Integrin β_3 Cytoplasmic Tail Is Necessary and Sufficient for Regulation of $\alpha_5\beta_1$ Phagocytosis by $\alpha_v\beta_3$ and Integrin-associated Protein

Scott D. Blystone, Frederik P. Lindberg, Susan E. LaFlamme,* and Eric J. Brown

Department of Medicine, Infectious Diseases Division, Washington University School of Medicine, St. Louis, Missouri 63110; and *Department of Physiology and Cell Biology, Albany Medical College, Albany, New York

Abstract. Using a K562 cell transfection model, we have previously described a novel relationship between the integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$. $\alpha_v\beta_3$ ligation was able to inhibit $\alpha_5\beta_1$-mediated phagocytosis without effect on $\alpha_5\beta_1$-mediated adhesion. The $\alpha_v\beta_3$-dependent inhibition apparently required a signal transduction cascade as it was reversed by inhibitors of serine/threonine kinases. Now, we have studied the mechanisms of signal transduction in this system and have found that the β_3 cytoplasmic tail is both necessary and sufficient for initiation of the signal leading to inhibition of $\alpha_5\beta_1$ phagocytosis. Ligation of integrin-associated protein (IAP), which has been implicated in $\alpha_v\beta_3$ signal transduction, mimics the effects of $\alpha_v\beta_3$ ligation only when the β_3 integrin with an intact cytoplasmic tail is present. Although fibronectin-mediated phagocytosis requires the high affinity conformation of $\alpha_5\beta_1$, ligation of $\alpha_v\beta_3$/IAP does not prevent acquisition of this high affinity state. We conclude that $\alpha_v\beta_3$/IAP ligation initates a signal transduction cascade, dependent upon the β_3 cytoplasmic tail, which inhibits the phagocytic function of $\alpha_5\beta_1$ at a step subsequent to modulation of integrin affinity.

INTEGRIN functions are modulated in many cells. This is best understood in the regulation of leukocyte adhesion and in platelet activation (9, 16, 24, 37). In these cell types, integrins have at least two conformational states. One, exhibiting a low affinity for ligand, is expressed by unactivated cells. The second state, which has a much higher affinity for ligand, is expressed by cells after activation. The signals which initiate the transition from low to high affinity states include thrombin and ADP stimulation in platelets (17, 19, 37), and a variety of serpentine receptor ligands in leukocytes, including the bacterial peptide f-Met-Leu-Phe, the complement activation fragment C5a, the product of arachidonate metabolism LTB_4, and chemokines such as MCP-1 (7, 16, 20, 27, 36).

In leukocytes, integrin function also can be regulated by ligation of another integrin. For example, $\alpha_5\beta_1$ ligation activates $\alpha_2\beta_1$ collagen binding in monocytes (39). β_3 integrins can influence the function of other receptors as well. Adhesion and activation studies suggest the hypothesis that on phagocytes, the ligation of β_3 integrins may be an early event in inflammation, directly affecting the function of other proinflammatory receptors (21, 42, 49). For example, β_3 integrin ligation activates the ability of the β_2 integrin Mac-1 to bind its ligands in both neutrophils and monocytes (26, 44). This β_3-initiated signal transduction requires a second plasma membrane protein, a member of the immunoglobulin superfamily with five membrane-spanning domains, known as integrin-associated protein (IAP)[1] (32, 49). The molecular mechanism(s) involved in regulation of leukocyte integrin function by β_3/IAP are incompletely understood.

Recently, we have developed a model in which to study the mechanism of β_3 "crosstalk" with other integrins. In K562 cells transfected with $\alpha_v\beta_3$, ligation of this integrin inhibits the phagocytic function of the integrin fibronectin receptor, $\alpha_5\beta_1$, which is endogenously expressed in K562 (2). This effect requires signal transduction, since it can be reversed by the serine/threonine kinase inhibitor H7 and the more specific protein kinase C inhibitor Calphostin C. Surprisingly, $\alpha_v\beta_3$ ligation affects phagocytosis without affecting the adhesive functions of $\alpha_5\beta_1$, suggesting that the signal transduction discretely inhibits high affinity $\alpha_5\beta_1$ function (phagocytosis) without altering the low affinity receptor function (adhesion) (18).

We now have used this model to dissect the roles of the integrin α and β chains and IAP in initiation of the signal transduction cascade. We show that $\alpha_{IIb}\beta_3$ is as effective as $\alpha_v\beta_3$ for initiation of this signal. Deletion of the β_3 cytoplasmic tail prevents signaling, while expression of a chimeric molecule consisting of the IL2 receptor α chain extracellular and transmembrane domains and the β_3 cytoplasmic tail constitutively prevents $\alpha_5\beta_1$ phagocytosis. This probably represents continuous signal transduction from the autonomous β_3 cytoplasmic domain rather than com-

Please address all correspondence to E. J. Brown, Infectious Diseases, Campus Box 8051, Washington University School of Medicine, St. Louis, MO 63110. Tel.: (314) 362-2125. Fax: (314) 362-9230.

1. *Abbreviations used in this paper:* Fg, fibrinogen; Fn, fibronectin; IAP, integin-associated protein; LPA, lysophosphatidic acid; PI, phagocytic index; Vn, vitronectin.

petition for cytoskeletal or other cytoplasmic components, because inhibition of phagocytosis is rapidly reversed by H7, and because the chimera has no effect on $\alpha_5\beta_1$ mediated adhesion to fibronectin (Fn)-coated surfaces. These data show that the β_3 cytoplasmic tail is both necessary and sufficient for initiation of the signal transduction cascade. IAP ligation only inhibits $\alpha_5\beta_1$ phagocytosis when $\alpha_v\beta_3$ is expressed, and, surprisingly, anti-IAP loses its inhibitory effect in cells expressing β_3 without its cytoplasmic tail. Finally, neither constitutive nor ligand-dependent β_3-initiated signal transduction affect the number of $\alpha_5\beta_1$ expressing the high affinity conformation. From these data, we hypothesize that ligation of the $\alpha_v\beta_3$/IAP complex initiates a signal transduction cascade mediated by the β_3 cytoplasmic tail which affects $\alpha_5\beta_1$ function at a step beyond regulation of integrin conformation.

Materials and Methods

Cells

The human erythroleukemia cell line K562 was maintained in Iscove's Modified Dulbecco's Medium (GIBCO, Gaithersburg, MD), containing 10% FBS (Hyclone, Logan, UT) and 1.0 μg/ml Gentamicin (Sigma Chem. Co., St. Louis, MO) and propagated in a 37°C, 5% CO_2, humidified incubator.

Truncation of the β_3 Integrin Subunit

The integrin β_3 subunit cytoplasmic domain was truncated at residue 728 by PCR mutagenesis to permit direct comparisons with previous work by other authors (11, 38). A 2,640-bp fragment of the β_3 cDNA subcloned into pRc/RSV (termed pIAP93) (2) was purified by restriction with AflII and HindIII and used as a PCR template. To terminate coding at residue 728 and introduce a novel SpeI restriction site the mutant oligonucleotide (5′-ATC CTG GTG TAA GAC TAG TCA GTG -3′, bases 2171-2194) and its reverse complement were used in two separate PCR reactions with oligonucleotides exactly matching either a 5′ upstream sequence overlapping and encoding the AflII restriction site (5′-GAA AGA GCT TAA GGA CAC TGG CAA-3′, bases 2020-2044); or a 3′ downstream sequence overlapping and encoding the HindIII restriction site (5′-GCC CAA GCT TTA GCT ATT CGA ACG -3′, bases 4640-4663). The two PCR products resulting from these reactions were subjected to restriction with AflII/SpeI and SpeI/HindIII, respectively. These products were purified and ligated back into the AflII/HindIII digested and purified pIAP93 restoring all 3′ untranslated sequence from the original construct resulting in a cDNA encoding a β_3 protein which terminates after amino acid 728, which we named β_3NT.

Protein Purifications

Fibronectin and fibrinogen were provided by Dr. John E. Kaplan, Albany Medical College (Albany, NY) and prepared as described (3). Vitronectin was prepared as described (48) by denaturation and adsorption on heparin. Casein was purchased in solution from Pierce (Rockford, IL). Purity of all protein reagents was determined to be greater than 99% by SDS-PAGE.

Antibodies

Polyclonal antibody against human $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins were purchased from GIBCO BRL. The $\alpha_v\beta_5$-specific monoclonal antibody (mAb) P1F6 (45) was the gift of Dr. Dean Sheppard (UCSF, San Francisco, CA). The $\alpha_v\beta_3$-reactive mAb LM609 (12) was a gift of Dr. David Cheresh (Scripps Research Institute, La Jolla, CA). The mAb BIIG2 and AIIB2, reactive with human integrin subunits α_5 and β_1, respectively (22, 46), were the kind gift of Dr. Caroline Damsky (UCSF, San Francisco, CA). The human integrin α_5-reactive mAb 16 (14) was the gift of Dr. Kenneth Yamada (NIDH/NIH, Rockville, MD). mAb IB4 (47) was the gift of Dr. Sam Wright (Rockefeller University, New York, NY). The β_1 integrin activating antibodies A1A5 (23) and 8A2 (28) were generously provided by Dr. Martin Hemler (Dana-Farber Cancer Institute, Boston, MA) and Drs. Nick Kovach and John Harlan (University of Washington, Seattle, WA), respectively. The $\alpha_{IIb}\beta_3$ specific monoclonal antibodies 10E5 and 7E3 were the gift of Dr. Barry Coller (Mt. Sinai School of Medicine). In our studies, these antibodies recognize $\alpha_{IIb}\beta_3$, but not $\alpha_v\beta_3$ when expressed in K562 as assessed by flow cytometry and immunoprecipitation. The β_1-reactive mAb P5D2 (8) was purchased from the Developmental Studies Hybridoma Bank (Iowa City, IA). mAb 9EG7 (31), which recognizes the high affinity state of β_1 integrins was the kind gift of Dr. Dietmar Vestweber, Max Planck Institute for Immunobiology. The HLA reactive mAb W6/32 (1) was purchased from the Amer. Type Culture Collection (Rockville, MD). The monoclonal antibody 4E3, reactive against the gp 55 (Tac) subunit of the IL2 receptor was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). mAbs IG114 against human α_4, B6H12 and 2D3 against human integrin associated protein (IAP), 7G2 specific for human integrin β_3, and 3F12 and 1C12 specific for human integrin α_v originated in this laboratory and were made as described (21). Fluorescein-labeled antibodies directed against rat or mouse IgG were purchased from Sigma Chem. Co. All antibody used as inhibitor were as purified IgG at 5.0 μg/ml unless specified.

cDNA Clones and Cell Transfection

The human β_5 integrin subunit cDNA was the gift of Dr. Sarah Bodary (Genentech, Inc., San Francisco, CA) (35). Human integrin α_v cDNA (33) was a gift of Dr. Joseph C. Loftus (Scripps Research Institute, La Jolla, CA). Human β_3 integrin cDNA was the gift of Dr. Jeffrey Smith (Scripps Research Institute, La Jolla, CA). Human α_{IIb} cDNA (40) was the gift of Dr. Joel Bennett (University of Pennsylvania). For transfection, the β_5 and β_3 cDNA were cloned into the stable expression vector pRc/RSV (Invitrogen, San Diego, CA) (β_5) or a derivative of pRc/RSV, termed pIAP58, with the cloning sites HindIII and XbaI in reverse orientation, (β_3) (32). The α_v and α_{IIb} cDNA's were subcloned into pCDM8 (Invitrogen). K562 cells were cotransfected with either α_v and β_3, α_{IIb} and β_3, α_v and β_5, or α_v and β_3NT by electroporation at 200 V, 600 μf using a Gene Pulser (Bio Rad Labs. Richmond, CA) cDNA encoding chimeras of the extracellular domain of the IL-2 receptor, gp 55, Tac subunit linked to the cytoplasmic tail domain of the β_3 and β_5 integrins (described in references 29, 30) were cotransfected with a neomycin selectable marker from pRc/RSV under the same transfection conditions. Transfected cell populations used in this study are KRc/RSV (vector alone), K$\alpha_v\beta_3$ (α_v and β_3), K$\alpha_v\beta_5$ (α_v and β_5), K$\alpha_{IIb}\beta_3$ (α_{IIb} and β_3), K$\alpha_v\beta_3$NT (α_v and tailless β_3 construct), K$\alpha_M\beta_2$ (α_{Mac1} and β_2), KTacβ_3 (IL2-β_3 chimera), KTacβ_5 (IL2-β_5 chimera), and KTacNT (IL2 tailless receptor construct). Transfected cells were selected in media containing 1,200 μg/ml G418 (Genticin, Gibco), and populations of transfectants expressing high levels of the transfected integrin were obtained by fluorescence cell sorting. For selection of integrin chimera expressing clones, cells were subjected to limiting dilution cloning after transfection and high expressing clonal populations were selected by FACS analysis using antibody 4E3 (Boehringer Mannheim) directed against the gp 55 subunit of the IL-2 receptor. K562 transfected with cDNA encoding the α_4 subunit (K$\alpha_4\beta_1$) were kindly provided by Dr. Martin Hemler, Dana Farber Cancer Institute (34).

FACS Analysis of Transfected and Endogenous Receptor Expression

Receptor expression on macrophages and K562 cells was analyzed by fluorescent flow cytometry as described (5, 32). Table I shows the relative expression levels (mean channel fluorescence) of transfected and endogenous integrin receptors as assessed by the specific antibodies indicated and flow cytometry for cells used in these studies. For all cells used, the mean channel fluorescence of irrelevant, isotype-matched antibodies did not exceed 0.10. Expression of $\alpha_5\beta_1$, demonstrated by mAb 16 and P5D2 fluorescence, directed against α_5 and β_1, respectively, remained at levels comparable to untransfected K562 when additional integrins were expressed. Also, as assessed by IC12, antibody specific for α_v, the relative expression of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ were equivalent in the transfectants expressing these respective integrins.

Flow Cytometric Phagocytosis Assay

Direct measurement of FITC-ligand opsonized particle phagocytosis by endogenous and transfected integrins in K562 cells was performed by flow cytometry as previously described (2) and expressed as a Phagocytic Index, the number of beads internalized per 100 cells (PI) (21).

Cell Adhesion

Adhesion of transfected K562 cells to ligand coated substrate was performed as described (2). Briefly, 96-well microtiter plates (Dynatech, Chantilly, VA) were coated with ligand (fibronectin, vitronectin, or casein) at 50 μg/ml in PBS at 4°C for 6 h. Nonadherent protein was removed by washing three times with PBS and wells blocked by the addition of 1% BSA in PBS for 30 min at room temperature. Cells were washed in HBSS, labeled with Calcein AM fluorescent dye (Molecular Probes, Eugene, OR) according to manufacturer's instructions and suspended in HBSS with 1.0 mM each of Ca^{++}, Mg^{++}, and Mn^{++} and 0.5% HSA. Cells were added to plates in 200 μl at 10^6/ml and incubated for 1.5 h at 37°C. Plates were rinsed twice gently with PBS and adhesion measured in a fluorescence plate reader (Cytofluor 2300, Millipore, Marlborough, MA). In some studies Lysophosphatidic Acid (LPA, Sigma Chem. Co.) was added along with cells at 5.0 μg/ml in PBS. Adhesion data is presented as percent of control (KRc\RSV) adhesion to fibronectin.

Reagents

H7 and HA-1004 were purchased from LC Laboratories (Woburn, MA). All other reagents were purchased from Sigma Chem. Co.

Data Presentation

Data are presented as either the mean ± SEM or as a representative study from at least three yielding equivalent results. Significance was determined by analysis of variance followed by Duncan's comparison testing. A minimum confidence interval of 95% was used in all analyses.

Results

Integrin Expression and Integrin-mediated Adhesion in Transfectants

Stable K562 transfectants expressing $\alpha_v\beta_3$ ($K\alpha_v\beta_3$), $\alpha_{IIb}\beta_3$ ($K\alpha_{IIb}\beta_3$), α_v and the tailless β_3 subunit ($K\alpha_v\beta_3NT$), $\alpha_v\beta_5$ ($K\alpha_v\beta_5$), and $\alpha_4\beta_1$ ($K\alpha_4\beta_1$) were used in these studies. Expression of transfected receptors as quantitated by FACS is shown in Table I. Expression of the endogenous K562 integrin, $\alpha_5\beta_1$, was unaffected by transfection of the additional integrins (Table I). Adhesion of the various integrin transfectants to fibronectin (Fn)-coated surfaces was equivalent (Fig. 1 *A*). $K\alpha_{IIb}\beta_3$, $K\alpha_v\beta_3$, and $K\alpha_v\beta_3NT$ attached equivalently to vitronectin (Vn)-coated surfaces; as expected, the other transfectants did not adhere to Vn, since they did not express a Vn receptor.

$\alpha_{IIb}\beta_3$ Ligation Regulates $\alpha_5\beta_1$ Phagocytosis

Previous work showed that ligation of $\alpha_v\beta_3$, but not $\alpha_v\beta_5$, inhibited $\alpha_5\beta_1$ phagocytosis. This suggested a requirement for β_3 in the signal transduction event (2). To determine whether there was α chain specificity as well for the signaling event, K562 cells were stably transfected with cDNAs encoding the $\alpha_{IIb}\beta_3$ receptor ($K\alpha_{IIb}\beta_3$). The expressed $\alpha_{IIb}\beta_3$ was functional in binding assays (Fig. 2). Unlike controls, including vector-transfected K562 (Fig. 2 *A*) and

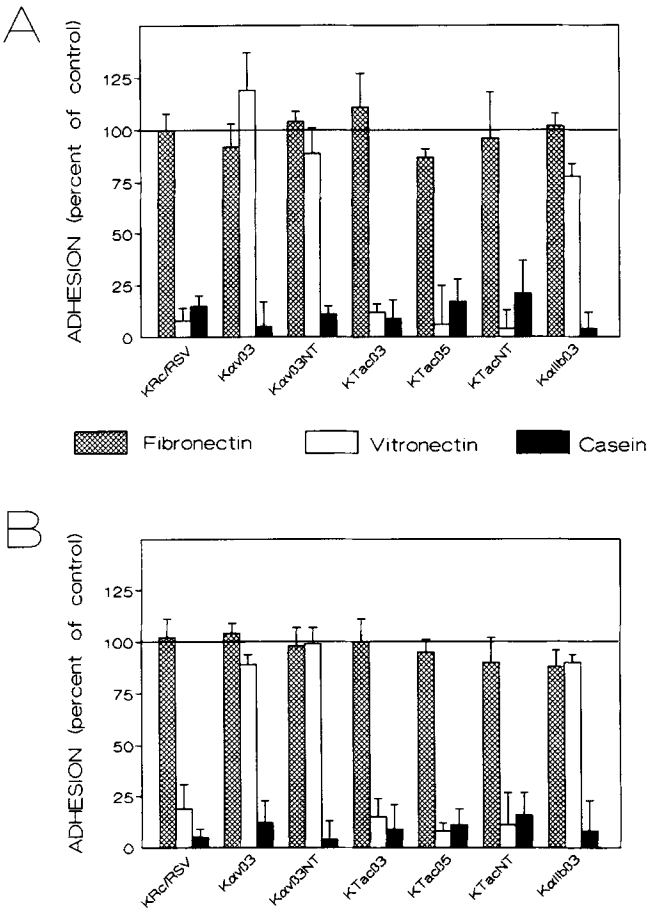

Figure 1. Adhesion of K562 cells expressing transfected integrins to ligands. K562 transfected with cDNAs encoding integrins, mutant integrins, or chimeric integrins were assessed for the ability to attach to fibronectin- (*hatched bars*), vitronectin- (*open bars*), or casein- (*filled bars*) coated substrate as described in Materials and Methods in the absence (*A*) or presence (*B*) of 5.0 μg/ml lysophosphatidic acid (LPA). Data are expressed as the percent of KRc/RSV adhesion to fibronectin in the absence of LPA. Shown is a summary of three separate experiments.

Table I. Integrin Expression in Transfected K562 Cells

	P5D2 (β1)	IC12 (αv)	LM609 (αvβ3)	P1F6 (αvβ5)	IB4 (β2)	IGII4 (α4)	mAb 16 (α5)	10E5 (αIIb)
KRc/RSV	9.83	0.21	0.19	0.17	0.26	0.32	14.8	0.31
Kαvβ3	10.1	25.2	27.8	0.22	0.31	ND	13.9	ND
Kαvβ5	9.61	26.7	0.23	39.5	0.18	ND	14.1	ND
KαIIbβ3	10.2	0.35	0.17	0.28	0.22	0.19	13.6	23.35
Kα4β1	10.7	0.17	ND	ND	ND	34.2	12.5	ND
KαMβ2	11.3	0.21	ND	0.30	9.46	ND	14.1	ND
Kαvβ3NT	9.66	31.2	28.8	0.17	ND	ND	14.1	ND
Human macrophages	15.8	12.3	17.6	8.62	ND	11.2	15.8	ND

Cultured human macrophages and K562 cells transfected with cDNA encoding various integrin subunits or mutant subunits were prepared and assessed for receptor expression by flow cytometry as described in Materials and Methods. Reactivity of monoclonal antibodies is indicated in parentheses below each antibody. Shown are the log mean channel (MCF) fluorescence from a representative study. For each antibody, cells were stained in parallel, permitting relative quantitation of receptor expression between cell types for any specific antibody. The MCF for irrelevant isotype-matched control antibodies did not exceed 0.10.

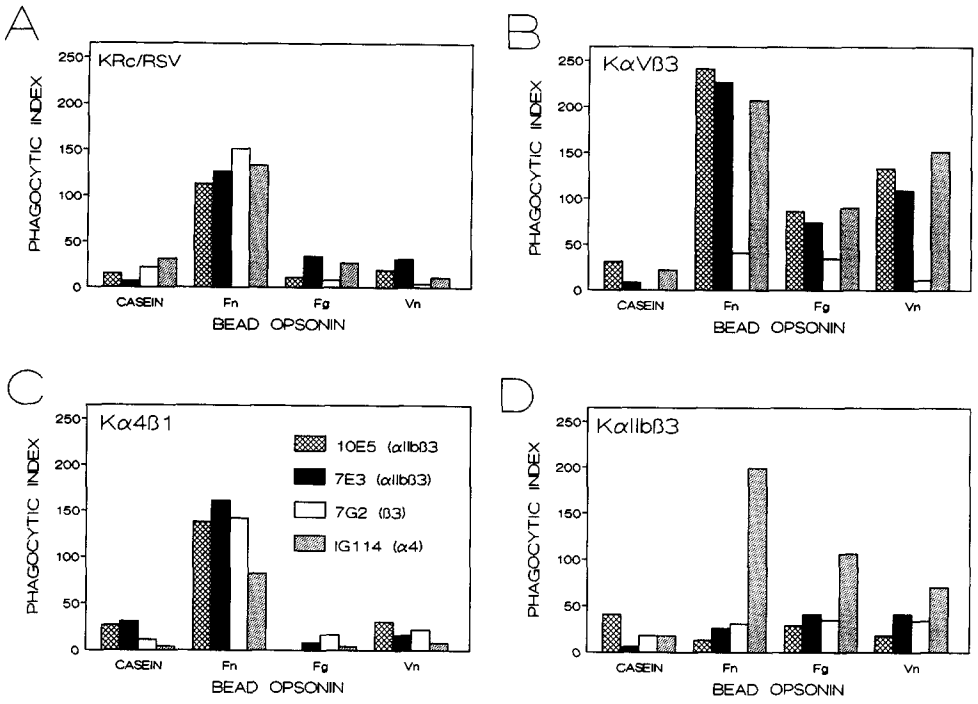

Figure 2. Phagocytosis of opsonized beads by transfected K562 cells. K562 transfected with vector alone (*A*) or cDNAs encoding $\alpha_v\beta_3$ (*B*), α_4 (*C*), or $\alpha_{IIb}\beta_3$ (*D*) were presented with beads coated with the indicated opsonin in the presence of monoclonal antibodies directed against integrin subunits, and phagocytosis was assessed by flow cytometry as described in Materials and Methods. Monoclonal antibody reactivities are indicated in parentheses and the key for all graphs is shown in panel *C*. Shown is a representative study of four yielding identical results. Data are presented as a Phagocytic Index as described in Materials and Methods.

$K\alpha_4\beta_1$ (Fig. 2 *C*) and $K\alpha_M\beta_2$ and $K\alpha_v\beta_5$ (2), $K\alpha_{IIb}\beta_3$ bound and ingested fibrinogen (Fg)-opsonized beads (Fig. 2 *D*). Fg-mediated phagocytosis by these cells was blocked by both α_{IIb}- and β_3-specific mAb. $K\alpha_v\beta_3$ also bound and ingested Fg-opsonized beads, and this was blocked by the β_3-specific but not by the α_{IIb}-specific mAb 10E5 (Fig. 2 *B*). mAb 7E3, reactive against $\alpha_{IIb}\beta_3$, has been reported to inhibit $\alpha_v\beta_3$ function in platelets. However, this Ab does not react with $\alpha_v\beta_3$ expressed in K562 as assessed by flow cytometry or with human macrophages as assessed by immunoprecipitation. Additionally, 7E3 has no effect on phagocytosis in K562 expressing $\alpha_v\beta_3$, but does inhibit in $K\alpha_{IIb}\beta_3$. Thus while mAb 7E3 can recognize platelet β_3, the recognition epitope is unlikely available in $\alpha_v\beta_3$ expressed on human macrophages or K562 cells. As previously shown for $K\alpha_v\beta_3$, $K\alpha_{IIb}\beta_3$ phagocytosis of Fn-opsonized beads was partially inhibited by mAb recognizing either α_5 or β_1 (not shown). Importantly, for both $K\alpha_v\beta_3$ and $K\alpha_{IIb}\beta_3$, mAb 7G2 to β_3 completely inhibited Fn bead phagocytosis (Fig. 2, *B* and *D*). Anti-α_{IIb} 10E5 also blocked Fn bead phagocytosis in the $\alpha_{IIb}\beta_3$ transfectants. This complete inhibition did not occur upon ligation of transfected receptors in $K\alpha_M\beta_2$ or $K\alpha_v\beta_5$ (2) or $K\alpha_4\beta_1$ (Fig. 2 *C*). Thus, ligation of either of two different β_3 receptors inhibited $\alpha_5\beta_1$-mediated phagocytosis.

The β3 Cytoplasmic Tail Is Required for β3 Integrin Inhibition of α5β1 Phagocytosis

To determine the domain(s) of β_3 required for regulation

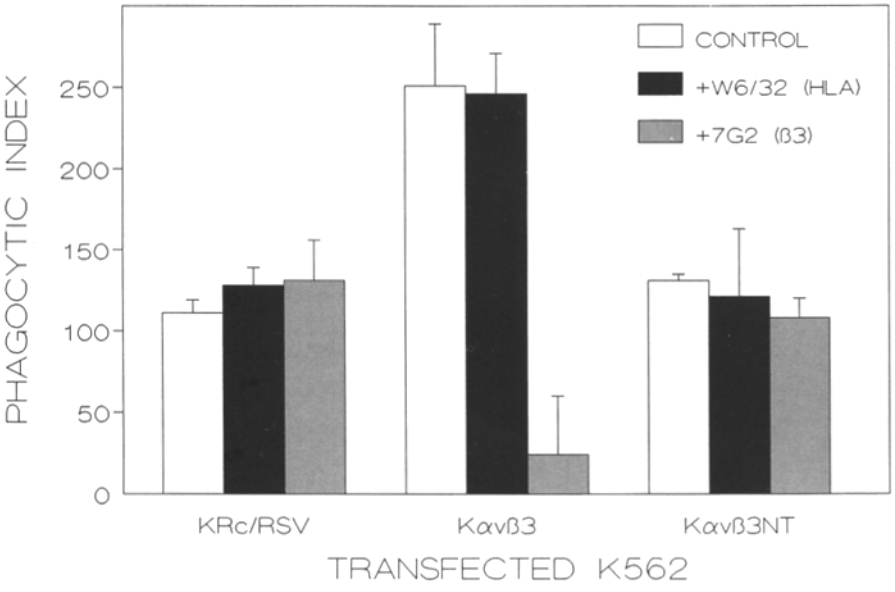

Figure 3. Effects of cytoplasmic β3 tail deletion upon αvβ3 regulation of fibronectin bead phagocytosis. Phagocytosis of fibronectin-opsonized beads by KRc/RSV, $K\alpha_v\beta_3$, and $K\alpha_v\beta_3$NT was quantitated by FACS, in the presence of antibody against HLA class I (W6/32) or β_3 (7G2). Shown is the summary of four experiments. Data are presented as a Phagocytic Index (PI). PI of casein opsonized beads by all cell types was less than 15 in all studies.

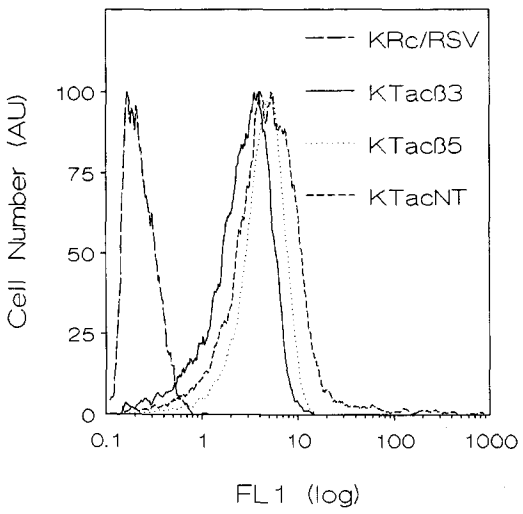

Figure 4. Expression of chimeric integrins in transfected K562. K562 were transfected with cDNA encoding chimeras of the Interleukin 2 receptor α subunit extracellular and transmembrane domains linked to the cytoplasmic domain of either β_3 (KTacβ_3), β_5 (KTacβ_5), or lacking a cytoplasmic tail (KTacNT) along with neomycin selectable vector pRc/RSV or vector alone (KRc/RSV). Shown is the expression of transfected chimeras as assessed by flow cytometry using the IL2 receptor specific monoclonal antibody 4E3 performed. Fluorescence profile of an irrelevant antibody was identical to 4E3 staining of KRc/RSV.

of $\alpha_5\beta_1$-mediated phagocytosis, we examined K$\alpha_v\beta_3$NT, which express β_3 truncated at amino acid 728 and which therefore lack the entire β_3 cytoplasmic tail after the HDRRE sequence conserved in many integrin β cytoplasmic tails. Although ligation of $\alpha_v\beta_3$ with the anti-β_3 mAb 7G2 completely blocked Fn-bead phagocytosis by K$\alpha_v\beta_3$, it had no effect on $\alpha_5\beta_1$-mediated ingestion in K$\alpha_v\beta_3$NT (Fig. 3). This indicates that the cytoplasmic tail of the β_3 subunit is required for the signaling which inhibits $\alpha_5\beta_1$ phagocytosis.

The β_3 Cytoplasmic Tail Is Sufficient for Inhibition of $\alpha_5\beta_1$ Phagocytosis

To determine if the β_3 tail is sufficient for signaling to $\alpha_5\beta_1$, K562 cells were transfected with chimeric DNA constructs encoding the extracellular and transmembrane domains of the Interleukin 2 receptor small subunit gp 55 (Tac) fused with the cytoplasmic tail domains of β_3 (KTacβ_3) or β_5 (KTacβ_5) or without a cytoplasmic tail (KTacNT). Expression of these constructs in stably transfected K562 cells was approximately equivalent, as assessed by flow cytometry using antibody 4E3, specific for the Tac antigen (Fig. 4). Several studies have shown that expression of isolated integrin tail domains can disrupt adhesion of cells to substrate (30, 43). However, KTacβ_3 and KTacβ_5 both adhered equivalently to Fn-coated surfaces as KTacNT or the control K562 transfected with vector alone (Fig. 1 *A*). Addition of lysophosphatidic acid has been reported to uncover an inhibitory effect on adhesion of these autonomous integrin β chain cytoplasmic domain chimeras (43). However, addition of 5.0 μg/ml lysophosphatidic acid did not affect KTacβ_3 or KTacβ_5 adhesion to Fn (Fig. 1 *B*). Possibly, failure of these isolated β chain cytoplasmic domains to inhibit adhesion reflects the fact that K562 adhesion to Fn is unassociated with cell spreading, and thus may not depend on the same integrin-cytoskeletal associations as cells spreading on the Fn substrate.

In contrast to their lack of effect on adhesion, expression of the autonomous β_3 cytoplasmic tail in K562 had a marked inhibitory effect on Fn-bead phagocytosis (Fig. 5). Expression of Tacβ_3, but not Tacβ_5 or TacNT decreased Fn-bead phagocytosis to the background level of casein bead phagocytosis. Surprisingly, inhibition by Tacβ_3 was reversed by the addition of the serine/threonine kinase inhibitor H7, while the structurally related inhibitor HA1004 had no effect (Fig. 6) (25). The Fn-bead phagocytosis seen in H7-treated KTacβ_3 was completely blocked by antibodies against $\alpha_5\beta_1$ (data not shown). Together with the lack of effect of $\alpha_v\beta_3$NT on Fn-bead phagocytosis, these results demonstrate that the β_3 cytoplasmic tail is both necessary

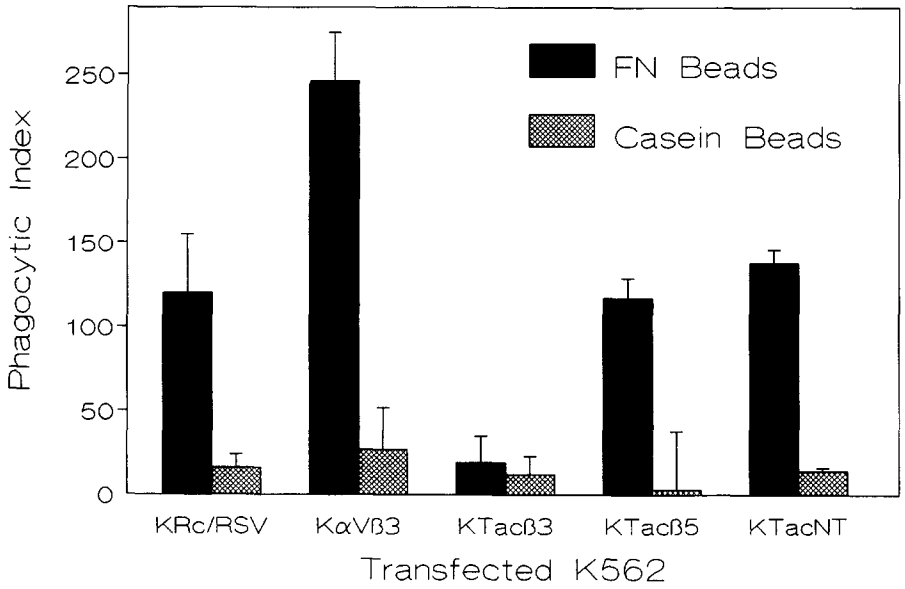

Figure 5. Effect of isolated integrin cytoplasmic tail expression upon fibronectin bead phagocytosis. Phagocytosis of Fn- and casein-opsonized beads by KRc/RSV, K$\alpha_v\beta_3$, KTacβ_3, KTacβ_5, and KTacNT was determined by FACS. Data are presented as Phagocytic Index and represent the summary of four separate experiments.

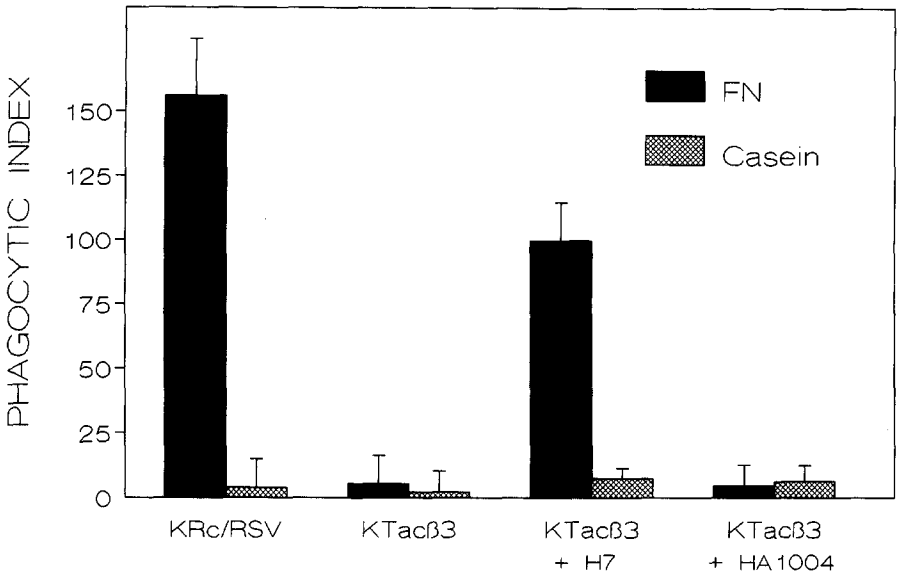

Figure 6. Reversal of $\alpha_5\beta_1$ phagocytosis inhibition by H7. KRc/RSV and KTacβ_3 phagocytosis of fibronectin- or casein-opsonized beads in the absence or presence of 50 nM H7 or 100 nM HA-1004 was assessed by FACS. Data are presented as Phagocytic Index and represent the summary of four separate experiments.

and sufficient for the inhibition of $\alpha_5\beta_1$-mediated phagocytosis. Moreover, the data suggest that the inhibitory effect of expression of the autonomous β_3 cytoplasmic tail results from constitutive activation of a kinase, rather than competition with endogenous β_1 for a cytosolic protein.

IAP Inhibition of $\alpha_5\beta_1$ Phagocytosis Requires β_3 Integrin

IAP has been implicated in β_3-dependent signal transduction in leukocytes and endothelial cells (13, 41, 49). It has been hypothesized that IAP and $\alpha_v\beta_3$ form a single signal transduction complex, but evidence in favor of that hypothesis has been limited to studies with antibodies on primary cells (49). Although K562 express no endogenous $\alpha_v\beta_3$, they express $>10^5$ copies/cell of IAP (not shown). We examined the effect of IAP ligation on Fn-bead phagocytosis in the K562 transfectants, using anti-IAP mAbs B6H12 and 2D3 (Fig. 7). B6H12 has been shown to be an inhibitory mAb in several assays, while 2D3 binds IAP with equal affinity, but is not inhibitory (6,42,49). Ligation of IAP by B6H12 inhibited $\alpha_5\beta_1$-mediated Fn-bead phagocytosis in K$\alpha_v\beta_3$ and K$\alpha_{IIb}\beta_3$, but not in K$\alpha_v\beta_5$ or in K562 transfected with the neo resistance vector alone (Fig. 7). As expected, 2D3 had no effect on $\alpha_5\beta_1$-mediated phagocytosis in any transfectant. Inhibition by B6H12 was reversed by the addition of H7 (data not shown). Thus, B6H12 inhibition of $\alpha_5\beta_1$-mediated ingestion required expression of the β_3 chain. Moreover, B6H12 had no effect on $\alpha_5\beta_1$-mediated phagocytosis in the K$\alpha_v\beta_3$NT cells (Fig. 8), demonstrating that the inhibitory effect of anti-IAP mAb on $\alpha_5\beta_1$-mediated phagocytosis required the β_3 cytoplasmic tail.

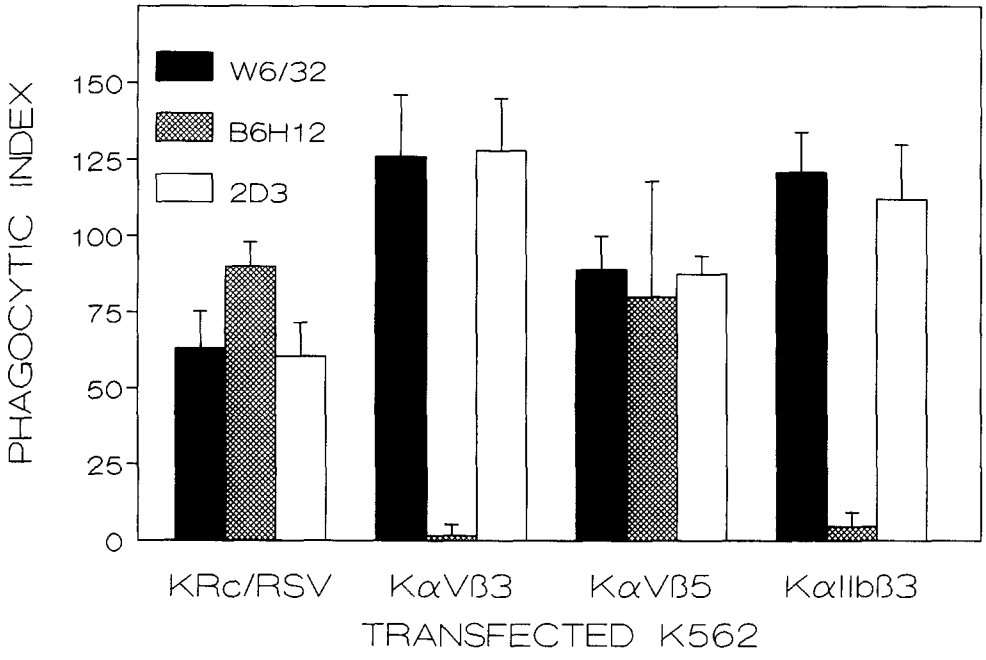

Figure 7. IAP can regulate $\alpha_5\beta_1$ through β_3 integrins. Phagocytosis of fibronectin-opsonized beads by KRc/RSV, K$\alpha_v\beta_3$, K$\alpha_v\beta_5$, K$\alpha_{IIb}\beta_3$ in the presence of monoclonal antibodies against HLA Class I (W6/32, *filled bars*), IAP inhibitory epitope (B6H12, *hatched bars*), or IAP noninhibitory epitope (2D3, *open bars*) was evaluated by FACS and are presented as a Phagocytic Index. Shown is the summary of three experiments. PI for casein-opsonized beads was always less than 18 for all transfectants, with or without antibodies.

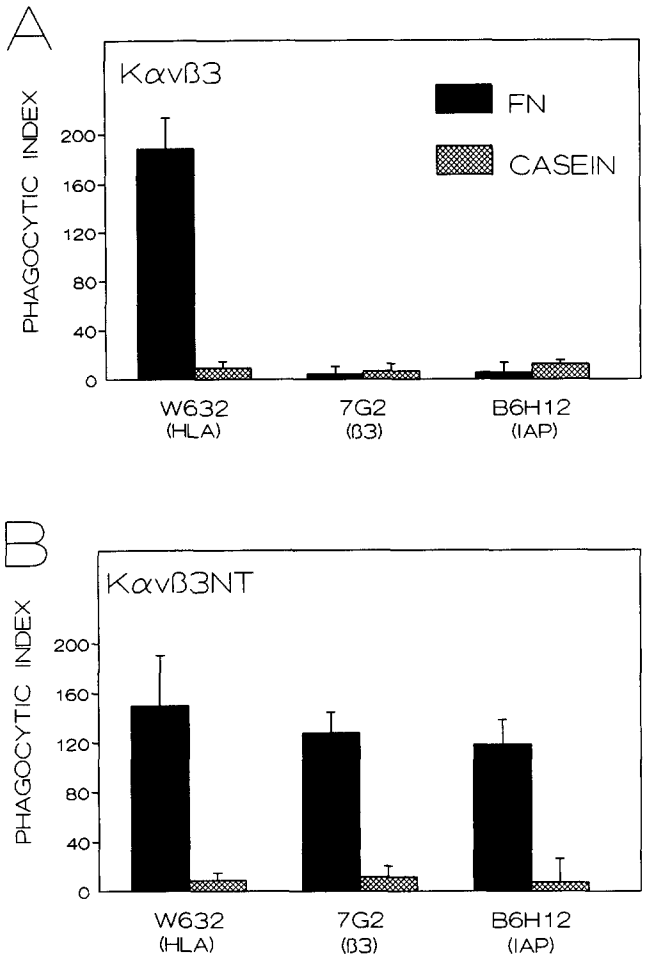

Figure 8. IAP regulation of $\alpha_5\beta_1$ phagocytosis is dependent upon a competent β_3 integrin. Phagocytosis of fibronectin- (*filled bars*) and casein-opsonized (*hatched bars*) beads by $K\alpha_v\beta_3$ (*A*) and by $K\alpha_v\beta_3NT$ (*B*) was evaluated in the presence of monoclonal antibodies against HLA class I (W6/32), IAP inhibitory epitope (B6H12), or β_3 (7G2). Shown are summaries of three separate experiments. Antibody reactivities are shown in parentheses.

Table II. Detection of High Affinity $\alpha_5\beta_1$ Epitopes on K562 Transfectants

	None		7G2 (anti-β3)		Stimulus
Detecting Ab	Control	2.0 mM Mn	Control	2.0 mM Mn	Conditions
W6/32 (HLA)	13.7	12.9	12.8	13.1	
mAb 16 (Total α5)	10.2	10.3	10.2	10.4	
9EG7 (High Affinity α5)	0.47	22.1	0.45	21.6	

$K\alpha_v\beta_3$ were assessed for the expression of HLA class I, total α_5 integrin, and high affinity state $\alpha_5\beta_1$ by flow cytometry using monoclonal antibodies W6/32, mAb 16, and 9EG7, respectively, under control conditions (HBSS, 1.0 mM Ca^{++}, 1.0 mM Mg^{++}) or with the addition of 2.0 mM Mn^{++} in the presence or absence of 7G2, monoclonal antibody reactive against β_3. Shown is the log of the mean channel fluorescence for a representative study. Staining was performed in parallel under all conditions and stimuli to permit direct comparison between conditions for any one antibody.

$\alpha_v\beta_3$ Inhibition of $\alpha_5\beta_1$ Phagocytosis Occurs Without a Change in $\alpha_5\beta_1$ Affinity for Ligand

In our previous study we demonstrated that Fn-bead phagocytosis in K562 required a high affinity conformation of $\alpha_5\beta_1$ which can bind soluble fibronectin (2). Expression of $Tac\beta_3$ in CHO cells has been shown to prevent $\alpha_{IIb}\beta_3$ from achieving its high affinity conformation (10). Therefore, it was possible that the mechanism by which ligation of $\alpha_v\beta_3$ inhibited $\alpha_5\beta_1$ phagocytosis involved preventing $\alpha_5\beta_1$ from achieving the high affinity state, making it incapable of phagocytosis. We tested this hypothesis by two methods. First, we examined the binding of the mAb 9EG7, which binds only to the high affinity state of β_1 integrins (31), to K562 transfectants under conditions in which Fn-bead phagocytosis was supported or inhibited (Table II). Induction of the high affinity state of $\alpha_5\beta_1$ in $K\alpha_v\beta_3$ by addition of 2 mM Mn^{2+} had no effect on the expression of the HLA class I molecule or the total number of α_5 integrin on these cells. However, the addition of Mn^{2+} greatly increased the 9EG7 binding, demonstrating that at least some $\alpha_5\beta_1$ had altered their conformation to the high affinity state. Addition of the anti-β_3 mAb 7G2 had no effect on 9EG7 epitope expression, which suggests that the affinity of $\alpha_5\beta_1$ is not changed upon ligation of $\alpha_v\beta_3$. To confirm that $\alpha_v\beta_3$ ligation affects $\alpha_5\beta_1$ phagocytosis through an event subsequent to ligand binding, we examined the phagocytosis of mAb 16-opsonized beads by $K\alpha_v\beta_3$. mAb 16 is specific for the α_5 subunit and its binding to $K\alpha_v\beta_3$ is unaffected by Mn^{2+} or by $\alpha_v\beta_3$ ligation. Therefore, binding of mAb16-coated beads to $\alpha_5\beta_1$ should be independent of the conformation of the receptor. Nonetheless, phagocytosis of mAb 16 was markedly inhibited in $KTac\beta_3$ compared to $KTac\beta_5$ (Fig. 9 *A*), demonstrating that $\alpha_5\beta_1$ phagocytosis of this ligand also was inhibited by constitutive expression of the β_3 cytoplasmic tail. Moreover, ligation of $\alpha_v\beta_3$ in $K\alpha_v\beta_3$ with 7G2 abolished the phagocytosis of mAb 16 opsonized beads, whereas control antibody against HLA (W6/32) had no effect (Fig. 9 *B*). Inhibition of phagocytosis of the mAb 16–coated beads by $\alpha_v\beta_3$ ligation or expression of $Tac\beta_3$ was reversed by the addition of H7 (data not shown). These data demonstrate that β_3 inhibits the phagocytic function of high affinity $\alpha_5\beta_1$ receptors by interference with an event downstream from ligand binding.

Discussion

In addition to its role as an adhesive ligand in extracellular matrix, fibronectin is a major opsonin in human serum which functions in both host defense and wound repair. While this aspect of Fn function has been known for many years, the molecular aspects of Fn's role as an opsonin, rather than as a conventional adhesion molecule, are not known. Among the reasons for this lack of understanding is the large number of different potential fibronectin receptors on macrophages, the major effector cells in recognition of fibronectin-opsonized bacteria and debris. Moreover, several different experimental approaches have suggested that there may be communication among the Fn-binding integrins expressed on phagocytic cells which further complicates analysis of the role for individual Fn receptors.

To study the mechanisms involved in integrin crosstalk in the regulation of interaction with Fn, we have used a model of transfection of different integrins into K562 cells,

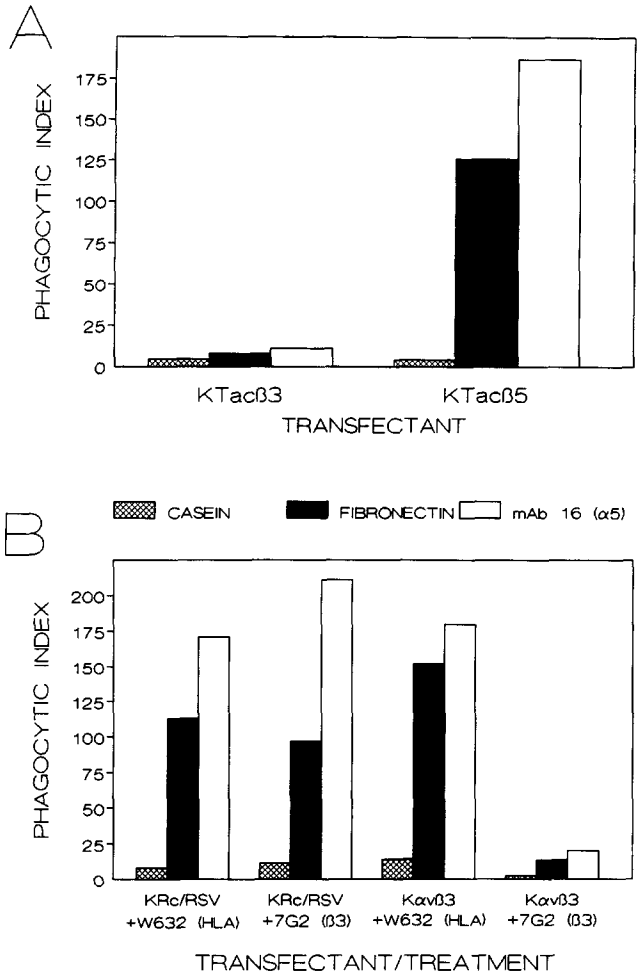

Figure 9. K562 transfectant phagocytosis of anti-$\alpha_5\beta_1$ opsonized beads. Casein- (*hatched bar*), fibronectin- (*filled bar*), or mAb 16– (*open bar*) opsonized beads were presented to $KTac\beta_5$ and $KTac\beta_3$ (*A*) and to KRc/RSV or $K\alpha_v\beta_3$ in the presence of antibody to HLA class I (W6/32) or β_3 (7G2) (*B*), and phagocytosis was quantitated. Data shown are representative of three studies yielding identical results. Antibody reactivities are shown in parentheses.

which endogenously express only $\alpha_5\beta_1$. In our previous report (2) we showed that $\alpha_v\beta_3$ ligation differentially regulates the phagocytic and adhesive properties of $\alpha_5\beta_1$ in K562 cells. While expression of $\alpha_v\beta_3$ had no effect on $\alpha_5\beta_1$ function as an adhesion receptor, ligation of $\alpha_v\beta_3$ with ligand or antibody completely blocked $\alpha_5\beta_1$ recognition and phagocytosis of Fn-opsonized particles. This effect was specific for $\alpha_v\beta_3$, since neither $\alpha_v\beta_5$ nor $\alpha_M\beta_2$ transfected into K562 modulated $\alpha_5\beta_1$ function at all. Because $\alpha_v\beta_3$-mediated inhibition of $\alpha_5\beta_1$ phagocytosis could be reversed by pharmacologic inhibitors of protein kinase C, we hypothesized that $\alpha_v\beta_3$ ligation initiated a signal transduction cascade which modulated $\alpha_5\beta_1$ function.

The current studies have extended this model of integrin crosstalk to begin to understand the initiation of signal transduction and the mechanism of inhibition. We have shown that expression of the β_3 cytoplasmic tail is necessary and sufficient for inhibition of $\alpha_5\beta_1$-mediated phagocytosis. The data for this conclusion are (*a*) $\alpha_{IIb}\beta_3$ is equally effective at modulation of $\alpha_5\beta_1$ phagocytosis as $\alpha_v\beta_3$; (*b*) ligation of $\alpha_v\beta_3$NT, in which the β_3 cytoplasmic tail has been truncated a few amino acids after the transmembrane domain, has no effect on $\alpha_5\beta_1$ function; and (*c*) the isolated β_3 cytoplasmic tail, expressed as a transmembrane protein with irrelevant transmembrane and extracellular domains, constitutively inhibits $\alpha_5\beta_1$ phagocytosis. This last effect is not reproduced by the β_5 cytoplasmic tail, again suggesting specificity for β_3 in the inhibition. Since the β_3 cytoplasmic domain is only 47 amino acids and unlikely to have enzymatic activity on its own, it is reasonable to speculate that there are cytosolic proteins in K562 cells which interact specifically with intracytoplasmic sequences in β_3.

Our data are reminiscent of studies which show that β_1 and/or β_3 cytoplasmic tails can inhibit focal contact formation, cell adhesion, and activation of the high affinity state of $\alpha_{IIb}\beta_3$ (10, 30, 43). These studies have been interpreted to demonstrate that these cytoplasmic tails compete for cytoplasmic proteins which link integrins to the cytoskeleton or to the integrin-activation complex. However, there are important differences between our data and these studies. First, $KTac\beta_3$, which have no $\alpha_5\beta_1$-mediated phagocytosis, adhere normally to Fn. This suggests that the β_3 cytoplasmic tail differentially regulates these functions of $\alpha_5\beta_1$ in K562 cells. Second, the inhibitory effect of the β_3 cytoplasmic tail is rapidly reversed by H7, a serine/threonine kinase inhibitor, suggesting that β_3 is not acting as a simple competitive inhibitor of the $\alpha_5\beta_1$ integrin by binding a cytosolic protein which is necessary for phagocytosis and would otherwise bind to the ligated β_1 integrin. The alternative hypothesis, that the isolated β_3 cytoplasmic tail constitutively activates a signal transduction cascade which results in inhibition of $\alpha_5\beta_1$ phagocytosis, but not adhesion, is more likely (30).

The ability of the β_3 cytoplasmic tail to initiate signal transduction was somewhat surprising because of previous experiments demonstrating an apparently central role for IAP in $\alpha_v\beta_3$ signaling (41). Experiments demonstrating that antibody to IAP could block β_3-induced activation of phagocytes and β_3-dependent increase in $[Ca^{2+}]_i$ in endothelial cells suggested that IAP would have an essential role in $\alpha_v\beta_3$-initiated signal transduction. Moreover, the complex structure of IAP suggested that it would be a good candidate for linking the integrin to signal transduction cascades. To investigate the role of IAP, we tested the effect of anti-IAP mAb on $\alpha_5\beta_1$ phagocytosis. In the absence of $\alpha_v\beta_3$, anti-IAP had no effect on $\alpha_5\beta_1$ function. This is genetic evidence which supports the hypothesis that β_3 integrins and IAP form a single signaling complex. This signaling complex requires the β_3 cytoplasmic tail, because its removal in $K\alpha_v\beta_3$NT abolished the regulatory effect of the anti-IAP mAb. This result, together with the fact that the $Tac\beta_3$ is constitutively active, suggests that IAP does not have an essential role in linking the integrin to intracellular signaling molecules. Instead, at least in this model, IAP appears to have an auxiliary role, perhaps by altering $\alpha_v\beta_3$ conformation in a way that facilitates its activation of the signal transduction cascade. Alternatively, intracytoplasmic sequences in IAP may interact with the β_3 cytoplasmic tail to initiate signal transduction. Unfortunately, we are unable to test whether IAP is essential for signal transduction by intact β_3 integrins in this model, be-

cause we do not have K562 cells without high expression of IAP.

We previously demonstrated that phagocytosis by $\alpha_5\beta_1$ required activation to the high affinity state through the addition of manganese or conformation stabilizing antibodies. Several investigators have demonstrated that $\alpha_5\beta_1$-mediated adhesion to fibronectin is mediated by the low affinity state of the integrin (18). Thus we hypothesized that β_3 specifically inhibited functions of $\alpha_5\beta_1$ which required its high affinity state. This suggested the possibility that β_3-initiated signal transduction might inhibit the ability of $\alpha_5\beta_1$ to achieve high affinity for ligand. Tacβ_3 chimeras have been shown to have precisely this effect on $\alpha_{IIb}\beta_3$ transfected into CHO cells (10). However, we found that ligation of $\alpha_v\beta_3$ had no effect on the expression of the epitope for 9EG7, a mAb which recognizes only the high affinity state of β_1 integrins (31). Second, we found that phagocytosis of beads opsonized with mAb 16, which recognizes α_5 equivalently in both conformations of the receptor, also was inhibited by ligation of $\alpha_v\beta_3$. This inhibition was reversed by the addition of H7 (data not shown), demonstrating that inhibition of phagocytosis of mAb-opsonized beads and Fn-opsonized beads proceeded by the same signaling pathway. Thus we conclude that β_3-dependent inhibition of $\alpha_5\beta_1$ phagocytosis affects an event in phagocytosis subsequent to ligand recognition by $\alpha_5\beta_1$.

In summary, we have shown that there is "crosstalk" between integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ in K562 cells and likely in human phagocytes (2). Ligation of the $\alpha_v\beta_3$/IAP complex activates a signal transduction cascade which inhibits phagocytosis, but not adhesion, mediated by $\alpha_5\beta_1$. This integrin crosstalk requires the β_3 cytoplasmic tail, and isolated β_3 cytoplasmic domain constitutively downregulates the phagocytic function of $\alpha_5\beta_1$. The crosstalk is not bidirectional, since ligation of $\alpha_5\beta_1$ does not affect $\alpha_v\beta_3$ phagocytic function ([2] and data not shown). Although $\alpha_5\beta_1$ phagocytosis requires the high affinity state of the integrin, β_3 regulation of phagocytosis is not via modulation of $\alpha_5\beta_1$ affinity for ligand. This suggests that β_3 cytoplasmic domain initiates a signal transduction cascade, potentially involving protein kinase C, which has as its target proteins involved in $\alpha_5\beta_1$-mediated phagocytosis. While the significance of this signaling pathway for cellular responses to Fn is not yet known, it is likely to have an important effect on the phenotype of macrophages, endothelial cells, platelets, and other cells expressing β_3 integrins. Because these cell types are often involved in inflammation and repair, this pathway may be especially significant in host defense and wound healing.

The authors wish to thank all contributors of cDNAs and antibodies listed in Materials and Methods and the members of the Brown laboratory for suggestions and advice on the performance of these studies.

This work was supported by grants AI24674 and GM38330 from the National Institutes of Health. Dr. Blystone is a recipient of National Research Service Award AI08990-02 and a grant from the Lucille P. Markey Foundation. Dr. Lindberg is a postdoctoral fellow of the Howard Hughes Medical Institute.

Received for publication 10 March 1995 and in revised from 25 April 1995.

References

1. Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens—new tools for genetic analysis. *Cell.*14:9–17.
2. Blystone, S. D., I. L. Graham, F. P. Lindberg, and E. J. Brown. 1994. Integrin α-v β-3 differentially regulates adhesive and phagocytic functions of the fibronectin receptor α-5 β-1. *J. Cell Biol.* 127:1129–1137.
3. Blystone, S. D., L. K. Weston, and J. E. Kaplan. 1991. Fibronectin dependent macrophage fibrin binding. *Blood.* 78:2900–2907.
4. Brooks, P. C., A. M. P. Montgomery, M. Rosenfeld, R. A. Reisfeld, T. Hu, G. Klier, and D. A. Cheresh. 1994. Integrin $\alpha_v\beta_3$ antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell.* 79:1157–1164.
5. Brown, E. J., and J. L. Goodwin. 1988. Fibronectin receptors of phagocytes: characterization of the Arg-Gly-Asp binding proteins of human monocytes and polymorphonuclear leukocytes. *J. Exp. Med.* 167:777–793.
6. Brown, E. J., L. Hooper, T. Ho, and H. D. Gresham. 1990. Integrin-associated protein: a 50-kD plasma membrane antigen physically and functionally associated with integrins. *J. Cell Biol.* 111:2785–2794.
7. Carlos, T. M., and J. M. Harlan. 1994. Leukocyte-endothelial adhesion molecules. *Blood.* 84:2068–2101.
8. Carter, W. G., M. C. Ryan, and P. J. Gahr. 1991. Epiligrin, a new cell adhesion ligand for integrin α3β1 in epithelial basement membranes. *Cell.* 65: 599–610.
9. Chen, Y.-P., I. Djaffar, D. Pidard, B. Steiner, A.-M. Cieutat, J. P. Caen, and J.-P. Rosa. 1992. Ser-752 → Pro mutation in the cytoplasmic domain of integrin β_3 subunit and defective activation of platelet integrin $\alpha_{IIb}\beta_3$ (glycoprotein IIb-IIIa) in a variant of Glanzmann thrombasthenia. *Proc. Natl. Acad. Sci. USA.* 89:10169–10173.
10. Chen, Y.-P., T. E. O'Toole, T. Shipley, J. Forsyth, S. E. LaFlamme, K. M. Yamada, S. J. Shattil, and M. H. Ginsberg. 1994. "Inside-out" signal transduction inhibited by isolated integrin cytoplasmic domains. *J. Biol. Chem.* 269:18307–18310.
11. Chen, Y.-P., T. E. O'Toole, J. Ylänne, J.-P. Rosa, and M. H. Ginsberg. 1994. A point mutation in the integrin β_3 cytoplasmic domain ($S^{752}\rightarrow P$) impairs bidirectional signaling through $\alpha_{IIb}\beta_3$ (platelet glycoprotein IIb-IIIa). *Blood.* 84:1857–1865.
12. Cheresh, D. A. 1987. Human endothelial cells synthesize and express an Arg-Gly-Asp-directed adhesion receptor involved in attachment to fibrinogen and von Willebrand factor. *Proc. Natl. Acad. Sci. USA.* 84: 6471–6475.
13. Cooper, D., F. P. Lindberg, J. R. Gamble, E. J. Brown, and M. A. Vadas. 1995. The transendothelial migration of neutrophils involves integrin associated protein (CD47). *Proc. Natl. Acad. Sci. USA.* 92:3978–3982.
14. Darribere, T., K. Guida, H. Larjava, K. E. Johnson, K. M. Yamada, J.-P. Thiery, and J.-C. Boucaut. 1990. In vivo analysis of integrin β1 subunit function in fibronectin matrix assembly. *J. Cell Biol.* 110:1813–1823.
15. Delannet, M., F. Martin, B. Bossy, D. A. Cheresh, L. F. Reichardt, and J.-L. Duband. 1994. Specific roles of the αVβ1, αVβ3 and αVβ5 integrins in avian neural crest cell adhesion and migration on vitronectin. *Development.* 120:2687–2702.
16. Diamond, M. S., and T. A. Springer. 1993. A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. *J. Cell Biol.* 120:545–556.
17. Du, X., E. F. Plow, A. L. Frelinger, III, T. E. O'Toole, J. C. Loftus, and M. H. Ginsberg. 1991. Ligands "activate" integrin $\alpha_{IIb}\beta_3$ (platelet GPIIb-IIIa). *Cell.* 65:409–416.
18. Faull, R. J., N. L. Kovach, J. M. Harlan, and M. H. Ginsberg. 1993. Affinity modulation of integrin α5β1: regulation of the functional response by soluble fibronectin. *J. Cell Biol.* 121:155–162.
19. Ginsberg, M. H., J. Forsyth, A. Lightsey, J. Chediak, and E. F. Plow. 1983. Reduced surface expression and binding of fibronectin by thrombin-stimulated thrombastenic platelets. *J. Clin. Invest.* 71:619–624.
20. Graham, I. L., J. B. Lefkowith, D. C. Anderson, and E. J. Brown. 1993. Immune complex-stimulated neutrophil LTB4 production is dependent on β2 integrins. *J. Cell Biol.* 120:1509–1517.
21. Gresham, H. D., J. L. Goodwin, D. C. Anderson, and E. J. Brown. 1989. A novel member of the integrin receptor family mediates Arg-Gly-Asp-stimulated neutrophil phagocytosis. *J. Cell Biol.* 108:1935–1943.
22. Hall, D. E., L. F. Reichardt, E. Crowley, B. Holley, H. Moezzi, A. Sonnenberg, and C. H. Damsky. 1990. The α1/β1 and α6/β1 integrin heterodimers mediate cell attachment to distinct sites on laminin. *J. Cell Biol.* 110:2175–2184.
23. Hemler, M. E., F. Sanchez-Madrid, T. J. Flotte, A. M. Krensky, S. J. Burakoff, A. K. Bhan, T. A. Springer, and J. L. Strominger. 1984. Glycoproteins of 210,000 and 130,000 m.w. on activated T-cells: cell distribution and antigenic relation to components on resting cells and T cell lines. *J. Immunol.* 132:3011–3018.
24. Hibbs, M. L., H. Xu, S. A. Stacker, and T. A. Springer. 1991. Regulation of adhesion to ICAM-1 by the cytoplasmic domain of LFA-1 integrin β subunit. *Science (Wash. DC).* 251:1611–1613.
25. Hidaka, H., M. Inagaki, S. Kawamoto, and Y. Sasaki. 1984. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochemistry.*23:5036–5041.
26. Ishibashi, Y., S. Claus, and D. A. Relman. 1994. *Bordetella pertussis* filamentous hemagglutinin interacts with a leukocyte signal transduction

complex and stimulates bacterial adherence to monocyte CR3 (CD11b/CD18). *J. Exp. Med.* 180:1225–1233.
27. Jiang, Y., J. F. Zhu, F. W. Luscinskas, and D. T. Graves. 1994. MCP-1-stimulated monocyte attachment to laminin is mediated by β2 integrins. *Am. J. Physiol.* 267:1112–1118.
28. Kovach, N. L., T. M. Carlos, E. Yee, and J. M. Harlan. 1992. A monoclonal antibody to β_1 integrin (CD29) stimulates VLA-dependent adherence of leukocytes to human umbilical vein endothelial cells and matrix components. *J. Cell Biol.* 116:499–509.
29. LaFlamme, S. E., S. K. Akiyama, and K. M. Yamada. 1992. Regulation of fibronectin receptor distribution. *J. Cell Biol.* 117:437–447.
30. LaFlamme, S. E., L. A. Thomas, S. S. Yamada, and K. M. Yamada. 1994. Single subunit chimeric integrins as mimics and inhibitors of endogenous integrin functions in receptor localization, cell spreading and migration, and matrix assembly. *J. Cell Biol.* 126:1287–1298.
31. Lenter, M., H. Uhlig, A. Hamann, P. Jenö, B. Imhof, and D. Vestweber. 1993. A monoclonal antibody against an activation epitope on mouse integrin chain β_1 blocks adhesion of lymphocytes to the endothelial integrin $\alpha_6\beta_1$. *Proc. Natl. Acad. Sci. USA.* 90:9051–9055.
32. Lindberg, F. P., H. D. Gresham, E. Schwarz, and E. J. Brown. 1993. Molecular cloning of integrin-associated protein: an immunoglobulin family member with multiple membrane spanning domains implicated in α-v, β-3-dependent ligand binding. *J. Cell Biol.* 123:485–496.
33. Loftus, J. C., T. E. O'Toole, E. F. Plow, A. Glass, A. L. Frelinger, and M. H. Ginsberg. 1990. A β3 integrin mutation abolishes ligand binding and alters divalent cation-dependent conformation. *Science (Wash. DC).* 249: 915–918.
34. Masumoto, A., and M. E. Hemler. 1993. Multiple activation states of VLA-4. Mechanistic differences between adhesion to CS1/fibronectin and to vascular cell adhesion molecule-1. *J. Biol. Chem.* 268:228–234.
35. McLean, J.W., D. J. Vestal, D. A. Cheresh, and S. C. Bodary. 1990. cDNA sequence of the human integrin β5 subunit. *J. Biol. Chem.* 265:17126–17131.
36. Monk, P. N., and P. Banks. 1991. The role of protein kinase C activation and inositol phosphate production in the regulation of cell-surface expression of Mac-1 by complement fragment C5a. *Biochim. Biophys. Acta. Mol. Cell Res.* 1092:251–255.
37. O'Toole, T. E., J. C. Loftus, X. Du, A. A. Glass, Z. M. Ruggeri, S. J. Shattil, E. F. Plow, and M. H. Ginsberg. 1990. Affinity modulation of the $\alpha_{IIb}\beta_3$ integrin (platelet GPIIb-IIIa) is an intrinsic property of the receptor. *Cell Regulation.* 1:883–893.
38. O'Toole, T. E., D. Mandelman, J. Forsyth, S. J. Shattil, E. F. Plow, and M. H. Ginsberg. 1991. Modulation of the affinity of integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) by the cytoplasmic domain of α_{IIb}. *Science (Wash. DC).* 254: 845-847.
39. Pacifici, R., J. Roman, R. Kimble, R. Civitelli, C. M. Brownfield, and C. Bizzarri. 1994. Ligand binding to monocyte α 5 β 1 integrin activates the α 2 β 1 receptor via the α 5 subunit cytoplasmic domain and protein kinase C. *J. Immunol.* 153:2222–2233.
40. Poncz, M., R. Eisman, R. Heidenreich, S. M. Silver, G. Vilaire, S. Surrey, E. Schwartz, and J. S. Bennett. 1987. Structure of the platelet membrane glycoprotein IIb. Homology to the α subunits of the vitronectin and fibronectin membrane receptors. *J. Biol. Chem.* 262:8476–8482.
41. Schwartz, M. A., E. J. Brown, and B. Fazeli. 1993. A 50 kDa integrin-associated protein is required for integrin-regulated calcium entry in endothelial cells. *J. Biol. Chem.* 268:19931–19934.
42. Senior, R. M., H. D. Gresham, G. L. Griffin, E. J. Brown, and A. E. Chung. 1992. Entactin stimulates neutrophil adhesion and chemotaxis through interactions between its Arg-Gly-Asp (RGD) domain and the leukocyte response integrin (LRI). *J. Clin. Invest.* 90:2251–2257.
43. Smilenov, L., R. Briesewitz, and E. E. Mercantonio. 1994. Integrin β-1 cytoplasmic domain dominant negative effects revealed by lysophosphatidic acid treatment. *Mol. Biol. Cell.* 5:1215–1223.
44. Van Strijp, J. A. G., D. G. Russell, E. Tuomanen, E. J. Brown, and S. D. Wright. 1993. Ligand specificity of purified complement receptor type 3 (CD11b/CD18, Mac-1, αM β2): indirect effects of an Arg-Gly-Asp sequence. *J. Immunol.* 151:3324–3336.
45. Wayner, E. A., R. A. Orlando, and D. A. Cheresh. 1991. Integrins αvβ3 and αvβ5 contribute to cell attachment to vitronectin but differentially distribute on the cell surface. *J. Cell Biol.* 113:919–929.
46. Werb, Z., P. M. Tremble, O. Behrendtsen, E. Crowley, and C. H. Damsky. 1989. Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. *J. Cell Biol.* 109:877–889.
47. Wright, S. D., P. E. Rao, C. Wesley, W. C. van Voorhis, L. S. Craigmyle, K. Iida, M. A. Talle, E. F. Westberg, G. Goldstein, and S. C. Silverstein. 1983. Identification of the C3bi receptor of human monocytes and macrophages by using monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 80: 5699–5703.
48. Yatohgo, T., M. Izumi, H. Kashiwagi, and M. Hayashi. 1988. Novel purification of vitronectin from human plasma by heparin affinity chromatography. *Cell Struct. Funct.* 13:281–292.
49. Zhou, M.-J., and E. J. Brown. 1993. Leukocyte response integrin and integrin associated protein act as a signal transduction unit in generation of a phagocyte respiratory burst. *J. Exp. Med.* 178:1165–1174.