Integrin-associated Protein Immunoglobulin Domain Is Necessary for Efficient Vitronectin Bead Binding

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Abstract. Integrin-associated protein (IAP/CD47) is physically associated with the αvβ3 vitronectin (Vn) receptor and a functionally and immunologically related integrin on neutrophils (PMN) and monocytes. Anti-IAP antibodies inhibit multiple phagocyte functions, including Arg-Gly-Asp (RGD)-initiated activation of phagocytosis, chemotaxis, and respiratory burst; PMN adhesion to entactin; and PMN transendothelial and transepithelial migration at a step subsequent to tight intercellular adhesion. Anti-IAP antibodies also inhibit binding of Vn-coated particles to many cells expressing αvβ3. However, prior studies with anti-IAP did not directly address IAP function because they could not distinguish between IAP blockade and antibody-induced signaling effects on cells. To better determine the function of IAP, we have characterized and used an IAP-deficient human cell line. Despite expressing αv integrins, these cells do not bind Vn-coated particles unless transfected with IAP expression constructs. Increasing the level of αvβ3 expression or increasing Vn density on the particle does not overcome the requirement for IAP. All known splice variants of IAP restore Vn particle binding equivalently. Indeed, the membrane-anchored IAP Ig variable domain suffices to mediate Vn particle binding in this system, while the multiply membrane-spanning and cytoplasmic domains are dispensable. In all cases, adhesion to a Vn-coated surface and fibronectin particle binding through αvβ1 fibronectin receptors are independent of IAP expression. These data demonstrate that some αv integrin ligand-binding functions are IAP independent, whereas others require IAP, presumably through direct physical interaction between its Ig domain and the integrin.

Integrins are heterodimeric cell surface receptors that mediate binding to other cells and to ligands in the extracellular matrix. Integrin-dependent ligand binding and clustering-induced signal transduction lead to changes in cell morphology and activation state. Conversely, signals inside the cell can result in changes in integrin ligand affinity and avidity. One of the proteins implicated in these regulatory events is integrin-associated protein (IAP).1

IAP is a unique Ig family member with an amino-terminal Ig variable (IgV) domain, a multiply membrane-spanning domain, and a short alternatively spliced carboxy-terminal cytoplasmic tail (Lindberg et al., 1993; Reinhold et al., 1995). IAP is identical to OA-3, an ovarian carcinoma antigen, and to CD47, an erythrocyte membrane protein decreased in Rhnull disease (Campbell et al., 1992; Lindberg et al., 1994; Mawby et al., 1994). Since IAP is ubiquitously expressed (Brown et al., 1990; Rosales et al., 1992; Reinhold et al., 1995), its function has been inferred from the effect of antibody inhibition, mainly in myeloid and endothelial cells.

IAP was first identified as the target of antibodies that block extracellular matrix-induced activation of neutrophils (PMN) and monocytes (Brown et al., 1990). Anti-IAP mAbs also inhibit PMN transendothelial migration, PMN and endothelial cell chemotaxis to RGD-containing proteins, and PMN adhesion to entactin (Cooper et al., 1995; Senior et al., 1992). In addition, anti-IAP mAbs inhibit an increase in intracellular calcium concentration seen when endothelial cells spread on vitronectin (Vn) in an αv-dependent manner (Schwartz et al., 1993). These studies all suggest that IAP interacts functionally with the integrin αvβ3, a hypothesis supported by physical association of the two molecules (Brown et al., 1990). Moreover,
anti-IAP mAb inhibits the binding of Vn-coated particles to αβ integrin on a variety of cells in addition to myeloid and endothelial cells (Lindberg et al., 1993).

Studies on the mechanisms by which IAP cooperates with αβ or by which anti-IAP mAb inhibits various αβ functions have been hampered by the ubiquity of IAP on tissue-culture cells. We have used an IAP-deficient ovarian carcinoma cell line, OV10, to better understand IAP-αβ cooperation. Although adhesion of these cells to a Vn-coated surface is normal, they bind Vn-opsonized beads only if they are made to express IAP by transfection. We show that Vn-bead binding is IAP and integrin dependent and correlates with the level of both integrin and IAP expression. We also demonstrate that the IAP IgV domain is sufficient for this function. As OV10 adhesion to Vn on a surface is unaffected by expression of IAP, our data demonstrate a clear distinction between two different functions of α integrins: binding of Vn-beads, which is IAP dependent, and adhesion to a Vn-coated surface, which is IAP independent.

Materials and Methods

Chemicals, Reagents, and Antibodies

All reagents were of the highest grade commercially available and purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. PBS and HBSS were purchased as 10X stocks from Life Technologies/GIBCO BRL (Gaithersburg, MD) or BioWhittaker, Inc. (Walkersville, MD). Negative control mAb 6F2, anti-αβ mAb 7G2, and anti-IAP mAbs B6H12 and 2D3 are all mouse IgG1 mAbs and have been described previously (Gresham et al., 1989; Brown et al., 1990). None of these mAbs bind the corresponding murine antigens. Anti-α, mAb L230 (Weinacker et al., 1994) was kindly provided by Dr. Dean Sheppard (University of California at San Francisco), and anti-LIBS1 (Fremlinger et al., 1990) was kindly provided by Dr. Mark Ginsberg (The Scripps Research Institute, La Jolla, CA). Control IgG1 mAb MOPC21 was from Organon Teknika (Durham, NC). Anti-αβ mAb 4F10 (IgG1) and anti-αβ, mAb P1F6 (IgG7) (Wayner et al., 1991), as well as rabbit polyclonal anti-human vitronectin receptor, were from Life Technologies/GIBCO BRL. Vn was purchased from Life Technologies/GIBCO BRL and Calbiochem-Novabiochem Corp. (La Jolla, CA). Fluorescinated anti-CD4-IgVt mAb OKT4A was purchased from Ortho Diagnostics (Raritan, NJ).

Tissue Culture

OV1 and OV10 were maintained in Iscove’s modified Dulbecco’s medium (IMDM) (Life Technologies/GIBCO BRL) with 10% FCS (HyClone Laboratories, Logan, UT) and 50 µg/ml gentamicin. Transfectants based on pRC/RSV or pAP58 (Invitrogen, San Diego, CA) (Lindberg et al., 1993) were isolated by limiting dilution 24 h after transfection. Several clones of the vector and IAP-2 transfectants were assayed with similar results. In some cases, clones were FACS® sorted to move populations of lower expression. The clones presented in this study are S8A2 (vector), 71E3 (IAP-1), 45B10 (IAP-2), 131G7 (IAP-3), 120B9 (IAP-4), and 83E7 (mAP).

IAP Expression Constructs and Transfectants

IAP alternative splice variants are referred to as IAP-1 through IAP-4 in order of increasing cytoplasmic tail length (Reinhold et al., 1995). The pRC/RSV-based vector pAP58 and the pAP58-derived expression constructs pAP45 (IAP-2), pAP71 (IAP-1), and pAP83 (mAP, murine IAP-2) have been described previously (Lindberg et al., 1993). The IAP-4 expression construct pAP120 was constructed in analogy with pAP71, except that the 5’-portion of the cDNA was derived from an IAP-4 clone. The 5’-fragment of the IAP-3 expression construct pAP131 was constructed from the IAP-4 version by PCR-mediated deletion of the 3’-end of the coding sequence, to correspond to the published IAP-3 sequence (Lindberg et al., 1993; Campbell et al., 1992; Reinhold et al., 1995). The IAP coding sequence of all constructs has been verified by dideoxynucleotide chain-termination sequencing (Sanger et al., 1977). The constructs were transfected into OV10 using the lipofection method (Life Technologies/GIBCO BRL). Clones were isolated by limiting dilution 24 h after transfection. Several clones of the vector and IAP-2 transfectants were assayed with similar results. In some cases, clones were FACS® sorted to move populations of lower expression. The clones presented in this study are S8A2 (vector), 71E3 (IAP-1), 45B10 (IAP-2), 131G7 (IAP-3), 120B9 (IAP-4), and 83E7 (mAP).

The IAP-glycosylphosphatidylinositol (GPI) construct pAP148 was constructed by cloning the IAP-5’ end up to including the IgV domain as a XbaI-PvuII fragment and a PvuII-HindIII fragment encoding the human decay-accelerating factor (DAF) GPI signal (Medof et al., 1987) (kindly provided by Dr. Douglas Lublin, Washington University, St. Louis, MO) into XbaI-HindIII-digested pAP58 vector. The fragment encoding the GPI signal was generated by PCR using the following oligonucleotides: AACCTCGAGCTCGCATGAAACACCCCA and CAACTGAAGCTTCTAATGAGAG. Conversely, the sequence encoding for the IAP-2 membrane and cytoplasmic domains was isolated from pAP70 (Lindberg et al., 1993) and fused with a PCR-generated HindIII-PvuII fragment of the 5’-end of human CD4 (kindly provided by Dr. Andre Shaw) up to and including the first IgV domain, yielding the plasmid pAP147. The oligonucleotides used were: TGCTTCTAGGCTACTGCTCAGCC and GTCAATCCGCTGCAGCTGAATTGCACCTCCTG.

Selection of an IAP-deficient Cell Line

The ovarian carcinoma line OVM1, kindly provided by Dr. Stimson (Strathclyde University, Glasgow, UK), was described as CD47 deficient by Campbell et al. (1992). The cells we obtained contained a small proportion (0.5-1%) of IAP-positive cells. Therefore, we cloned this cell line and selected numerous IAP-deficient clones from it. After tests of transfectability, the clone OV10 was chosen for further study. OV10 is IAP deficient by FACS® and immunoprecipitation with all our IAP mAbs, negative by immunoprecipitation with polyvalent rabbit anti-IAP carboxy-terminal peptide antisera (Lindberg et al., 1993), and negative for IAP mRNA by Northern blot and reverse-transcriptase PCR assays (not shown). We have been unable to detect spontaneous IAP-positive cells in OV10 populations. OV10 express a small amount of αβ, a moderate amount of αβ, (Table I), and a large amount of αβ (not shown).

Flow Cytometry and Cell Sorting

Cells were removed from subconfluent tissue-culture flasks with PBS-2 mM EDTA, washed twice in complete medium, and counted. 1-3 million cells were resuspended in 25 µl complete medium with 1 µg goat anti-mouse IgG-PITC (F-7856; Sigma Chemical Co.), and then incubated an additional 30 min. After a wash, cells were analyzed on a flow cytometer (Epics-XL; Coulter Electronics, Inc., Hialeah, FL). For cell sorting, 15 million cells were stained with proportionately larger amounts of medium and antibodies resuspended at 5 million cells per ml and sorted on a fluorescein-activated cell sorter (Epics 742; Coulter Electronics, Inc.).
CCACCAAGCTGACTAA from pIAP1 [Lindberg et al., 1993] and cloned into pBSReEN, yielding pIAP323. In both cases, a single round of FACTS sorting yielded a population with high expression in a narrow distribution. These populations were designated IAP-2(hi) (pIAP315) and IAP-TP (pIAP23).

To obtain OV10 transfectants with increased expression of integrin αβ, a β expression plasmid was constructed by ligating the human integrin βc DNA as an Xbal fragment into Nhel-digested pREP11 (Invitrogen). Transfecting this construct into OV10 with or without a 20-fold excess of pCDM8-α (Loftus et al., 1990), and selecting for hygromycin resistance, resulted in equivalent average levels of αβ expression with a corresponding increase in surface expression of α5 (as assayed by FACTS with mAbs AP3 and L230; Table I). This suggests that β chain synthesis limits αβ expression. One clone of pIAP164-transfected OV10 (OV0164-10) was chosen and transfected in a second step with previously described IAP constructs, followed by genetin selection and cloning. The transfected clones used were: βc vector (pIAP164 + pIAP58B3), βc/IAP-2 (pIAP164 + 4582), and βc/IAP-GPI (pIAP164 + pIAP148C2), to yield cells expressing high amounts of αβ without IAP, with wild-type IAP, or with the IAP IgV domain on a GPI anchor, respectively.

Bead Binding
Confluent monolayers of OV10 transfectants were removed with trypsin-EDTA, washed in complete medium, and resuspended at 20,000 cells per ml in RPMI-based selection media (RPMI with 10% FCS, 400 μg/ml G418, and/or 100 μg/ml hygromycin, depending on the transfectant). Cell suspensions were added to glass 8-chamber LabTek slides and cultured for 24 h at 37°C in 5% CO2. Vn-bead binding was optimal in a buffer of HBSS containing 10 mM Hepes, 0.1 mM MnCl2, and 1% human serum albumin (HSA) (bead-binding buffer), which was used for all studies. Adherent cells were washed twice with buffer and incubated with antibodies in 250 μl buffer for 20 min at room temperature. The antibodies used were: anti-IAP mAb 2D3 and B6H12 (Brown et al., 1990), anti-α3 mAb 7G2 (Gresham et al., 1989; Brown et al., 1990), and anti-αv mAb P1F6 (Wayner et al., 1991) all at 10 μg per well, as well as anti-β1 P4C10 (1:50 dilution of ascites). After incubation with the antibodies, the adherent cells were washed twice, and 250 μl buffer was added to each well. Vn- and HSA-coated beads were made exactly as described (Lindberg et al., 1993), except that the source of the Vn was Life Technologies/GIBCO BRL or Calbiochem-Novabiochem Corp. The cells were incubated with either 20 μl Vn- or HSA-coated beads. For βc transfectants in Fig. 5, A and C, the volume of beads was reduced to 5 μl to facilitate counting of attached beads. After 1 h at 37°C, unbound beads were washed away, and the number of beads bound was assessed by fluorescence microscopy. Binding was quantitated as an attachment index, the number of beads bound per 100 cells.

Assay for Ligand-induced Binding Sites
OV10 transfectants were harvested in PBS–2 mM EDTA, washed twice in HBSS without divalent cations, and resuspended to 2 million cells per ml in bead-binding buffer or in bead-binding buffer with 5 mM MgCl2 rather than 0.1 mM MnCl2. Human IgG (500 μg/ml) was used to further reduce nonspecific binding. 100-μl aliquots were incubated 30 min at room temperature with anti-LIBS1 (10 Iz/ml) (Frelinger et al., 1990) and KYAVT-GRGD at varying concentrations. After two washes, cells were stained with 3 μM calcein-AM (Molecular Probes, Eugene, OR). To analyze the structure–function relationships in IAP, we isolated an IAP-deficient clone, OV10, from the ovarian carcinoma cell line OV11 (see Materials and Methods). OV10 cells express large amounts of αβ and α3 (not shown), a moderate amount of αv and β3, and a small amount of β1 (Table I). When assayed for Vn-bead binding, OV10 cells showed attachment indices only marginally above those seen with beads coated with HSA. However, transfectants expressing IAP-2 (Fig. 1), the predominant form of IAP in myeloid cells, showed a markedly enhanced capacity to bind Vn-beads (Fig. 2 A). Binding was increased with increasing Vn density on the bead, but it remained IAP dependent up to saturating Vn concentrations (~12.5 μg/ml). The IAP-mediated enhancement of Vn-bead binding was completely reversed by the functional anti-IAP mAb B6H12 (Fig. 2 A). The IAP dependence was Vn specific, as binding to fibronectin (Fn)–opsonized beads was independent of IAP and unaffected by the presence of anti-IAP mAb B6H12 (Fig. 2 B). Vn-bead binding was entirely dependent on αβ and α3, as shown by its sensitivity to anti-β1 mAb 7G2 (Fig. 2 D) and anti-α3 mAb P1F6 (Fig. 2 C), and was independent of β1 integrins, as shown by resistance to inhibition by anti-β1 mAb P4C10 (Fig. 2 C). In contrast, Fn-bead binding was almost completely inhibited by P4C10, but unaffected by 7G2 (Fig. 2 B). Anti-IAP mAb 2D3 binds IAP with similar affinity as B6H12, but it

Phosphoinositide Phospholipase C Treatment
Confluent monolayers of cells were removed with trypsin-EDTA, washed in complete IMDM with 10 mM EDTA, and resuspended in the same medium to 3 million cells per ml. 0.5-ml aliquots were incubated with or without the addition of 0.1 U of phosphoinositide phospholipase C (PI-PLC) (Boehringer Mannheim Biochemicals) for 1 h at 37°C. Volumes of 0.1 ml were pelleted in microtiter plates, washed, and processed for flow cytometry after staining with negative control mAb 6F2 or anti-IAP mAbs 2D3 or 1F7. Both anti-IAP mAbs gave identical results.

IAP-β3, Comunonprecipitation
Cells were grown to subconfluence, harvested by scraping, and washed three times in HBSS-. 40 million cells ( routinely ~98% intact by trypsin blue exclusion) were lysed in 1 ml 0.25 M sucrose/50 mM Hepes (pH 7.4)/ 100 mM octylglucoside with protease inhibitors (5 mM soybean trypsin inhibitor, 1 mM PMSF, 50 μg/ml aprotinin, 50 μg/ml leupeptin) by rotation for 2 h at 4°C. Lysates were cleared by a 30-min centrifugation (15,000 g) at 4°C. Equal fractions of the lysate were rotated at 4°C with 50 μl anti-IAP (2D3), anti-β3 (7G2), or anti-αv (P1F6) Sepharose. Sepharose was prepared by binding 2 mg mAb to 1 ml cyanogen bromide-activated Sepharose (Pharmacia, Uppsala, Sweden) as recommended by the manufacturer. The affinity Sepharose was washed with 10 mM Tris-HCl (pH 8.0)/0.1% Tween-20 and 150 mM NaCl. Bound protein was eluted by incubation for 10 min at 65°C with nonreducing SDS sample buffer (Laemmli, 1970). Equivalent fractions of the total sample were electrophoresed on 7.5% SDS-PAGE, transferred to Immobilon P (Millipore Corp.), and reacted with anti-β3 mAb 7G2. Detection was by enhanced chemiluminescence according to the manufacturer's recommendations (ECL: Amersham Corp., Arlington Heights, IL).

Results
Vn-Bead Binding Is Deficient in OV10, But Can Be Restored by IAP Transfection
To analyze the structure–function relationships in IAP, we isolated an IAP-deficient clone, OV10, from the ovarian carcinoma cell line OV11 (see Materials and Methods). OV10 cells express large amounts of αβ and α3 (not shown), a moderate amount of αv and β3, and a small amount of β1 (Table I). When assayed for Vn-bead binding, OV10 cells showed attachment indices only marginally above those seen with beads coated with HSA. However, transfectants expressing IAP-2 (Fig. 1), the predominant form of IAP in myeloid cells, showed a markedly enhanced capacity to bind Vn-beads (Fig. 2 A). Binding was increased with increasing Vn density on the bead, but it remained IAP dependent up to saturating Vn concentrations (~12.5 μg/ml). The IAP-mediated enhancement of Vn-bead binding was completely reversed by the functional anti-IAP mAb B6H12 (Fig. 2 A). The IAP dependence was Vn specific, as binding to fibronectin (Fn)–opsonized beads was independent of IAP and unaffected by the presence of anti-IAP mAb B6H12 (Fig. 2 B). Vn-bead binding was entirely dependent on αβ and α3, as shown by its sensitivity to anti-β1 mAb 7G2 (Fig. 2 D) and anti-α3 mAb P1F6 (Fig. 2 C), and was independent of β1 integrins, as shown by resistance to inhibition by anti-β1 mAb P4C10 (Fig. 2 C). In contrast, Fn-bead binding was almost completely inhibited by P4C10, but unaffected by 7G2 (Fig. 2 B). Anti-IAP mAb 2D3 binds IAP with similar affinity as B6H12, but it
Adhesion to a Vn-coated Surface Is IAP Independent

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To determine the relative contributions of αvβ3 and αvβ5, we tested the effect of anti-αvβ3 mAb P1F6 on Vn- and Fn-bead binding. P1F6 completely inhibited Vn-bead binding in a manner similar to anti-β3 mAb 7G2 (Fig. 2 C). Surprisingly, testing multiple preparations from several sources, we consistently found inhibition of Fn-bead binding by P1F6 as well (Fig. 2 B). This suggests that P1F6 ligation of αβ3 may affect the function of multiple OV10 integrins, perhaps through "integrin cross talk" (Blystone et al., 1994, 1995). Thus, our data indicate that expression of IAP specifically enhances Vn-bead binding, but we cannot determine the relative contributions of αvβ3 and αvβ5.

Adhesion to a Vn-coated Surface Is IAP Independent

To investigate the role of IAP in adhesion to a Vn-coated surface, we adhered different transfectants to microtiter plate wells coated with different concentrations of Vn (1 ng/ml to 10 μg/ml) for 60 min, using the same conditions as for the Vn-bead binding. Adhesion was maximal at a coating concentration of 100 ng/ml Vn, and only marginally above background at 3 ng/ml (not shown). Anti-β3 7G2 does not inhibit adhesion in other clearly αvβ3-dependent systems, and it failed to do so here as well. Therefore, anti-αv mAb L230 was used in combination with anti-αvβ3 mAb P1F6 to dissect integrin dependence. At a Vn concentration of 50 ng/ml, binding was inhibited completely by L230 and almost completely by P1F6. Only minimal differences in binding between IAP-positive and IAP-negative cells were seen (Fig. 3), which correlated with small differences in binding between αvβ3-expressing and αvβ3-negative cells (Table I), which correlated with small differences in αvβ3 expression (Table I). OV10 expressing high levels of β3, from transfected β3 CDNA (β3/vector and β3/IAP-2) maintained significant binding in the presence of 10 μg/ml anti-αvβ3 mAb P1F6. This suggests that binding under these conditions was mainly αvβ3 mediated in the low β3 cells (vector and IAP-2) and mediated by both αv integrins in the high β3 expressors. Both in the presence and absence of P1F6, there was no effect of anti-IAP B6H12 even at 150 μg/ml (Fig. 3; not shown). Thus, under these conditions, IAP is uninvolved either in β3, integrin-dependent binding of particles coated with Fn or in adhesion to a Vn-coated surface.

The IAP Cytoplasmic Domain Is Irrelevant for Vn-Bead Binding

IAP exists as four different forms generated by alternative splicing that differ only in their carboxy-terminal cytoplasmic extensions. These forms are called IAP-1, IAP-2, IAP-3, and IAP-4 (Fig. 1), in order of increasing cytoplasmic tail length (Reinhold et al., 1995). While IAP is ubiquitously

| Antibody Antigen | 6F2 (control) | B6H12 | AP3 | P1F6 αβ3 | L230 αv | OKT4A CD4 | W6/32 MHC |
|------------------|-------------|-------|-----|---------|--------|----------|---------|
| Name | Plasmid | Mean fluorescence* |
| --- | --- | --- |
| IAP-2 | plAP164 + 45 | 0.80 ± 0.05 | 3.77 ± 0.13 | 7.01 ± 0.42 | 1.95 ± 0.32 | 12.53 ± 0.41 | ND | 17.73 ± 1.48 |
| IAP-GPI | plAP148 | 0.66 ± 0.03 | 7.64 ± 0.30 | 0.73 ± 0.06 | 2.49 ± 0.40 | 3.57 ± 0.21 | ND | 20.80 ± 4.70 |
| IAP-2 (hi) | plAP315 | 0.62 ± 0.07 | 36.93 ± 0.81 | 0.66 ± 0.05 | 2.32 ± 0.15 | 4.45 ± 0.16 | ND | 20.03 ± 1.47 |
| IAP-CD4 | plAP147 | 0.75 ± 0.00 | 0.68 ± 0.02 | 0.82 ± 0.03 | 2.18 ± 0.07 | 3.92 ± 0.12 | 3.10 ± 0.08 | 27.75 ± 0.39 |
| IAP-GPI | plAP58 | 0.64 ± 0.07 | 1.71 ± 0.19 | 0.66 ± 0.05 | 2.14 ± 0.47 | 3.16 ± 0.28 | ND | 13.77 ± 2.23 |
| IAP-4 | plAP120 | 0.79 ± 0.13 | 9.40 ± 0.99 | 0.77 ± 0.04 | 2.52 ± 0.26 | 4.90 ± 0.82 | ND | 23.50 ± 5.87 |
| IAP-TM | plAP323 | 0.57 ± 0.05 | 11.68 ± 1.42 | 0.64 ± 0.04 | 2.72 ± 0.12 | 3.49 ± 0.12 | ND | 13.43 ± 0.80 |
| β3/vector | plAP164 + 58 | 0.78 ± 0.08 | 0.82 ± 0.09 | 4.18 ± 1.86 | 1.54 ± 0.24 | 10.42 ± 0.36 | ND | 20.80 ± 4.70 |
| β3/IAP-2 | plAP164 + 45 | 0.80 ± 0.05 | 3.77 ± 0.13 | 7.01 ± 0.42 | 1.95 ± 0.32 | 12.53 ± 0.41 | ND | 17.73 ± 1.48 |
| β3/IAP-GPI | plAP164 + 148 | 0.70 ± 0.05 | 6.06 ± 0.31 | 4.29 ± 0.10 | 1.89 ± 0.36 | 7.70 ± 0.28 | ND | 20.03 ± 1.47 |

*Mean and standard error from two to three determinations with saturating amounts of antibody are shown. Multiple assays throughout this study gave similar relative results. In all cases, the cell populations yielded narrow single peaks, except for IAP-2, IAP-GPI, and IAP-2 (hi) where a small population (<10%) of low positive cells could be seen with anti-IAP, and β3/vector where a small population (<10%) of cells could be seen with slightly higher staining with AP3 and L230. The murine IAP-2 expressing clone (mIAP) was not assayed in this experiment. In separate experiments, integrin expression on mIAP was not different from untransfected or vector transfected OV10 (~ and vector, respectively).

†The name given is that used in all of the figures and the text. For the clone name, see Materials and Methods.

‡The plasmid used to transfected the OV10 parental cell line. Where two plasmids are listed, the first plasmid was transfected into OV10, a clone selected, and this clone was then used as a parent of all transfectants harboring this plasmid.

§The IAP-GPI clone shown is 148A10. IAP expression of clone 148C11 is similar, and that of clone 148D12 is ~50% higher (not shown).

MHC, major histocompatibility complex class I antigen.
Figure 1. Predicted structure of IAP variants used. Amino acids are shown in single-letter amino acid code or as lines. Ovals represent membrane-spanning segments, and Ω denotes Ig domains. IAP-derived segments are shown as solid lines, ovals, and letters without underlining, while other segments are shown as broken lines (the CD4 IgV domain), open ovals (the CD7 membrane segment), or underlined letters (sequences derived from CD4, CD7, or created in the fusion). The GPI anchor is shown as a circle connected to the serine residue to which the GPI anchor is linked. Residues shown within parentheses are removed as the GPI anchor is added. IAP-1, IAP-2, IAP-3, and IAP-4 correspond to naturally occurring splice variants of IAP. The expression of the correct tail has been verified for forms to which specific antisera have been raised (IAP-2 and IAP-4). IAP-2 and IAP-2(hi) differ only in the level of expression and the expression vector used. Murine IAP (mIAP) has the murine IAP-2 tail (not shown).

Expressed, the alternative splicing is under strict cell type-specific control, leading to the almost exclusive expression of IAP-2 in myeloid cells and endothelium, and virtually exclusive expression of IAP-4 in neuronal tissue (Reinhold et al., 1995). To determine the role of the IAP cytoplasmic tail in Vn-bead binding, we used transfectants expressing the different splice forms of IAP in the bead binding assay. As shown in Fig. 2, C and D, expression of any of the four forms of IAP in OV10 led to enhancement of Vn-bead binding. This included IAP-1, which has only

Figure 2. Efficient binding of vitronectin-coated but not fibronectin-coated particles is dependent on IAP. (A) OV10 transfected with vector alone (vector) or an IAP-2 expression construct (IAP-2) were assayed for Vn-bead binding, expressed as beads bound per 100 cells (Attachment Index). Before the assay, cells were preincubated for 20 min with the indicated mAbs (40 μg/ml), followed by two washes. Vn-beads prepared in different concentrations of vitronectin were used. Increasing the Vn concentration increased specific binding, but binding remained IAP dependent and B6H12 inhibitible. All binding was sensitive to anti-β1 mAb 7G2 (not shown). (B) The experiment was performed as in A, except that beads were coated with 10 μg/ml fibronectin. IAP expression, anti-IAP mAbs B6H12, and anti-β1 mAb 7G2 were all without effect (40 μg/ml), whereas anti-β1 mAb P4C10 (1:50 dilution of ascites) inhibited binding. Surprisingly, anti-αβ6 mAb P1F6 (40 μg/ml) inhibited binding to an extent comparable to anti-β1 mAb P4C10 (see text). (C) OV10 transfected with vector alone (vector) or constructs encoding IAP alternative splice forms with successively longer cytoplasmic tails (IAP-1, IAP-2, IAP-3) were grown in Labtek chambers, and binding of Vn- or HSA-coated latex beads was assayed as in A. All three forms of IAP conferred the Vn-bead binding phenotype, which is sensitive to inhibition by anti-IAP mAb B6H12. Vn-bead binding was inhibited by anti-αβ6 mAb P1F6, whereas anti-β1 mAb P4C10 was without effect. Data are mean +SEM of three experiments performed under identical conditions. (D) Transfectants expressing the longest form of IAP (IAP-4) showed enhanced bead binding inhibited by anti-IAP mAb B6H12, whereas the nonfunctional anti-IAP mAb 2D3 had no effect. In all cases, Vn-bead binding was completely inhibited by anti-β1 mAb 7G2. (E) OV10 transfected with vector alone, or constructs encoding human IAP-2 (IAP-2) or murine IAP-2 (mIAP) were assayed for Vn-bead binding. Both human and murine IAP mediated the enhancement of Vn-bead binding. Anti-IAP mAb B6H12 does not bind murine IAP. While it inhibited Vn-bead binding mediated by human IAP, it had no effect on binding mediated by murine IAP.
Vn adhesion is IAP independent. OV10 overexpressing IAP-2 and β2/β2/IAP-2, IAP-2 alone (IAP-2), β2 alone (β2/vector), or neither (vector) were assayed for adhesion to microtiter plate wells coated with Vn (50 ng/ml) in the presence or absence of various mAbs (B6H12 and 2D3 at 150 μg/ml, L230 and P1F6 at 40 μg/ml). Maximal adhesion was 70–80%, and background was 0–2%. Means ± SEM for triplicate wells from a representative experiment are shown. Experiments varying the Vn-coating concentration (1 ng/ml to 10 μg/ml) or incubation time (15–120 min), or the anti-IAP concentration (10 μg/ml to 150 μg/ml), failed to show any effect of IAP on the level of adhesion.

The IAP IgV Domain on a GPI Anchor Is Sufficient to Mediate the IAP Effect on Vn-Bead Binding

We next sought to determine the roles for the IAP extracellular and multiply membrane-spanning domains in Vn-bead binding. For this purpose, we made a construct encoding the IAP IgV domain fused to the human DAF GPI anchor signal (IAP-GPI), as well as the “inverse” construct encoding the human CD4 IgV1 domain fused to the IAP-2 membrane and cytoplasmic domains (CD4-IAP) (Fig. 1). Clones of the corresponding transfectants expressed the recombinant proteins (Table I). Surprisingly, IAP-GPI enhanced Vn-bead binding, while CD4-IAP failed do so (Fig. 4 A). To confirm this unexpected finding, two additional transfectant clones of IAP-GPI were tested

Figure 4. The IAP IgV domain is sufficient to mediate vitronectin bead binding. (A) Vitronectin- and HSA-coated latex particle binding were assayed as in Fig. 2. OV10 cells were transfected with vector alone (vector), wild-type IAP (IAP-2), a construct encoding the IAP IgV domain linked to a DAF GPI signal (IAP-GPI), or the CD4-IgV1 domain linked to the IAP-2 membrane and cytoplasmic domain (CD4-IAP). Surprisingly, IAP-GPI mediated Vn-bead binding, suggesting that the IAP IgV domain is sufficient to complement the bead binding phenotype, and that the membrane and cytoplasmic domains are dispensable for this function. Data are mean ± SEM of three experiments performed under identical conditions. (B) Another two independent IAP-GPI transfectants (148D12 and 148C11) were compared to IAP-2 and the original IAP-GPI clone (IAP-GPI) with similar results (representative of two experiments). (C) OV10 clones expressing IAP-GPI (IAP-GPI, 148C11, 148D12) and a control clone expressing IAP-2 were trypsinized and incubated for 1 h at 37°C in complete medium with 2 mM EDTA with or without 0. 2 U/ml PI-PLC, as described in Materials and Methods. After the digestion, cells were washed and stained with anti-IAP mAb 2D3 (no PI-PLC and 2 U/ml PI-PLC) followed by FITC-goat anti-mouse IgG and analysis by flow cytometry. As a negative control, untreated cells were stained with mAb 6F2 (control mAb). PI-PLC-treated cells gave identical reactivity with the control mAb and are therefore not shown. The PI-PLC treatment completely removed surface IAP reactivity from the IAP-GPI clones, whereas it had no effect on wild-type IAP, showing that the clones indeed expressed the expected IAP form (representative of two experiments). Data obtained using anti-IAP mAb 1F7 were identical (not shown).
for Vn-bead binding, yielding similar results (Fig. 4 B).
Furthermore, we confirmed that IAP–GPI was in fact GPI
linked by demonstrating that essentially all anti-IAP reac-
tivity could be removed from the surface of IAP–GPI by
PI-PLC, whereas expression of wild-type IAP-2 was unaf-
fected by this enzyme (Fig. 4 C). Thus, the GPI-anchored
IAP IgV domain is sufficient for the restoration of Vn-
bead binding to IAP-deficient cells.

The IAP IgV Domain Functions Irrespective of the
Nature of the Domain Anchoring It to the Membrane

GPI-anchored proteins exist in specialized membrane do-
 mains in which they are associated with intracellular src ki-
nases (Brown, 1993). Cross-linking of several GPI-linked
proteins leads to kinase activation (Brown, 1993). It is
therefore theoretically possible that the IAP multiply
membrane-spanning domain functions in a manner that
can be duplicated by these special properties of the GPI
anchor. To exclude this possibility, we generated cells ex-
pressing the IAP-IgV domain linked to the single trans-
membrane segment of human CD7 (IAP–TM) (Fig. 1). As
shown in Fig. 5 A, this construct enhanced Vn-bead bind-
ing as well as IAP-2 and IAP–GPI, showing that the mem-
brane-anchored IAP-IgV domain suffices for this pheno-
type, irrespective of the exact nature of the membrane
anchor.

Increases in Either IAP or αvβ3 Expression Lead to
Increased Vn-Bead Binding

We observed that the extent of Vn-bead binding corre-
lated with the level of IAP expression regardless of splice
form. This suggested that IAP expression was limiting to
Vn-bead binding. To test this hypothesis, we made a con-
struct encoding IAP-2 using a more efficient promoter
(see Materials and Methods). A transfectant of this con-
struct (IAP-2[hi]) expressed IAP at significantly higher
levels than the control clone (IAP-2) (Table I) and bound
Vn-beads at a significantly higher level than the IAP-2
construct (attachment index of 373 vs 181) (Fig. 5 B).
Thus, the extent of bead binding correlated with IAP ex-
pression.

It was surprising that Vn-bead binding was inhibited by
anti-β3 mAb 7G2, even though OV10 expresses only mini-
mal levels of integrin αvβ3 (Table I). To investigate this
further, and to test the hypothesis that IAP dependence
could be overcome by increased surface expression of the
integrin, we derived a series of clones with high αvβ3 ex-
pression by transfection with β3-encoding constructs (Ma-
terials and Methods). Even with high expression of the
αvβ3, Vn-bead binding was markedly enhanced by IAP
(Fig. 5 C). Clones overexpressing both the integrin and
IAP (β3/IAP-2) bound Vn-beads to a much greater extent
than even the IAP-2(hi) clone (Fig. 5 B), although they
had significantly lower IAP surface expression than IAP-
2(hi) (3.77 ± 0.13 vs 36.93 ± 0.81; see Table I). Thus, both
αvβ3 expression and IAP expression are limiting for Vn-
bead binding by OV10. In the αvβ3-overexpressing clones,
the membrane-anchored IAP IgV domain alone was still
sufficient to restore Vn-bead binding, since expression of
IAP–GPI was as efficient as IAP-2 (Fig. 5 C); lower attach-
ment indices in this experiment reflect the lower bead con-
centrations used in this experiment). As with the original
OV10 IAP transfectants, the enhancement of Vn-bead
binding in the αvβ3-overexpressing cells was completely
reversed by anti-IAP mAb B6H12 and completely inhib-
ited by anti-β3 mAb 7G2. Despite overexpression of αvβ3,
anti-α5 mAb P1F6 also completely inhibited Vn-bead binding (Fig. 6).

**Induction of β3 Ligand–induced Binding Sites Is Not Influenced by IAP**

To determine if IAP alters the affinity state of αβ3, we determined the generation of the ligand-induced β3 LIBS1 epitope by increasing concentration of peptide KYAVT-GRGDS on OV10 expressing high amounts of αβ3 with or without IAP-2 (see Materials and Methods). Over a range of 1–1,000 μM ligand, there was clear induction of the LIBS1 epitope, with a higher ligand sensitivity in 0.1 mM MnCl2 than in 5 mM MgCl2. However, we observed no difference between IAP-positive and IAP-negative cells in these experiments (not shown).

**Comimmunoprecipitation of β3 and IAP**

The dependence of Vn-bead binding on the plasma membrane concentration of both IAP and αβ3 suggested that the relevant binding structure contained both molecules, as has been shown previously (Brown et al., 1990; Zhou and Brown, 1993). Thus, we examined whether the two proteins could be coprecipitated in transfected OV10 cells. When either IAP-2 or IAP–GPI was immunoprecipitated, coprecipitated β3 could be detected by Western blotting (Fig. 7), showing that the αβ3 association can be mediated by the IAP IgV domain alone. No β3 was coprecipitated with αβ5. Coprecipitated β3 was a small minority of total β3, suggesting that the majority of both IAP and αβ3 was not in a stable complex.

**Discussion**

Antibody to integrin-associated protein (IAP/CD47) has been shown to affect a large number of cell functions associated with αβ3 and αβ3-like integrins, such as Vn-bead binding, RGD activation of Fc-receptor–mediated phagocytosis, chemotaxis, and oxidative burst, as well as PMN migration through epithelial or endothelial barriers (Parks et al., 1991, 1996; Cooper et al., 1995; Gresham et al., 1992; Senior et al., 1992). Because of the ubiquity of IAP expression, it has been difficult to determine if the effects of anti-IAP mAb were to inhibit IAP function, or if mAb binding to IAP generated a negative signal that then inhibited function. Indeed, the transfection of human IAP into CHO cells expressing hamster IAP made Vn-bead binding sensitive to the anti-human IAP mAb B6H12, raising the possibility that the antibody generated a signal that specifically regulated αv integrin function (Lindberg et al., 1993). In this paper, we have directly tested whether or not IAP is necessary for Vn-bead binding by αv integrins. We have shown that a cell line lacking IAP fails to bind Vn-beads and that expression of IAP on these cells confers the ability to bind Vn-coated particles. Increased integrin expression led to increased binding, but failed to obviate the IAP requirement. Thus, the phenotype of IAP-deficient cells is similar to that of anti-IAP–treated, IAP-expressing cells. The requirement for IAP does not reflect direct binding of Vn-beads by IAP, since in all cases it is inhibited completely by anti-β3 and αβ3 mAbs. Expression of IAP does not influence β3 integrin bead binding, since αβ3 integrin–mediated binding of Fn-beads is unaffected by the absence of IAP.

In requiring IAP, Vn-bead binding differs from adhesion to a Vn-coated surface, which is IAP independent. Similarly, αv-dependent spreading of endothelial cells on Vn is IAP independent, whereas the concomitant rise in intracellular calcium concentration can be inhibited by
anti-IAP mAbs (Schwartz et al., 1993). A difference in Fn-bead binding and adhesion to a Fn-coated surface has been described for K562 cells, which was attributed to the requirement for a high affinity \( \alpha_v\beta_3 \) in Fn-bead binding (Blystone et al., 1994). Possibly, IAP is necessary for a higher avidity of \( \alpha_v\beta_3 \), which might be required for Vn-bead binding. Whether this reflects a dependence on IAP for generation of high affinity \( \alpha_v\beta_3 \) or another mechanism for creating increased avidity, such as receptor clustering or altered receptor interaction with the cytoskeleton, is unclear. However, while RGD peptide induced the activation-independent LIBS1 epitope on \( \alpha_v\beta_5\)-overexpressing OV10, this was independent of IAP expression. Whatever the mechanism, these studies clearly demonstrate an important distinction between bead binding and adhesion. Thus, studies using bead binding as a model for adhesion to a surface (Miyamoto et al., 1995a,b) need to be interpreted with some caution.

The extent of Vn-bead binding is dependent on the levels of both IAP and \( \alpha_v\beta_3 \) expression, but even increasing \( \alpha_v\beta_3 \) expression 10-fold did not make bead binding IAP independent. Previous work in K562 cells has shown that Vn-bead binding also requires expression of \( \alpha_v \) integrins (Blystone et al., 1994). Since Vn-bead binding is sensitive to the concentrations of both IAP and \( \alpha_v\beta_3 \) in the cell membrane, it is likely that bead binding requires a physical complex between IAP and the integrin. As previously shown for platelets (Brown et al., 1990), integrin \( \beta_3 \) coimmunoprecipitates with IAP in OV10 transfected. However, under the conditions of the assay, most IAP on the cell surface is not associated with \( \beta_3 \) integrin, and most \( \alpha_v\beta_3 \) is not associated with IAP. Using the mild detergent conditions required to show coprecipitation, IAP is relatively poorly solubilized, whereas harsher conditions disrupt IAP-integrin association. It is clear, since IAP can be expressed on cells in the absence of \( \alpha_v \) integrins (Blystone et al., 1994), and \( \alpha_v \) integrins can be expressed without IAP in OV10, that transport to the plasma membrane does not require complex formation between these proteins. Instead, the complex probably forms on the plasma membrane, presumably in equilibrium with free IAP and \( \alpha_v\beta_3 \). The complex has ligand-binding properties distinct from free \( \alpha_v\beta_3 \). A simple explanation for the requirement of IAP in \( \alpha_v \) integrin bead binding would be that IAP is necessary for generation of the high affinity form of the integrin. However, we found no evidence for any IAP-dependent change in ligand affinity, using the display of the LIBS1 epitope as assay. This does not rule out the possibility that a small but functionally important subset of \( \alpha_v \) integrin molecules are affinity regulated by IAP, but it is more likely that IAP acts in some other way to alter the function of \( \alpha_v \) integrins. It will be interesting and important to determine which of the integrin’s functions in migration, neovascularization, metastasis, etc. (Leavesley et al., 1992, 1993; Brooks et al., 1994; Filardo et al., 1995) are actually dependent on its association with IAP.

OV10 cells express much more \( \alpha_v\beta_3 \) than \( \alpha_v\beta_5 \) (Table 1). Since Vn-bead binding is dependent on IAP, and on K562 cells (which express IAP), \( \alpha_v\beta_3 \) can bind Vn-beads in the absence of \( \alpha_v\beta_5 \) (Blystone et al., 1994), it is very likely that \( \alpha_v\beta_3 \) also interacts functionally with IAP. We could not coimmunoprecipitate IAP with \( \alpha_v\beta_5 \), but this may reflect only a slightly lower interaction between the two molecules in detergent solution than between IAP and \( \alpha_v\beta_3 \). Both anti-\( \beta_3 \) mAb 7G2 and anti-\( \alpha_v\beta_3 \) mAb P1F6 completely inhibit Vn-bead binding regardless of the relative amounts of \( \alpha_v\beta_3 \) and \( \alpha_v\beta_5 \). Furthermore, P1F6 but not 7G2 blocked Fn-bead binding. In contrast, P1F6 had no effect on adhesion to Vn (Fig. 3) or Fn (not shown). While the basis of these effects of \( \alpha_v \) integrin antibodies on bead binding is unknown, they may reflect interintegrin signaling (cross talk) (Blystone et al., 1994, 1995). Alternatively, inhibition may reflect clustering of several integrins at sites of cell–bead contact, with resulting steric inhibition of binding by either mAb. However, IAP would reasonably be expected to be present in such an aggregate, and a purely steric inhibition is hard to reconcile with the lack of effect of the noninhibitory anti-IAP mAb 2D3.

The absence of IAP in OV10 cells allowed the first structure–function studies on IAP. These studies demonstrated that the amino-terminal IgV domain was necessary and sufficient for Vn-bead binding. The importance of the IgV domain of IAP for interaction with \( \alpha_v \) integrins is intriguing because interactions between Ig family members and integrins are well-described mechanisms for cell–cell interaction in the immune system. ICAM-1 interaction with \( \beta_2 \) integrins (Diamond et al., 1991; Staunton et al., 1990), PECAM-1 with \( \alpha_v\beta_3 \) (Piali et al., 1995; Buckley et al., 1996), VCAM-1 with \( \alpha_v\beta_1 \) (Elices et al., 1990), and MadCAM with \( \alpha_v\beta_1 \) (Berlin et al., 1993; Briskin et al., 1996) all have roles in transendothelial migration of leukocytes. In contrast to these cell–cell adhesive interactions, the IAP-integrin interaction must involve an IgV domain on the same cell as the integrin. IAP interaction with \( \alpha_v \) integrins would represent an extension of the paradigm of Ig family–integrin binding to interactions between molecules on a single plasma membrane.

The cell type–specific expression of different cytoplasmic tail forms of IAP would suggest that this part of the molecule has a specific functional role. Also, the anti-IAP inhibitable inward calcium flux in endothelial cells spreading on Vn (Schwartz et al., 1993) makes the IAP multiply membrane-spanning domain a potential candidate for a channel. Surprisingly, both these parts of the IAP molecule are dispensable for Vn-bead binding in our transfection system. It is theoretically possible that a mutated form of IAP is expressed by OV10 and is able to interact with IAP-GPI and IAP–TM, reconstituting a functional but noncovalently associated IAP molecule. However, we have been unable to detect in OV10 any IAP message by reverse transcriptase–PCR, any IAP-2 or IAP-4 cytoplasmic tail by immunoblot or immunoprecipitation with specific antisera, or any IAP Ig immunoglobulin domain with monoclonal and polyclonal anti-IAP antibodies by immunoprecipitation or FACS® (not shown). This makes the existence of any mutant IAP protein in OV10 highly unlikely. More likely, while interaction with \( \alpha_v \) integrins is mediated by the IgV domain, other, as yet undefined functions of IAP require these other domains.

Recently, IAP has been shown to be the receptor for the carboxy-terminal cell-binding domain of thrombospondin-1 (TS-1) (Gao et al., 1996). TS-1 is a 450-kD trimeric matrix protein that, in addition to the cell-binding domain, has several other cell- and matrix-interaction domains, includ-
ing a RGD sequence capable of ligating αvβ3. This suggests the possibility that the Vn used may contain small amounts of TS-1, and that this contaminant is responsible for the IAP effect on Vn-bead binding. However, binding of IAP-αβ complex represents an extension of the integrin-Ig family adhesive interactions to molecules on a single plasma membrane for the purpose of modulating integrin function. The structural details and biological consequences of this modulation, as well as the functions of the more complex membrane-spanning and cytoplasmic domains of IAP, remain to be determined.

We thank Matthew Williams and Alexander Zheleznyak for excellent technical assistance, and Dr. Scott Blystone for critically reviewing this manuscript.

This research was funded grant GM38330 from the National Institutes of Health (to E.J. Brown), a Howard Hughes Physici-Scienct fellow-ship (to F.P. Lindberg), an Arthritis Investigator Award (to F.P. Lindberg), the Monsanto-Washington University Agreement (to E.J. Brown), and the Medical Research Service of the Department of Veterans Affairs (to H.D. Gresham). The Journal of Cell Biology, Volume 134, 1996

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Received for publication 17 May 1996 and in revised form 2 July 1996.

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