The Role of p21\textsuperscript{ras} in CD28 Signal Transduction:
Triggering of CD28 with Antibodies, but Not the Ligand B7-1, Activates p21\textsuperscript{ras}

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Summary
CD28 is a 44-kD homodimer expressed on the surface of the majority of human T cells that provides an important costimulus for T cell activation. The biochemical basis of the CD28 accessory signals is poorly understood. Triggering of the T cell antigen receptor (TCR) activates the p21\textsuperscript{ras} proteins. Here we show that ligation of CD28 by a monoclonal antibody (mAb) also stimulates p21\textsuperscript{ras} and induces Ras-dependent events such as stimulation of the microtubule-associated protein (MAP) kinase ERK2 and hyperphosphorylation of Raf-1. One physiological ligand for CD28 is the molecule B7-1. In contrast to the effect of CD28 mAb, the present studies show that interactions between CD28 and B7-1 do not stimulate p21\textsuperscript{ras} signaling pathways. Two substrates for TCR-regulated protein tyrosine kinases (PTKs) have been implicated in p21\textsuperscript{ras} activation in T cells: p95\textsuperscript{Vav} and a 36-kD protein that associates with a complex of Grb2 and the Ras exchange protein Sos. Triggering CD28 with both antibodies and B7-1 activates cellular PTKs, and we have exploited the differences between antibodies and B7-1 for p21\textsuperscript{ras} activation in an attempt to identify critical PTK-controlled events for Ras activation in T cells. The data show that antibodies against TCR or CD28 induce tyrosine phosphorylation of both Vav and p36. B7-1 also induces Vav tyrosine phosphorylation but has no apparent effect on tyrosine phosphorylation of the Grb2-associated p36 protein. The intensity of the Vav tyrosine phosphorylation is greater in B7-1 than in TCR-stimulated cells. Moreover the kinetics of Vav tyrosine phosphorylation is prolonged in the B7-1-stimulated cells. These studies show that for CD28 signaling, the activation of p21\textsuperscript{ras} correlates more closely with p36 tyrosine phosphorylation than with Vav tyrosine phosphorylation. However, the experiments demonstrate that Vav is a major substrate for B7-activated PTKs and hence could be important in CD28 signal transduction pathway.

Lymphocyte activation is initiated when the T cell encounters antigen/major histocompatibility molecules presented to the T cell on the surface of an APC. The signals generated by the TCR ensure the immune specificity of T cell activation, but for a full immune response there is a requirement for additional signals triggered by accessory molecules present on the surface of the APC (1, 2). A large number of molecules participate in the interaction between the T cell and the APC, including molecules like CD4 and CD8, as well as adhesion molecules and integrins such as CD2 and LFA-1. However, there is considerable evidence that CD28, a 44-kD homodimeric glycoprotein expressed by most mature T lymphocytes, is one of the more important receptors for the generation of the "second signal" for T cell activation (3). Two physiological ligands for CD28 have been described: B7-1 (B7/BB1) and B7-2 (B70) (4–8). These B7 molecules are expressed by APCs and their interactions with CD28 have been shown to costimulate T cells in cooperation with engagement of the TCR (8, 9).

Triggering of the TCR/CD3 complex initiates a cascade of biochemical events, the earliest of which is activation of intracellular protein tyrosine kinases (PTKs)\textsuperscript{1} (10). This activation of PTKs appears to be absolutely required for all subsequent T cell responses (11, 12). One substrate for TCR-stimulated PTKs is an inositol lipid-specific phospholipase C, PLC\textsubscript{y1} (13). Hence PTKs enable the TCR to regulate the hydrolysis of membrane phosphoinositides (PtdIns),

\textsuperscript{1} Abbreviations used in this paper: EGFR, epidermal growth factor receptor; GST, glutathione S-transferase; MAP, microtubule-associated protein; MBP, myelin basic protein; PDBu, phorbol 12, 13-dibutyrate; PKC, protein kinase C; PLC, phospholipase C; PtdIns, phosphoinositides; PTK, protein tyrosine kinase; SH, Src homology.
releasing diacylglycerols (DAG) that activate the serine/threonine kinase, protein kinase C (PKC) (14–16), and inositol polyphosphates that regulate intracellular Ca^{2+}.

The TCR also stimulates the guanine nucleotide binding proteins p21^{ras} (17, 18), which then couple the TCR to the MAP kinase ERK2 and hence to the regulation of transcriptional factors such as AP-1, NFAT, and the IL-2 gene (19–21). TCR activation of p21^{ras} requires cellular PTK function and recent studies have identified two PTK substrates, Vav and a 36-kD membrane protein, as potential mediators of the TCR/p21^{ras} link (22, 23). In fibroblasts, PTKs stimulate p21^{ras} by regulating the activity of Ras GTP/GDP exchange proteins. T cells express two potential Ras guanine nucleotide exchange proteins: Sos, a homologue of the Drosophila Son of Sevenless gene product (24), and Vav, which has in vitro guanine nucleotide exchange activity. Vav is expressed in a 36-kD membrane protein that is substrate for the TCR-induced PTKs (23). This complex is analogous to the Sos/Grb2 epidermal growth factor receptor (EGFR) complex that localizes Sos to the plasma membrane in fibroblasts.

Little is known about the CD28 costimulatory signals relative to the wealth of knowledge about TCR signal transduction. CD28 signal transduction appears to be controlled by PTKs and it has been described that CD28 regulates PLCγ1 tyrosine phosphorylation, PtdIns hydrolysis, and intracellular Ca^{2+} levels (30–32). However, calcium signaling pathways in T cells are sensitive to inhibition by the immunosuppressive drugs cyclosporin A (CsA) and FK506 and one characteristic of CD28 costimulatory signals is that they are not susceptible to inhibition by CsA (33). The role of Ras in CD28 signaling has never been studied, although recent studies have shown unequivocally that p21^{ras} function is essential for T cell activation. Moreover, T cells transfected with v-Ha-ras, a constitutively activated Ras mutant, are hyperresponsive to TCR ligation (20, 21). This latter observation in particular has prompted us to examine the effect of CD28 on the p21^{ras} pathway.

The present data show that there is the potential for CD28 to couple to p21^{ras} because ligation of CD28 with specific antibodies induces the accumulation of Ras/GTP complexes, the hyperphosphorylation of a MAP kinase kinase kinase (MAPKKK) Raf-1 and the activation of a MAP kinase ERK2. CD28 antibodies also induce the tyrosine phosphorylation of Vav and the Sos/Grb2-associated protein, p36. In contrast, the costimulatory interaction between CD28 and its natural ligand B7-1 did not induce the Ras signaling pathway. B7-1 was able to induce tyrosine phosphorylation of Vav and the failure to activate Ras was correlated with a failure of B7-1 to induce tyrosine phosphorylation of p36. Studies of Vav tyrosine phosphorylation revealed that TCR ligation induces a relatively weak, rapid, and transient phosphorylation of Vav. In contrast, the Vav tyrosine phosphorylation monitored in B7-1–activated cells is markedly stronger and prolonged.

### Materials and Methods

**Cell Lines.** The subclone of the Jurkat T leukemia cells, J.H6.2 was maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS. The Jurkat J.H6.2 cell line has been described (34) and this cell line does not express the other ligand for B7 molecules, CTLA-4 (data not shown). huB7+L cells were obtained by transfection of the human B7-1 cDNA in murine L cells (A. Truneh, manuscript in preparation) and selected by G418 resistance. These cells were maintained in DMEM supplemented with 10% FCS, penicillin, streptomycin, and glutamine. For T cell stimulations, L cells were detached from the tissue culture flasks by incubation with trypsin/EDTA, washed once in PBS then once in RPMI medium, and resuspended in RPMI medium. Untransfected L cells were used as control cells.

**Antibodies and Fusion Proteins.** mAb UCHT1 reactive with the e chain of the human TCR/CD3 antigen complex and mAb CD28.2 or CD28.5 reactive with the human CD28 molecule (34) were purified from hybridoma supernatants by protein A–Sepharose. To stimulate cells, UCHT1 or CD28.2 were used at 10 μg/ml and CD28.5 at 50 μg/ml. The ascites of the anti-B7 mAb 104 is a kind gift of J. Banchereau (Schering Plough, Dardilly, France) and was used at 1 in 100.

The mAb Y13-259 was used to immunoprecipitate p21^{ras} (17). The rabbit antisera 66 was used to detect p74^{K} in immunoblotting (35) and the rabbit antisera 122 was used to immunoprecipitate and to detect p42^{MAPK} (36). The monoclonal antiphosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The monoclonal anti-Grb2 antibody was purchased from AFFINITI (Nottingham, UK). The antipeptide antisera VAV-1 used to immunoprecipitate and detect in immunoblotting the p95^{Vav} protein was described previously (37).

The fusion protein encoding glutathion-s-transferase (GST) mSos1 COOH terminus (residues 1135–1336) was coupled to glutathione beads and used to purify Grb2 as described (23, 26).

**Assay of p21^{ras} Activation.** p21^{ras} proteins were immunoprecipitated with the antibody Y13-259 from cells in which guanine nucleotides were labeled biosynthetically with $[^{32}P]3$ orthophosphate (17). Labeled guanine nucleotides bound to p21^{ras} proteins were eluted, separated by TLC, and then quantitated by direct scanning for β emissions (AMBIS Systems Inc., San Diego, CA). Results are expressed as the percentage of p21^{ras} proteins bound to GTP relative to total guanine nucleotides complexed to the protein.

**Immunoprecipitation and Immunoblotting.** The Jurkat T cells were removed from culture, washed twice in RPMI 1640, and suspended at $4 \times 10^6$ cells/ml in RPMI medium. The cells were prewarmed at 37°C for 3 min. Cells were stimulated at 37°C, then pelleted in a microcentrifuge, and lyzed in a buffer containing 50 mM Hepes (pH 7.4), 1% NP-40, 150 mM NaCl, 20 mM NaF, 20 mM iodoacetamide, 1 mM PMSF, 1 μM protease inhibitors (leupeptin, pepstatin A, chymotrypsin), and 1 mM Na3VO4.

Acetone-precipitated proteins from cell lysates corresponding to $4 \times 10^6$ Jurkat cells were resolved in either 10% SDS-PAGE (for detection of ERK2) or 8% SDS-PAGE standard gels (for detection of other proteins).

For immunoprecipitation, lysates were clarified and incubated with purified anti-Vav antibody coupled to protein A–Sepharose or 5 μg of GST fusion protein immobilized on glutathione beads for 2 h at 4°C. Immunoprecipitates or precipitates were washed...
four times in 1 ml of lysis buffer and then boiled in reducing SDS gel sample buffer for 5 min. Samples were resolved by 8% SDS-PAGE standard gels.

For immunoblotting, membranes were blocked and probed with specific antibodies. Blots were then incubated with the appropriate second antibodies, anti-rabbit IgG or anti-mouse IgG (Amersham International, Little Chalfont, UK), all conjugated with horseradish peroxidase. Immunoreactive bands were visualized by enhanced chemiluminescence (Amersham International).

Analysis of MAP Kinase Activity in ERK2 Immunoprecipitates. ERK2 was immunoprecipitated with a specific antisera and kinase activity of ERK2 assessed as described previously (36).

Briefly 4 x 10^6 Jurkat T cells were stimulated at 37°C as indicated, then pelleted and lysed in a buffer containing 50 mM Hepes (pH 7.4), 1% NP-40, 150 mM NaCl, 20 mM NaF, 20 mM iodoacetamide, 1 mM PMSF, 1 μg/ml protease inhibitors (leupeptin, pepstatin A, chymotrypsin), and 1 mM Na3VO4. After 10 min of preclarifying with protein A insoluble suspension (Sigma Chemical Co., St. Louis, MO), then 10 min of preclarifying with protein A-Sepharose beads (Pharmacia LKB, Uppsala, Sweden), lysates were incubated for 2 h at 4°C with 10 μl of the 122 antisera precoupled to 50 μl of a 50% suspension of protein A-Sepharose beads.

Immunoprecipitates were washed three times with lysis buffer and once with kinase assay buffer (30 mM Tris [pH 8], 20 mM MgCl2, and 2 mM MnCl2). In vitro kinase assay buffer was carried out for 30 min at 30°C in 20 μl of kinase assay buffer supplemented with 10 μM ATP, 5 μCi γ-[32P]ATP, and 10 μg m-ethyl basic protein as a substrate. The kinase reaction was stopped with 20 μl of 2 × SDS sample buffer, and samples were run in 15% SDS-PAGE gels. Quantitation of 3P incorporated into the MBP protein band was done by β radiation scanning of dried gels using a scanner (AMBIS Systems Inc.).

IL2 Production. Jurkat cells were washed several times in culture medium, then resuspended at a concentration of 0.5 x 10⁶/ml. Duplicate cultures (200 μl) in 96-well flat-bottomed plates were set up in the presence of saturating concentrations of mAbs or PHA (10 μg/ml). Purified PHA was supplied by Industries Biologiques Française (Villeneuve-la-Garenne, France). The L cells were used at a concentration of 0.25 x 10⁶/ml. After 18 h, supernatants were collected and stored at −80°C for IL2 assay. IL2 concentrations were measured by ELISA, using a commercially available kit (Immunotech S.A., Marseille, France) according to the manufacturer's instructions.

Results

CD28 Triggering Activates p21ras in Jurkat Cells. T cells can be activated via the CD28 pathway by triggering cells with CD28 antibodies. To determine whether the p21ras guanine nucleotide binding cycle is regulated via the CD28 molecule, p21ras/GTP loading experiments were performed. Endogenous guanine nucleotides were metabolically labeled with [32P]orthophosphate and p21ras protein was immunoprecipitated from Jurkat cells exposed to antibodies against CD28 or the CD3/TCR complex, and the phorbol ester phorbol 12,13-dibutrylate (PDBu) that directly activates PKC.

The mAb used to trigger the CD3/TCR complex was UCHT1. Two CD28 mAbs were used, CD28.2, which can induce a IL-2 secretion in Jurkat cells and CD28.5, which is nonfunctional in this respect, and that apparently binds to CD28 without triggering CD28 signal transduction (34).

The proportion of p21ras bound to GTP (the ratio between GTP and GTP plus GDP-labeled nucleotides on p21ras) was evaluated by β emission scanning of TLC-resolved guanine nucleotides eluted from immunoprecipitated ras proteins. Fig. 1 A shows that PKC stimulation with PDBu increased the level of GTP bound to ras from 11% in unstimulated Jurkat cells to 40% (fourfold). CD28 triggering with CD28.2 resulted in a threefold increase in the proportion of GTP-bound ras (from 11 to 29%), this increase is similar to the increase after CD3/TCR triggering with UCHT1. The CD28 mAb, CD28.5 did not induce an increase in the proportion of GTP-bound ras. The kinetics of the increase in the proportion of GTP-bound ras are shown in Fig. 1 C. The TCR response peaked after 1 min, whereas the CD28 response was maximal at 5 min. Both TCR and CD28 responses could still be detected 30 min after CD3/TCR or CD28 stimulation.

CD28 Triggering Induces the Phosphorylation of ERK2 and RAF. In many cell systems, p21ras couples receptor stimulated PTKs to Raf-1 and hence to the MAP kinase cascade. The predominant MAP kinase in T cells is ERK2 and p21ras plays a crucial role in the TCR/ERK2 link. The activation of ERK2 can be monitored by Western blot analysis with an ERK2-specific antiseraure because the activation of this kinase requires its phosphorylation on threonine and tyrosine residues and the phosphorylated "active" ERK2 has a reduced mobility on SDS-PAGE gels compared with nonphosphorylated, "inactive" ERK2 (36). The experiment in Fig. 2 shows the reduced mobility of ERK2 in Jurkat cells treated with the PKC activator, PDBu, a CD3 mAb UCHT1 and CD28 mAbs compared with control unstimulated cells. Stimulation with UCHT1 or CD28.2 rapidly induced a mobility shift in the band corresponding to ERK2 that was maximal between 5 and 30 min of exposure to the antibody. PDBu also induced a rapid shift in ERK2 mobility in Jurkat cells.

Previous studies have shown that Raf-1 is activated rapidly in TCR- and phorbol ester-stimulated T cells (38). It is also well documented that Raf-1 is hyperphosphorylated in response to T cell activation (39, 40) and this hyperphosphorylation of Raf-1 correlates with activation of the enzyme although it is not directly involved in mediating the increase in Raf-1 enzymatic activity (41). The stimulation of Raf-1 has been recently shown in peripheral blood T cells after CD28 mAb cross-linking (38). Hyperphosphorylated Raf-1 has reduced mobility on SDS-PAGE gels hence to determine whether Raf-1 is hyperphosphorylated in Jurkat cells, a Western blot of cell lysates prepared from cells treated with UCHT1, CD28 mAbs, or PDBu was probed with a Raf-1 antisera. The data in Fig. 2 show the UCHT1, CD28.2, and PDBu induce a shift in Raf-1 mobility that is comparable with the ERK2 response. The CD28 mAb, CD28.5, which does not costimulate T cells, did not induce a detectable hyperphosphorylation of Raf-1 or ERK2.

B7-1-Transfected L Cells Induce Tyrosine Phosphorylation of Cellular Substrates and IL-2 Secretion, but Not RAS Activation or RAF and ERK2 Phosphorylations. It has been shown that the natural ligand for CD28, B7-1, expressed on the surface of Chinese hamster ovary cells can provide costimulation for T cells, replacing the effects seen with CD28 mAbs (9).
determine whether IL-2 secretion could be induced by ligand of CD28 with B7-1 in Jurkat cells, the culture supernatants from cells stimulated with PHA in the presence of L cells transfected with human B7-1 (huB7 + L cells) or CD28.2 mAb were collected and assayed for IL-2 production. A small amount of IL-2 was detected when the Jurkat cells were stimulated with PHA alone (Fig. 3). When CD28.2 or huB7 + L cells were added to PHA-stimulated Jurkat cells, IL-2 secretion was markedly increased (20-fold). The addition of anti-B7 mAb 104 blocked the stimulation of IL-2 production by huB7 + L cells.

The experiment in Fig. 4 A compares the effect of huB7 + L cells, UCHT1, CD28.2, and PDBu on Ras/GTP loading. The data show that PDBu, UCHT1, and CD28.2 induced an increase in cellular levels of active Ras/GTP complexes, whereas huB7 + L cells have no effect. In parallel experiments (Fig. 4 B), the phosphorylation of Raf-1 and ERK2 in B7-1–stimulated T cells was monitored. UCHT1, CD28.2, or PDBu induced an increase of the apparent molecular weight of p74raf-1 or p42erk2. However, over a 30-min time course, there was no detectable effect of huB7 + L cells on Raf-1 or ERK2 electrophoretic mobility.

To examine the consequences of CD28 ligation on ERK2 activity, MBP kinase assays were carried out on MAP kinase immunoprecipitates. The data in Fig. 4 C show that UCHT1, PDBu, and CD28.2 induced an approximate two- to three-fold increase in ERK2 kinase activity in Jurkat T cells, whereas huB7 + L cells have no effect.

It has been described previously that CD28 ligation activates cellular PTKs (42, 43). Accordingly, we probed the Raf-1 and ERK2 blots with an antiphosphotyrosine antibody. The data in Fig. 4 D show that a 95-kD tyrosine phosphoprotein was detected in Jurkat cells within 5 min after contact of the cells with huB7 + L cells. The results of the antiphosphotyrosine Western blot show that the failure to detect an effect of huB7 + L cells on Raf-1 or ERK2 was not due to the failure of B7-1 to trigger CD28. In these experimental conditions, we can not detect tyrosine phosphoproteins in lysates from L cells alone and the pattern of tyrosine phosphorylations in Jurkat cells was the same with paraformaldehyde-fixed huB7 + L cells or not fixed huB7 + L cells (data not shown). The 95-kD tyrosine phosphoprotein

Figure 1. p21ras is activated in Jurkat cells stimulated by the CD28 mAb CD28.2. (A) TLC of the nucleotides eluted from immunoprecipitates of p21ras from [32p]orthophosphate-labeled Jurkat cells. The cells were unstimulated (control) or stimulated for 10 min with a CD3 mAb UCHT1 (10 μg/ml), CD28 mAbs, (CD28.2 [10 μg/ml] or CD28.5 [50 μg/ml]), and PDBu (100 ng/ml) before lysis and immunoprecipitations of p21ras with Y13-259 as described. The position at which GTP and GDP standards ran is indicated. (B) The figure shows the quantitation of guanine nucleotides of p21ras by direct scanning of β radiation from the TLC in A. Data are expressed as percentage of GTP to ras with respect to the total amount of guanine nucleotide on p21ras. The data given are representative of the results obtained from at least five experiments. (C) Kinetics of p21ras activation after T cell activation with UCHT1 or CD28.2. Time-course showing the nucleotides bound to Ras upon stimulation of Jurkat cells with UCHT1 (10 μg/ml) or CD28.2 (10 μg/ml). Nucleotides were separated by TLC and quantitated by direct scanning for β radiation.
was also detected after stimulation of T cells with UCHT1 or CD28.2. However, the pattern and intensity of tyrosine phosphorylation in TCR- or CD28-activated T cells was different. Some tyrosine phosphoproteins were common to TCR- and CD28-induced cells but in general the level of phosphorylation was stronger in the TCR-activated cells. There was one tyrosine phosphoprotein of approximately 65 kD that was detected in the B7-1 stimulated cells but not in TCR-stimulated Jurkat. This molecule could be a unique substrate for CD28-activated PTKs.

Stimulation of Jurkat Cells through the CD28 Molecule by a Specific mAb or the Ligand B7-1 Induces a Tyrosine Phosphorylation of VAV. One candidate for the 95-kD tyrosine phosphoprotein in the TCR- and CD28-activated cells is p95\textsuperscript{Vav}, which is known to be tyrosine-phosphorylated in response to TCR triggering (37, 44). The stimulation of p21\textsuperscript{ras} requires cellular PTK function, and since Vav has been described to act as a guanine nucleotide exchange protein for ras in vitro, it has been proposed that Vav tyrosine phosphorylation is an important event for Ras regulation (22). We therefore compared the tyrosine phosphorylation of Vav in TCR- or CD28-activated cells. For these experiments, antiphosphotyrosine immunoblots were performed on Vav immunoprecipitates. In the experiment shown in Fig. 5 A, a weak basal tyrosine phosphorylation of Vav was detectable in Jurkat cells. UCHT1 and CD28.2 both induced a rapid increase in Vav tyrosine phosphorylation that was maximal at 1 min in the TCR-activated cells and 5 min in the CD28.2-stimulated population. There was no change in Vav tyrosine phosphorylation in cells treated with CD28.5.

The data in Fig. 5 B show the effect of the CD28 ligand B7-1 on the tyrosine phosphorylation of Vav. In this experiment, the immunoblots were probed with an antiphosphotyrosine antibody and then reprobed with an anti-Vav antibody to control the presence of Vav in all immunoprecipitates. The data show that huB7\textsuperscript{+} L cells do not express Vav but are able to induce a strong tyrosine phosphorylation of Vav in T cells, that was detectable within 30 s and maintained for 10 min. Untransfected L cells did not have this effect. The level of tyrosine phosphorylated Vav in cells triggered by B7-1 was markedly higher than the maximal phosphorylation seen in cells activated with UCHT1 or CD28.2 (Fig. 5 B). In addition, the increase in Vav tyrosine phosphorylation was a more prolonged response in the B7-1-stimulated cells.

The COOH-terminal Domain of SOS Complexes with the p36 Tyrosine Phosphoprotein in Jurkat Cells Stimulated by CD28 mAb but Not by B7-1 Ligation. Very recently, it was shown that TCR activation of ras might be controlled, at least in part, by a mechanism involving the formation of a complex of the Ras exchange protein Sos, Grb2, and a membrane-bound protein of 36 kD that is tyrosine-phosphorylated in TCR-activated cells (23). The data in Fig. 6 compare the ability of CD28 ligation and TCR triggering to induce the tyrosine phosphorylation of the 36-kD Grb2-associated protein. Grb2 is composed of two SH3 domains that bind to Sos and an SH2 domain that interacts with tyrosine phosphoproteins. To look at the ability of tyrosine-phosphorylated proteins to interact with the Grb2-SH2 domain, we have used a GST fragment of the proline-rich COOH-terminal domain of Sos (amino acids 1135–1336) immobilized on glutathione agarose beads to precipitate Grb2- and Grb2-binding proteins from the lysates of resting and stimulated Jurkat cells. The Grb2 complexes are then analyzed by Western blotting with an antiphosphotyrosine antibody. The GST/Sos fragment used

**Figure 2.** CD28 mAb CD28.2 induces a hyper-phosphorylation of Raf-1 and a phosphorylation of MAPK Erk2. Jurkat cells were stimulated with UCHT1 (10 μg/ml), CD28.2 (10 μg/ml), CD28.5 (50 μg/ml), or PDBu (100 ng/ml) for the indicated time. Whole cell lysates were probed with the Raft antibody 66 (top) or the ERK2 antibody 122, which recognizes p42/44 Erk2 (bottom). This experiment was performed three times with identical results.

**Figure 3.** IL-2 production in response to CD28 mAb or B7-1–expressed L cells in PHA-stimulated Jurkat cells. Jurkat cells were stimulated by PHA alone or with CD28 mAb CD28.2 (10 μg/ml) or B7-1–expressed L cells (huB7\textsuperscript{+} L cells at a ratio of 1:20). Controls were added using untransfected L cells or huB7\textsuperscript{+} L cells preincubated with anti-B7-1 mAb, 104 (1:100 of ascites) for 5 min. After 18 h, supernatants were assayed for IL-2 as described in Materials and Methods. The experiment shown is representative of three separate experiments. Results are presented as means ± standard errors of the means of duplicate determinations.
Figure 4. Interaction of CD28 with huB7+ L cells induces tyrosine phosphorylation of cellular proteins, but not Ras activation, Erk2 activation, Raf-1 and Erk2 phosphorylations. L cells were detached from tissue culture plates and washed once in PBS then twice in RPMI medium. Jurkat cells were unstimulated (control) or stimulated with UCHT1 (10 μg/ml), CD28.2 (10 μg/ml), PDBu (100 ng/ml) for 10 min and L cells expressing B7-1 (huB7+ L cells) or not expressing B7-1 (L cells) (at a ratio of 1:2) for the indicated time. (A) Before stimulation, Jurkat cells were labeled with [32p]orthophosphate. After stimulation, an assay of p21<sup>ras</sup> activation was performed. The figure shows the quantitation of guanine nucleotides of p21<sup>ras</sup> by direct scanning of β radiation. Data are expressed as percentage of GTP to ras with respect to the total amount of guanine nucleotide on p21<sup>ras</sup>. (B) Whole cell lysates were probed with the anti-Raf antibody 66 (top) or the anti-Erk antibody 122 (bottom). (C) Regulation of Erk2 in Jurkat cells. The cells were unstimulated (control) or stimulated with UCHT1 (10 μg/ml), CD28.2 (10 μg/ml), PDBu (100 ng/ml), L cells (at a ratio of 1:2) for 10 min and L cells expressing B7-1 (huB7+ L cells) (at a ratio of 1:2) for the indicated time. A MBP phosphorylation assay was performed as indicated in Materials and Methods. Data are expressed as cpm of 32p incorporated into MBP as assessed by Ambis β scanning. (D) Immunoblots from B were stripped of antibody and redevetoped with the antiphosphotyrosine 4G10 antibody. The position of molecular mass markers are shown on the left; arrowheads indicate the position of 65- and 95-kD phosphoproteins.

in these experiments binds to the SH3 domains of Grb2 and competes for the binding of endogenous Sos and thereby allows the precipitation of endogenous Grb2 and any proteins complexed to the Grb2-SH2 domain (23).

The interaction between Grb2 and GST fusion protein of the COOH-terminal region of Sos is shown from Jurkat cell lysates, using an anti-Grb2 antibody in immunoblotting (Fig. 5, bottom). When the Grb2 precipitates are probed with antiphosphotyrosine antibodies in a Western blot analysis (Fig. 6, top), a 36-kD tyrosine phosphoprotein, p36, is seen in the Sos/Grb2 complexes isolated from TCR-stimulated Jurkat cells, p36 is also present in the Sos/Grb2 complex after stimulation with CD28.2, but was not detectable when the Jurkat cells were unstimulated. The kinetics of p36 tyrosine phosphorylation in TCR-stimulated cells are comparable with the kinetics of Vav tyrosine phosphorylation. The huB7+ L cells
trigger a strong tyrosine phosphorylation of Vav (Fig. 5) but did not induce the tyrosine phosphorylation of p36. The data in Fig. 6 (top) show antiphosphotyrosine immunoblots of Grb2 isolated from Jurkat cells exposed to huB7+ L cells alone. p95 was detected with antiphosphotyrosine 4G10 antibody (top, A and B) or anti-Vav antibody, VAV-1 (bottom, A and B).

Discussion

The stimulation of the CD28 molecule delivers important costimulatory signals for T cell activation (3). CD28 signaling pathways and the biochemical basis of this costimulatory function are poorly understood. Recent studies have shown that p21ras proteins have an important function in TCR signal transduction (20, 21). The object of the present study was to determine whether p21ras is involved in CD28 signaling. The activation of p21ras involves the accumulation of Ras-GTP complexes. Accordingly, the experiments herein compared the ability of CD28 mAbs or the human B7-1 molecule to stimulate p21ras and p21ras-dependent functions such as the hyperphosphorylation of Raf-1 and the activation of the MAP kinase ERK2. The data presented show that there is the potential for CD28 to regulate the Ras signaling pathway. Thus CD28, a functionally agonistic CD28 mAb, induces a rapid conversion of p21rasGDP to p21rasGTP. CD28.2 also induces a hyperphosphorylation of a MAPKKK, Raf-1, and stimulates the MAP kinase ERK2. One natural ligand for CD28 is the B7-1 molecule which, when expressed in fibroblasts, can provide costimulatory signals for IL-2 secretion in T cells (9). The present data show that CD28 interactions with the B7-1 molecule do not induce any increase in the accumulation of p21rasGTP. Moreover, huB7+ L cells were not able to induce a hyperphosphorylation of Raf-1 or ERK2 activation in T cells. Collectively the GTP loading data and the Raf-1, ERK2 phosphorylations and ERK2 activation data...
indicate that unlike the CD28 antibody CD28.2, B7-1 does not initiate a Ras signaling pathway.

TCR stimulation of p21<sup>ras</sup> is dependent on tyrosine kinase function (45). Two substrates for TCR-stimulated PTKs have been identified that may mediate the PTK effects on Ras, p95<sup>tyr</sup> and p36, a protein that complexes to the SH2 domain of the Grb2 adapter molecule, thus recruiting the guanine nucleotide exchange protein Sos (22, 23). CD28 ligation with both the CD28 mAb CD28.2 and the ligand B7-1 apparently activated cellular PTK pathways as judged by Western blot analysis with an antiphosphotyrosine antibody. We therefore explored whether the differences between CD28.2 and B7-1 with regard to p21<sup>ras</sup> activation could be explained by differences in the ability of these CD28 ligands to modulate the tyrosine phosphorylation of Vav or p36.

The data show that Vav is tyrosine phosphorylated in T cells activated via the TCR or CD28 irrespective of whether the CD28 mAb CD28.2 or the ligand B7-1 was used to trigger the CD28 receptor. The overall intensity of tyrosine phosphoproteins in the TCR-activated cells was markedly higher than in the B7-1-activated cells. In particular, in the antiphosphotyrosine blots of total cell lysates a 95-kD protein that migrates at a similar position to Vav is strongly tyrosine phosphorylated in TCR-activated cells and more weakly in T cells activated with huB7<sup>+</sup> L cells (Fig. 4 D). This band probably represents multiple 95-kD tyrosine phosphoproteins because when Vav tyrosine phosphorylation was monitored directly, the relative effect of the TCR and B7-1 on Vav phosphorylation was the opposite of that suggested by the analysis of the 95-kD protein in total cell lysates. For direct analysis of Vav tyrosine phosphorylation, Vav immunoprecipitates were probed with an antiphosphotyrosine antibody. These data confirm previous observations that triggering of the TCR stimulation induces a rapid but transient tyrosine phosphorylation of Vav in T cells (37, 44). The ligand for CD28, B7-1, also induced Vav tyrosine phosphorylation but with an apparently higher stoichiometry than the TCR stimulus. Moreover, compared with the TCR response, the B7-1-induced tyrosine phosphorylation of Vav was a rapid but prolonged event. Previous studies have identified a 100-kD protein (pp100) as a substrate for CD28-activated PTKs in PMA-treated Jurkat cells (42, 43, 46). It is not known whether this pp100 protein is Vav or another substrate. The 95-kD tyrosine substrate seen in the present study may be the same as pp100. In the antiphosphotyrosine immunoblots of total lysates, a 65-kD protein appeared selectively in the B7-1-activated cells, which supports the hypothesis that CD28 and the TCR may regulate different PTKs. The present study illustrates a second major difference between TCR and CD28/PTK pathways by a difference in the magnitude and kinetics of p95<sup>tyr</sup> tyrosine phosphorylation.

Tyrosine phosphorylation of Vav regulates its in vitro guanine nucleotide exchange activity for Ras (22). However, one conclusion from the present data is that Vav tyrosine phosphorylation is not sufficient to ensure activation of p21<sup>ras</sup>. In vivo, this does not necessarily exclude that Vav tyrosine phosphorylation plays a role in Ras regulation. First, it is possible that regulation of Vav GTP exchange activity requires tyrosine phosphorylation of particular tyrosine residues and in the present study it was not established that Vav is phosphorylated on identical sites in the TCR- and CD28-activated cells. Second, Vav tyrosine phosphorylation may be important for Ras regulation but there may be a simultaneous requirement for other signals that are triggered by the TCR and not by CD28.

Sos, another guanine exchange factor for Ras, is detected in T cells and interacts with an adapter protein Grb2 (23, 47). The complex of Sos/Grb2 is present in resting and active T cells (Fig. 6) (23) and the crucial regulatory step is the recruitment of the Grb2/Sos complex to the plasma membrane. Grb2 is translocated to the membrane by interactions between the Grb2-SH2 domain and tyrosine-phosphorylated proteins. The first example of this phenomenon was in fibroblasts, where EGF promotes the formation of a heterotrimeric complex of EGF receptor, Grb2 adaptor protein and Sos (26, 27). Upon TCR triggering a tyrosine-phosphorylated p36 protein complexes to the Grb2-SH2 domain and it is proposed that this p36 interaction recruits Grb2 and Sos to the membrane. The current data show that T cell activation with the CD28 mAb CD28.2 can also induce tyrosine phosphorylation of a p36 Grb2-associated protein. However, stimulation of T cells with huB7<sup>+</sup> L cells does not have this effect. Thus the Grb2-associated p36 protein is not a substrate for B7-1-stimulated PTKs. Furthermore the tyrosine phosphorylation of p36 appears to correlate with activation of the Ras signaling pathway which supports the hypothesis that p36 is crucial for Ras activation.

In fibroblasts another protein that can complex with the Grb2 SH2 domain is Shc (26). Shc can bind to the TCR-<gamma>-chain and when it is tyrosine phosphorylated in TCR-activated cells, the formation of a Grb2/Shc complex has been observed (47). In CD3-stimulated T cells, it seems difficult to detect tyrosine-phosphorylated Shc in association with Sos (23). This seems to reflect that the stoichiometry of Shc tyrosine phosphorylation in TCR activated cells is low. Thus the only tyrosine phosphorylation of particular tyrosine residues and in the present study it was not established that Vav is phosphorylated on identical sites in the TCR- and CD28-activated cells. Second, Vav tyrosine phosphorylation may be important for Ras regulation but there may be a simultaneous requirement for other signals that are triggered by the TCR and not by CD28.

### Table 1. A Comparison of Signal Transduction Pathways by a CD28 mAb CD28.2 and the CD28 Ligand B7-1

<table>
<thead>
<tr>
<th>Inducible events</th>
<th>CD28 stimulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody</td>
<td>Ligand</td>
</tr>
<tr>
<td>Tyrosine phosphorylation of p95&lt;sup&gt;tyr&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td>Tyrosine phosphorylation of p36 associated with the complex Sos/Grb2</td>
<td>+</td>
</tr>
<tr>
<td>p21&lt;sup&gt;ras&lt;/sup&gt; activation</td>
<td>+</td>
</tr>
<tr>
<td>Raf kinase hyperphosphorylation*</td>
<td>+</td>
</tr>
<tr>
<td>MAP kinase phosphorylation*</td>
<td>+</td>
</tr>
<tr>
<td>MAP kinase activation†</td>
<td>+</td>
</tr>
<tr>
<td>Costimulatory effects on IL-2 secretion</td>
<td>+</td>
</tr>
</tbody>
</table>

Rating scale: -, not detectable; +, positive.
* Determined by altered molecular weight on SDS-PAGE gels.
† Determined by MBP phosphorylation assay.
References


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