Integrin-associated Protein: A 50-kD Plasma Membrane Antigen Physically and Functionally Associated with Integrins

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Abstract. Phagocytosis by monocytes or neutrophils can be enhanced by interaction with several proteins or synthetic peptides containing the Arg-Gly-Asp sequence. Recently we showed that an mAb, B6H12, specifically inhibited this enhancement of neutrophil phagocytosis by inhibiting Arg-Gly-Asp binding to the leukocyte response integrin (Gresham, H. D., J. L. Goodwin, P. M. Allen, D. C. Anderson, and E. J. Brown. 1989. *J. Cell Biol.* 108:1935–1943). Now, we have purified the antigen recognized by B6H12 to homogeneity. Surprisingly, it is a 50-kD molecule that is expressed on the plasma membranes of all hema-topoietic cells, including erythrocytes, which express no known integrins. On platelets and placenta, but not on erythrocytes, this protein is associated with an integrin that can be recognized by an anti-β_3 antibody. In addition, both the anti-β_3 and several mAbs recognizing the 50-kD protein inhibit Arg-Gly-Asp stimulation of phagocytosis. These data demonstrate an association between integrins and the 50-kD protein on several cell types. For this reason, we call it Integrin-associated Protein (IAP). We hypothesize that IAP may play a role in signal transduction for enhanced phagocytosis by Arg-Gly-Asp ligands.

PHAGOCYTOSIS is a regulated function in professional phagocytes. Unstimulated phagocytes show decreased capacity for ingestion compared with cells stimulated with a variety of cytokines, phorbol esters, or extracellular matrix (ECM)[1] proteins (Pommier et al., 1983, 1984a,b; Wright et al., 1983; Bohnsack et al., 1985; Gresham et al., 1988; Graham et al., 1989). The ECM–phagocyte interaction may be proinflammatory for other functions as well, such as printing for an enhanced, prolonged respiratory burst (Nathan et al., 1989) or inducing degranulation (Wachtfogel et al., 1988). We have hypothesized that the interaction of ECM with phagocytes provides a signal that the cells have moved out of the vasculature and into an area of inflammation or infection (Brown, 1986). Subsequent work has shown that at least some of the effects of the ECM are mediated through recognition of the Arg-Gly-Asp (RGD) sequence in ECM proteins by phagocyte integrin receptors (Wright and Meyer, 1985; Brown and Goodwin, 1988). Our previous work suggested that a heterodimer on PMN and monocytes that bound to RGD-Sepharose was a prime candidate for the integrin receptor through which ECM enhanced the phago-cytic function of these cells (Brown and Goodwin, 1988; Gresham et al., 1989). This heterodimer had characteristics which suggested that it was related to the β_3 integrin family.

1. *Abbreviations used in this paper:* ECM, extracellular matrix; fmoc, 9-fluorenylmethyloxycarbonyl; IAP, integrin-associated protein; LRI, leu-kocyte response integrin; NPGB, paranitrophenyl paraaguanidinobenzoate.

Because it mediated phagocytic responses to several RGD-containing proteins, we termed this receptor the Leukocyte Response Integrin (LRI).

Recently, we described an mAb, B6H12, that inhibited the RGD-dependent enhancement of neutrophil IgG-mediated phagocytosis (Gresham et al., 1989). This mAb inhibited phagocytosis enhancement by fibronectin, collagen type IV, von Willebrand's factor, fibrinogen, and vitronectin as well as by synthetic RGD-containing ligands, but had no effect on laminin-enhanced phagocytosis. Because B6H12 was able to inhibit RGD-dependent binding of the synthetic ligands and immunoprecipitated a PMN receptor similar in behavior on SDS-PAGE the heterodimer we had purified on RGD-Sepharose, we hypothesized that it recognized LRI, the cell surface integrin involved in phagocytosis enhancement.

Here we report that the antigen recognized by B6H12 is not an integrin but a 50-kD protein functionally and perhaps physically associated with LRI. The antigen is widely distributed, including expression on erythrocytes, a cell with no known integrins. Using immunoprecipitation and affinity purification of the 50-kD protein with B6H12 antibody, an associated integrin was found in platelets and placenta, but not in erythrocytes. As we had shown for LRI (Gresham et al., 1989), the associated integrin β had immunologic characteristics consistent with β_3. In addition, mAb recognizing both the 50-kD protein and β_3 inhibited Arg-Gly-Asp-stimulated ingestion by both PMN and monocytes. These data show that the 50-kD protein can modulate LRI function

in intact cells and may physically associate with it. For this reason, we have called this membrane protein, Integrin-associated Protein (IAP). We postulate that IAP is part of the signal transduction mechanism for some ECM–integrin interactions. Because IAP is expressed on erythrocytes independently of known integrins, it may also represent a molecule involved in signal transduction for other receptors.

Materials and Methods

Reagents

All reagents were obtained as previously described (Gresham et al., 1989). B6H12 antibody was obtained as described (Gresham et al., 1989); IgG was purified from B6H12 ascites by octanoic acid precipitation (Steinbuch and Audran, 1969) and DEAE-Sephacel chromatography. 7G2 antibody is an IgG$_1$ mAb which recognizes the integrin β_3 band by Western blot and which immunoprecipitates gpIIb/IIIa and vitronectin receptor (Gresham et al., 1989), and which gives a typical staining pattern for β_3 on osteoclasts and platelets. B3F12 (3F12) is a mAb that recognizes α_v (Gresham et al., 1989); 3D9 is a mAb that recognizes complement receptor type I (O'Shea et al., 1985); W6/32 is a mAb that recognizes a monomorphic determinant on HLA class I, and HYPC 1643 is an antiphosphorylcholine mAb. For these mAbs, IgG was obtained from ascites fluid by octanoic acid precipitation. mAbs IgG were coupled to cyanogen bromide–Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) at 5 mg/ml. Fibronectin was purified as described (Pommier et al., 1983).

Cells

Monocytes (Pommier et al., 1983), PMN (Gresham et al., 1988, 1989), and platelets (Brown and Goodwin, 1988) were purified as previously described. Erythrocytes were obtained from the dextran sulfate pellet during PMN purification, and washed three times in HBSS. The myelomonocytic cell lines HL60 and U937 were maintained in Iscove's Medium with 10% FBS.

Phagocytosis Assays

Sheep erythrocytes opsonized with either IgG (EIgG) or complement (EC3b) were prepared as phagocytic targets as previously described (Pommier et al., 1983). Fibronectin stimulation of ingestion by uncultured, elutriated peripheral blood monocytes was performed essentially as described (Pommier et al., 1983) using monocytes adherent to Labtek chambers (Miles Scientific, Naperville, IL), and fibronectin added in solution with target EC3b. Assay of EIgG ingestion by PMN was performed in suspension, as previously described (Gresham et al., 1988, 1989). Phagocytosis assays for both PMN and monocytes were performed in the presence of catalase (5,000 U/assay; Sigma Chemical Co., St. Louis, MO) (Gresham et al., 1989). Phagocytosis was quantitated as a phagocytic index (PI, no. of erythrocytes ingested/100 phagocytes).

Synthetic Peptide

In some cases, phagocytosis was stimulated with 5 μg/ml of a synthetic peptide based on the Arg-Gly-Asp (RGD) sequence. This peptide was a branched peptide, prepared using 9-fluorenylmethyloxycarbonyl (fmoc) chemistry and the Ramps (Dupont) Corp., Wilmington, DE) system, based on the synthetic concept of Posnett et al. (1988). The peptide was an eightfold repeat of GRGDSPA (Fig. 1). We have found that oligomerization of the RGD sequence is necessary for RGD-dependent stimulation of phagocytosis.

Purification of the B6H12 Antigen

Placental membranes were prepared, solubilized, incubated with wheat germ agglutinin-agarose and with an irrelevant γ-IFN-affinity column as described (Calderon et al., 1988). The WGA-binding, γ-IFN nonbinding fractions were pooled and incubated with B6H12 Sepharose, which was sequentially washed with buffer A (150 mM NaCl, 25 mM Hepes, 3 mM Ca^{2+}, 1 mM Mg^{2+}, 25 μM paranitrophenyl paraguanidinobenzoate (NPGB), 10 mM CHAPS, pH 7.4) and buffer B (buffer A containing 500 mM NaCl). Bound proteins were eluted with buffer E (500 mM NaCl, 100 mM glycine, 10 mM CHAPS, pH 3.3) and immediately neutralized with 1/10 vol 1 M
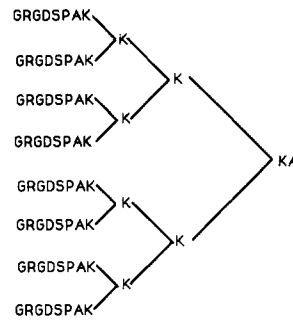

Figure 1. Structure of the branched peptide used to stimulated PMN ingestion of opsonized erythrocytes. The branched peptide (branched RGD) was synthesized according to the conception of Posnett et al. (1988), using fmoc chemistry.

Tris. After dialysis against buffer A, the eluted proteins were reapplied to the B6H12 affinity column, washed, and eluted identically.

B6H12 antigen was purified from erythrocytes, platelets, and HL60 and U937 cells following essentially an identical scheme. Cells were initially solubilized in a buffer identical to buffer A, except with 1% Triton X-100 instead of CHAPS, and containing 20 mM iodoacetamide and 10 μg/ml each of leupeptin, pepstatin A, and aprotinin as additional protease inhibitors.

Tryptic Peptides of IAP

100 μg of purified placental IAP was carboxymethylated as described (Crestfield et al., 1963), diluted to 4× vol to reduce the guanidine concentration and incubated for 24 h at 37°C with 5 μg/ml TPCK-trypsin. The reaction was stopped by injection onto a C18 reverse phase HPLC column equilibrated with 0.05% trifluoracetic acid. Peptides were eluted with a 2–80% gradient of acetonitrile. Peptides were sequenced by automated Edman degradation by the Washington University Protein Chemistry Laboratory. Each reported peptide sequence was obtained from two different purifications of IAP. Peptide sequences were compared to sequences in the National Biomedical Research Foundation database (version 23) using the PSQ programs of the Protein Information Resource.

Preparation of mAbs

Mice were immunized with purified antigen from placenta obtained by affinity chromatography as above. The spleen of a single mouse was removed and single cells were fused with the nonsecreting myeloma cell line P3X63AG8.6.5.3. Hybridoma supernatants were screened for antigen reactivity using an ELISA in which wells were coated with 100 ng of purified protein. All cloned hybridoma supernatants used in subsequent experiments gave absorbance values at 490 nm between 1.0 and 1.5. mAbs were screened for crossreactivity with B6H12 by incubating 100 μl of varying dilutions of antibody-containing tissue culture supernatant together with 200 ng of biotinylated B6H12 antibody on ELISA wells coated with purified antigen. Bound B6H12 was detected with avidin-peroxidase (E-Y Laboratories, San Mateo, CA).

Immunoprecipitation

Platelets were surface radiolabeled as described for erythrocytes (Thompson et al., 1987), washed three times with HBSS and then solubilized at a concentration of 1–5 × 10^{10}/ml in a lysis buffer consisting of HBSS with 20 mM Hepes, 10 mM iodoacetamide, 10 mg/ml ovalbumin, 5 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 50 μM paranitrophenyl paraguanidinobenzoate, and 0.2% Triton-X 100. After 30 min at 4°C, lysates were cleared of insoluble material by centrifugation at 15,000 g for 10 min, and supernatants were cleared of nonspecifically binding material by incubation with gelatin-Sepharose overnight at 4°C. Precleared lysates were then precipitated by incubation with Sepharose-bound antibodies for 3 h at room temperature. The immunoaffinity beads were then washed with lysis buffer without ovalbumin four times, and specifically bound material was eluted with 0.05 M diethylamine, 0.5% Triton-X 100, pH 11.3. The eluate was neutralized with 1/20 vol of 1 M NaH$_2$PO$_4$, and immediately prepared for SDS-PAGE by incubation with an equal volume of sample buffer at 60°C for 15 min.

For some experiments, an octylglucoside extract of placental proteins (Calderon et al., 1988) was labeled with Na^{125}I using chloroglycoluril
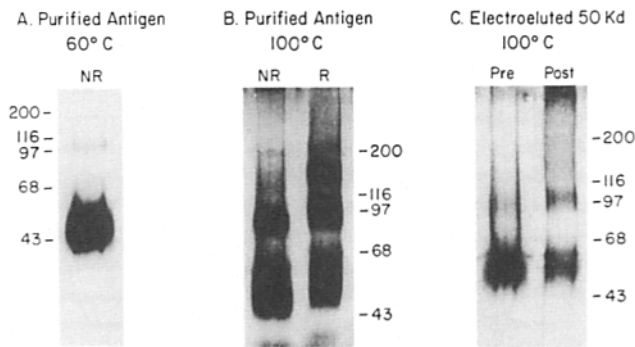
Figure 2. Purification of IAP. The placental protein recognized by B6H12 was purified as described in Materials and Methods, run on SDS-PAGE, and silver stained. When prepared for SDS-PAGE at 60°C, the antigen had an apparent M_r of 50,000 (A). A faint band at 100 kD was also visible. When prepared for SDS-PAGE at 100°C, the 100-kD band became much more prominent, and even higher apparent molecular mass forms appeared when 50 mM DTT was added to the 100°C incubation (B). When a 100°C-incubated sample was run on SDS-PAGE (C, pre) and the 50-kD band cut out and electroeluted from the gel, it again showed two bands on subsequent SDS-PAGE (C, post). These data suggest that the B6H12 antigen is a 50-kD species that aggregates to higher relative molecular mass forms at least in part during incubation at elevated temperatures in SDS-PAGE sample buffer.

(Markwell and Fox, 1978), and labeled proteins separated from free iodide on Sephadex G25 equilibrated in a buffer of 0.15 M NaCl, 0.02 M Hepes, 3 mM Ca^{2+}, 1 mM Mg^{2+}, 10 mM CHAPS, and 50 μM NPGB. Aliquots of labeled protein were immunoprecipitated with B3F12, B6H12 or 3D9 using 10 μg/ml of antibody, as described (Gresham et al., 1989). Supernatants of these immunoprecipitations were then reprecipitated with 7G2 (10 μg/ml), followed by anti-mouse Ig Sepharose (Cooper Biomedical, Inc., Malvern, PA). After thorough washing, precipitated proteins were eluted from the anti-mouse Ig Sepharose with SDS-PAGE sample buffer and analyzed by SDS-PAGE.

Other Techniques

Fluorescent flow microcytometry (Brown and Goodwin, 1988), Western blotting (Brown and Goodwin, 1988), and Staphylococcus V8 protease cleavage and peptide analysis (Cleveland et al., 1977) were all performed as previously described. SDS-PAGE was performed essentially as described (Maizel, 1971). Some samples were prepared by heating at 60°C for 15 min rather than at 100°C or 5 min, because 100°C incubation resulted in significant aggregation of the purified protein (see Results).

Results

Purification of IAP

Initially, we purified antigen from placental membranes by wheat germ agglutinin chromatography, removal of nonspecifically adsorbing proteins on an irrelevant affinity column, and two rounds of B6H12 antibody-Sepharose affinity chromatography. SDS-PAGE of the resultant protein revealed a major diffuse band at M_r ~50,000 (Fig. 2). On reduction with DTT, it migrated at slightly higher apparent molecular mass. There was a minor band at 100 kD. Several pieces of data suggested that the 100-kD band represented a dimer of the 50-kD protein. Within a single purified preparation, the ratio of higher band to lower band increased as more antigen was loaded onto the gel, and higher apparent molecular mass

bands were especially marked if the sample was heated at 100°C, rather than at 60°C. Slower migrating bands appeared on reduction and were consistent in apparent molecular mass with still higher order aggregates (Fig. 2). In addition, SDS-PAGE of the B6H12 antigen followed by electro-elution of the lower band and reelectrophoresis still revealed two bands in approximately the same ratio as the original material (Fig. 2). Reduction and alkylation of antigen before electrophoresis did not decrease the amount of the higher band; in fact, reduction tended to increase the aggregation (Fig. 2). Two-dimensional nonreduced, reduced SDS-PAGE showed that aggregation of the lower band during electrophoresis in the second dimension led to a band at exactly the apparent molecular mass of the higher band (not shown). The amino termini of both the higher and the lower bands were blocked, precluding direct determination of amino-terminal sequence. Finally, both bands were recognized by B6H12 antibody on Western blot. Based on all the evidence, we concluded that the higher apparent molecular mass band represented an aggregate of the lower, which arose at least in part during sample preparation for SDS-PAGE and during electrophoresis. Thus, the apparent molecular mass of the monomer IAP on SDS-PAGE is ~50,000 and the antigen increases slightly in M_r to ~55,000 upon reduction.

Purified IAP was cleaved with trypsin and several major peptide peaks were partially sequenced (Table I). The sequences were not contained within any proteins in the NBRF data base and specifically were different from any peptides within the deduced protein sequences of integrin chains α_v, gpIIb, α_5, α_4, α_2, α_M, α_x, α_L, β_1, β_2, and β_3.

Characterization of Monoclonal and Polyclonal Antibodies to Purified IAP

Because the antigen that we purified with B6H12, the antibody that inhibited RGD-stimulated ingestion, was apparently not an integrin, we needed to explore further its relationship to RGD-stimulated phagocytosis. We considered the possibility that B6H12 antibody cross-reacted with a cell surface molecule involved in RGD binding that was otherwise unrelated to the antigen purified by affinity chromatography. To examine this possibility, monoclonal and polyclonal antibodies were prepared using the purified placental protein as immunogen. These antibodies initially were examined for their ability to compete with B6H12 for binding in an ELISA. We examined six independently selected mAbs as well as a polyclonal antiserum. Of the six mAbs, four competed with B6H12 for binding by ELISA, suggesting that

Table I. Tryptic Peptide Sequences from IAP

No.	Sequence
1	IQPSH
2	LVPTNF
3	AVSHTG
4	VPTDFSSAK
5	SAVSVTGXYTEVTXL
6	IYTFDGAL
7	AVPQLITSN
8	YFDVTE
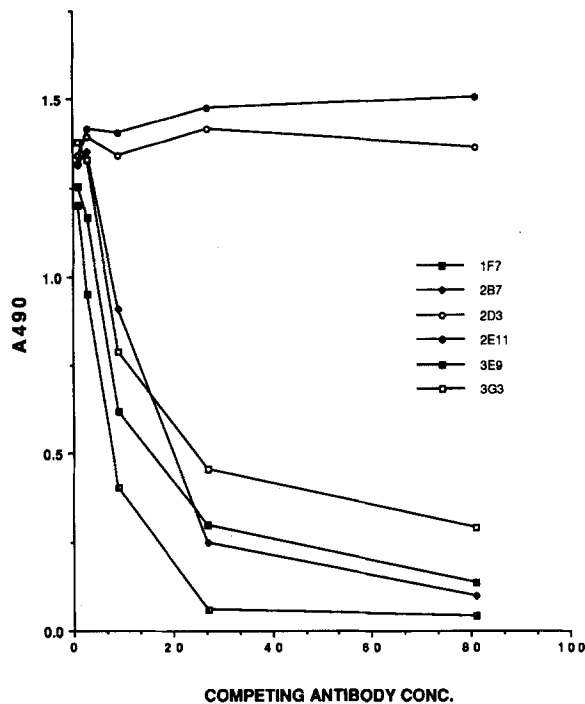
Figure 3. Competitive inhibition of B6H12 binding by mAbs directed against IAP. Biotinylated B6H12 (200 ng/well) was incubated on ELISA plates coated with 100 ng of purified 50-kD protein with 100 μl of various dilutions of tissue culture supernatants containing mAbs that recognize IAP. After 2 h incubation, B6H12 binding was quantitated using avidin-peroxidase, followed by o-phenylenediamine substrate. Data are plotted as optical density developed in each well versus competing antibody concentration (antibody concentration = 81 corresponds to undiluted tissue culture supernatant). 2E11 and 2D3 did not inhibit B6H12 binding, whereas 1F7, 2B7, 3E9, and 3G3 did. Representative of two experiments.

this was an immunodominant epitope (Fig. 3). The two which did not compete (2D3 and 2E11) both recognized the 50-kD protein on Western blot (Fig. 4), demonstrating that their reactivity in the ELISA was with the purified antigen and not with a trace contaminant.

IAP Is Expressed on Many Cell Types

Next, we undertook a survey of antigen expression on various cell types. We found that B6H12 bound to all hematopoietic cells, including erythrocytes, lymphocytes, platelets, and monocytes (Fig. 5) as well as neutrophils (Gresham et al., 1989), and to the promonocytic and promyelocytic cell lines U937 and HL60 (not shown). The polyclonal antibody also recognized each of these cells (data not shown). 2E11 and 2D3, the mAbs that did not crossreact with B6H12 by ELISA, also recognized all cell types tested (Fig. 6). Thus, at least two, and probably three, distinct epitopes of the 50-kD antigen were broadly and strongly displayed on all hematopoietic cell types and at least two cell lines. This suggested that the antigen purified by B6H12 affinity chromatography is expressed at the plasma membrane.

Next, platelets were surface labeled with ¹²⁵I, solubilized, and subjected to affinity chromatography on B6H12 Sepharose as described in Materials and Methods for IAP purification. Under these conditions, a prominent 50-kD band was

seen (Fig. 7). This also suggests that IAP can be expressed on the plasma membrane. Despite its abundance as determined by immunofluorescence and antibody binding, IAP labels very poorly with a variety of iodination techniques. For example, on U937 cells, which express ∼100,000 B6H12 binding sites (Brown, E., unpublished data), <0.01% of protein bound ¹²⁵I (as judged by TCA precipitability after solubilization) was specifically bound to the B6H12 affinity column. Thus, far, we have found platelets, from which we recover 20–60 × 10³ IAP molecules/cell by affinity chromatography, to be the cell on which IAP labels best by surface labeling techniques.

To test whether the membrane molecule recognized by B6H12 was similar in all cells, antigen was purified by B6H12 affinity chromatography from platelets and erythrocytes. The purified antigen from both cell types displayed a band pattern on silver strain similar to that of the placental antigen, showing a major silver-stained band at 50 kD, with a minor band at 100 kD (Fig. 8 A). The same 50-kD band was seen after B6H12 affinity chromatography of lysates from the monocytoid cell line U937 and from the myelomonocytic cell line HL60 as well (data not shown). Staphylococcus V8 protease digests of erythrocyte and placental 50-kD antigen showed similar gel patterns (Fig. 8 B). Thus we concluded that IAP, the 50-kD protein recognized by B6H12, is a widely distributed molecule or closely related family of molecules, which can be expressed at the plasma membrane on a variety of hematopoietic cells.

## Physical Association between IAP and β₃ Integrins

We considered the possibility that IAP itself bound to the RGD sequence. This might have been why it originally bound to KYAVTGRGDS-Sepharose (Gresham et al., 1989). Therefore, purified, radiolabeled IAP was incubated with KYAVTGRGDS-Sepharose (Brown and Goodwin, 1988). Less than 0.5% of the IAP bound to and eluted from the column. As a control, placental vitronectin receptor (VnR) was purified on 7G2 (anti-β₃) Sepharose by a protocol identical to the one used to purify IAP on B6H12 Sepharose. 20% of this radiolabeled vitronectin receptor bound to and eluted from the KYAVTGRGDS-Sepharose. Thus, affinity-purified IAP did not itself recognize the RGD sequence.

Next, we considered the possibility that B6H12 recognized
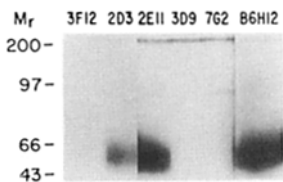
Figure 4. Western blot of placental IAP. Solubilized placental proteins were precleared on γ-IFN sepharose and then IAP purified by affinity chromatography on B6H12 Sepharose. Purified antigen was incubated at 60°C in sample buffer and then electrophoresed in 7.5% SDS-PAGE. Various lanes were subjected to Western blotting with 3F12 (=B3F12), anti-α_v; 2D3, 2E11, and B6H12, anti-IAP; 3D9, anti-CR1; and 7G2, anti-β₃. The anti-IAP antibodies were used at 1 μg/ml, the others at 10 μg/ml. Blots were developed by subsequent incubation with rabbit anti-mouse Ig and ¹²⁵I-protein A. The anti-IAP antibodies recognize a 50-kD protein; 7G2 faintly recognizes a 90-kD protein; the 3F12 and 3D9 lanes are blank.
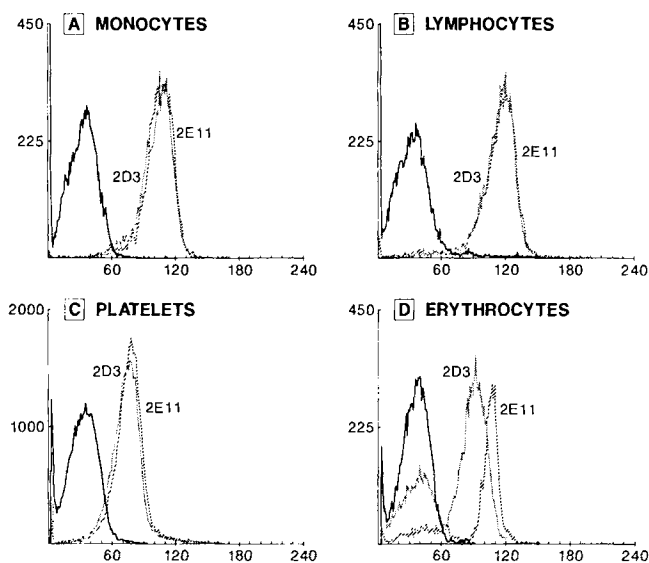
Figure 5. Fluorescent analysis of B6H12 binding to hematopoietic cells. B6H12 binding to monocytes, lymphocytes, platelets, and erythrocytes was tested by fluorescent flow cytometry, as described (Brown and Goodwin, 1988). Fluorescence distribution for each cell type after incubation with an isotype control antibody is shown in the solid lines; fluorescence distribution after B6H12 staining is shown with the stippled lines. All cell types tested are reactive, as are PMN.

VnR in addition to the 50-kD protein. To assess this, two experiments were performed. First, aliquots of radiolabeled placental proteins were incubated with B3F12 (anti-α_v), B6H12 or an irrelevant (3D9, anti-CR1) antibody. The precleared lysates were then precipitated with the anti-β_3 mAb,
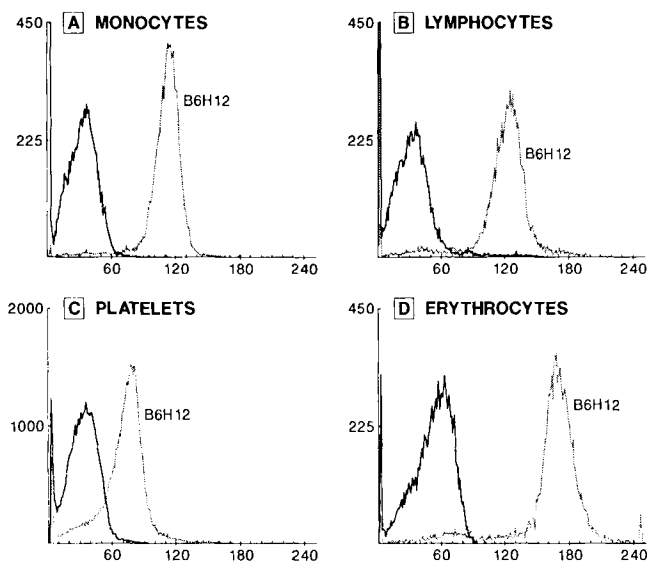
Figure 6. Fluorescent analysis of anti-IAP mAb binding. Fluorescence distribution of cells after incubation with control mAb (*solid lines*), mAb 2D3 (*stippled lines*), or mAb 2E11 (*hatched lines*) is shown for monocytes, lymphocytes, platelets, and erythrocytes. All cell types shown stain with these two mAbs, which do not recognize the same epitope as B6H12. PMN, U937, and HL60 cells are also stained with these antibodies. Methods as in Fig. 4.
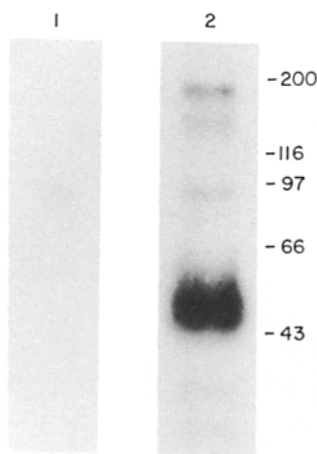

Figure 7. Immunoaffinity isolation of IAP from surface iodinated platelets. Intact platelets were iodinated as described in Materials and Methods, solubilized in 100 mM octylglucoside, and incubated with either B6H12-Sepharose, or 3D9 (anti-CR1 [O'Shea et al., 1985])-Sepharose as a control. Antibody-Sepharose columns were then washed and eluted as described for the purification of IAP. Eluates from 3D9-Sepharose (lane *1*) and B6H12-Sepharose (lane *2*) were incubated with SDS-PAGE sample buffer at 60°C before electrophoresis and autoradiography. A broad band at 50 kD is present in the eluate from B6H12-Sepharose, consistent with the pattern seen on silver stain of purified material (see Fig. 2).

7G2. As shown in Fig. 9 *A*, while preincubation with B3F12 cleared most of the VnR from the solubilized placental protein mixture, preincubation with B6H12 had no effect on subsequent precipitation of VnR by 7G2. Thus B6H12 did not immunoprecipitate VnR under these conditions. Second, placental VnR was purified by affinity chromatography on 7G2 Sepharose and subjected to Western blotting with B6H12 and several other mAbs that recognize the 50-kD antigen. None recognize VnR by Western blot (Fig. 9 *B*). Thus, B6H12, which was raised against a placental antigen purified on RGD-Sepharose did not bind VnR directly, nor did its apparent antigen bind to the RGD sequence.

For these reasons, we considered the hypothesis that the 50-kD protein could, under some circumstances, interact with an RGD-binding integrin. Immunoprecipitation of surface labeled PMN with B6H12 showed a heterodimer consistent with an integrin structure (Gresham et al., 1989). Furthermore, some binding of 7G2 (anti-β_3) to a 90-kD band was observed in Western blots of IAP purified from placenta by B6H12 affinity chromatography (Fig. 4). These data suggested that IAP might be physically associated with PMN and placental integrins. To obtain further evidence for a physical association between IAP and integrins, surface iodinated platelets were solubilized in a low concentration of Triton X-100 and immunoprecipitated with B6H12, 2D3, and the polyclonal anti-IAP antibody. Immunoprecipitation with all three anti-IAP antibodies revealed two bands not present in the control, at 90 and 175 kD, in addition to the diffuse band at 50-kD, which is the B6H12 antigen (Fig. 10). The two higher apparent molecular mass bands are consistent with the noncovalent heterodimeric structure of an integrin. 2D3 does not compete with B6H12 for binding to IAP (see Fig. 3); thus antibodies against two different epitopes of IAP coprecipitate the high apparent molecular mass bands. To test the hypothesis that these bands are an integrin, platelet antigen isolated on B6H12 was subjected to Western blot with 7G2 (Fig. 11 *A*). 7G2 recognized a 90-kD band in this preparation. A similar experiment performed by isolating IAP on 2D3 Sepharose also revealed a coprecipitating 90-kD band that was not seen when platelet lysates were chromatographed on W6/32 (anti-HLA Class I) Sepharose (Fig.
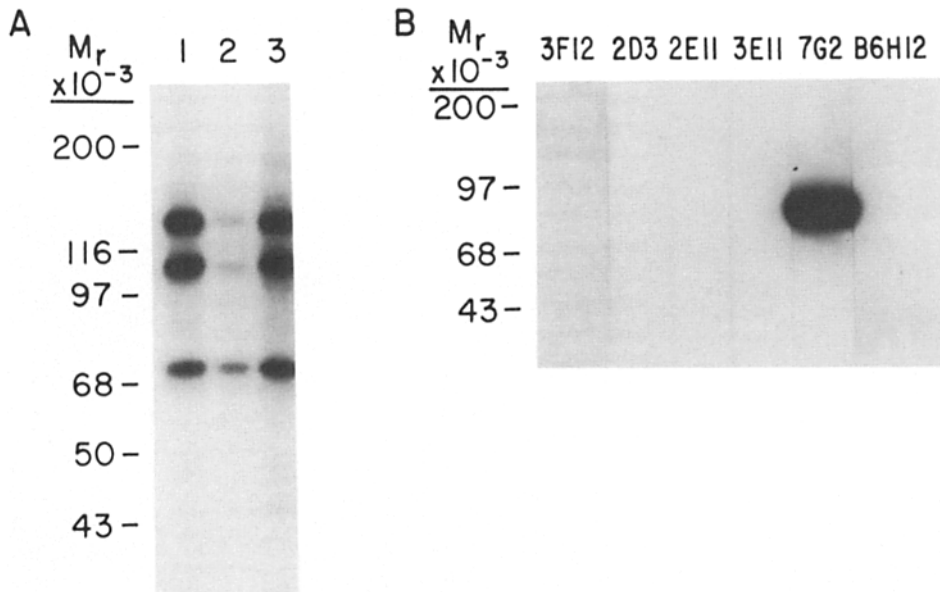
A. 50 Kd PROTEIN

Platelet   Erythrocyte

-200
-116
-97
- 68
- 43

B. V8 DIGEST

Platelet   Erythrocyte
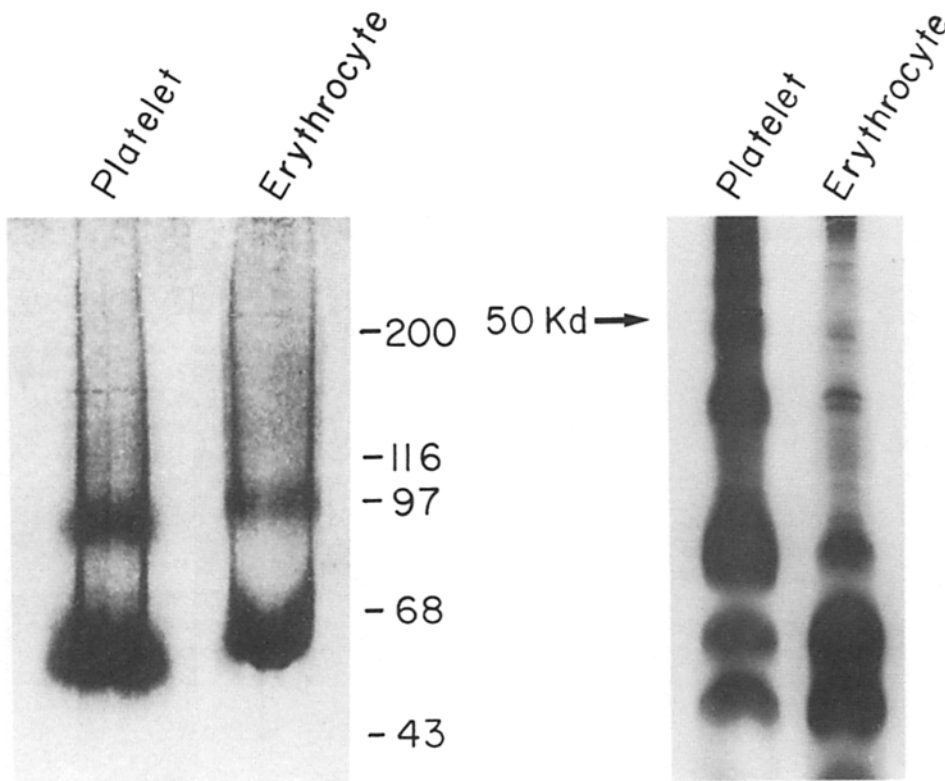
50 Kd→

Figure 8. Purification of 50 kD antigen from platelets and erythrocytes. The 50-kD antigen was purified from platelets and erythrocytes essentially as described for placenta in Fig. 2, and prepared for SDS-PAGE by incubation in sample buffer at 100°C, and electrophoresed on 5–10% gradient gels, followed by silver staining (*A*). Purified antigen was radiolabeled, digested with *Staphylococcus aureus* V8 protease, and run on 15% SDS-PAGE (*B*). Peptides were detected by autoradiography. Purified antigens from platelets and erythrocytes appear similar in relative molecular mass and in proteolytic fragments and are similar to placental antigen in relative molecular mass and potential to aggregate on heating.

11 *B*). Thus, the 90-kD band seen in immunoprecipitations is recognized by 7G2 on Western blots and thus is gpIIIa or an immunologically related protein. The 50-kD band purified on 2D3 Sepharose is recognized by B6H12 (Fig. 11 *B*) and thus is IAP. These data show that β_3 integrins can be copurified and coprecipitated with IAP.

To determine whether this association between IAP and β_3 integrins was a general phenomenon, IAP was partially purified from various cells or tissues by affinity chromatography of octylglucoside lysates on B6H12 Sepharose. The eluted proteins were used in a Western blot with either B6H12 antibody (Fig. 12 *A*) or 7G2 antibody (Fig. 12 *B*).

A

M_r
$\times 10^{-3}$

1 2 3

200 –

116 –
97 –

68 –

50 –

43 –

B

M_r
$\times 10^{-3}$

3F12 2D3 2E11 3E11 7G2 B6H12

200 –

97 –

68 –

43 –

Figure 9. Anti-IAP antibodies do not recognize VnR. (*A*) ^{125}I-labeled placental lysate was precleared by immunoprecipitation with: irrelevant antibody 3D9 anti CR-1 (lane *1*); anti-α_v antibody B3F12 (lane *2*); or anti-IAP B6H12 (lane *3*), as described in Materials and Methods. Supernatants were then immunoprecipitated with 7G2 (anti-β_3). Whereas B3F12 removed most of the 7G2-precipitatable material from placenta, preclearance with B6H12 had no effect on subsequent VnR immunoprecipitation. (*B*) VnR from unlabeled placental lysates was affinity purified with 7G2-Sepharose as described in Materials and Methods. Eluted proteins were then prepared for SDS-PAGE at 60°C, electrophoresed, and Western blotted with 3F12 (*B3F12*), anti-

α_v; 7G2, anti-β_3, and four anti-IAP antibodies, 2D3, 2E11, 3E11, and B6H12. The faint staining of a ∼150-kD α_v band by 3F12, a poor reagent for Western blotting, is barely visible. None of the anti-IAP mAbs recognize VnR by Western blotting.
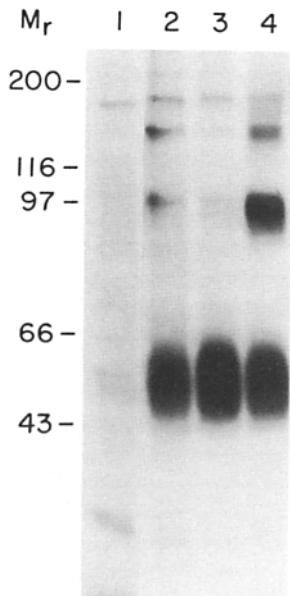
Figure 10. Immunoprecipitation from platelets. Platelets were surface radiolabeled and solubilized in 0.2% Triton X-100-containing buffer. After preclearing with gelatin-Sepharose, the labeled lysates were incubated with: lane 1, anti-phosphorylcholine (HYPC 1643)-Sepharose; lane 2, polyclonal rabbit anti-IAP Sepharose; lane 3, 2D3 (anti-IAP) Sepharose; lane 4, B6H12 (anti-IAP) Sepharose. Specifically immunoprecipitated bands at 90 and 175 kD, in addition to the broad band at 50 kD, are seen in the lanes incubated with anti-IAP antibodies.

B6H12 recognized a 50-kD band from erythrocytes, platelets, and placenta. A weaker band was seen at ∼100 kD, consistent with the dimerization seen on silver stain, since this preparation was incubated at 100°C before SDS-PAGE (see Fig. 2). 7G2, the mAb to β_3, blotted a copurifying 90-kD band from platelets and placenta, but not from erythrocytes. Thus, in two of the three tissues examined, the 50-kD B6H12 antigen was associated with a 90-kD chain that has antigenic similarity to integrin β_3.
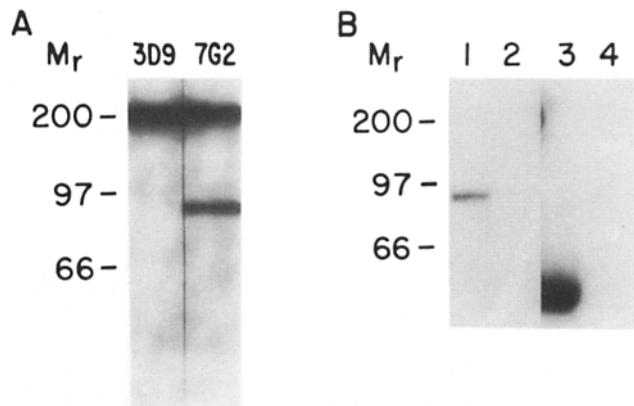
Figure 11. Western blot of platelet proteins binding to anti-IAP affinity columns. Platelets were solubilized in 100 mM octylglucoside and chromatographed on B6H12 Sepharose (A), or 2D3 (anti-IAP) or W6/32 (anti-HLA Class I) Sepharose (B). Eluted proteins were incubated at 60°C and electrophoresed on 7.5% SDS-PAGE. In A, 7G2 (anti-β_3) is shown to recognize a 90-kD band that is not recognized by the irrelevant 3D9 (anti-CR1) antibody. In B, lanes 1 and 3 are Western blots of proteins eluted from 2D3 Sepharose; lanes 2 and 4 are Western blots of proteins eluted from W6/32 Sepharose. Lanes 1 and 2 have been developed with 7G2 (anti-β_3) antibody; lanes 3 and 4 have been developed with B6H12 antibody. 7G2 recognizes a 90-kd band in the 2D3 eluate which is not present in the W6/32 eluate. B6H12 recognizes a 50-kd protein in the 2D3 eluate that is similarly not purified on the anti-HLA class I column.
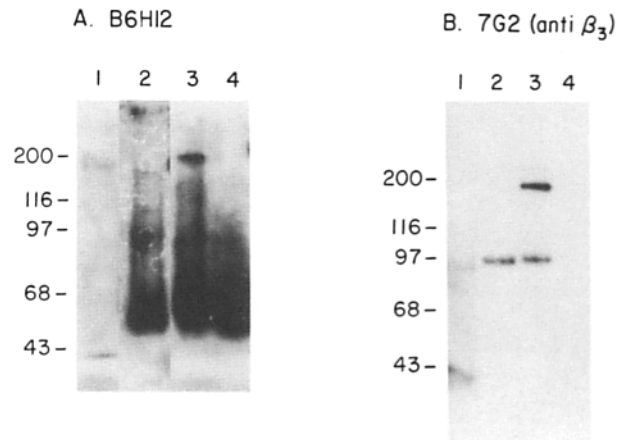

Figure 12. Western blotting of affinity-purified protein from platelets, placenta, and erythrocytes. Detergent-solubilized platelets, placenta, and erythrocytes were incubated with B6H12-Sepharose, and the column washed and eluted as described in Materials and Methods. Platelet lysate was also incubated with Sepharose bearing the anti-MHC class I mAb W6/32 as a control. 2 μg of each eluate was incubated at 100°C with SDS-PAGE sample buffer, electrophoresed on 5-10% gradient gels, and transferred to Immobilon (Millipore Corp., Bedford, MA) membranes. In A, the blots were probed with B6H12 antibody and in B with 7G2 antibody. Lane 1, W6/32 eluate from platelets; lane 2, B6H12 eluate from platelets; lane 3, B6H12 eluate from placenta; lane 4, B6H12 eluate from erythrocytes. A 90-kD band recognized by 7G2 coelutes with IAP from platelets and placenta, but not erythrocytes. The 200-kD band present in lane 3 in A and B is nonspecific, since it can be detected even in the absence of mAb incubation (for comparison, see Figs. 4 and 11 A).

Antibodies to IAP Inhibit LRI-dependent Stimulation of Phagocytosis

To test whether IAP was involved in LRI-dependent stimulation of ingestion, all six new mAb were tested for their ability to inhibit RGD-stimulated phagocytosis by PMN. Three of the antibodies which inhibited B6H12 binding inhibited branched RGD enhancement of EIgG ingestion by PMN; interestingly, one (3G3) did not (Fig. 13). Importantly, one (2E11) of the two antibodies which did not compete with B6H12 also inhibited RGD-dependent phagocytosis; the other (2D3) did not (Fig. 13). The polyclonal antiserum also inhibited RGD-stimulated ingestion. None of these antibodies, including B6H12, affected unstimulated or phorbol ester-stimulated EIgG ingestion by PMN, showing that these antibodies were not generally toxic to the phagocytic process. Thus, antibodies directed against two distinct epitopes of IAP could specifically inhibit RGD-stimulated phagocytosis. This was evidence against the possibility that the effects of B6H12 on phagocytosis enhancement represented an incidental cross-reactivity of the antibody. Thus, RGD-stimulated ingestion by PMN is affected by antibodies to IAP.

Our previous data showed that RGD-dependent phagocytosis was mediated by an integrin, the LRI. Studies with polyclonal antibodies suggested that LRI, like the copurifying integrins from platelets and placenta, was related to the β_3 integrin family (Gresham et al., 1989). To confirm this, we used the anti-β_3 mAb 7G2 which inhibits VnR ($\alpha_v\beta_3$)-dependent ingestion (Savill et al., 1990). Consistent with the polyclonal data, 7G2 inhibited Fn-stimulated ingestion by
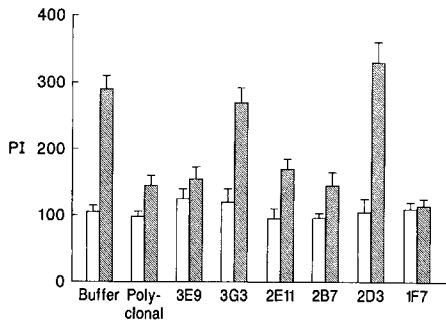
Figure 13. Inhibition of RGD-stimulated phagocytosis by anti-IAP antibodies. Branched RGD peptide was used to stimulate ingestion of EIgG and antibodies were tested for inhibition of stimulation as described (Gresham et al., 1989). 100,000 PMN were incubated with 1:20 dilutions of serum-free tissue culture supernatants or 1.65 μg of purified polyclonal antibody in the presence of 5,000 U of catalase for 15 min at room temperature before addition of EIgG. Unstimulated ingestion is shown in the open bars; stimulated ingestion in the hatched bars. Branched peptide led to a threefold increase in phagocytosis (*buffer columns*). mAb 3E9, 2E11, 2B7, and 1F7, and the polyclonal antibody all prevented the RGD-dependent enhancement of ingestion without affecting unstimulated ingestion. 2D3, which did not compete with B6H12 for binding to IAP (see Fig. 3) and 3G3, which did, affected neither unstimulated or stimulated ingestion. Data are the mean ± SEM of three to four determinations for each antibody.

PMN (Fig. 14). This inhibition was not dependent on recognition of VnR by 7G2, since PMN have no detectable VnR (Gresham et al., 1989). Thus antibodies to both β_3 and IAP inhibit RGD-stimulated ingestion by PMN, suggesting that there is a functional association between LRI and IAP.

To determine whether the same functional association exists on monocytes, we tested the ability of B6H12 antibody to inhibit complement receptor–mediated phagocytosis stimulated by fibronectin. As shown in Fig. 15, both B6H12 and 7G2 inhibited monocyte phagocytosis of C3b opsonized erythrocytes. B3F12, (anti-α_v) which inhibits VnR-dependent ingestion by macrophages (Savill et al., 1990), neither bound to uncultured monocytes (data not shown), nor inhibited fibronectin stimulation of phagocytosis. Thus both B6H12 and 7G2 were able to inhibit fibronectin-stimulated phagocytosis by both PMN and monocytes, mediated by either IgG Fc or complement receptors. These data demonstrate that both LRI and IAP are involved in RGD-dependent stimulation of phagocytosis for PMN and monocytes.

Discussion

Previously, we produced B6H12, an mAb that inhibited RGD-stimulated ingestion by PMN, and presented evidence that the stimulation was dependent on RGD interaction with a cell surface integrin (Gresham et al., 1989). In the current study, we have shown that the antibody also inhibits fibronectin stimulated ingestion by monocytes, suggesting that it recognizes a molecule involved generally in RGD effects on phagocytosis. We have now purified the antigen recognized by B6H12. It is a 50-kD protein, originally purified from placenta, but equivalent to a plasma membrane protein of all hematopoietic cells. This protein appears not to be a proteo-

lytic fragment of an integrin, but an independent molecule which coassociates with specific β_3 or β_3-like integrins on PMN, platelets, and placenta. For this reason, we have called it IAP. Limited sequencing of tryptic peptides from IAP show no relationship to known integrins, and, in fact, show no identity to any sequences in the NBRF/PIR data base. This unexpected finding led us to consider the possibility that the B6H12 antibody crossreacted on intact cells with a plasma membrane integrin which was lost during our purification procedure, or that the 50-kD molecule independently recognized the RGD sequence. However, B6H12 did not immunoprecipitate VnR or recognize it by Western blot. Although we cannot be certain it was functionally active, affinity-purified IAP did not bind to an RGD peptide column. Several monoclonal and polyclonal antibodies against purified IAP stained the plasma membranes of hematopoietic cells, and antibodies against two distinct epitopes inhibited RGD-stimulated ingestion. Initial studies with B6H12 suggested that there might be a physical link between IAP, its antigen, and a β_3 integrin. B6H12 coprecipitated additional higher apparent molecular mass chains with IAP, and the 90-kD band was recognized by the β_3-specific mAb 7G2. Similar studies were performed with 2D3, an mAb prepared against IAP, but that recognized a distinct epitope from B6H12 and which had no effect on RGD-stimulated phagocytosis. Immunoprecipitation of surface radiolabeled platelets with 2D3 coprecipitated similar high apparent molecular mass bands as B6H12, and the 90-kD band was again recognized by 7G2 on Western blot. These data greatly decreased the likelihood that the original observations with B6H12 resulted from a fortuitous crossreactivity, and we have concluded that IAP indeed is involved in RGD-stimulated ingestion. At this point we are not certain of the stoichiometry or the functional significance of the apparent physical association of IAP with β_3 or β_3-like integrins. IAP itself labels very poorly and variably on intact cells with ^{125}I, by a variety of labeling
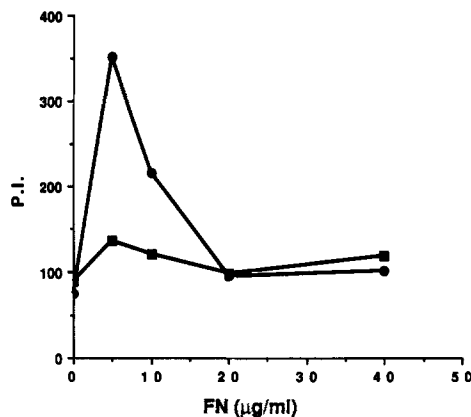
Figure 14. Effect of anti-β_3 mAb 7G2 on Fn-stimulated ingestion by PMN. The effect of Fn on phagocytosis of EIgG by PMN in the presence of 7G2 (■) or the anti-α_v antibody B3F12 (●) as a control was assessed as described in Fig. 13. 100,000 PMN were incubated with 0.4 μg of purified 7G2 or B3F12 in the presence of 5,000 U catalase for 1 h at 4°C before addition of EIgG and warming to 37°C. Fn induced a fourfold increase in phagocytosis in the presence of B3F12. 7G2 almost completely inhibited enhancement. Data are representative of two experiments.
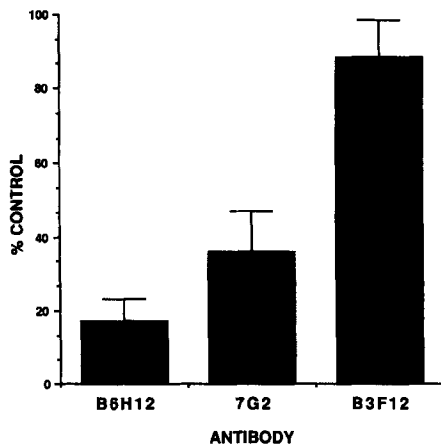
Figure 15. Effect of B6H12 and 7G2 on fibronectin stimulation of monocyte CR1-mediated phagocytosis. Freshly elutriated monocytes (90–98% esterase positive) were adhered to LabTek chambers and incubated with 10 μg/ml mAb or with buffer for 15 min at room temperature before warming to 37°C with addition of EC3b and 40 μg/ml fibronectin. Phagocytosis in antibody-containing wells is compared with buffer as percent control = PI (mAb well)/PI (buffer well). 7G2 and B6H12 both inhibit fibronectin-stimulated, CR1-mediated ingestion, whereas B3F12 (anti-α_v) does not. Data represent mean ± SEM of six experiments.

techniques. This may explain why it was not discerned in our original immunoprecipitations from PMN (Gresham et al., 1989).

IAP does not associate with all integrins. In preliminary work we have shown that B6H12 antibody does not affect β_2-dependent functions on PMN, such as phorbol ester-stimulated phagocytosis (Graham et al., 1989), or PMN adhesion to protein-coated plastic, or VLA dependent adhesion to fibronectin or laminin. We considered the possibility that IAP is a fragment of an α chain that associates with β_3. This hypothesis would require that IAP be part of a new α chain, since neither gpIIb nor α_v is expressed on PMN or uncultured monocytes nor has a known 50-kD cleavage fragment. Moreover, sequence data from tryptic peptides of IAP do not contain gpIIb or α_v sequences. The distribution of the 50-kD antigen does not correspond to that of β_3, since it is present on erythrocytes, a cell type which contains no β_3 integrin. It might be possible that the putative undescribed α chain could associate with two different β chains to explain its broad cell distribution (Cheresh et al., 1989). Again, the strong expression of IAP on erythrocytes is against this hypothesis, since erythrocytes express no known integrin β chains. It is most likely that the antigen recognized by B6H12 is a membrane molecule that is associated with integrin function on some cells, but that is expressed independently of the integrins themselves. At this point, we cannot determine whether the specificity of the association between IAP and LRI is related to the integrin α or β chain. It is possible that IAP interacts with the LRI α or possibly α_v. However, an alternative hypothesis is that the β_3-like molecule associated with IAP is not exactly equivalent to gpIIIa. Two forms of intracytoplasmic tail of β_3 have been identified, generated by alternative splicing of mRNA (van Kuppevelt et al., 1989). If IAP is associated specifically with an integrin β chain, it may be with only one of these or other forms of integrin β cross-reactive with β_3.

What role does IAP play in RGD-stimulated ingestion? One possibility is that is is merely closely associated with the integrin receptor and that mAbs to it inhibit RGD ligand binding by steric blockade; mAbs that do not inhibit stimulated phagocytosis might be direct at epitopes of IAP distant from its site of association with integrins. Against this "passive bystander" hypothesis is the fact that mAb 3G3 binds to an epitope very close to B6H12, since it inhibits binding in the ELISA, but does not affect RGD-stimulated ingestion. Moreover, both B6H12 and 2E11, which inhibit RGD-stimulated ingestion at low concentration, enhance ingestion on their own at higher concentrations (Gresham et al., 1989). Anti-IAP mAbs that do not inhibit ingestion do not have this effect. This would suggest that sufficient antibody binding induces the "activated" state of the integrin. This would be hard to explain by simple steric inhibition of ligand binding and implies that there is a functionally important relationship between IAP and the RGD-binding integrin.

A more intriguing possibility is that IAP is a part of the signal transduction mechanism by which RGD ligands communicate the signal for enhanced ingestion to the cell interior. If IAP selectively associated with only some integrins, its involvement in signal transduction may be associated only with very specific functions. There is now reasonable evidence that at least some integrins transduce signals that alter cell behavior (van Noesel et al., 1988; Gresham et al., 1989; Groux, et al., 1989; Wacholtz et al., 1989; Werb et al., 1989). In many cases, however, this concept has been difficult to investigate, since ligand binding by integrins often leads to cell attachment and spreading on surfaces, an effect that in itself may markedly alter cell responses. Our system is particularly amenable to the study of signal transduction through integrins, since the LRI on phagocytes regulates ingestion even when it is occupied by a soluble ligand and the phagocytes are nonadherent. Thus, signal transduction in this system can be completely separated from any effects of cell adhesion and spreading on two dimensional surfaces. If this is the role for IAP, its signal transducing role is intimately involved with ligand binding, since B6H12 inhibits RGD binding as well as enhanced phagocytosis. This would suggest that antibodies which inhibit RGD-stimulated phagocytosis may do so by stabilizing a conformation of the LRI-IAP complex with decreased affinity for ligand. Sufficient aggregation of receptors in this conformation by high concentrations of antibody might be enough to induce signal transduction for increased phagocytosis. Finally, it is intriguing to speculate that since IAP is expressed on cells independent of integrin expression (e.g., on erythrocytes), it may represent a molecule involved in signal transduction for other receptors as well. The γ chain of the high affinity IgE receptor recently has been shown also to associate with certain IgG receptors and participate in their expression and function (Ra et al., 1989; Hibbs et al., 1989).

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