Integrin β3 Cytoplasmic Tail Is Necessary and Sufficient for Regulation of α5β1 Phagocytosis by αvβ3 and Integrin-associated Protein

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Abstract. Using a K562 cell transfection model, we have previously described a novel relationship between the integrins αβ1 and αβ3, αβ3 ligation was able to inhibit αβ1-mediated phagocytosis without effect on αβ3-mediated adhesion. The αβ3-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 β3 cytoplasmic tail is both necessary and sufficient for initiation of the signal leading to inhibition of αβ1 phagocytosis. Ligation of integrin-associated protein (IAP), which has been implicated in αβ3 signal transduction, mimics the effects of αβ3 ligation only when the β3 integrin with an intact cytoplasmic tail is present. Although fibronectin-mediated phagocytosis requires the high affinity conformation of αβ1, ligation of αββ/IAP does not prevent acquisition of this high affinity state. We conclude that αββ/IAP ligation initiates a signal transduction cascade, dependent upon the β3 cytoplasmic tail, which inhibits the phagocytic function of αβ1 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 LTB4, 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, αβ ligation activates αβ collagen binding in monocytes (39). β integrins can influence the function of other receptors as well. Adhesion and activation studies suggest the hypothesis that on phagocytes, the ligation of β3 integrins may be an early event in inflammation, directly affecting the function of other proinflammatory receptors (21, 42, 49). For example, β3 integrin ligation activates the ability of the β3 integrin Mac-1 to bind its ligands in both neutrophils and monocytes (26, 44). This β3-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) (32, 49). The molecular mechanism(s) involved in regulation of leukocyte integrin function by β/IAP are incompletely understood.

Recently, we have developed a model in which to study the mechanism of β3 "crosstalk" with other integrins. In K562 cells transfected with αβ, ligation of this integrin inhibits the phagocytic function of the integrin fibronectin receptor, αβ, 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, αβ ligation affects phagocytosis without affecting the adhesive functions of αβ, suggesting that the signal transduction discretely inhibits high affinity αβ 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 αββ is as effective as αβ for initiation of this signal. Deletion of the β3 cytoplasmic tail prevents signaling, while expression of a chimeric molecule consisting of the IL2 receptor α chain extracellular and transmembrane domains and the β3 cytoplasmic tail constitutively prevents αβ phagocytosis. This probably represents continuous signal transduction from the autonomous β3 cytoplasmic domain rather than com-

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petition for cytoskeletal or other cytoplasmic components, because inhibition of phagocytosis is rapidly reversed by H7, and because the chimera has no effect on αβ integrin mediated adhesion to fibronectin (Fn)-coated surfaces. These data show that the β3 cytoplasmic tail is both necessary and sufficient for initiation of the signal transduction cascade. IAP ligation only inhibits αβ integrin-mediated adhesion to fibronectin when αβ integrin is expressed, and, surprisingly, anti-IAP loses its inhibitory effect in cells expressing β3 without its cytoplasmic tail. Finally, neither constitutive nor ligand-dependent β3-initiated signal transduction affect the number of αβ integrin expressing the high affinity conformation. From these data, we hypothesize that ligation of the αβ/IAP complex initiates a signal transduction cascade mediated by the β3 cytoplasmic tail which affects αβ integrin 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% CO2, humidified incubator.

**Truncation of the β3 Integrin Subunit**

The integrin β3 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 β3 cDNA subcloned into pRc/RSV (termined plAP93) (2) was purified with restriction with AffilI 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'-GCC

**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 αβ integrin subunits α and β integrins were purchased from GIBCO BRL. The αβ subunit-specific monoclonal antibody (mAb) P1F6 (45) was the gift of Dr. Dean Sheppard (UCSF, San Francisco, CA). The mAb II2G2 and IAIB2, reactive with human integrin subunits α and β, respectively (22, 46), were the kind gift of Dr. Caroline Damsky (UCSF, San Francisco, CA). The human integrin αβ-reactive mAb 16 (14) was the gift of Dr. Kenneth Yamada (NIH/NIH, Rockville, MD). mAb B4 (47) was the gift of Dr. Sam Wright (Rockefeller University, New York, NY). The β3 integrin activating antibodies A1A5 (23) and 8A2 (28) were generously provided by Dr. Martin Hemler (Dana-Farber Cancer Institute, Boston, MA) and Dares Nick Kovach and John Harlan (University of Washington, Seattle, WA), respectively. The αβ integrin specific monoclonal antibodies 10E5 and 7E3 were the gift of Dr. Barry Coiler (Mt. Sinai School of Medicine). In our studies, these antibodies recognize αβ integrin, but not αβ integrin when expressed in K562 as assessed by flow cytometry and immunoprecipitation. The β3-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 αβ 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 gp55 Tc1 subunit of the IL-2 receptor was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). mAbs IG114 against human αβ, B6H12 and 2D3 against human integrin associated protein (IAP), 7G2 specific for human integrin, and 3F12 and 1C12 specific for human integrin 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 β3 integrin subunit cDNA was the gift of Dr. Sarah Bodary (Genentech, Inc., San Francisco, CA) (35). Human integrin αC cDNA (33) was a gift of Dr. Joseph C. Lofts (Scripps Research Institute, La Jolla, CA). Human β3 integrin cDNA was the gift of Dr. Jeffrey Smith (Scripps Research Institute, La Jolla, CA). Human αBβ2 cDNA (40) was the gift of Dr. Joel Bennet (University of Pennsylvania). For transfection, the β3 and β3 cDNA were cloned into the stable expression vector pRc/RSV (Invitrogen, San Diego, CA) (β3) or a derivative of pCDMA (Invitrogen). K562 cells were cotransfected with either αβ, αBβ2 and β3, αβ, and β3 was the gift of Dr. Martin Hemler (Dana-Farber Cancer Institute, Boston, MA) and Drs. Nick Kovach and John Harlan (University of Washington, Seattle, WA), respectively. The αβ specific monoclonal antibodies 10E5 and 7E3 were the gift of Dr. Barry Coiler (Mt. Sinai School of Medicine). In our studies, these antibodies recognize αβ integrin, but not αβ integrin when expressed in K562 as assessed by flow cytometry and immunoprecipitation. The β3-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 αβ 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 gp55 Tc1 subunit of the IL-2 receptor was purchased from Boehringer Mannheim Corp. (Indianapolis, IN). mAbs IG114 against human αβ, B6H12 and 2D3 against human integrin associated protein (IAP), 7G2 specific for human integrin, and 3F12 and 1C12 specific for human integrin 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.

**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 αβ integrin demonstrated by mAb 16 and P5D2 fluorescence, directed against αβ integrin, respectively, remained at levels comparable to untransfected K562 when additional integrins were expressed. Also, as assessed by IC12, antibody specific for αβ integrin expression. 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 αβ3 (Kαβ3), αIIIβ3 (KαIIIβ3), α, and the tailless β3 subunit (Kαβ3NT), αβ5 (Kαβ5), and αβ1 (Kαβ1) were used in these studies. Expression of transfected receptors as quantitated by FACS is shown in Table I. Expression of the endogenous K562 integrin, α5β1, was unaffected by transfection of the additional integrins (Table I). Adhesion of the various integrin transfectants to fibronectin (Fn)-coated surfaces was equivalent (Fig. 1A). KαIIIβ3, Kαβ3, and Kαβ3NT 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.

αIIIβ3 Ligation Regulates αβ3 Phagocytosis

Previous work showed that ligation of αβ3, but not αβ5, inhibited αβ1 phagocytosis. This suggested a requirement for β3 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 αIIIβ3 receptor (KαIIIβ3). The expressed αIIIβ3 was functional in binding assays (Fig. 2). Unlike controls, including vector-transfected K562 (Fig. 2A) and

Table 1. Integrin Expression in Transfected K562 Cells

| PSD2 (β1) | IC12 (αv) | LM609 (αvβ3) | P1F6 (αvβ5) | IB4 (β2) | lGHI4 (αx4) | mAb 16 (α5) | 10E5 (αIIb) |
|-----------|-----------|--------------|-------------|----------|-------------|-------------|-------------|
| KRC/RSV   | 9.83      | 0.21         | 0.19        | 0.17     | 0.26        | 0.32        | 14.8        | 0.31        |
| Kavβ3     | 10.1      | 25.2         | 27.8        | 0.22     | 0.26        | 0.32        | 13.9        | ND          |
| Kavβ5     | 9.61      | 26.7         | 0.23        | 39.5     | 0.18        | 0.22        | 0.22        | 14.1        | ND          |
| KalIB3    | 10.2      | 0.35         | 0.17        | 0.28     | 0.30        | 0.19        | 13.6        | 23.35       |
| Kavβ4     | 10.7      | 0.17         | ND          | ND       | ND          | ND          | 12.5        | ND          |
| KavβM2    | 11.5      | 0.21         | ND          | 0.30     | 9.46        | ND          | 14.1        | ND          |
| Kavβ3NT   | 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 αβ1 (B), αδ (C), or αβ1β3 (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.

Kαβ1 (Fig. 2 C) and Kαδβ2 and Kαμβ3 (2), Kαμβ3 bound and ingested fibrinogen (Fg)-opsonized beads (Fig. 2 D). Fg-mediated phagocytosis by these cells was blocked by both αμb- and β3-specific mAb. Kαμβ3 also bound and ingested Fg-opsonized beads, and this was blocked by the β3-specific but not by the αμb-specific mAb 10E5 (Fig. 2 B). mAb 7E3, reactive against αμbβ3, has been reported to inhibit αμ-β3 function in platelets. However, this Ab does not react with αμbβ3 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 αμbβ3 but does inhibit in Kαμbβ3. Thus while mAb 7E3 can recognize platelet β3, the recognition epitope is unlikely available in αμbβ3 expressed on human macrophages or K562 cells. As previously shown for Kαμb3, Kαμb3, phagocytosis of Fn-opsonized beads was partially inhibited by mAb recognizing either α5 or β1 (not shown). Importantly, for both Kαμb3 and Kαμbβ3, mAb 7G2 to β3 completely inhibited Fn bead phagocytosis (Fig. 2, B and D). Anti-αμb 10E5 also blocked Fn bead phagocytosis in the αμbβ3 transfecants. This complete inhibition did not occur upon ligation of transfected receptors in Kαμbβ3 or Kαμb3 (2) or Kαμb1 (Fig. 2 C). Thus, ligation of either of two different β3 receptors inhibited αμb-mediated phagocytosis.

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

To determine the domain(s) of β3 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, Kαβ3, and Kαβ3NT was quantitated by FACS, in the presence of antibody against HLA class I (W6/32) or β3 (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 β3 (KTacβ3), β5 (KTacβ5), 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 α3β3-mediated phagocytosis, we examined Kαββ3NT, which express β3 truncated at amino acid 728 and which therefore lack the entire β3 cytoplasmic tail after the HDRRE sequence conserved in many integrin β cytoplasmic tails. Although ligation of αβ3 with the anti-β3 mAb 7G2 completely blocked Fn-bead phagocytosis by Kαβ3, it had no effect on α3β3-mediated ingestion in Kαβ3NT (Fig. 3). This indicates that the cytoplasmic tail of the β3 subunit is required for the signaling which inhibits α3β3 phagocytosis.

The β3 Cytoplasmic Tail Is Sufficient for Inhibition of α3β3 Phagocytosis

To determine if the β3 tail is sufficient for signaling to α3β3, 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 β3 (KTacβ3) or β5 (KTacβ5) 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, KTacβ3 and KTacβ5 both adhered equivalently to Fn-coated surfaces as KTacNT or the control K562 transfected with vector alone (KRc/RSV, 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 KTacβ3 or KTacβ5 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 β3 cytoplasmic tail in K562 had a marked inhibitory effect on Fn-bead phagocytosis (Fig. 5). Expression of Tacβ3, but not Tacβ5 or TacNT decreased Fn-bead phagocytosis to the background level of casein bead phagocytosis. Surprisingly, inhibition by Tacβ3 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 KTacβ3 was completely blocked by antibodies against α3β1 (data not shown). Together with the lack of effect of α3β3NT on Fn-bead phagocytosis, these results demonstrate that the β3 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, Kαβ3, KTacβ3, KTacβ5, and KTacNT was determined by FACS. Data are presented as Phagocytic Index and represent the summary of four separate experiments.
and sufficient for the inhibition of α5β1-mediated phagocytosis. Moreover, the data suggest that the inhibitory effect of expression of the autonomous β3 cytoplasmic tail results from constitutive activation of a kinase, rather than competition with endogenous β1 for a cytosolic protein.

**IAP Inhibition of αβ1 Phagocytosis Requires β3 Integrin**

IAP has been implicated in β3-dependent signal transduction in leukocytes and endothelial cells (13, 41, 49). It has been hypothesized that IAP and αβ3 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 αβ3, 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 αβ1-mediated Fn-bead phagocytosis in Kαβ3 and Kαβ3β3, but not in Kαβ5 or in K562 transfected with the neo resistance vector alone (Fig. 7). As expected, 2D3 had no effect on αβ1-mediated phagocytosis in any transfectant. Inhibition by B6H12 was reversed by the addition of H7 (data not shown). Thus, B6H12 inhibition of αβ1-mediated ingestion required expression of the β3 chain. Moreover, B6H12 had no effect on αβ1-mediated phagocytosis in the KOtv133NT cells (Fig. 8), demonstrating that the inhibitory effect of anti-IAP mAb on αβ1-mediated phagocytosis required the β3 cytoplasmic tail.

**Figure 6.** Reversal of αβ1 phagocytosis inhibition by H7. KRc/RSV and KTac03 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.

**Figure 7.** IAP can regulate αβ1 through β3 integrins. Phagocytosis of fibronectin-opsonized beads by KRc/RSV, Kαβ3, Kαβ5, Kαβ5β3 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.
bodies against HLA class I (W6/32), IAP inhibitory epitope expression of Tacβ3 in CHO cells has been shown to prevent KC~vβ3NT (B) was evaluated in the presence of monoclonal anti-a competent β3 integrin. Phagocytosis of fibronectin-opsonized fibronectin-bead phagocytosis was supported or inhibited (Table II). Induction of the high affinity state of αβ1 in Karlβ3 by addition of 2 mM Mn²⁺ had no effect on the expression of the HLA class I molecule or the total number of α5 integrin on these cells. However, the addition of Mn²⁺ greatly increased the 9EG7 binding, demonstrating that at least some αβ1 had altered their conformation to the high affinity state. Addition of the anti-β3 mAb 7G2 had no effect on 9EG7 epitope expression, which suggests that the affinity of αβ1 is not changed upon ligation of αβ3. To confirm that αβ3 ligation affects αβ1 phagocytosis through an event subsequent to ligand binding, we examined the phagocytosis of mAb 16-opsonized beads by Koβ3. mAb 16 is specific for the α5 subunit and its binding to Koβ3 is unaffected by Mn²⁺ or by αβ3 ligation. Therefore, binding of mAb16-coated beads to αβ3 should be independent of the conformation of the receptor. Nonetheless, phagocytosis of mAb 16 was markedly inhibited in KTαcβ3 (Fig. 9 A), demonstrating that αβ3 phagocytosis of this ligand also was inhibited by constitutive expression of the β3 cytoplasmic tail. Moreover, ligation of αβ1 in Koβ3 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 αβ3 ligation or expression of Tacβ3 was reversed by the addition of H7 (data not shown). These data demonstrate that β3 inhibits the phagocytic function of high affinity αβ3 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,
which endogenously express only α3β1. In our previous report (2) we showed that α3β1 ligation differentially regulates the phagocytic and adhesive properties of αβ3 in K562 cells. While expression of α3β3 had no effect on α3β1 function as an adhesion receptor, ligation of α3β3 was blocked by antibody completely blocked α3β1 recognition and phagocytosis of Fn-opsonized particles. This effect was specific for α3β3, since neither α3β1 nor α3β2 transfected into K562 modulated α3β1 function at all. Because α3β3-mediated inhibition of α3β1 phagocytosis could be reversed by pharmacologic inhibitors of protein kinase C, we hypothesized that α3β3 ligation initiated a signal transduction cascade which modulated α3β1 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 β3 cytoplasmic tail is necessary and sufficient for inhibition of αβ3-mediated phagocytosis. The data for this conclusion are (a) α1β3 is equally effective at modulation of αβ1 phagocytosis as α3β3; (b) ligation of αβ3NT, in which the β3 cytoplasmic tail has been truncated a few amino acids after the transmembrane domain, has no effect on αβ3 function; and (c) the isolated β3 cytoplasmic tail, expressed as a transmembrane protein with irrelevant transmembrane and extracellular domains, constitutively inhibits αβ3 phagocytosis. This last effect is not reproduced by the β3 cytoplasmic tail, again suggesting specificity for β3 in the inhibition. Since the β3 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 β3.

Our data are reminiscent of studies which show that β1 and/or β3 cytoplasmic tails can inhibit focal contact formation, cell adhesion, and activation of the high affinity state of αβ3 (10, 30, 43). These studies have been interpreted to demonstrate that these cytoplasmic tails compete for cytosolic 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, KTacβ3, which have no α3β1-mediated phagocytosis, adhere normally to Fn. This suggests that the β3 cytoplasmic tail differentially regulates these functions of αβ3 in K562 cells. Second, the inhibitory effect of the β3 cytoplasmic tail is rapidly reversed by H7, a serine/threonine kinase inhibitor, suggesting that β3 is not acting as a simple competitive inhibitor of the αβ3 integrin by binding a cytosolic protein which is necessary for phagocytosis and would otherwise bind to the ligated β3 integrin. The alternative hypothesis, that the isolated β3 cytoplasmic tail constitutively activates a signal transduction cascade which results in inhibition of αβ1 phagocytosis, but not adhesion, is more likely (30).

The ability of the β3 cytoplasmic tail to initiate signal transduction was somewhat surprising because of previous experiments demonstrating an apparently central role for IAP in αβ3 signaling (41). Experiments demonstrating that antibody to IAP could block β3-induced activation of phagocytes and β3-dependent increase in [Ca2+]i in endothelial cells suggested that IAP would have an essential role in αβ3-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 αβ3 phagocytosis. In the absence of α3β3, anti-IAP had no effect on α3β3 function. This is genetic evidence which supports the hypothesis that β3 integrins and IAP form a single signaling complex. This signaling complex requires the β3 cytoplasmic tail, because its removal in Kαβ3NT abolished the regulatory effect of the anti-IAP mAb. This result, together with the fact that the Tacβ3 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 α3β3 conformation in a way that facilitates its activation of the signal transduction cascade. Alternatively, intracytoplasmic sequences in IAP may interact with the β3 cytoplasmic tail to initiate signal transduction. Unfortunately, we are unable to test whether IAP is essential for signal transduction by intact β3 integrins in this model, be-

![Figure 9. K562 transfected phagocytosis of anti-αβ1 opsonized beads. Casein (hatched bar), fibronectin (filled bar), or mAb 16 (open bar) opsonized beads were presented to KTacβ1 and KTacβ3 (A) and to KRC/RSV or Kαβ3 in the presence of antibody to HLA class I (W6/32) or β3 (7G2) (B), and phagocytosis was quantitated. Data shown are representative of three studies yielding identical results. Antibody reactivities are shown in parentheses.](image-url)
cause we do not have K562 cells without high expression of IAP.

We previously demonstrated that phagocytosis by αβ1 required activation to the high affinity state through the addition of manganese or conformation stabilizing antibodies. Several investigators have demonstrated that αβ1-mediated adhesion to fibronectin is mediated by the low affinity state of the integrin (18). Thus we hypothesized that β1 specifically inhibited functions of αβ1 which required its high affinity state. This suggested the possibility that β1-initiated signal transduction might inhibit the ability of αβ1 to achieve high affinity for ligand. Tacβ3 chimeras have been shown to have precisely this effect on αιNβ3 transacted into CHO cells (10). However, we found that ligation of αβ1 had no effect on the expression of the epitope for 9EG7, a mAb which recognizes only the high affinity state of β1 integrins (31). Second, we found that phagocytosis of beads opsonized with mAb 16, which recognizes α6 equivalently in both conformations of the receptor, also was inhibited by ligation of αβ1. 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 β1-dependent inhibition of αβ1 phagocytosis affects an event in phagocytosis subsequent to ligand recognition by αβ1.

In summary, we have shown that there is a ‘crosstalk’ between integrins αβ3 and αβ1 in K562 cells and likely in human phagocytes (2). Ligation of the αβ1/IAP complex activates a signal transduction cascade which inhibits phagocytosis, but not adhesion, mediated by αβ3. This integrin crosstalk requires the β1 cytoplasmic tail, and isolated β3 cytoplasmic domain constitutively downregulates the phagocytic function of αβ3. The crosstalk is not bidirectional, since ligation of αβ3 does not affect αβ1 phagocytic function (2) and data not shown). Although αβ1 phagocytosis requires the high affinity state of the integrin, β1 regulation of phagocytosis is not via modulation of αβ1 affinity for ligand. This suggests that β3 cytoplasmic domain initiates a signal transduction cascade, potentially involving protein kinase C, which has as its target proteins involved in αβ1-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 β3 integrins. Because these cell types are often involved in inflammation and repair, this pathway may be especially significant in host defense and wound healing.

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