Cell spreading distinguishes the mechanism of augmentation of T cell activation by integrin-associated protein/CD47 and CD28

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Abstract

Integrin-associated protein (IAP/CD47) is a 50 kDa transmembrane protein initially defined as a regulator of β3 integrin-mediated functions in neutrophils. IAP also can synergize with the TCR in T cell activation independent of β3 integrins. To analyze the mechanism for IAP synergy with TCR, we expressed in Jurkat cells a chimeric molecule, consisting of the CD16 extracellular domain, the CD7 transmembrane domain and the TCR ζ chain cytoplasmic tail (CD16-7-ζ), which on its own is unable to induce IL-2 production. Ligation of IAP acted in synergy with TCR to induce IL-2 transcription and synthesis, but failed to synergize with the signal generated by CD16-7-ζ, while CD28 was a potent co-stimulator with both TCR and CD16-7-ζ. The failure of IAP to activate Jurkat together with CD16-7-ζ correlated with a lack of c-Jun N-terminal kinase, but not extracellular-signal-regulated kinase activation. Jurkat adhesion to anti-IAP, but not anti-CD28, induced cell spreading and the same domains of IAP required for augmentation of T cell activation were required to induce cell spreading. IAP synergy with TCR signaling likely results from its ability to stimulate adhesion to a ligand-expressing surface or antigen-presenting cell (APC), rather than from initiation of a novel signaling cascade. We conclude that a major role for ligation of IAP in T cell activation is to enhance the efficiency of TCR signaling by causing T cells to spread on an APC or surface.

Introduction

Activation of T lymphocytes from a quiescent to a proliferating state requires not only the engagement of the TCR complex but also the participation of accessory or co-stimulatory molecules (1–3). Lack of co-stimulation prevents cytokine production and clonal expansion, and can induce a state of unresponsiveness or anergy (2,4). CD28 is a well-described co-stimulatory molecule, which contributes to T cell activation by inducing the activation of signaling events that do not occur when TCR is ligated alone (3,5). The ability of CD28-deficient T cells to develop normally (6) suggests that there may be other T cell surface molecules that can, in some circumstances, serve the same co-stimulatory function as CD28.

Ligation of a number of cell surface molecules can enhance T cell activation by suboptimal concentrations of TCR ligand. Among these are LFA-1, heat stable antigen, and integrin fibronectin, collagen, vitronectin and laminin receptors (7–10). However, whether these molecules act as co-stimulators similar to CD28 or synergize with TCR signals by some other mechanism is unknown. Integrin-associated protein (IAP, CD47) also can augment TCR signal transduction, enhancing IL-2 production and proliferation with suboptimal anti-CD3 (11–13). Moreover, ligation of IAP can change altered peptide TCR ligands that give partial or antagonistic signals for T cell activation into fully activating antigens, a property not shared by CD28 (11). For this reason, we have studied the...
mechanism of IAP synergy with TCR signaling and compared it to CD28 co-stimulation.

IAP is a 50 kDa protein, which has three domains: an Ig variable (IgV)-like N-terminal domain, a domain containing multiple membrane-spanning segments and a short cytoplasmic tail with four alternatively spliced forms (14, 15). IAP was identified by co-immunoprecipitation with β3 integrins (16) and later shown to be identical to the erythrocyte antigen CD47 (17, 18). IAP has a role in several β3-mediated functions, such as binding of vitronectin-coated beads to cells expressing α2β3 (19), polymorphonuclearophil activation by and chemotaxis to Arg-Gly-Asp (RGD)-containing ligands (20), and an increase in intracellular [Ca2+]i in endothelial cells during adhesion to fibronectin (21), as shown by both antibody inhibition and genetic analysis. IAP is more broadly expressed than α2β3, suggesting it may have additional physiologic functions. This is supported by the finding that IAP’s role as an accessory molecule in T cell proliferation appears to be independent of its association with β3 (11, 12).

To analyze the mechanism of synergy between the TCR complex and IAP, we simplified the TCR by substitution with a chimeric molecule consisting of the CD16 extracellular domain, the CD7 transmembrane domain and the TCR ζ chain cytoplasmic tail (16-7-ζ). Previous studies have shown that this chimera is sufficient to induce T cell activation events, such as an increase in [Ca2+]i and induction of cytolytic activity (26). CD28, but not IAP, was able to synergize with 16-7-ζ to activate IL-2 synthesis and stimulate N-terminal jun kinase (JNK) in Jurkat cells. In contrast, adhesion of Jurkat cells to surfaces coated with anti-IAP, but not anti-CD28 mAb, induced cell spreading. These data suggest that ligation of IAP results in increased interaction of adherent cells with the antigen-presenting surface, thereby enhancing the efficiency of TCR signaling, but is unable to confer new signaling capability on the TCR. This mechanism for augmenting TCR signaling is completely distinct from CD28, but may be shared by other receptors which synergize with TCR through adhesion.

Methods

Cell culture

Jurkat and the IAP-deficient Jurkat clone JinB8 were maintained in culture in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 0.1 mM NEAA, 50 μM 2-mercaptoethanol and 0.1% gentamcin. Transfected Jurkat were maintained in the same media in the presence of 1.5 mg/ml geneticin (Gibco/BRL, Gaithersburg, MD). Cells were cloned by limiting dilution.

Generation of IAP-deficient Jurkat cells

The Jurkat T cell line was obtained from the ATCC (Rockville, MD). Briefly, Jurkat cells were cultured at a concentration of 0.5×10^6 cells/ml in RPMI medium supplemented with 10% FCS. Ethyl-methanesulfonate (200 μg/ml) (Sigma, St Louis, MO) was added for 18 h. Cells were washed 3 times and allowed to recover for 5 days. IAP-expressing cells were eliminated by seven rounds of negative selection using the mAb BRIC126 (1 μg/ml) (International Blood Group Reference Laboratory, Bristol, UK) and non-toxic rabbit complement (Behring, Marburg, Germany). Finally, cells were labeled with FITC-rabbit anti-mouse F(ab’)2, and IAP- cells were sorted using FACS sorting (FACStar; Becton Dickinson, Mountain View, CA).

DNA constructs

Standard techniques were used for nucleic acid manipulations. The CD16-7-ζ construct was a kind gift of Dr Brian Seed (Harvard Medical School, Boston, MA). The cDNA was subcloned into pRC/CMV using HindIII and NotI sites. To generate the epitope tagged FLAG-TM2 construct, the following oligonucleotides containing the FLAG sequence were made: 5′-AACAGCTAGCGACTACAAAGACGATGACGATAAGAGATC-3′ and 5′-GTTGATTCCGAGATCTTTATCGCATCGTTTTGTAGTC-3′, which include a BamHI and Nehel site. The primers were allowed to anneal, and the annealed product was cut with BamHI and Nehel. The purified fragment was then ligated into plAP401 cut with BamHI and Nehel, which contains the CD5 signal sequence 5′ of the BamHI site (kind gift from Dr Brian Seed), generating plAP332. The Xhol–BamHI fragment of plAP332 containing the CD5 signal sequence 5′ of the FLAG sequence was ligated together with a BamHI–NheI fragment from plAP331 encoding the IAP transmembrane domain and form 2 tail into pBRSRaNEN cut with Xhol and XbaI.

DNA Transfection

cDNA constructs were transfected into wild-type Jurkat clones or the IAP-deficient Jurkat clone JinB8 by electroporation. For stable transfections, Jurkat clones (5×10^5/500 μl) were mixed with 15 μg cut plasmid DNA in fresh RPMI medium, incubated at room temperature for 10 min and then electroporated at 300 V, 1000 μF in a 0.4 cm cuvette using the Invitrogen Electroporator II. After electroporation, cells were immediately placed on ice for 10 min and then resuspended in 10 ml RPMI complete medium for 24 h before transfer into selection media (RPMI plus 2 mg/ml geneticin). In all experiments, bulk FACS-sorted transfecants were tested. For transient transfections, 2×10^7 Jurkat cells/well were transfected as described above and resuspended in 10 ml RPMI complete medium for 24 h before stimulation.

mAb and reagents

The following mAb were used in these studies: 2E11, 2D3 [IgG1, murine anti-huIAP (16,22)]; W6/32 [IgG1, murine anti-HLA (23)]; OKT3(IgG2a, murine anti-huCD3, purchased from the ATCC); 9.3 IA1 (IgG2a, murine anti-huCD28 was provided by Dr Jeffrey Ledbetter, Bristol-Myers Squibb, Seattle, WA); M2 (IgG1, murine anti-FLAG; Eastman Kodak, New Haven, CT); IB4 [IgG1, murine anti-huCD18 (24)]; 3G8 [IgG1, murine anti-huCD16 (25)]; MOPC-21 (mouse IgG1 κ purchased from Sigma, St Louis, MO); mouse anti-EKR1/2 7D8 (IgG1; purchased from Zymed, San Francisco, CA); and affinity-purified rabbit polyclonal phospho-specific c-Jun (Ser63) antibody (purchased from New England Biolab, Beverly, MA).The IL-2-LUC reporter construct was provided by Dr Kenneth M. Murphy (Washington University, St Louis, MO).

Flow cytometry

Cells were stained with saturating concentrations of antibody, then incubated with fluorescein-conjugated goat anti-mouse...
or goat anti-rat antibody before analysis in a FACSscan (Epics XL; Coulter) as previously described (16).

**Preparation of antibody-coated tissue culture plates**

Plates were coated as previously described. Briefly, flat-bottomed microtiter (3595; Costar, Cambridge, MA) and six-well plates (3046; Becton Dickinson Labware, Franklin Lakes, NY) were precoated overnight at 4°C with 5 µg/ml goat antimouse IgG antibodies (Organon Teknika, Durham, NC) (70 µl/well or 2 ml/well) in 20 mM sodium bicarbonate buffer, pH 9.0. Additional protein binding sites were blocked by treatment with 2% BSA overnight in RPMI 1640 at 4°C. After washing plates for 3 times with PBS, the individual stimulating antibodies were added in a 100 µl or 1 ml volume (final volume per well was 200 µl or 2 ml) and incubated overnight at 4°C. OKT3 supernatant was used in 10-fold dilutions. All other antibodies were used at 1 µg/ml or as indicated.

**Production of IL-2**

IL-2 production was measured by incorporation of [3H]-thymidine (0.4 µCi/well, 6.7 Ci/mmol sp. act.; ICN) by CTLL-2 cells as previously described (11).

**Cell spreading**

Jurkat cells were resuspended at 2.5×10⁵ cells/ml in RPMI-1640 and allowed to spread on mAb-coated tissue culture wells for 2–5 h at 37°C in 5% CO₂.

**Luciferase assay**

Jurkat cells were stimulated on antibody-coated plates for 6 h at 24 h after transfection. Following stimulation, the cells were washed 3 times with PBS and resuspended in 1 ml TEN buffer [40 mM Tris (pH 7.8), 1 mM EDTA, 150 mM NaCl], centrifuged, resuspended in 150 µl of 250 mM Tris (pH 8.0) and freeze-thawed 3 times (dry ice to 37°C bath). Cell debris were removed by centrifugation at top speed in a microfuge tube and 75 µl of the cleared extract was measured using an Optilocomp II (MGM) Luminometer.

**Preparation of total cell lysates and extracellular-signal-regulated (ERK) mobility shift**

Jurkat cells (5×10⁶) were applied to antibody-coated plates and incubated at 37°C for the times indicated in the text. Cells were then lysed in 500 µl ice cold lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM H₂O₂-activated Na₃VO₄, 50 mM NaF, 1 mM p-nitrophenyl phosphate, 50 mM calyculin A, 1 mM PMSF, and 10 µg/ml aprotinin and leupeptin] and insoluble material was removed by centrifugation at 13,000 g for 5 min. Then 50 µl of lysate was electrophoresed on a 15% SDS-polyacrylamide gel, blotted onto nitrocellulose and incubated with 0.2 µg/ml anti-ERK mAb. The antibody–antigen complexes were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

**Solid-phase JNK assay and immunoblot**

The solid-phase JNK assay was performed using a kit according to the manufacturer’s instructions (New England Biolob, Beverly, MA). Briefly, 2×10⁶ cells/well were lysed with cell lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM Na pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin and 1 mM PMSF]. The cell extracts were mixed with 2 µg of c-Jun fusion protein beads and incubated with gentle rocking overnight at 4°C. Pellets were washed twice with 500 µl lysis buffer and twice with 500 µl kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na₃VO₄ and 10 mM MgCl₂] and suspended in 50 µl kinase buffer containing 100 µM ATP. After 30 min at 30°C the reaction was stopped by the addition of 20 µl 3×SDS sample buffer. Phosphorylated proteins were resolved on a 15% SDS–polyacrylamide gel, transferred to nitrocellulose membranes and incubated with a 1:1,000 dilution of rabbit polyclonal phospho-specific c-Jun (Ser63) antibody. Detection of the protein–antibody complexes was done as described above. Before addition of the primary antibody, membranes were stained with Coomassie blue to confirm equal loading of sample in each lane.

**Results**

**IAP co-stimulation requires the TCR complex**

We recently have shown that IAP can enhance T cell activation in conjunction with suboptimal anti-CD3. To further study the interaction between TCR and IAP in this synergy, we transfected Jurkat cells with a chimeric molecule consisting of the extracellular domain of CD16 (FcyRIII, an irrelevant Ig family member), the transmembrane domain of CD7 and the TCRζ chain cytoplasmic tail (CD16-7-ζ) (26). Aggregation of this construct leads to its tyrosine phosphorylation, and to cell activation events such as increased [Ca²⁺], and cytotoxicity (26 and data not shown). Surprisingly, Jurkat cells transfected with the CD16-7-ζ chimera failed to produce significant levels of IL-2 at all concentrations of anti-CD16 tested (Fig. 1A). Furthermore, none of the anti-IAP mAb was able to co-stimulate IL-2 production with cross-linking of the CD16 chimera. In contrast, anti-CD28 co-stimulated very efficiently (Fig. 1A). In these same CD16-7-ζ transfected cells, all anti-IAP mAb tested enhanced IL-2 production in conjunction with suboptimal anti-CD3 mAb (Fig. 1B). These data show that IAP is unable to co-stimulate IL-2 production in conjunction with the incomplete TCR signal furnished by the CD16-7-ζ chimera. To confirm this result, we transiently transfected Jurkat cells with an IL-2-LUC reporter, containing the regulatory region of the IL-2 gene fused to the luciferase (LUC) reporter gene (27). Ligation of IAP with plate-bound mAb synergized with low concentrations of anti-CD3 to up-regulate IL-2 transcription, demonstrating that transcriptional activation of the IL-2 gene is involved in IAP synergy with TCR (Fig. 2A). The level of induction mediated by suboptimal anti-CD3 plus anti-IAP was similar to that seen for high concentrations of anti-CD3 (Fig. 2A). As with IL-2 synthesis, no enhancement of IL-2 transcription was observed at any concentration of anti-CD16 mAb tested and no anti-IAP mAb could synergize with anti-CD16 to induce IL-2 transcription, whereas anti-CD28 mAb did so efficiently (Fig. 2B). These experiments support the model that IAP’s role is to enhance the efficiency of productive ligation of the TCR complex. In contrast, CD28’s mechanism of action is distinct, since it can efficiently enhance IL-2 transcription and synthesis together with CD16-7-ζ.
IAP synergizes with TCR but not CD16-7-ζ to activate ERK kinase

To define the reason for the inability of IAP to synergize with CD16-7-ζ, we compared the signaling pathway induced by co-ligation of the TCR complex or CD16-7-ζ with both IAP and CD28. First, we determined the ability of IAP and CD28 to augment ERK activation, which is required for IL-2 transcription and synthesis (28). ERK activation was measured by retardation of migration on SDS–PAGE, reflecting increased phosphorylation, as previously described (29). mAb against IAP and CD28 both synergized with low-concentration anti-CD3 to activate ERK, as compared to the negative control mAb (Fig. 3A). Adhesion to either anti-IAP or anti-CD28 mAb alone did not enhance ERK activation (data not shown). No enhancement of ERK activation by cell adhesion to anti-IAP was seen in conjunction with a suboptimal concentration of anti-CD16 mAb (Fig. 3B), whereas anti-CD28 mAb 9.3 efficiently co-stimulated ERK activation (Fig. 3B). Importantly, coating plates with a high concentration of either anti-CD3 or anti-CD16 induced activation of ERK kinases without ligation of either CD28 or IAP (Fig. 3A and B). This differs from the induction of IL-2 transcription and synthesis, where anti-CD16 was inactive at any concentration. Thus, although IAP can co-stimulate ERK activation with suboptimal anti-CD3, ERK activation alone is insufficient to initiate IL-2 synthesis.

Synergistic activation of JNK by anti-CD3 and anti-IAP

Recently, JNK has been identified as a distinct member of the MAP kinase group (30). This kinase selectively phosphorylates the N-terminal transactivating domain of the c-Jun protein, thereby increasing its transcriptional activity and ultimately leading to enhanced IL-2 synthesis. Experiments by Su et al. (27) showed that activation of JNK is dependent on the integration of at least two signals. In model systems, these signals have been generated either by treatment with phorbol ester and ionomycin or by cross-linking with anti-CD3 and anti-CD28 mAb. To determine whether IAP ligation synergizes
with TCR in JNK activation, we measured the phosphorylation of Gst–c-Jun by lysates of cells adherent to various surfaces. Both anti-IAP mAb and anti-CD28 mAb efficiently augmented JNK activation with suboptimal anti-CD3 (Fig. 4A). Adhesion of Jurkat cells to plates coated with high-concentration anti-CD3 alone also activated JNK (data not shown). In contrast, anti-CD16 failed to activate JNK without co-stimulation at any concentration tested (Fig. 4B and data not shown). However, ligation with high- or low-concentration anti-CD16 in the presence of anti-CD28 mAb efficiently activated JNK (Fig. 4B and data not shown). Co-ligation of CD16 and IAP did not cause JNK activation (Fig. 4B). These data suggest that activation of JNK requires signals mediated by the TCR complex which cannot be generated by the CD16-7-ζ construct. These signals are provided by co-stimulation with anti-CD28, which has been shown to be involved in JNK activation (27), but are not provided by co-ligation of IAP.

**Anti-IAP mAb induces Jurkat cell spreading**

One difference between IAP and CD28 in T cell activation is that while CD28 and TCR can be engaged on opposite cell surfaces, IAP synergy requires ligation on the antigen-presenting surface (11). A further difference between these two activation stimuli is the ability of IAP, but not CD28, to augment early signaling events arising from TCR ligation (11). Together with IAP’s inability to enhance IL-2 production in conjunction with the incomplete TCR signal provided by CD16-7-ζ, these findings suggest that IAP’s role could be to increase the efficiency of the TCR signal, perhaps by increasing effective ligation of the TCR complex by suboptimal ligand. For this reason we investigated if ligation of IAP on Jurkat cells induced cell spreading, since increased cell area in contact with ligand could enhance presentation to TCR. Jurkat cells were allowed to adhere to plates coated with anti-IAP (2E11, 2D3, B6H12 or 2B7), anti-CD28 (9.3) or control (W6/32; IB4) mAb, either with or without co-immobilized low-concentration anti-CD3 (0.05 µg/ml) or a low concentration of anti-CD16 (0.05 µg/ml) co-immobilized with anti-IAP, anti-CD28 or control (MOPC) mAb. Cell lysates were incubated with 2 µg Gst–c-Jun (1–89) agarose beads for 12 h at 4°C. After extensive washing, the beads were incubated in kinase buffer for 30 min at 30°C, after which proteins were separated by SDS–PAGE followed by Western blotting with phospho-specific c-jun (Ser63) antibody. (B) Jurkat clones transfected with CD16-7-ζ were stimulated for 30 min at 37°C with a high concentration of anti-CD16 (5 µg/ml) or a low concentration of anti-CD16 (0.05 µg/ml) co-immobilized with anti-CD28, anti-IAP or control (MOPC) mAb. Cells were treated for 15 min at 37°C with 50 µg/ml phorbol myristate acetate and 1 mM ionomycin for positive control. Activation of JNK kinase activity was analyzed as described in (A). Experiments shown are representative of more than three with similar results.
IAP and CD28 in T cell activation

Fig. 5. Spreading of Jurkat cells on anti-IAP, but not anti-CD28. Jurkat cells were allowed to spread at 37°C on plates coated with anti-IAP (2E11; B6H12), anti-CD28 (9.3) or control anti-β2 (IB4) mAb. Pictures were taken 2 h after cells were added to the plate.

The IAP extracellular and transmembrane domains are required for spreading of Jurkat cells

To determine which domain(s) of IAP were required for spreading we utilized an IAP-deficient Jurkat cell line, which was generated by chemical mutagenesis (see Methods). An IAP-deficient subclone of these cells, JinB8, was used for subsequent studies. This clone expressed no IAP by FACS analysis (Fig. 6). Expression levels of other cell surface molecules, such as MHC class I (W6/32) or the β2 integrin (IB4), were not affected (Fig. 6). Expression level of the TCR complex declined when the clones were maintained in culture for an extended period of time and transfection of these cells with IAP did not stabilize cell surface expression of TCR (data not shown). All cells were sorted for high levels of CD3 expression prior to analysis (Fig. 6). JinB8 cells were transfected with wild-type human IAP (huIAP form 2), huIAP form 1, which has only 4 amino acids in its cytoplasmic tail, and huIAP/CD7, which consists of the IAP extracellular domain anchored into the membrane via the CD7 transmembrane domain (see Fig. 7A). Expression levels of the transfected constructs were similar as determined by FACS staining (Fig. 7B). Transfection of this clone with the expression vector alone failed to generate IAP⁺ cells, confirming that the IAP expressed in the transfectants was a product of the input DNA. Anti-IAP mAb 2E11 and 2D3 synergized with suboptimal concentrations of anti-CD3 to enhance IL-2 synthesis in cells transfected with huIAP form 1 and form 2, but not in cells transfected with the huIAP–CD7 construct (Table 1 and data not shown). These data are entirely consistent with the structure–function studies previously reported for human IAP transfected into the murine 3L2 T cell hybridoma (11).

When JinB8 cells expressing huIAP form 1 or form 2 adhered to plates coated with anti-IAP mAb [2E11 (Fig. 8A), 2B7 and B6H12 (not shown)] with or without anti-CD3, they were able to spread. No spreading was observed on plates coated with anti-CD28 [9.3 (Fig. 8B) or anti-MHC class I (not shown) mAb] with or without anti-CD3. In contrast, JinB8 cells transfected with huIAP/CD7 did not spread on anti-IAP mAb (Fig. 8C) with or without anti-CD3. Similarly, Jurkat cells transfected with FLAG-TM2, a chimeric molecule in which the entire IAP extracellular domain was replaced by the FLAG epitope (Fig. 7), were unable to spread when allowed to adhere to plates coated with anti-FLAG mAb (Fig. 8F), whether or not anti-CD3 was co-immobilized. Furthermore, no IL-2 synthesis was observed when these same transfected Jurkat clones were allowed to adhere to plates coated with sub-
optimal anti-CD3 plus anti-FLAG mAb (Table 1). These Jurkat transfectants could still respond to anti-IAP (Table 1). The FLAG epitope was accessible to the antibody, as shown by FACS (Fig. 7B). These data show that both the IAP extracellular and transmembrane domains are required for spreading, while the cytoplasmic tail is dispensable. Thus, the domains required for IAP’s ability to induce cell spreading and to enhance IL-2 production (11) (Table 1) with suboptimal anti-CD3 were identical, suggesting a close correlation between IAP’s ability to induce cell spreading and its synergy with TCR. These data suggest the hypothesis that the main effect of IAP ligation is to increase the frequency of productive ligation of the TCR by increasing T cell spreading on the antigen-presenting surface.

Discussion

Full activation of T cells requires the participation of accessory or co-stimulatory molecules. Recently, we have shown that IAP can function to augment T cell activation (11). In recent years, much has been learned about the signal mediated by the TCR. In contrast, the mechanisms by which co-stimulators function, as well as their signaling pathways, are still poorly understood. This is true even of CD28, the most extensively studied co-stimulator to date. The object of the present study was to further analyze the role of IAP in T cell activation and to determine its relationship to CD28 and TCR signals.

Detailed analysis of signaling events showed that IAP enhances IL-2 production in the presence of low concentrations of anti-CD3 by activation of the MAP kinases ERK1/2 and JNK, resulting in increased IL-2 transcription. Unlike CD28, which has identical biochemical effects, IAP may augment activation by increasing the efficiency of antigen receptor signaling. Supporting this hypothesis is (i) IAP co-stimulation of early events in TCR signaling (11), (ii) IAP conversion of antagonist to agonist peptides (11), suggesting an enhancement of signal strength (a property not shared by CD28), and (iii) as demonstrated in this study, the inability of IAP, unlike CD28, to generate new signals when co-ligated with CD16-7ζ. A possible mechanism by which IAP ligation may enhance the efficiency of antigen receptor activation is suggested by the discovery that Jurkat cells will spread on a surface coated with anti-IAP. When the surface is coated with anti-CD3 as well, it is likely that increased CD3 engagement will occur either because the cell surface in contact with the activating ligand is increased or because adhesion to the activating surface is tighter. On an appropriate antigen-presenting cell (APC), ligand for IAP also could induce an increased interaction between T cell and APC. Actin filament-dependent spreading induced by IAP ligation could prolong the time of contact between individual TCR molecules and the MHC–peptide complex on the APC as well as the number of contacts attained. In its ability to induce cell spreading, IAP ligation differs dramatically from CD28, which co-stimulates without causing cell spreading. It may be that IAP shares this mechanism of action with other adhesion-dependent accessory molecules, such as LFA-1 or integrin extracellular matrix receptors (10,32,33).

To test this hypothesis, we determined which IAP domains were required for Jurkat spreading. A previous study had demonstrated a requirement for both the Ig and the multiply membrane-spanning domain of IAP in T cell activation (11).
If spreading on the antigen-coated surface was important in synergy with TCR, the same domains should be required for spreading. Indeed, our structure–function studies on IAP demonstrated a requirement for both the extracellular IgV and the multiply membrane-spanning domains for cell spreading, without need for the IAP cytoplasmic tail, providing a link...
between spreading and IAP–TCR synergy. The fact that TCR-
deficient Jurkat cells spread on anti-IAP-coated plates and
that normal Jurkat cells do not spread on the low concen-
trations of anti-CD3 used in these experiments rules out an
essential role for the TCR in IAP-mediated cell spreading.
The requirement for the IAP multiply membrane-spanning
domain in cell spreading suggests that it may mediate con-
tacts with cytosolic or other membrane proteins to signal actin filament assembly. How this occurs is not yet known. While interaction with lymphocyte integrins is an appealing hypo-
thesis, failure of divergent cation chelators to affect IAP-
dependent spreading does not support this model.

The requirement for the Ig domain of IAP in spreading is
quite surprising, since this domain is separated from the
cytoskeleton by a plasma membrane. The only known roles
for the IAP Ig domain are in binding thrombospondin (34)
and in lateral interaction with α, integrins (19). The requirement
for the Ig domain in both T cell activation and spreading
suggests either that there are additional unknown lateral
interactions of the IAP Ig domain or that the Ig domain
is required for appropriate conformation and cytoskeletal
association of the multiply membrane-spanning domain. Fur-
ther work will be required to distinguish these possibilities.

The TCR complex is composed of six different polypeptide
chains, including a ligand binding heterodimer (αβ or γδ)
and the non-polymorphic CD3 (ε, γ, δ) and ζ chains. The complexity
of the TCR may be means for amplifying the TCR signal
(35), since chimeric molecules containing a single functional
domain of ζ are sufficient, although less potent, to induce signaling (36). Why the CD16-7-ζ construct used in this study
does not enhance IL-2 production is uncertain. It is possible
that, since this construct exists as a monomer on the cell
surface, whereas the TCR ζ chain naturally exists as a
dimer, it fails to generate a fully activating signal because of
differences in antibody-induced clustering. Chimeras in which
the ζ chain has been linked to the CD8 or IL-2 receptor
extracellular and transmembrane domains do form dimers
and are able to induce IL-2 production after antibody cross-
linking, albeit less efficiently than the TCR complex (37,38).
An alternative explanation for the failure of CD16-7-ζ cross-
linking to signal IL-2 transcription may be that ITAMs are
bound differentially by different signaling molecules, such as
Zap70, phospholipase Cγ1 or shc (36,39,40) and that some
essential component is not recruited by cross-linking the
chimeric receptor. Whatever its basis, the finding that activa-
tion with high concentrations of anti-CD16 mAb did not activate JNK or induce IL-2 production provided a mechanism
for further distinguishing the adhesion-dependent effect of
IAP on T cell activation from CD28.

The defect in signaling from the CD16-7-ζ chimera is its
inability to activate JNK, which presumably leads to failure
to activate c-Jun and therefore to form the AP-1 transcription
complex which is required for IL-2 transcription. Our data
clearly demonstrate that co-ligation of CD28 provides a
sufficient signal for JNK activation by CD16-7-ζ, while co-
ligation of IAP does not. An essential role for CD28 in co-
stimulation of JNK has been suggested previously (27). Importantly, cross-linking of CD28 alone does not activate
JNK, so signals from ligation of the chimeric ζ chain
receptor are clearly required for this activation. This is
consistent with the model that JNK is involved in the
integration of TCR and CD28 signals during T cell activation,
leading to enhanced IL-2 production (27). Furthermore, the
ability of CD28 to synergize with the chimeric construct
shows that co-stimulation with CD28 imparts new signaling
activities to cross-linked CD16-7-ζ rather than simply
enhancing the efficiency of the signaling from the chimeric
molecule, a property which further distinguishes the mechan-
ism of co-stimulation by IAP and CD28. It is possible that
upon IAP ligation, IAP associates with specific signaling
molecules or regulators of cytoskeletal organization. These
proteins may then be able to relay the signal into the cell,
resulting in cytoskeletal reorganization. In support of this
hypothesis is the observation that IAP has been found to
associate with a src kinase family member (12) and Ge
subunits (41). However, as proven by the failure of IAP to
co-stimulate the CD16-7-ζ chimera, any putative IAP-
derived signals cannot contribute directly to the T cell
mitogenic pathway.

In summary, a detailed study of the pathways arising
from ligation of IAP or CD28 with TCR demonstrates that
they differ in several fundamental aspects. CD28 can co-
stimulate IL-2 synthesis together with a CD16-7-ζ chimera,
while IAP cannot, suggesting that CD28 has an ability to
complement the ζ chain signal not shared by IAP. In
contrast, IAP augments early events in T cell activation and
induces cell spreading, properties not shared by CD28.
These data are completely consistent with the hypothesis
that IAP ligation induces increased T cell contact with an
antigen-presenting surface but that the mitogenic signal
arises entirely from TCR ligation. Thus, unlike CD28, IAP
can increase the sensitivity of even the earliest events to
suboptimal TCR ligation. On the other hand, ligation of IAP
and TCR cannot reconstitute a mitogenic signal from a

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### Table 1. IL-2 synthesis by JinB8 and Jurkat clone transfectants

<table>
<thead>
<tr>
<th>Transfectant</th>
<th>Anti-CD3lo</th>
<th>Anti-CD3lo + IAP</th>
<th>Anti-CD3lo + control</th>
<th>Anti-CD3lo + M2</th>
</tr>
</thead>
<tbody>
<tr>
<td>JinB8-huIAP1</td>
<td>3521 ± 645</td>
<td>14624 ± 1445</td>
<td>5522 ± 1330</td>
<td></td>
</tr>
<tr>
<td>JinB8-huIAP/CD7</td>
<td>5298 ± 1136</td>
<td>4598 ± 1412</td>
<td>6170 ± 3072</td>
<td></td>
</tr>
<tr>
<td>Jurkat–FLAG-TM2</td>
<td>17209 ± 2090</td>
<td>6290 ± 798</td>
<td>6218 ± 360</td>
<td></td>
</tr>
</tbody>
</table>

JinB8 and Jurkat cell transfectants were tested for IL-2 synthesis when allowed to adhere to plates coated with anti-CD3lo (1) with or without anti-IAP (2E11), control (W6/32) or anti-FLAG (M2) mAb. Experimental conditions are as in Fig. 1 and the values shown represent c.p.m. of triplicates of [3H]thymidine incorporation by the CTLL-2 cells in one experiment of more than three with similar results.
IAP and CD28 in T cell activation

Fig. 8. Both the IAP extracellular and transmembrane domain are required for spreading on antibody-coated surfaces. JinB8 (A–D) or wild-type Jurkat clones (E and F), transfected with huIAP form 1 (A and B), IAP/CD7 (C and D) or FLAG-TM2 (E and F), were allowed to spread on surfaces coated with anti-IAP (2E11; A, C and E), anti-CD28 (9.3; B and D) or anti-FLAG (M2) mAb (F) in conjunction with low concentrations of anti-CD3 (OKT3) mAb. Both the Ig domain and the multiply membrane-spanning domains of IAP are necessary for spreading on anti-IAP.

signaling-deficient TCR. It will be important to determine by examining other adhesion-dependent co-activators such as ICAM-1, fibronectin and collagen, whether this difference between IAP and CD28 is the fundamental distinction between those co-stimulators that act by enhancing adhesion and those that generate TCR-independent signals.
Acknowledgements

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Abbreviations

- APC: antigen-presenting cell
- ERK: extracellular-signal-regulated kinase
- IAP: integrin-associated protein
- IgV: Ig variable
- JNK: c-Jun N-terminal kinase

References


