Costimulation of T Cell Activation by Integrin-associated Protein (CD47) Is an Adhesion-dependent, CD28-independent Signaling Pathway

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Summary

The integrin-associated protein (IAP, CD47) is a 50-kD plasma membrane protein with a single extracellular immunoglobulin variable (IgV)-like domain, a multiply membrane-spanning segment, and alternatively spliced short cytoplasmic tails. On neutrophils, IAP has been shown to function in a signaling complex with β_3 integrins. However, the function of IAP on T cells, which express little or no β_3 integrin, is not yet defined. Here, we show that mAbs recognizing IAP can enhance proliferation of primary human T cells in the presence of low levels of anti-CD3, but have no effect on T cell proliferation on their own. Together with suboptimal concentrations of anti-CD3, engagement of IAP also enhances IL-2 production in Jurkat cells, an apparently integrin-independent function of IAP. Nonetheless, costimulation by IAP ligation requires cell adhesion. IAP costimulation does not require CD28. Furthermore, anti-IAP, but not anti-CD28, synergizes with suboptimal anti-CD3 to enhance tyrosine phosphorylation of the CD3 ζ chain and the T cell–specific tyrosine kinase Zap70. Ligation of human IAP transfected into the hemoglobin-specific 3.L2 murine T cell hybridoma costimulates activation for IL-2 secretion both with anti-CD3 and with antigenic peptides on antigen-presenting cells (APCs). Moreover, ligation of IAP but not CD28 can convert antagonist peptides into agonists in 3.L2 cells. Using costimulation by IAP ligation as an assay to analyze the structure–function relationships in IAP signaling, we find that both the extracellular and multiply membrane-spanning domains of IAP are necessary for synergy with the antigen receptor, but the alternatively spliced cytoplasmic tails are not. These data demonstrate that IAP ligation initiates an adhesion-dependent costimulatory pathway distinct from CD28. We hypothesize that anti-IAP generates the costimulatory signal because it modulates interactions of the IgV domain with other plasma membrane molecules; this in turn activates effector functions of the multiply membrane-spanning domain of IAP. This model may have general significance for how IAP functions in cell activation.

Integrin-associated protein (IAP)[1] is a 50-kD highly hydrophobic cell surface glycoprotein that was originally copurified with the $\alpha_v\beta_3$ vitronectin receptor from placenta (1) and later shown to be the antigen recognized by CD47-specific mAb (2). Abs that recognize IAP inhibit some β_3 integrin-mediated functions, including binding of vitronectin coated beads to cells, PMN activation by and chemotaxis to Arg–Gly–Asp (RGD)-containing ligands, and endothelial $[Ca^{2+}]_i$ increase during adhesion to fibronectin or vitronectin (3, 4). IAP has a broader cellular distribution than β_3 integrins, suggesting that it may have functions other than those associated with β_3. Recently, IAP has been shown to have a role in PMN migration across both endothelial and epithelial barriers (5, 6) and to bind the large multifunctional glycoprotein thrombospondin (7), all functions without an obvious role for $\alpha_v\beta_3$. Molecular cloning of IAP cDNAs from mouse and human revealed that it is an unusual Ig family member, with an Ig variable (IgV)-like amino terminal extracellular domain, a domain containing multiple membrane spanning segments, and a short cytoplasmic tail (CT) with four alternatively spliced forms (8, 9). This three-domain structure raises the possibility that each domain plays a discrete role in IAP function, but nothing is known about structure–function relationships of

[1]*Abbreviations used in this paper:* CT, cytoplasmic tail; IAP, integrin-associated protein; IgV, immunoglobulin variable.

IAP. The ubiquity of IAP expression on continuous cell lines has hampered a systematic approach to this question.

Although IAP is highly expressed on peripheral T lymphocytes, which express little if any $\alpha_v\beta_3$, its function in these cells is not known. A potential role in T cell costimulation has been suggested by recent experiments (10). Whereas definitions of costimulation vary, in this work we have defined a costimulatory molecule as one that enhances T cell activation in response to a suboptimal antigen receptor–initiated signal. Studies using mAbs directed against potential receptors for the costimulatory signal have identified more than 20 different T cell surface receptors, including multiple adhesion molecules, which can augment lymphocyte mitogenesis initiated by TCR engagement (11). The costimulatory receptors not only strengthen the adhesion between the antigen-responsive T cell and the APC, but also deliver crucial costimulatory signals to facilitate cytokine production and clonal expansion (12–14). Although CD28 is the most intensively studied costimulatory receptor, even in this case, the specific molecular events induced by ligation of CD28 required for costimulation remain uncertain.

Although they retain the ability to bind to the MHC of the APC, antigenic peptides with mutations in the amino acids that interact with the TCR, may be unable to activate fully antigen-specific T cells (15, 16). These peptides, called altered peptide ligands (APLs) can act as antagonists of T cell activation by wild-type peptides even without competing for MHC binding. How engagement of costimulatory molecules affects T cell signals resulting from recognition of APLs is unknown.

In the present study, we confirm that IAP can function as a costimulator in T cell activation in both human and mouse T cells and show that the costimulatory activity of IAP is distinct from that of the well-defined costimulator CD28. Furthermore, IAP ligation can convert antagonist APL peptides into full T cell agonists, a property not shared by CD28. Finally, using IAP costimulation as an assay to understand structure–function relationships in IAP signaling, we find that the extracellular and multiply membrane-spanning domains of IAP are required for synergy with the TCR, but the alternatively spliced cytoplasmic tails are not. These studies demonstrate that T cell IAP may have important immunomodulatory effects by a mechanism distinct from that initiated by CD28 ligation.

Materials and Methods

Cell Culture. Jurkat (provided by Dr. M. Thomas, Washington University School of Medicine, St. Louis, MO) and 3.L2 cells (17) were maintained in culture in RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine, 0.1 mM NEAA, 50 mM 2-ME, 0.1% gentamicin. Transfected Jurkat and 3.L2 clones were maintained in the same media in the presence of 2 mg/ml or 1.5 mg/ml Geneticin (GIBCO BRL, Gaithersburg, MD), respectively. Jurkat and 3.L2 cells were cloned by limiting dilution.

DNA Constructs. Standard techniques were used for nucleic acid manipulations. PCR was performed to amplify the human IAP transmembrane domain plus cytoplasmic tail and the cytoplasmic tail alone from human IAP form 2 in pBS (pIAP3) (8). The IAP transmembrane plus cytoplasmic tail segment was obtained using primers containing NheI and XbaI–SacI cloning sites (sense oligonucleotide, 5′-GTTTCATGGGCTAGCCCAAATGAAAATATTCTT-3′; anti-sense oligonucleotide, 5′-ATCGAGCTCATGGTTCTAGAACACAAGTGT-3′). The IAP cytoplasmic tail segment was obtained using primers containing SalI and XbaI–SacI cloning sites (sense oligonucleotide, 5′-TTACTTGGACTAGGTCGACTGAAATTTGTG-3′; anti-sense oligonucleotide as above; cloning sites are underlined). These fragments were digested with NheI and SacI or SalI and SacI, respectively. The cut fragments were ligated into a CD8-expressing plasmid, CD8-8-45 in pBS (18), using the SpeI and SacI or SalI and SacI sites. These chimeric cDNAs were cloned into the expression vector BSRαEN (gift of Dr. A. Shaw, Washington University School of Medicine, St. Louis, MO) using the XhoI and XbaI sites. This generated two cDNAs encoding chimeric proteins. One encodes the extracellular Ig domain of CD8 and the multiply membrane-spanning and cytoplasmic domains of IAP form 2 (CD8MC2, Fig. 1). The other encodes a chimera of the extracellular and transmembrane domain of CD8, and the cytoplasmic tail of IAP form 2 (CD8C2; Fig. 1). The CD8-8-* (Fig. 1) construct was generated using the following primers, 5′-CGATTAATCTAGAGAGCT-3′ and 5′-CTCTAGATTAAT-3′, which generate, upon annealing, a stop codon immediately followed by an XbaI site (underlined) plus ClaI and SacI overhangs (double underlined). Upon annealing, this fragment was ligated into ClaI and SacI cut CD8-8-45 in pBS, and the Xho and Xba fragment was then subcloned into BSRαEN.

To generate the IAP–CD7 construct (Fig. 1), CD16–CD7–syk (19) was used as a template for PCR amplification of the CD7

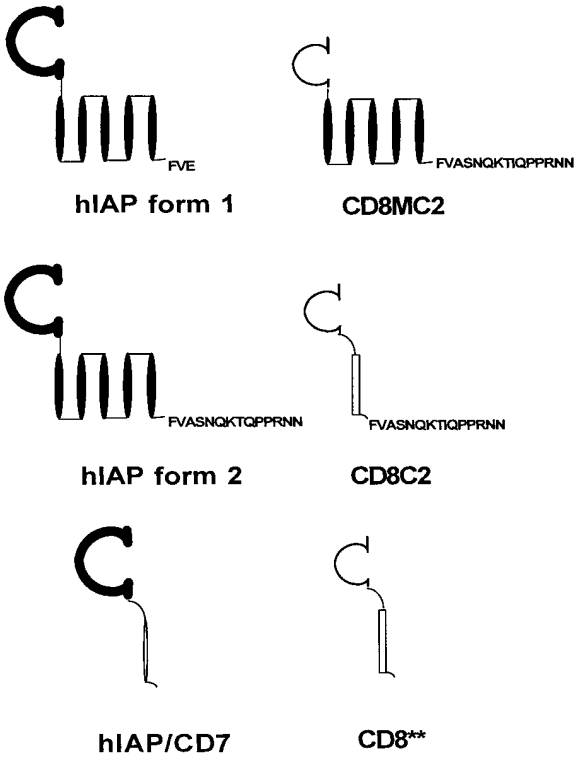

Figure 1. Schematic representation of the native and chimeric molecules used in this study. Generation of the individual chimeras is described in Materials and Methods.

transmembrane domain using primers that contain BamHI and XbaI cloning sites (sense oligonucleotide, 5′-CCTGGGGCGGATCCACCAAGGGCCTCTGCC-3′, anti-sense oligonucleotide, 5′-ACTGTCTGCCATCTAGAGCGTCCTCGCCAG-3′; cloning sites are underlined). The extracellular domain of IAP was generated as a XhoI and BamHI fragment from pIAP 419 (unpublished data). Upon digestion of the PCR fragment with BamHI and XbaI, both fragments were ligated into BSRαEN cut with XhoI and XbaI. All PCR amplified DNA segments were verified by DNA sequencing.

DNA Transfection. cDNA constructs were transfected into two independent clones each of both the Jurkat and 3.L2 cells. Transfection of Jurkat and 3.L2 clones was conducted by electroporation. Jurkat clones (5×10^6/500 μl) were mixed with 15 μg plasmid DNA in fresh RPMI medium at room temperature for 10 min, then electroporated at 300 V, 1,000 μF in a 0.4-cm cuvette using the Invitrogen Electroporater II. After electroporation, cells were immediately placed on ice for 10 min, then resuspended in 10 ml RPMI medium for 24 h before transfer into selection media (RPMI plus 2 mg/ml Geneticin). 3.L2 clones were washed two times with cold PBS and 5×10^6/500 μl were mixed with 15 μg plasmid DNA in PBS and kept on ice for 10 min, then electroporated at 250 V, 1,000 μF in a 0.4-cm cuvette using the Invitrogen Electroporater II. After electroporation, cells were immediately placed on ice for 10 min, then resuspended in 10 ml RPMI medium for 24 h before transfer into selection media (RPMI plus 1.5 μg/ml Geneticin). In all experiments, bulk FACS®-sorted transfectants of both transfected clones (for either Jurkat or 3.L2) were tested. In all cases, data obtained from both clones were similar. Thus, data from single transfected clones are presented. In some assays, transfected cell populations were sorted a second time to obtain populations with high level of expression of transfected molecules.

Isolation of T Cells. PBMCs from healthy donors were separated by Ficoll–HyPaque density gradient centrifugation. Adherent cells were eliminated by culture for several hours on tissue culture–treated plastic. B cells were deleted by immunomagnetic negative selection using Dynabeads M-450 coupled to the pan B cell antigen CD19 (Dynal, Inc., Great Neck, NY). The purity of the isolated T cells was >90% as assessed by immunofluorescent analysis using anti-CD3 and anti-CD19 and fluorescent flow cytometry (EPICS XL; Coulter Corp., Hialeah, FL).

mAbs. The following mAbs were used in these studies: 2E11, 2D3, B6H12 (IgG1, murine anti–huIAP; 1, 20); W6/32 (IgG1, murine anti–HLA; 21); OKT3 (IgG2a, murine anti–huCD3); and 53.67 (IgG2a, rat anti–muCD8α) were purchased from the American Type Culture Collection (Rockville, MD); 9.3 IA1 (IgG2a, murine anti–huCD28 was provided by Dr. J. Ledbetter, Bristol-Myers, Squibb, Seattle, WA); 37.51 (IgG, hamster anti–muCD28, purchased from PharMingen, San Diego, CA); 145-2C11(IgG, hamster anti–muCD3; 22); IB4 (IgG1, murine anti–huCD18; 23); YTS 213.1 (IgG2a, rat anti–muCD18; purchased from BioSource, CA); YTS105.18 and KT15 (IgG2a, rat anti–muCD8; purchased from Serotec, Ltd., Oxford, England); 3D9 (IgG1, murine anti–CD35; 24); 7G2 (IgG1, murine anti–huβ$_3$; 20); P5D2 (IgG1, murine anti–huβ$_1$; Developmental Studies Hybridoma Bank, Iowa City, IA; 25); rabbit anti–ζ chain peptide antiserum (P. Allen, unpublished data); rabbit anti-Zap70 peptide antiserum (provided by Dr. A. Chan, Washington University, St. Louis, MO); 2E11, 2D3, IB4 IgG were purified from ascites using octanoic acid as described (26). SDS-PAGE of all purified IgG preparations showed them to be >90% IgG.

Flow Cytometry. Cells were stained with saturating concentrations of antibody, then incubated with fluorescein-conjugated goat anti–mouse or goat anti–rat Ab before analysis in a FACScan® (EPICS XL; Coulter) as previously described (1). 3.L2 cells were precoated with saturating concentration of human IgG to block FcR expressed on these cells.

Preparation of Antibody-coated Microtiter Plates. Flat-bottomed microtiter plates (3595 Costar, Cambridge, MA) were precoated overnight at 4°C with 5 μg/ml of either goat anti–mouse or a mixture of goat anti–mouse plus goat anti–rat or anti–hamster IgG antibodies (Organon Teknika, Durham, NC) (70 μl/well in 20 mM sodium bicarbonate buffer, pH 9.0). Additional protein binding sites were blocked by overnight treatment with 2% BSA in RPMI-1640 at 4°C. Plates were washed three times with PBS and individual stimulating Abs were added in a 100 μl volume (final volume per well was 200 μl) and incubated overnight at 4°C. OKT3 supernatant was used in 10-fold dilutions for dose–response curves. Anti-IAP or control anti-KLH were used at 10-fold dilutions of supernatant. All other Abs were used at 1 μg/ml or as indicated.

Proliferation Assays. Proliferation assays were performed using standard techniques. In brief, 40,000 purified T cells/microtiter well in 100 μl were cultured in the mAb precoated plates for 3 d in RPMI media and pulsed with a [^{3}H]thymidine solution (1 μCi/well, 6.7 Ci/mmol specific activity, ICN) during the last 18 h before harvesting.

Production of IL-2. 1×10^5 Jurkat or 3.L2 cells/well were cultured for 24 h in the mAb precoated 96-well microtiter plates, after which the supernantants from these cultures were collected and added to the IL-2-dependent CTLL-2 line for 48 h and pulsed over the last 18 h with [^{3}H]thymidine (0.4 μCi/well, 6.7 Ci/mmol specific activity, ICN) (27). For activation of the 3.L2 hybridoma in the presence of APC, 1×10^5 3.L2 cells were cultured in 200 μl of RPMI media for 24 h with APC (2×10^4, CH27), mAbs and the stated peptide concentrations. Supernatants (100 μl) were removed and added to the IL-2 dependent CTLL-2 line and assayed as above. In some assays, dilutions of IL-2 containing supernatants were used and compared with an IL-2 standard curve to quantitate IL-2 production.

Immunoprecipitations and Immunoblots. Jurkat cells (10^6cells/pt for ζ chain and 5×10^6 cells/pt for Zap70) were incubated on Ab-coated surfaces at 37°C for 5–15 min, as indicated in the text. Cells were lysed in 1% NP-40, 0.5% DOC, 50 mM Hepes (pH 7.5), 150 mM NaCl, 20 mM NaF, 1 mM EDTA, 10 μg/ml leupeptin and aprotinin, 10 mM betaglycerophosphate, 50 nM calyculin, and 250 μM sodium vanadate. Insoluble material was removed by centrifugation at 13,000 *g* for 5 min. Prepared cell lysates were immunoprecipitated for 3 h by incubation at 4°C with Ab and protein A–Sepharose (CL-4B; Pharmacia), followed by washing of the immunoprecipitates with lysis buffer before further analysis. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blots were performed as described (28) and developed with 4G10 antiphosphotyrosine mAb (UBI, Lake Placid, NY) or with polyclonal antiprotein antibodies to assure equal loading of sample into each lane. ζ chain and Zap70 tyrosine phosphorylation were quantitated by densitometric scanning of the exposed x-ray films. In all cases, experimental results were compared with control lanes on the same gel of ζ or Zap70 from cells adherent to the noncostimulatory combination of low concentration anti-CD3 together with anti-HLA. In each experiment, this control level of tyrosine phosphorylation was assigned a density of 1 and experimental values compared with this density. Means of independent experiments were obtained using these values, and comparisons

between costimulatory and noncostimulatory conditions evaluated using a two-tailed Student's *t* test.

Results

Anti-IAP and Anti-CD3 Costimulate Human PBL Proliferation and IL-2 Production by Jurkat and HUT78. To investigate the function of IAP on lymphocytes, we evaluated its role in T cell activation. When human T lymphocytes were purified from peripheral blood, no concentration of anti-IAP alone stimulated proliferation (data not shown). In contrast, when combined with a suboptimal concentration of anti-CD3, three different anti-IAP mAbs enhanced proliferation of purified peripheral blood T cells. Using the same low concentration of anti-CD3, the nonbinding isotype-matched negative control 3D9 (anti-CR1/CD35) (Fig. 2 *A*) and mAb W6/32 (anti-HLA), which binds to a different cell surface antigen (data not shown), did not significantly enhance proliferation. In contrast with assays in which IAP has been shown to function with β_3 integrins, the anti-IAP mAb B6H12 was much less potent than anti-IAP 2D3, which recognizes a distinct epitope on the Ig domain (1) (data not shown). This suggested the possibility that the role for IAP is different in synergy with anti-CD3 than in cooperation with β_3 integrins. 2E11, an anti-IAP mAb recognizing a third distinct epitope, also was costimulatory. Anti-IAP mAbs 2E11 and 2D3 also synergized with suboptimal concentrations of anti-CD3 to increase IL-2 production in Jurkat cells, while anti-HLA did not (Fig. 2 *B*; data not shown). This synergy is unlikely to be dependent on IAP cooperation with an integrin, since mAbs against β1, β2, and β3 integrins did not increase T cell proliferation or Jurkat IL-2 production in combination with anti-CD3 (data not shown). The $\alpha_v\beta_3$ ligand vitronectin also was unable to costimulate T cell proliferation or Jurkat IL-2 production with suboptimal anti-CD3 (data not shown). These results imply that IAP enhancement of IL-2 production and T cell proliferation is independent of IAP association with integrins. For costimulation, anti-IAP and anti-CD3 had to be on the same surface. If either or both antibodies were used in solution, there was no costimulation even when the antibodies were cross-linked with a secondary antibody (data not shown). This suggests that the costimulatory signal arises from adhesion to a surface presenting ligands for both IAP and the TCR complex. When IL-2 production was quantitated, IAP-mediated enhancement of IL-2 production with low concentration of anti-CD3 was similar to that seen with the well characterized costimulator CD28 (Fig. 2 *B*). To determine whether costimulation by IAP required CD28, we tested whether IAP was able to augment IL-2 production in the CD28 deficient human cutaneous T cell lymphoma, HUT 78. Although without effect on their own, anti-IAP Abs enhanced IL-2 production with suboptimal anti-CD3 in HUT 78, equivalent to their effect in Jurkat cells (data not shown). Thus, anti-IAP-mediated costimulation does not require expression of CD28.

Costimulation with Anti-IAP Abs but Not Anti-CD28 Results in Enhanced TCR ζ Chain and Zap70 Phosphorylation. One of the earliest events in T cell activation is the tyrosine phosphorylation of the TCR ζ chain and the syk family tyrosine kinase Zap70. To determine whether co-

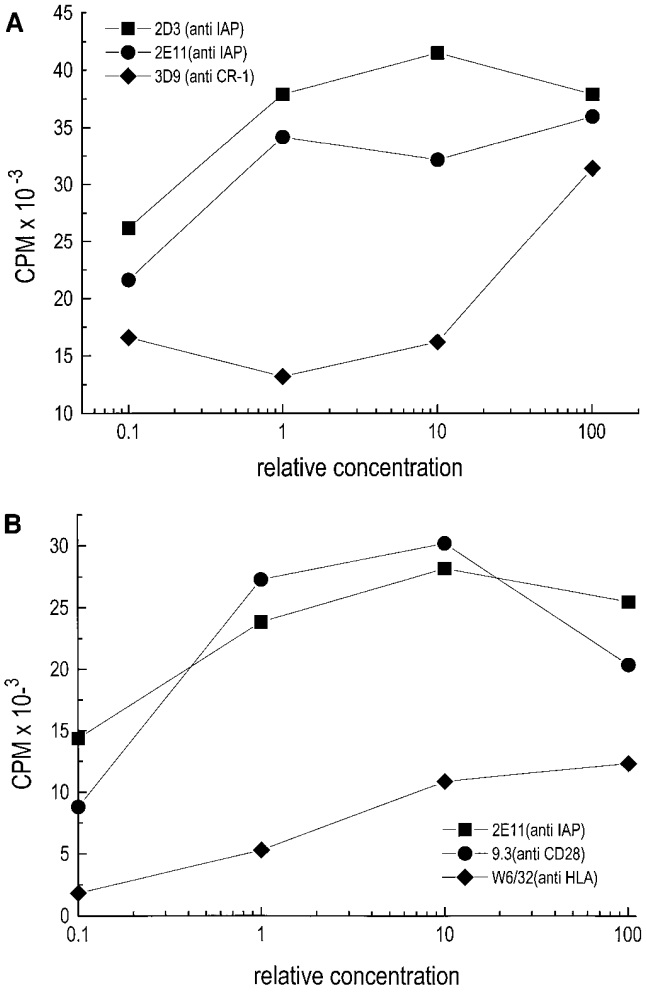

Figure 2. IAP synergy with CD3. (*A*) Human peripheral blood T cells were incubated on plates coated with a low concentration of anti-CD3 together with increasing concentrations of anti-IAP mAb 2E11 or 2D3, or anti-CR1 (3D9). Cells were pulsed with [^{3}H]thymidine for the last 16 h of a 90 h incubation. Shown are averages of triplicate wells from 1 experiment of >3 with similar results. Cells plated on 2E11, 2D3, and 3D9 alone had <1,000 CPM. Maximum stimulation by high concentration of anti-CD3 was 40,000 cpm. (*B*) Jurkat cells were incubated on plates coated with increasing concentrations of anti-CD3 and the same concentration of anti-IAP (2D3), anti CD28 (9.3), or anti-HLA (W6/32) mAbs. Supernatants were harvested after 24 h and IL-2 concentration measured by assay on CTLL-2 cells. The values shown represent triplicates of [^{3}H]thymidine incorporation by the CTLL-2 cells in 1 experiment of >3 with similar results. Quantitation of IL-2 concentration showed that stimulation of Jurkat cells by low levels of anti CD3 (1) in the presence of anti-IAP or anti-CD28 mAbs led to 1 U/ml, compared with 0.1 U/ml for the negative control mAb. Neither anti-IAP or anti-HLA caused detectable IL-2 secretion in the absence of anti-CD3. Additional mAbs that do not costimulate with anti-CD3 include anti-CD61 (integrin β3), anti-CD18 (integrin β2), and anti-CD29 (integrin β1).

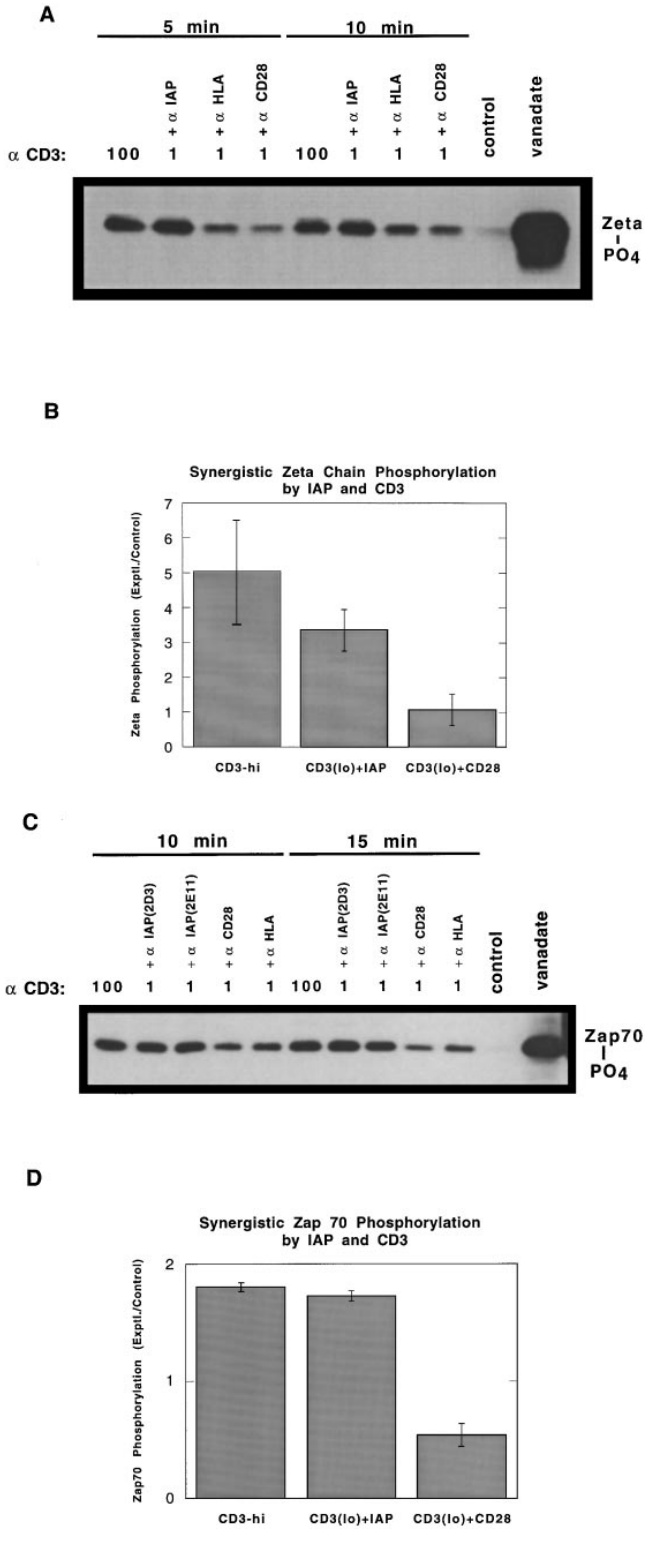

Figure 3. Costimulation with anti-IAP and not anti-CD28 results in enhanced ζ chain and Zap70 tyrosine phosphorylation. (*A* and *C*) Jurkat cells (10^6cells, *A*; 5×10^6 cells, *C*) were stimulated for the indicated timepoints with either an optimal high concentration of anti-CD3 (100) or a low concentration of anti-CD3 (1) coimmobilized with anti-IAP, anti-HLA, or anti-CD28. Cell lysates were immunoprecipitated with anti-ζ chain (*A*) or anti-Zap70 (*C*) polyclonal Abs and analyzed by SDS-PAGE followed by Western blotting with antiphosphotyrosine. (*B* and *D*) ζ chain and Zap70 tyrosine phosphorylation expressed as fold increase over control noncostimulatory conditions (low anti-CD3 plus anti-HLA). Bars represent the mean and SEM of three independent experiments at either 5 min (*B*) or 15 min (*D*). Phosphorylation of both ζ chain and Zap70 was increased by cell adhesion to the costimulatory combination of anti-CD3 and anti-IAP compared to control and compared with adhesion to anti-CD3 and anti-CD28 ($P < 0.05$ in all cases). In contrast, adhesion to the costimulatory combination of anti-CD3 and anti-CD28 did not stimulate ζ chain or Zap70 phosphorylation compared with control.

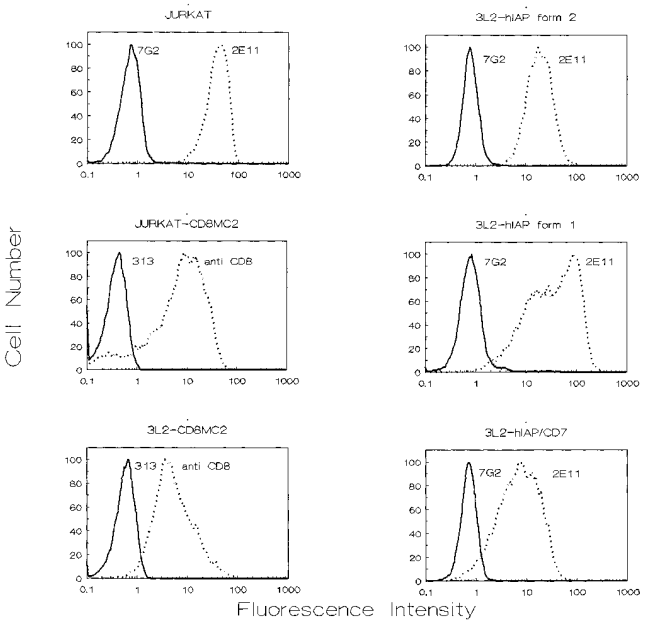

Figure 4. hIAP and chimera expression in Jurkat and 3.L2 subclones. Expression of native hIAP and chimeric constructs was determined by staining with mouse anti–hIAP IgG1 (2E11) or rat anti–murine CD8α (*dotted lines*), or isotype-matched control (7G2 or 313, respectively, *solid lines*) mAbs as described in Materials and Methods. Shown are profiles of one of the transfected Jurkat or 3.L2 clones, with similar levels of expression in the second clone transfected.

stimulation by IAP affects the phosphorylation status of ζ and Zap70, we analyzed ζ chain and Zap70 immunoprecipitates after activation of Jurkat clones under costimulatory (low anti-CD3 plus anti-IAP or anti-CD28) and control conditions (low anti-CD3 plus anti-HLA). We found that ζ chain tyrosine phosphorylation in the presence of anti-IAP mAbs was enhanced over control and was almost equivalent to optimal anti-CD3 (Fig. 3, *A* and *B*). In contrast, the costimulatory combination of anti-CD28 with anti-CD3 did not enhance ζ chain tyrosine phosphorylation. Similarly, Zap70 phosphorylation was equivalent for optimal anti-CD3 and the costimulatory combination of CD3 and IAP mAb. In contrast, anti-CD28 did not costimulate Zap70 phosphorylation above control levels (Fig. 3, *C* and *D*). These data demonstrate that IAP-mediated costimulation occurs by a signaling pathway distinct from that initiated by CD28.

Transfected Human IAP Costimulates a Murine T Cell Hybridoma. To begin to understand IAP function in T cell

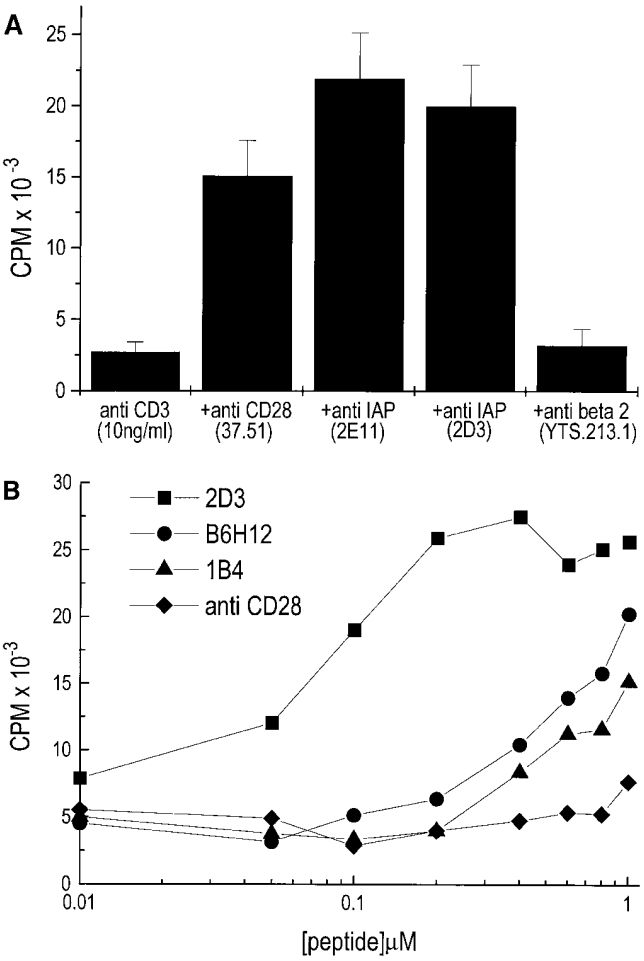

Figure 5. IL-2 production by 3.L2 clones transfected with hIAP form 2. (*A*) Anti-CD3 was coimmobilized at the indicated concentration with anti-CD28, anti-hIAP (2E11, 2D3), or control mAb (YTS 213.1). 3.L2 clones, transfected with hIAP (form 2) were plated at 1×10^5 cells/well. Supernatants were harvested after 24 h and IL-2 concentration measured as described in Fig. 2 *B*. (*B*) 3.L2 clones transfected with hIAP (form 2) at 1×10^5 cells/well were activated with the indicated amounts of Hb(64–76) peptide presented by CH27 cells (2×10^4 cells/well) in the presence of anti-IAP mAbs 2D3 or B6H12, anti CD28 (37.51) or a control mAb (IB4). T cell hybridoma activation was measured by IL-2 production after 24 h of culture as described in Fig. 2 *B*. Neither anti-IAP or control Ab alone caused detectable IL-2 production. The values shown represent averages of triplicates of 1 experiment of >3 with similar results.

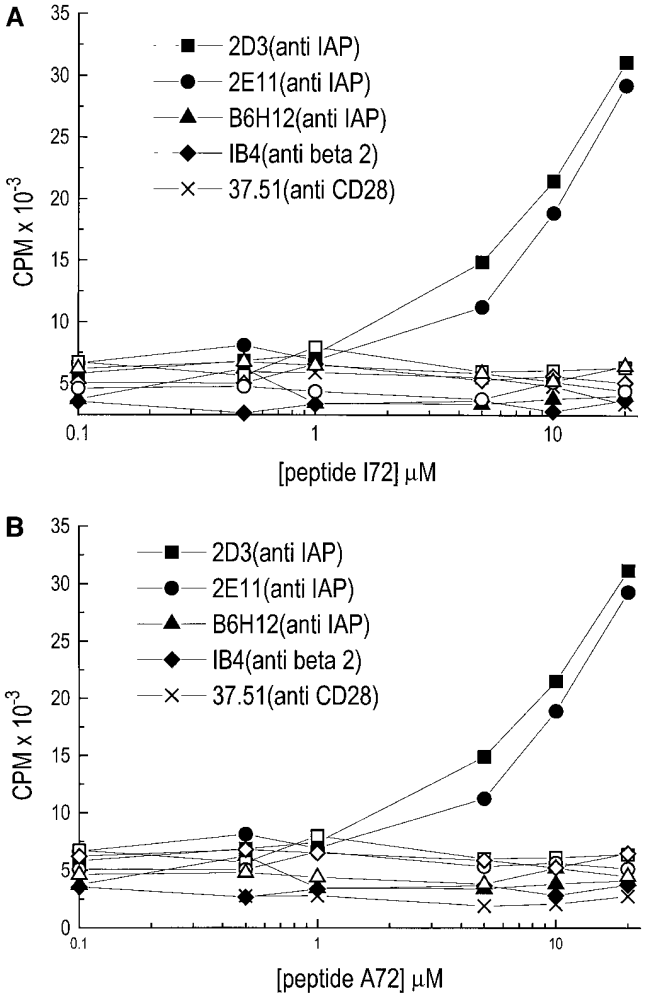

Figure 6. IAP can convert antagonist peptides I72 and A72 to agonists. 3.L2 clones transfected with hIAP form 2 (*closed symbol*) or IAP/CD7 (*open symbol*) were activated with the indicated concentration of the Hb(64–76)–I72 (*A*) or Hb(64–76)–A72 (*B*) peptide presented by CH27 cells in the presence of anti-IAP mAbs 2E11, 2D3 or B6H12, anti CD28 (37.51), or a control mAb (IB4). I72 and A72 have been shown previously to have significant antagonist but no activating effects on 3.L2 (30). T cell activation was measured as described in Fig. 2 *B*. The values shown represent averages of triplicates of 1 experiment of >3 with similar results.

costimulation, we transfected form 2 of human IAP (hIAP), which is the predominant IAP form found in leukocytes, into two independent clones of the hemoglobin-specific murine T cell hybridoma 3.L2 (Fig. 4) (17). Initial experiments using the 3.L2 clones showed that mAbs recognizing CD28 costimulated IL-2 production with suboptimal anti-CD3, indicating that this murine hybridoma was responsive to costimulatory signals. Coligation of hIAP with anti-hIAP mAbs, which do not cross-react with mouse IAP, and suboptimal anti-CD3 also resulted in enhanced IL-2 production over control mAb (Fig. 5 *A*).

Anti-IAP and Antigen Costimulate 3.L2 Transfectants. The TCR of the 3.L2 mouse hybridoma is known to recognize a peptide sequence, (Hb 64–76), from the murine β-minor chain of hemoglobin protein in the context of MHC class II (I-E^k) (17). This peptide, when presented by the B cell lymphoma CH27, stimulates a dose dependent induction of 3.L2 activation. When the 3.L2 clones transfected with human IAP form 2 were incubated with antigenic peptide, addition of anti-IAP mAbs 2D3 (and 2E11; data not shown) stimulated a marked increase in IL-2 production at low peptide concentrations (Fig. 5 *B*). No increase in IL-2 production was observed upon addition of an anti-CD28 mAb. At an optimal peptide concentration, anti-IAP had no costimulator effect. Thus, in both antigen- and anti-CD3-stimulated T cell activation, ligation of IAP alters the sensitivity of cell activation to TCR ligation, but does not affect maximal response.

$F(ab')_2$ of these anti-hIAP mAb failed to costimulate (data not shown), suggesting that binding to the APC via its FcR

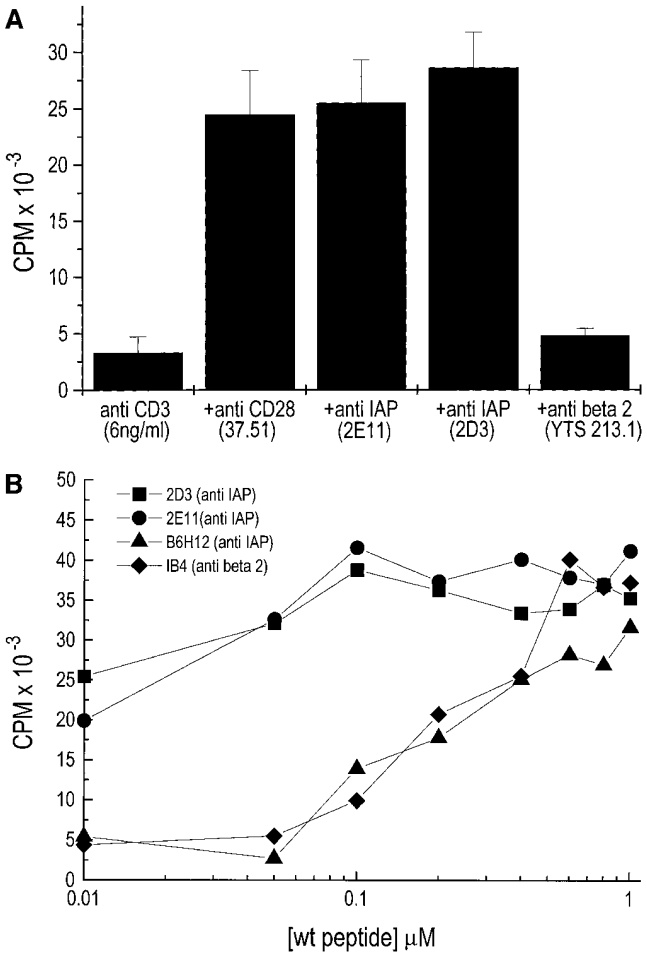

Figure 7. Anti-IAP costimulates IL-2 production in 3.L2 clones transfected with hIAP form 1. (*A*) 3.L2 clones, transfected with hIAP form 1, were cultured on surfaces coated with anti-CD3 at the indicated concentration plus anti-CD28 (37.51), anti-hIAP (2E11, 2D3), or control mAb (YTS 213.1) and IL-2 production was measured. (*B*) 3.L2 clones, transfected with hIAP form 1, were activated with the indicated concentration of Hb(64–76) peptide in the presence of anti-IAP mAbs 2E11, 2D3, or B6H12 or a control mAb (IB4). T cell activation was analyzed as described in Fig. 2 *B*. The values shown represent averages of triplicates of 1 experiment of >3 with similar results.

was required for the anti-IAP mAb to act as a costimulator. This is consistent with the observation in human cells that the signal for costimulation arises from adhesion to a surface expressing both antigen receptor (CD3) and IAP ligands. The increase in IL-2 production was not simply a result of enhanced interaction between the APC and the T cell, since a third anti-human IAP mAb, B6H12, which has equal affinity for human IAP as 2E11 or 2D3, did not enhance IL-2 production above background levels (Fig. 5 *B*). Stimulation by anti-IAP mAb 2D3 and failure of B6H12 to stimulate is in direct contrast with the effects of these mAbs on integrin β_3-dependent functions (8, 29), emphasizing the independence of T cell costimulation from IAP–integrin association.

IAP Ligation Alters 3.L2 Response to APL Peptides. To test whether the nature of the peptide ligand affected the ability of IAP to costimulate T cell activation, the effects of APL peptides on 3.L2 activation were tested. The two antagonist peptides chosen were previously shown to have no agonist effects on 3.L2 (30), because they did not induce any IL-2 production from 3.L2 cells on their own even at concentrations above 100 μM. When tested in combination with anti-IAP, both peptides induced the T cell hybridoma to make IL-2 (Fig. 6). In contrast, addition of anti-CD28 mAb did not result in IL-2 production by the 3.L2 clones. Thus, coligation of IAP but not CD28 with the antigen receptor gives a fully activating signal, even with peptides incapable of producing any activating signal on their own.

IAP Cytoplasmic Tail Is Not Required for Costimulation. To begin to understand the domains of IAP required for costimulation, we tested whether the cytoplasmic tail of IAP is required for costimulatory activity. IAP form 1, which has a cytoplasmic tail of only four amino acids, is a naturally occurring form of IAP expressed in keratinocytes and several transformed cell lines (9). 3.L2 clones transfected with hIAP form 1 (see Fig. 4) were tested for their ability to synergize with anti-CD3. All anti-human IAP mAbs tested (2E11, 2D3, or B6H12) were able to costimulate IL-2 production in these cells (Fig. 7 *A*). 3.L2 transfectants expressing the tailless form of IAP also were tested with antigenic peptide. In these transfectants, the addition of anti-hIAP mAbs 2E11 or 2D3 resulted in a marked increase in IL-2 production (Fig. 7 *B*). Thus, in two assays, the IAP cytoplasmic tail was not required for costimulation.

The IAP Multiply Membrane-spanning Domain Is Required for T Cell Costimulation. To determine whether the IAP Ig domain alone is able to enhance IL-2 production, we replaced the multiply membrane-spanning domain and cytoplasmic tail of IAP with the CD7 transmembrane domain (see Fig. 1). All anti-IAP mAbs recognized this chimeric protein when expressed in the 3.L2 clones (see Fig. 4). Moreover, this construct restored vitronectin bead binding when transfected into an IAP-deficient cell expressing $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (31). Thus, both mAb and functional data suggest that the Ig domain conformation was unaltered. Nonetheless, ligation of IAP/CD7 did not costimulate in either 3.L2 clone (Fig. 8 *A*). These results show that the extracellular domain of IAP is not sufficient to synergize with anti-CD3.

The multiply membrane-spanning domain was required for IAP costimulation in antigen presentation as well, since IAP/CD7 transfectants failed to enhance IL-2 production in response to either of the stimulating anti-human IAP mAbs in combination with low concentrations of stimulating peptide (Fig. 8 *B*). To eliminate the possibility that the lower level of expression of IAP/CD7 contributed to the inability of this chimera to costimulate, we generated 3.L2 clones expressing equivalent levels of IAP/CD7 and wild-type human IAP as determined by FACS® staining. Still, IAP/CD7 did not costimulate IL-2 production at all (data not shown). Thus, in a direct antigen stimulation assay, the Ig and multiply membrane spanning domain are sufficient to costimulate T cell activation (see Fig. 7 *B*), while the IAP Ig domain fails to activate (Fig. 8 *B*). Failure of costim-

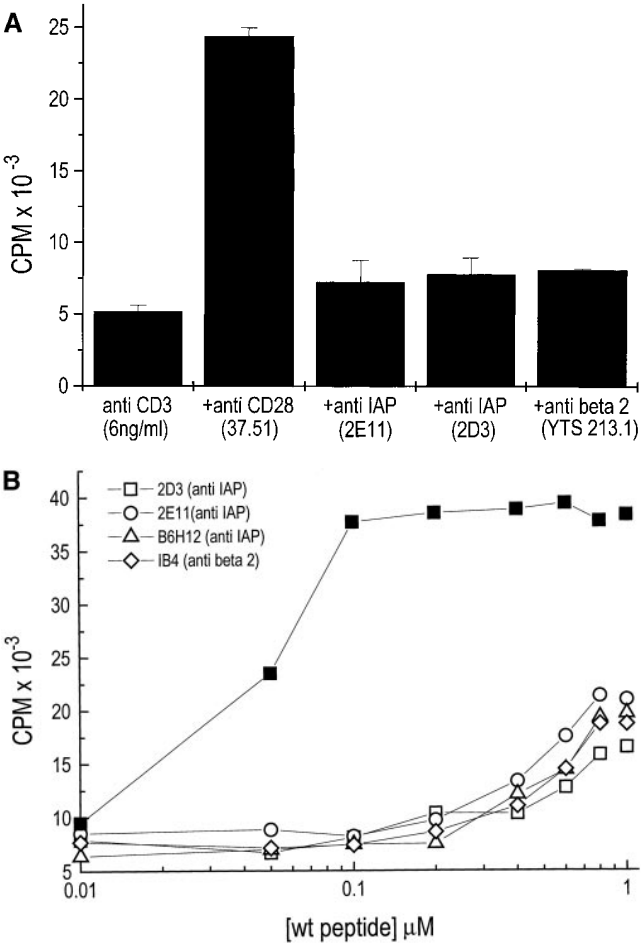

Figure 8. IAP/CD7 cannot costimulate IL-2 production. (*A*) 3.L2 clones, transfected with IAP/CD7, were plated on plates coated with CD3 at the indicated concentration in the presence of either anti-CD28 (37.51), anti-hIAP (2E11, 2D3), or control mAb (YTS 213.1). (*B*) IAP/CD7-transfected 3.L2 clones (*open symbol*) or hIAP form 2 (*closed symbol*) were activated with the indicated concentration of Hb(64–76) peptide in the presence of anti-IAP mAbs 2E11, 2D3, or B6H12, or a control mAb (IB4) and T cell activation was analyzed. The values shown represent averages of triplicates of 1 experiment of >3 with similar results.

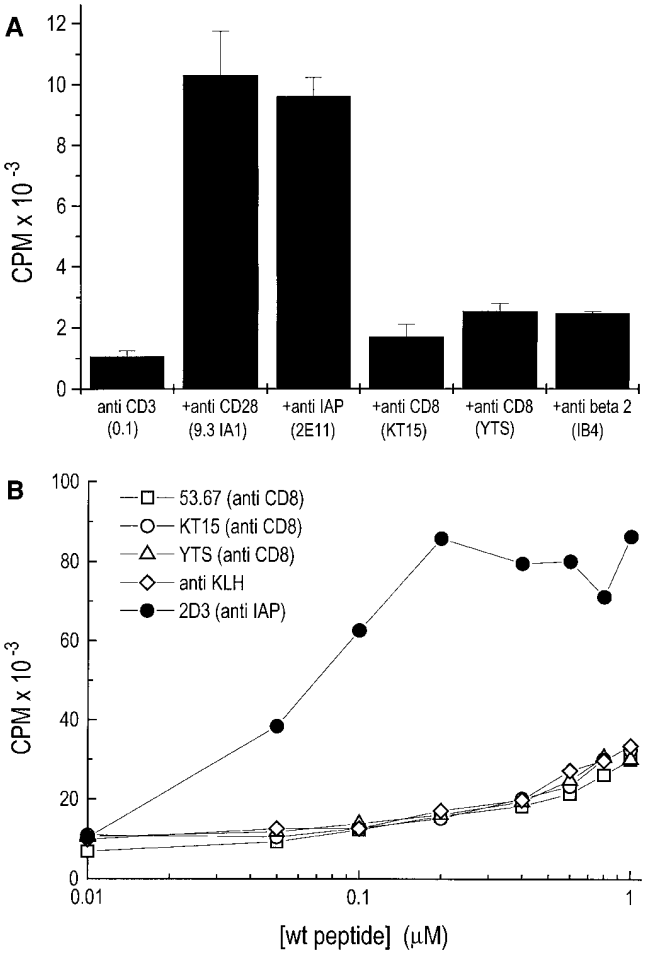

Figure 9. The multiply membrane-spanning domain of IAP is not sufficient for T cell costimulation. (*A*) Jurkat clones, transfected with CD8MC2, were cultured on plates coated with anti-CD3 plus anti-CD28 (9.3), anti-IAP (2E11), two different anti-CD8 (YTS, KT15), or control mAb (IB4). Supernatants were harvested after 24 h and IL-2 concentration measured using CTLL-2 as described in Fig. 2 *B*. A third anti-CD8 mAb (53.67) also failed to costimulate these transfected Jurkat cells. (*B*) 3.L2 clones transfected with CD8MC2 (*open symbols*) or hIAP form 2 (*closed symbol*) were activated by the indicated amounts of Hb(64-76) peptide presented by CH27 cells in the presence of anti-IAP (2D3), anti-CD8 (53.67; KT15; YTS), or control (anti-KLH) mAb and IL-2 production was measured. The values represent averages of triplicates of 1 experiment of >3 with similar results.

ulation by IAP/CD7 also is further evidence that mAb-mediated aggregation of APCs and T cells is not sufficient to account for the role of IAP in T cell activation.

The Multiply Membrane-spanning Domain of IAP Is Not Sufficient for T Cell Costimulation. To determine whether the multiply membrane-spanning domain of IAP was sufficient for IAP function, we replaced the IAP extracellular domain with that of mouse CD8α (CD8MC2). We used IAP form 2 for the chimeric construct because this is the endogenous form of IAP expressed in T cells and in the Jurkat cell line. Control chimeras consisting of the mouse CD8α extracellular and transmembrane domain with (CD8C2; see Fig. 1) and without (CD8*; see Fig. 1) the IAP cytoplasmic tail also were transfected (see Fig. 4; data not shown). Jurkat clones expressing the CD8 constructs did not show costimulatory activity when activated by a low concentration of anti-CD3 and any of three different anti-CD8 mAbs tested (Fig. 9 *A*; data not shown). As for all cDNAs, these chimeras were transfected into two independent Jurkat clones each, with identical results. Expression of the chimeric molecules did not prevent costimulation of Jurkat cells, since activation of the endogenous IAP with 2E11 mAb still resulted in elevated IL-2 levels (Fig. 9 *A*). To test the CD8MC2 chimera in the antigen-induced activation, it was transfected into two 3.L2 clones (see Fig. 4). Anti-CD8 mAbs failed to enhance IL-2 production over background in this assay as well (Fig. 9 *B*). To rule out the possibility that low expression of the chimera led to failure to costimulate, a transfectant population was selected stably expressing fivefold more CD8MC2. Although these clones

expressed the chimera at a level equivalent to expression of the wild-type human IAP, anti-CD8 still failed to costimulate IL-2 production (data not shown).

Discussion

IAP (CD47) is an Ig family member highly expressed on lymphocytes, but without known function on these cells. In the present work, we describe a role for IAP in costimulation of T cell activation in combination either with low dose anti-CD3 or with antigen. Costimulation is a fundamental requirement for optimal T cell activation. The best understood costimulatory signal comes from the interaction of B7 with its receptor CD28. This interaction leads to intracellular signals that result in enhanced IL-2 production and the prevention of anergy (32). In addition to CD28, other molecules on the surface of T cells have been implicated in costimulation. These include a variety of adhesion receptors, including integrins recognizing fibronectin, ICAM-1, and laminin (33–35). However, little is known about the mechanism by which adhesion receptors cooperate with the antigen receptor, or about structural requirements for adhesion receptor function in this important biological response. In particular, previous experiments have failed to demonstrate rigorously that costimulation by adhesion molecules requires signaling through the adhesion receptor. It is possible that the enhancement of the antigen receptor signal simply represented increased efficiency of interaction of the antigen receptor with ligand or antibody due to enhanced contact with the activating surface.

The close relationship between IAP and the integrin $\alpha_v\beta_3$ led us to examine whether IAP could costimulate T cell activation like other adhesion receptors. Indeed, this was the case, as we have shown both in an assay using anti-CD3 and another involving presentation of specific antigen in the context of MHC. Despite the requirement for cell adhesion in IAP costimulation, two lines of evidence suggest that the effect of IAP ligation in these assay is independent of integrins. First, in Jurkat cells, ligation of IAP costimulated IL-2 production, but this effect was not mimicked by ligation of any integrin on the cell. This is also true in 3.L2 cells, in which ligation of transfected human IAP effectively costimulated proliferation, while ligation of endogenous β_1 or β_2 integrins did not (data not shown). Second, the effects of specific anti-IAP mAb were very different in T cell costimulation than in β_3 integrin-dependent functions. Multiple studies have shown that anti-IAP mAb B6H12 is functionally active, whereas the mAb 2D3, which has the same affinity for IAP, is not (8, 29). Since B6H12 and 2D3 both recognize the IAP Ig domain but have different noncompetitive epitopes, these data suggest that B6H12 recognizes a site on the extracellular domain necessary for functional interaction with β_3 integrins, whereas 2D3 does not. In contrast, both in the antigen-dependent assay for IAP costimulation and in the anti-CD3-dependent costimulation of peripheral blood T cells, B6H12 was less effective than 2D3. These data suggest that the IAP Ig domain has a different role in T cell costimulation than in functional association with β_3 integrins.

The adhesion-dependent costimulatory activity of IAP is distinct from CD28. First, expression of CD28 is not required for IAP to enhance IL-2 production, as shown by the CD28-deficient T cell lymphoma HUT 78. Second, although IAP or CD28 can synergize equally well with low concentration of anti-CD3 to enhance IL-2 production in Jurkat cells, only IAP synergizes with CD3 to promote ζ chain and Zap70 phosphorylation. Thus, the mechanism of IAP costimulation is quite different from CD28. This distinction is supported by the finding that addition of anti-IAP, but not anti-CD28 mAbs, converted the antagonistic APL peptides into agonists, resulting in efficient IL-2 production by the 3.L2 T cell hybridoma.

Studies of costimulation by chimeric molecules containing IAP domains demonstrate that molecules lacking either the Ig domain or the multiply membrane-spanning domain fail to costimulate. This suggests that each domain is involved in a function required for costimulation. On the other hand, there appears to be no essential role for the IAP cytoplasmic tail in costimulation, since IAP form 1 (which has only four amino acids in its cytoplasmic tail) is as effective as form 2, the major leukocyte form of IAP, which has a 15–amino acid cytoplasmic tail. Of course, this does not rule out interaction of IAP with specific cytosolic proteins via the short hydrophilic sequences in the multiply membrane-spanning domain that link the transmembrane sequences. Importantly, the described studies do rule out the possibility that anti-IAP nonspecifically enhances interaction of CD3 with anti-CD3 or of TCR with peptide and MHC. Antibodies that are effective for costimulation on wild-type IAP fail to costimulate the IAP/CD7 chimera, despite expression of the identical epitope for the mAb in both molecules. Moreover, a mAb (B6H12) with equal affinity to these costimulatory antibodies fails to costimulate. Thus, ligation of IAP must generate a signal that cannot occur with suboptimal anti-CD3 or peptide, no matter how efficiently they are presented to the T cell. This conclusion is reinforced by the studies with altered peptide ligand that also cannot activate T cells on their own at any concentration, but that are effective stimulators in association with IAP ligation. Because ligation of IAP cannot lead to IL-2 synthesis on its own and can only effectively costimulate when presented on the same surface as anti-CD3 or activating peptide, the costimulatory effect of IAP apparently requires the physical proximity of the TCR complex. The observation that IAP enhances ζ chain and Zap70 phosphorylation under the costimulatory conditions suggests that IAP ligation may modify a TCR-generated signal; alternatively, the cytoplasmic domains of molecules in the TCR complex may serve as interaction sites for cytoplasmic molecules affected by the signal(s) generated from IAP ligation.

The requirement for the multiply membrane-spanning domain suggests that this region of IAP is involved in some signaling function of the molecule. A role for IAP as a

membrane Ca^{2+} channel has been proposed (4). If this is the mechanism by which IAP affects costimulation, the increase in $[Ca^{2+}]_i$ generated by IAP ligation would likely be only in a small part of the cell, given the requirement that IAP and CD3 must be in close proximity for effective costimulation. Alternatively, the multiply membrane spanning domain could provide a docking site for cytoplasmic molecules that are involved in antigen receptor–mediated signal transduction. A similar role has recently been proposed for CD20, a B cell surface antigen that cooperates with the B cell antigen receptor in cell activation. CD20 has a domain that is thought to have four membrane-spanning regions. This highly hydrophobic domain has been shown to associate with src family tyrosine kinases (36), which is thought to be the mechanism by which CD20 contributes to B cell activation.

The inability of the CD8 Ig domain to substitute for the IAP Ig domain is very surprising. Quite often in immune signaling, the effector domains are intracytoplasmic, or membrane and cytoplasmic, and ligand or antibody generates a signal by aggregation of these effector domains. In this model, the exact nature of the extracellular domain is irrelevant, as long as it can be aggregated by antibodies (19, 37, 38). This cannot be the case for IAP, because the CD8 extracellular domain does not substitute for the IAP Ig domain and some antibodies against the IAP Ig domain are ineffective at costimulation. This suggests that, despite the likely independence of IAP T cell costimulation from integrins, the IAP Ig domain may interact with another molecule in addition to the antibody presented on the activating surface or cell. The requirement for the IAP Ig domain exists even when the only cell in the assay is the responding T cell, suggesting that the IAP Ig domain recognizes another plasma membrane molecule on the same cell, as it does in forming a signaling complex with β_3 integrins. Because of the requirement for proximity to the TCR complex, some component of that complex is a good candidate for interaction with the IAP Ig domain. Alternatively, if IAP acts as a membrane channel, it is possible that the Ig domain interacts directly with the multiply membrane-spanning domain to regulate channel activity.

The exact role of IAP in the immune response remains to be determined. While we have found that anti-IAP can costimulate T cell responses, it is not known under what circumstances IAP ligation is necessary or aids T cell activation. Since anti-IAP stimulates activation with antigen presentation by CH27, we assume that these APCs do not express endogenous IAP ligand. The discovery that thrombospondin is an IAP ligand (7) suggests that its interaction with IAP might play a role in T cell activation at sites of inflammation, where thrombospondin is transiently a component of the extracellular matrix. This would provide a mechanism by which IAP, which is constituitively expressed, would only be engaged at inflammatory sites, leading to optimal T cell activation as required for an effective immune response.

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