was related to an inability to regulate CamKII, we evaluated CamKII activity after mAb-16 bead binding in K562 cells transfected with wild-type $\alpha_v\beta_3$ and the S752A and Y747F mutants. While ligation of $\alpha_v\beta_3$ with the

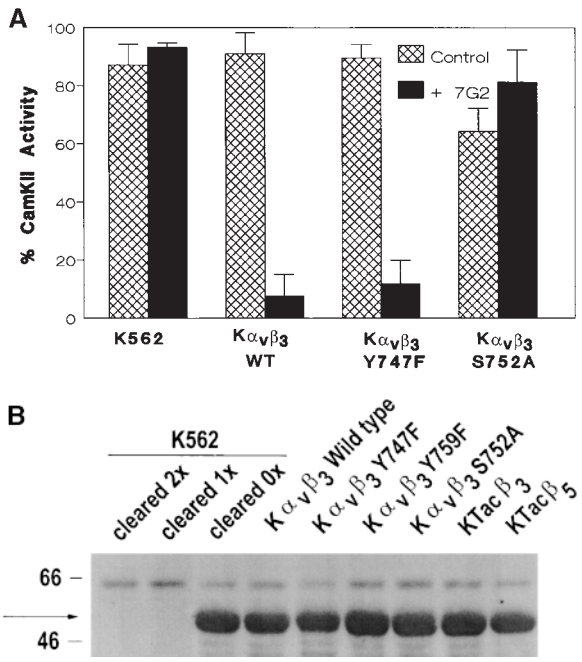

Figure 4. Role of β_3 Ser752 in $\alpha_v\beta_3$ regulation of CamKII. Untransfected K562 cells or K562 cells expressing transfected wild-type $\alpha_v\beta_3$ ($K\alpha_v\beta_3$) or $\alpha_v\beta_3$ in which Tyr747 was mutated to Phe ($K\alpha_v\beta_3Y747F$) or in which Ser752 was mutated to Ala ($K\alpha_v\beta_3S752A$) were assayed for CamKII activity (A) as described in Materials and Methods, after $\alpha_5\beta_1$ ligation with mAb-16 beads in the presence or absence of the $\alpha_v\beta_3$ ligand 7G2 (5.0 μg/ml). Shown are mean ± SEM of three determinations with no fewer than three replicates. Also as described in Materials and Methods, total cellular CamKII (see arrow) from transfected K562 cell populations used in this study was recovered by immunoprecipitation and revealed by Western blotting (B). For untransfected K562 cells, cell lysates were cleared by immunoprecipitation with anti-CamKII twice (cleared 2×) or once (cleared 1×), before analysis of the final immunoprecipitation to ensure that recovery of protein was complete. Shown is a representative study.

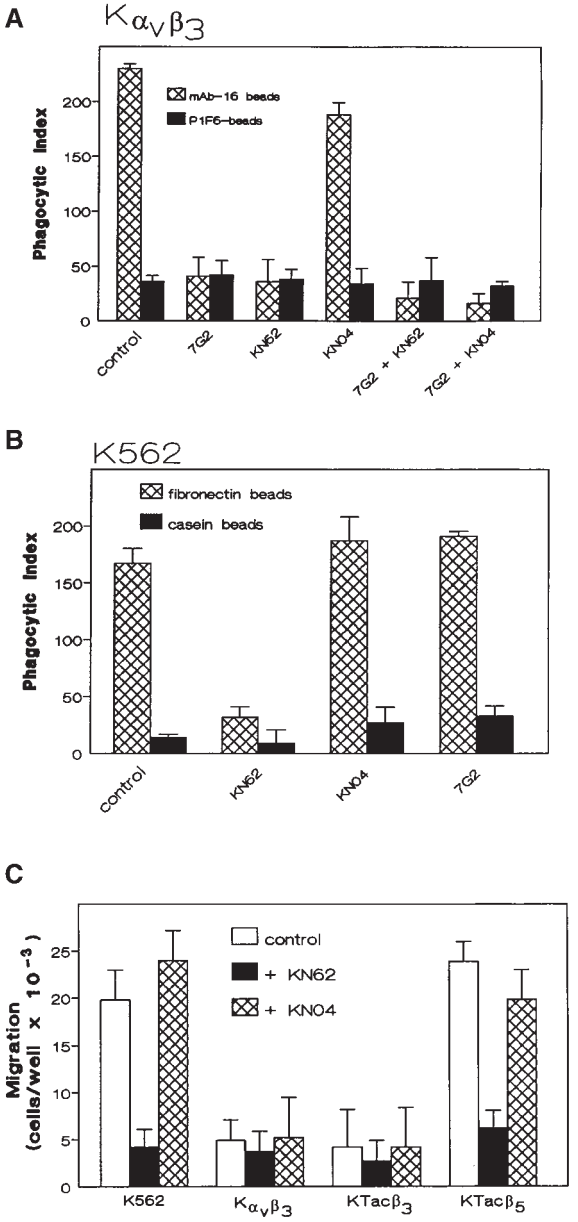

Figure 5. Effects of CamKII inhibitors on $\alpha_5\beta_1$ function and $\alpha_v\beta_3$ crosstalk. Phagocytosis of the $\alpha_5\beta_1$-specific target mAb-16 beads, or control target P1F6-beads was evaluated in K562 expressing $\alpha_v\beta_3$ in the presence and absence of $\alpha_v\beta_3$ ligation by mAb 7G2 (5.0 μg/ml) and/or the CamKII inhibitor KN62 (2.5 μM) or control KN04 (5.0 μM) (A) as described in Materials and Methods. Shown are mean ± SEM of three determinations with four replicates each. $\alpha_5\beta_1$-mediated phagocytosis of FN beads was evaluated in untransfected K562 cells in the presence and absence of KN62 (2.5 μM), KN04 (5.0 μM), or the β_3-specific mAb 7G2 (5.0 μg/ml) (B) as described in Materials and Methods. Shown are the mean ± SEM of two determinations with three replicates each. In C, the migration of untransfected K562 cells, $K\alpha_v\beta_3$, $KTac\beta_3$, and $KTac\beta_5$ to FN was assessed in the presence and absence of KN62 (2.5 μM) or KN04 (5.0 μM) as described in Materials and Methods. Shown are mean ± SEM of four determinations with at least three replicates each.

β_3-specific mAb 7G2 suppressed mAb-16 bead-induced activation of CamKII, mutation of Ser752 of β_3 prevented the suppression of CamKII activity seen upon $\alpha_v\beta_3$ ligation (Fig. 4 A). However, mutation of Tyr747 or Tyr759 (data not shown) did not affect $\alpha_v\beta_3$ regulation of CamKII. Thus, Ser752 is required for both $\alpha_v\beta_3$ inhibitory crosstalk to $\alpha_5\beta_1$ (Fig. 2) and $\alpha_v\beta_3$ regulation of CamKII (Fig. 4).

To determine the effect of $\alpha_v\beta_3$ and mutant β_3 on expression of CamKII, immunoprecipitates of CamKII were analyzed by Western blot with CamKII-specific Ab. As shown in Fig. 4 B, cellular expression of CamKII (see arrow) was unchanged by the expression of $\alpha_v\beta_3$ and mutants in transfected K562 cells.

Role of CamKII in $\alpha_v\beta_3$ Crosstalk to $\alpha_5\beta_1$

Suppression of the $\alpha_5\beta_1$-dependent increase in CamKII activity by $\alpha_v\beta_3$ ligation or by $Tac\beta_3$ expression suggested that CamKII regulation could have a role in $\alpha_v\beta_3$ crosstalk

to $\alpha_5\beta_1$. To determine the role of CamKII in $\alpha_v\beta_3$ crosstalk to $\alpha_5\beta_1$, K$\alpha_v\beta_3$ cells were incubated with the $\alpha_5\beta_1$ phagocytosis target, mAb-16 beads, or control target, P1F6 (anti-$\alpha_v\beta_5$) beads. Phagocytosis was measured in the presence and absence of 7G2 to ligate $\alpha_v\beta_3$, the CamKII inhibitor KN62, or control KN04. As reported previously, phagocytosis via $\alpha_5\beta_1$ was inhibited upon $\alpha_v\beta_3$ ligation with mAb 7G2 (2, 3). $\alpha_5\beta_1$ phagocytosis also was inhibited by KN62 (Fig. 5 A), but not KN04. Combining 7G2 and KN62 resulted in no further decrease in $\alpha_5\beta_1$ phagocytosis. Under all conditions, there was no significant internalization of P1F6 beads.

To determine the dependence of $\alpha_5\beta_1$ phagocytosis on CamKII activation, we evaluated phagocytosis in untransfected K562 cells which express $\alpha_5\beta_1$, but not $\alpha_v\beta_3$. The absence of $\alpha_v\beta_3$ in these cells permitted the use of FN-coated beads as a phagocytosis target for $\alpha_5\beta_1$ rather than the more selective mAb-16 beads used when $\alpha_v\beta_3$ is present. K562 phagocytosis of FN-coated beads via $\alpha_5\beta_1$ was inhibited by the CamKII inhibitor KN62 (Fig. 5 B), but not the control KN04. Thus, enhanced CamKII activity, initiated by $\alpha_5\beta_1$ binding of mAb-16 beads, appears to be required for $\alpha_5\beta_1$ phagocytosis.

These data support the hypothesis that ligation of $\alpha_5\beta_1$ stimulates CamKII activity and that $\alpha_v\beta_3$-mediated suppression of this activity is at least in part responsible for its inhibition of $\alpha_5\beta_1$-mediated phagocytosis. To demonstrate that a similar mechanism was responsible for the inhibitory β_3 crosstalk to $\alpha_5\beta_1$ during migration, we evaluated the effects of the CamKII inhibitor KN62 on K562 cell migration in response to FN. KN62, but not the inactive analogue KN04, inhibited the FN-induced migration of mock transfected K562 cells and KTacβ_5 (Fig. 5 C). The presence of KN62 did not further attenuate the minimal migration of K$\alpha_v\beta_3$ or KTacβ_3 cells.

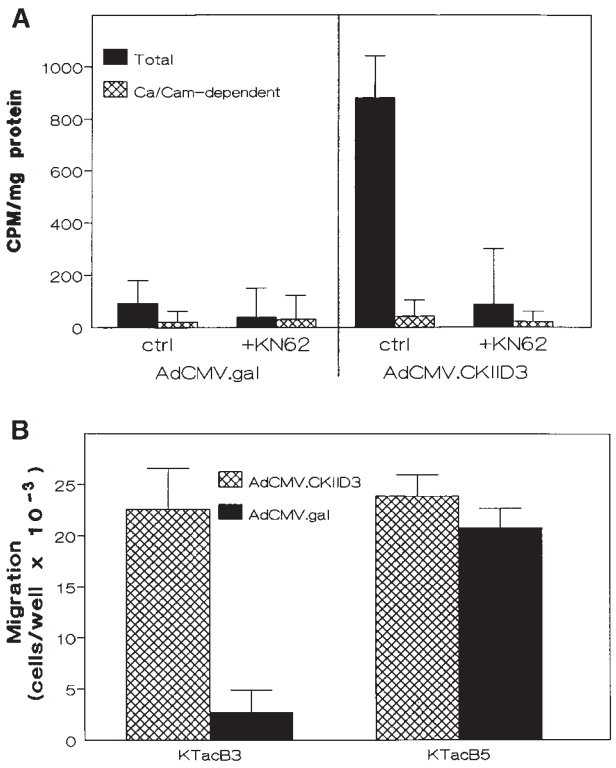

Figure 6. Constitutively active CamKII reverses $\alpha_v\beta_3$ inhibitory crosstalk. Untransfected K562 cells (A), or KTacβ_3 and KTacβ_5 (B) were infected with an adenovirus encoding either β-galactosidase (AdCMV.gal) or a constitutively active form of CamKII (AdCMV.CKIID3). CamKII activity against a synthetic substrate was assayed in the presence and absence of calmodulin and the CamKII inhibitor KN62 (2.5 μM) as described in Materials and Methods and reported as the CamKII activity per mg of total cellular protein (A). Migration of KTacβ_3 and KTacβ_5 expressing either β-galactosidase or constitutively active CamKII to FN was assessed as described in Materials and Methods (B). Shown are mean ± SEM of three determinations performed in triplicate.

Constitutively Active CamKII Overcomes $\alpha_v\beta_3$-Inhibitory Integrin Crosstalk

To test the hypothesis that $\alpha_v\beta_3$ crosstalk to $\alpha_5\beta_1$ was a result of CamKII downregulation by β_3, K562 cells were infected with an adenovirus-directing expression of a constitutively active form of CamKII (1). Expression of this construct in untransfected K562 cells resulted in an eightfold increase in CamKII activity over a control viral construct encoding β-galactosidase (Fig. 6 A).

Next, KTacβ_3 and KTacβ_5 infected with virus encoding either β-galactosidase or constitutively active CamKII were assayed for their ability to migrate in response to FN. Expression of the active kinase specifically overcame the constitutive inhibition of $\alpha_5\beta_1$-mediated migration in KTacβ_3, without any effect on migration in KTacβ_5 (Fig. 6 B). Thus, expression of active CamKII overcame $\alpha_v\beta_3$-mediated suppression of $\alpha_5\beta_1$ high affinity functions. Unfortunately, safety concerns precluded testing the effect of the constitutively active CamKII in the phagocytosis assay.

Discussion

Integrin crosstalk is an important mechanism for coordinating signals from multiple simultaneously ligated integrins on a single cell for a functional response to extracellular matrix. Although sometimes called "transdominant inhibition," crosstalk may induce, as well as suppress functions of the target integrin, so we believe the more general term, preferable (9). Although the number of examples of integrin crosstalk has rapidly expanded in the past few years, little is known concerning the molecular mechanisms by which one integrin affects the function of another. K562 cells have proved a valuable model for examination of integrin crosstalk because these cells express a single integrin, $\alpha_5\beta_1$, permitting a wide variety of genetic experiments exploring the basis of integrin crosstalk. In this system, we have previously shown that ligation of transfected $\alpha_v\beta_3$ inhibits the high affinity phagocytic function of $\alpha_5\beta_1$ without effect upon low affinity $\alpha_5\beta_1$-mediated adhesion and that the β_3 cytoplasmic tail is both necessary and sufficient for this effect. We now have used this model to explore the biochemical mechanisms involved in crosstalk. Based on a previous report, we examined a potential role for CamKII in $\alpha_v\beta_3$-mediated suppression of the high affinity functions of $\alpha_5\beta_1$, and performed struc-

ture-function analysis of the β_3 cytoplasmic tail to further delineate the required structures for this unique signaling event.

In this study, we show that either $\alpha_v\beta_3$ ligation or expression of the isolated β_3 cytoplasmic tail exerts an inhibitory effect upon $\alpha_5\beta_1$-mediated migration as well as phagocytosis. Since both $\alpha_5\beta_1$ migration and phagocytosis are events that require the high affinity state of the integrin, and since the $\alpha_v\beta_3$-mediated inhibition of $\alpha_5\beta_1$ is reversed by KN62 in both cases, these data suggest that a common signaling mechanism is responsible for these crosstalk events.

Based on the data in this report, we propose the hypothesis that CamKII, a ser/thr kinase with multiple intracellular substrates, is an important regulator of $\alpha_5\beta_1$ function and a target of integrin crosstalk. First, ligation of $\alpha_5\beta_1$ by specific antibody- or ligand-coated beads enhances the activity of CamKII in both macrophages and K562 cells. Second, activation of CamKII by ligation of $\alpha_5\beta_1$ is required for both phagocytosis and migration. In contrast CamKII inhibitors do not affect adhesion which can be effected by low affinity $\alpha_5\beta_1$. Thus, the requirement for CamKII activation appears to be specific for the high affinity functions of $\alpha_5\beta_1$.

Coligation of $\alpha_v\beta_3$, or exposure of the isolated β_3 cytoplasmic tail, prevents $\alpha_5\beta_1$-induced rise in CamKII activity. Since the β_3 integrin and the CamKII inhibitor have the same effect on $\alpha_5\beta_1$ function, the data suggest that suppression of the ability of $\alpha_5\beta_1$ to activate CamKII may be an important mechanism of integrin crosstalk. A role for CamKII suppression in integrin crosstalk is supported by the reversal of crosstalk inhibition of migration with constitutively active CamKII. Thus, our data support the hypothesis that $\alpha_5\beta_1$-mediated CamKII activation is required for the high affinity functions of migration and phagocytosis and that $\alpha_v\beta_3$-activated crosstalk suppresses these functions through inhibition of CamKII activation. Thus, $\alpha_5\beta_1$ and $\alpha_v\beta_3$ have opposing effects on CamKII activity.

Neither the upstream events regulating CamKII nor its downstream effector are yet known. Tyrosine kinase inhibitors have no effect either on the high-affinity functions of $\alpha_5\beta_1$ or on suppression by $\alpha_v\beta_3$, suggesting the possibility that the entire pathway is independent of the well-known effects of integrin ligation on several tyrosine kinases (2, 3). Indeed, the independence of integrin crosstalk from the phosphorylation of Tyr747 further suggests that the signaling involved in the regulation of CamKII may be completely independent of these pathways. A recent report by Wu et al. (25) demonstrates that ligation of $\alpha_5\beta_1$ and $\alpha_v\beta_3$ have opposite effects on plasma membrane calcium channel activity. Since calcium is an important regulator of CamKII, this voltage gated calcium channel may be important in the differential regulation of CamKII by these two integrins. Based on our preliminary pharmacologic data, one likely effector for CamKII in $\alpha_5\beta_1$ high affinity function is myosin light chain kinase (MLCK). MLCK inhibitors KT5926 and ML9 both reverse $\alpha_v\beta_3$ inhibition of $\alpha_5\beta_1$-mediated phagocytosis and migration without affecting inhibition of CamKII activation by $\alpha_v\beta_3$ ligation (Blystone, S.D., and E.J. Brown, unpublished data). MLCK phosphorylation by CamKII is known to inhibit MLCK activity, leading presumably to decreased myosin-induced cell traction (21). This integrin-mediated modulation of myosin function is consistent with the known role for myosin in phagocytosis and migration.

Analysis of structural requirements in the β_3 cytoplasmic tail in $\alpha_v\beta_3$-mediated crosstalk reveals that Ser752 of the β_3 cytoplasmic tail is required for inhibition of CamKII and for initiation of integrin crosstalk, while crosstalk is independent of either of the β_3 cytoplasmic tail tyrosines. The requirement for β_3 Ser752 in crosstalk is unexpected. The importance of Ser752 was suggested by a mutation to proline in a patient with Glanzmann's Thrombasthenia which abolished high affinity binding of fibrinogen by platelet $\alpha_{IIb}\beta_3$ (6, 8). However, detailed analysis has shown that mutation of Ser752 to Ala does not affect ligand binding by $\alpha_{IIb}\beta_3$ (8). The failure of the Ser752 to Ala β_3 mutation to affect ligand binding is supported by our studies in K562 which demonstrate normal adhesion, normal migration (Fig. 2, B and C), and normal generation of the ligand-induced binding site (LIBS) recognized by the antibody LIBS-1 in response to RGD peptide in this mutant (data not shown). In contrast, integrin crosstalk is entirely abolished by the S752A mutation, as it is by mutation to Pro (the original Glanzmann's mutation), to Glu (to mimic a potential phosphorylation), and to Cys (as a conservative mutation). Thus, it appears that Ser is absolutely required at this position. While this suggests the possibility of Ser phosphorylation in integrin crosstalk, we have been unable to demonstrate such phosphorylation so far. In contrast, Tyr747, which is absolutely required for stimulated adhesion and for $\alpha_v\beta_3$-mediated migration (Fig. 1) in K562 cells, is not involved in integrin crosstalk. Thus, these two amino acids, closely spaced in the relatively short cytoplasmic domain of one chain of an integrin, mediate two entirely distinct signaling cascades.

In a recent report, Bouvard et al. (5) showed that increased CamKII levels resulted in a decrease in the affinity of $\alpha_5\beta_1$ for FN. In this in vitro system, CamKII and the phosphatase calcineurin regulate $\alpha_5\beta_1$ affinity. Because the β_3 suppression of $\alpha_5\beta_1$ phagocytosis occurs subsequent to $\alpha_5\beta_1$ binding of ligand (3), it is possible that repeated cycling of $\alpha_5\beta_1$ affinity is required for phagocytosis and migration. Binding of ligand-coated beads to high affinity $\alpha_5\beta_1$ would activate CamKII, which would then decrease integrin affinity. This hypothesis predicts that integrin crosstalk from $\alpha_v\beta_3$ which blocks CamKII activation would prevent $\alpha_5\beta_1$ movement to the low affinity state. This is entirely consistent with reports of receptor activation rather than inactivation by integrin crosstalk (14, 24) which measured ligand binding rather than functions that require affinity modulation.

Finally, these data demonstrate that, while increased CamKII activity is required for $\alpha_5\beta_1$-mediated phagocytosis and migration, $\alpha_v\beta_3$ can perform these same functions independent of any increase in CamKII. This is a startling example of the diversity of signaling and function among the integrins. It suggests that there may be fundamental differences within this family of closely related receptors in how they mediate even their most basic functions. While many studies have emphasized common features of integrin α- and β-chains in association with cytoskeleton, calreticulin, and signaling molecules, the differences between $\alpha_v\beta_3$ and $\alpha_5\beta_1$ in requirements for phagocytosis and

migration suggest that there will be profound differences among integrins even as they perform similar functions.

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