Signal Transduction by CD28 Costimulatory Receptor on T Cells

B7-1 and B7-2 REGULATION OF TYROSINE KINASE ADAPTOR MOLECULES*

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This study compares the biochemical responses in T cells activated with the CD28 ligands B7-1 and B7-2. The patterns of tyrosine phosphorylation induced in T cells by these two CD28 ligands are identical, but clearly different from the tyrosine phosphorylation induced by the T cell receptor (TCR). The TCR regulates protein complexes mediated by the adapter Grb2 both in vivo and in vitro. In contrast, there is no apparent regulation of in vivo Grb2 complexes in response to B7-1 or B7-2. Rather, B7-1 and B7-2 both induce tyrosine phosphorylation of a different adapter protein, p62. The regulation of p62 is a unique CD28 response that is not shared with the TCR. These data indicate that B7-1 and B7-2 induce identical tyrosine kinase signal transduction pathways. The data show also that the TCR and CD28 couple to different adapter proteins, which could explain the divergence of TCR and CD28 signal transduction pathways during T cell activation.

T lymphocyte activation is controlled by the T cell antigen receptor (TCR)1 in combination with additional signals triggered by accessory molecules present on the surface of the antigen-presenting cells (1, 2). CD28, a 44-kDa homodimeric glycoprotein expressed by most mature T lymphocytes, is a costimulatory signal receptor for this process of T cell activation (3, 4). Two physiological ligands for CD28 have been described: B7-1 (CD80) and B7-2/B70 (also called CD86) (5–11). B7-2/B70 shares 25% sequence identity with the extracellular domains of B7-1 (6, 8, 9). B7-1 and B7-2/B70 have similar binding properties for CD28 and can provide apparently identical costimulatory signals for interleukin-2 production by T cells (12–14). However, these molecules are differentially expressed on antigen-presenting cells: B7-1 expression is detected only on activated antigen-presenting cells, whereas B7-2/B70 expression is detected on unactivated quiescent monocytes (6). It has also been reported that these molecules are differentially involved in the differentiation of Th1/Th2 subpopulations of T cells (14, 15). Thus, one crucial question in CD28 signal transduction is whether B7-1 and B7-2 use similar signal transduction pathways to costimulate T cells.

Triggering of the TCR-CD3 complex activates intracellular protein-tyrosine kinases (PTKs) that couple the TCR to phospholipase C, phospholipase C-γ1 (16), and the guanine nucleotide-binding protein p21ras (17). Triggering of the CD28 receptor with antibodies induces phospholipase C and Ras activation (18, 19). Previous studies using CD28 antibodies have suggested that PTKs involved in CD28 signal transduction include the Src kinases p56lck and p59fyn (20, 21) and the Tec family kinase ITK/EMT (22). The TCR regulates these PTKs, but also activates the T cell-specific kinase ZAP70. Triggering of CD28 with the natural ligand B7-1 also activates cellular PTKs, but with the pattern of phosphoproteins differs from that seen in CD28 antibody-activated cells (19, 23). In addition, CD28 stimulation by the natural ligand B7-1 does not induce phospholipase C activity or Ras activation, but is associated with activation of phosphatidylinositol 3kinase (19, 24).

Recently, there has been considerable analysis of the role of the adapter molecule Grb2/SEM5 in T cells (25). The importance of Grb2 for PTK signaling was first established in the Ras signaling pathway (26). Many receptors in a variety of cells regulate p21ras by stimulating the guanine nucleotide exchange protein Sos via a mechanism involving Grb2. Grb2 is composed of one SH2 domain and two SH3 domains. The Grb2 SH2 domains bind the carboxyl-terminal proline-rich domain of Sos, and the Grb2 SH2 domain binds to tyrosine-phosphorylated molecules. In TCR-stimulated cells, there is rapid formation of a complex between Sos/Grb2 and a 36-kDa membrane protein that is a substrate for TCR-induced PTKs (27). Grb2 and p36 may link the TCR to multiple signaling pathways. Thus, p36 is an apparent link between the TCR and both Grb2 and phospholipase C (28). Grb2 may also be important in coupling the TCR to more than just Sos/p21ras because additional Grb2 effector molecules have been identified in T cells. The best characterized of these novel Grb2 effector molecules is a 75-kDa molecule, SLP-76, that is constitutively associated with Grb2 SH3 domains (29–31) and is a substrate for TCR-activated PTKs.

There has been no analysis of CD28 links to adapter molecules analogous to the TCR studies, although such experiments will almost certainly afford insight into the mechanisms that transduce the signals generated by CD28-activated PTKs. In this context, it has been reported that CD28 cross-linking with antibodies can induce an association between tyrosine-phosphorylated CD28 and Grb2 (32). Also, ligation of CD28 with antibodies has been shown to induce tyrosine phosphorylation of the Grb2-associated p36 molecule, whereas triggering of CD28 with the natural ligand B7-1, which also activates cellular PTKs, does not (19). It thus seems that there is the potential for CD28 to regulate Grb2, but this potential is not always realized in response to ligation with the natural ligand for...
CD28, B7-1. Nevertheless, it is possible that the effects of the CD28 antibodies on p36 phosphorylation mimic the effects of the ligand B7-2/B70 triggering of CD28.

Accordingly, the object of this study was to compare the effects of CD28 triggering with antibodies or B7-1 or B7-2 on the regulation of Grb2 protein complexes. The data show that the CD28 ligands B7-1 and B7-2 induce tyrosine phosphorylation of apparently identical cellular substrates. There are recent data suggesting that B7-1 and B7-2 activate differentially Th1 and Th2 cytokines (14, 15). In this context, it is worth considering if B7-1 and B7-2 differ in their ability to regulate signal transduction pathways. These studies have identified proteins that are tyrosine-phosphorylated in response to either TCR or CD28 signaling. Thus, the p36 Grb2 SH2 domain-binding protein and SLP-76, the 75-kDa Grb2 SH3 domain-binding protein, are selective substrates for TCR (but not CD28) signaling pathways. Recent studies have identified a tyrosine-phosphorylated 62-kDa molecule, p62, as a multifunctional adapter molecule in many different cells and receptor systems (33). Herein, we show that this adapter molecule, p62, is a substrate for CD28-activated (but not TCR-regulated) PTKs, which implies that p62 may have a selective function in CD28 (but not TCR) signal transduction. The present data thus indicate that the regulation of adapter molecules is a point of divergence of CD28 and TCR signal transduction pathways.

**EXPERIMENTAL PROCEDURES**

Cell Lines—For the subclone of the J urkat T leukemia cells, J H6.2 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum. The J urkat J H6.2 cell line has been described previously (34). The B7-1, B7-2, and B70 L cells were obtained by transfection of the human B7-1, B7-2, and B70 cDNAs in murine L cells, respectively, and selected by G418 resistance. These cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine. CTLA4-Ig fusion protein staining was performed to control the human B7 expression at the transfectants surface. B7 expression is 10-fold less than B7-1 or B7-2 expression (data not shown). For T cell stimulations, L cells were detached from tissue culture flasks by incubation with trypsin/EDTA, washed once in phosphate-buffered saline and then once in RPMI 1640 medium, and resuspended in RPMI 1640 medium. Untransfected L cells were used as control cells.

Antibodies, Peptides, and Fusion Proteins—The monoclonal antibody (mAb) UCHT1 (reactive with the e-chain of the human TCR-CD3 antigen complex) and mAb CD28.2 (reactive with the human CD28 molecule) (34) were purified from hybridoma supernatants by protein A-Sepharose. To stimulate cells, UCHT1 or CD28.2 was used at 10 μg/ml. CD28 mAb CD28.2 was used for CD28 detection in immunoblotting as described previously (35).

The monoclonal anti-phosphotyrosine antibody 4G10 and the monoclonal anti-human GTPase-activating protein (GAP) antibody were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The monoclonal anti-grb2 antibody was purchased from Affinity (Nottingham, United Kingdom). The anti-peptide antiserum Vav-1 was used to immunoprecipitate and detect in immunoblotting the p95vav protein as described previously (36). The monoclonal anti-phosphotyrosine antibody 4G10 was used to immunoprecipitate tyrosine-phosphorylated proteins as described (37). The polyclonal Cbl antibody was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA).

The EGFR-pY1068 phosphotyrosine peptide has the sequence PVPEYINQS and was used to precipitate endogenous Grb2 via its SH2 domain as described (29).

The human CTLA4-Ig fusion protein (CTLA4-Ig) was a kind gift of Dr. P. S. Linsley (Oncogene, Seattle, WA). CTLA4 binds to the B7 molecules with high affinity (12); a preincubation of L cells expressing B7-1 or B7-2/B70 with CTLA4-Ig at 5 μg/ml prevents the interaction of B7 with the CD28 receptor. Glutathione S-transferase fusion proteins encoding full-length Grb2 (GST-Grb2) and double SH3 mutant GST-Grb2 49L/203R (named GST-Grb2SH3) as well as carboxy-terminal GST-mSos1 (where “m” is murine; residues 1135–1336) (GST-C-Sos) have been described (38). The isolated amino-terminal GST-huGrb2 (where “hu” is human) SH3 domain (residues 1–58) (N-SH3) and the carbonyl-terminal GST-huGrb2 SH3 domain (residues 159–217) (C-SH3) have been described (39).

**RESULTS**

B7-1 and B7-2 Can Induce Tyrosine Phosphorylation of Cellular Substrates in T Cells—We have reported previously that B7-1 L cells can induce tyrosine phosphorylation of cellular substrates in J urkat cells (19). To examine whether B7-2/B70 can induce tyrosine phosphorylation of cellular substrates, a Western blot of cell lysates prepared from cells treated with CD28 antibody CD28.2, or B7-1, B7-2, or B7 L cells, or from untransfected L cells was probed with an anti-phosphotyrosine antibody. The data in Fig. 1 show that B7-1 or B7-2/B70 ligation is able to induce a similar pattern of tyrosine phosphorylation. Under these experimental conditions, we could not detect tyrosine phosphoproteins in lysates from L cells alone, and the pattern of tyrosine phosphorylation in J urkat cells was the same with paraformaldehyde-fixed B7-1, B7-2, and B7 L cells or unfixed B7-1, B7-2, and B7 L cells (data not shown). The pattern of CD28-induced phosphorylation was compared with the tyrosine phosphorylation induced by the TCR-CD3 antibody UCHT1.

The data in Fig. 1 show that the patterns and intensities of tyrosine phosphorylation in TCR- and CD28-activated T cells are 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. A 95-kDa tyrosine phosphoprotein was detected in J urkat cells within 1 min of contact of the cells with B7-1, B7-2, or B7 L cells. The 95-kDa tyrosine phosphoprotein was also detected after stimulation of T cells with UCHT1 or CD28.2. One candidate for the 95-kDa tyrosine phosphoprotein in the TCR- and CD28-activated cells is p95vav, which is known to be tyrosine-phosphorylated in response to TCR, CD28 mAb, or B7-1 triggering (19, 22, 36, 40). Fig. 2A shows anti-phosphotyrosine Western blot analyses of p95vav immunoprecipitates. Weak basal tyrosine phosphorylation of p95vav was detectable in unstimulated J urkat cells. B7-2 or B7 L cells, like mAbs UCHT1 and CD28.2 or B7-1 L cells, induced an increase in p95vav tyrosine phosphorylation. The Vav tyrosine phosphorylation induced by B7-1 or B7-2/B70 was inhibited by preincubation of the B7-expressing L cells with CTLA4-Ig.

The data in Fig. 1 show that TCR or CD28 engagement leads to an increase in tyrosine phosphorylation of a 120-kDa band. A 120-kDa protein, p120cbl, has been described to be tyrosine-
phosphorylated upon TCR stimulation (41, 42). Fig. 2B shows anti-p120\textsuperscript{CA} Western blot analyses of anti-phosphotyrosine immunoprecipitates. Basal tyrosine phosphorylation of p120\textsuperscript{CA} was detectable in unstimulated Jurkat cells. Like mAb UCHT1, mAb CD28.2 and B7-1 and B7-2 L cells induced an increase in p120\textsuperscript{CA} tyrosine phosphorylation. The Cbl tyrosine phosphorylation induced by B7-1 or B7-2 was inhibited by preincubation of the B7-expressing L cells with CTLA4-Ig. There was one tyrosine phosphoprotein of 62 kDa that was detected in the B7-1- or B7-2/B70-stimulated T cells, but not in the TCR-stimulated Jurkat cells (Fig. 1).

Comparison of TCR-, B7-1-, and B7-2-induced Grb2 Complexes—Grb2 is a ubiquitously expressed adapter molecule involved in diverse signal transduction pathways in a variety of cells. TCR activation can induce tyrosine phosphorylation of multiple Grb2-binding proteins, including a 36-kDa molecule, a 75-kDa molecule that binds to Grb2 SH2 domains (27, 28), and a 116-kDa Grb2 SH3 domain-binding protein (31). We showed previously that the CD28 ligand B7-1 does not induce tyrosine phosphorylation of p36 (19), but it has not yet been determined whether B7-2 regulates p36 phosphorylation. Moreover, there has been no analysis of CD28 effects on tyrosine phosphorylation of Grb2 SH3 domain-binding proteins, and the possibility that CD28 might induce tyrosine phosphorylation of novel Grb2-binding proteins has not been explored.

Triggering of CD28 with the ligand B7-1 induced tyrosine phosphorylation of multiple cellular proteins. In initial experiments to determine whether these tyrosine phosphoproteins include Grb2-binding proteins, a series of binding experiments with GST-Grb2 fusion proteins were performed. Western blot analysis with anti-phosphotyrosine antibodies of GST-Grb2-binding proteins isolated from CD28-activated cells (Fig. 3) showed that CD28 induced a major tyrosine phosphorylation of Grb2-binding proteins of 62 and 120 kDa. The pattern of CD28-induced Grb2-binding proteins had some similarities, but also some differences from that observed in TCR-activated cells, where tyrosine phosphoproteins of 36, 75, 95, and 120 kDa were induced in response to ligation of the TCR complex.

Grb2 has a single SH2 domain and two SH3 domains. To explore the SH domain specificity of the CD28-induced tyrosine phosphoproteins, binding experiments were carried out with GST-Grb2\textsubscript{SH2}, which has an intact SH2 domain, but a single mutation in both the COOH- and NH\textsubscript{2}-terminal SH3 domains. The data in Fig. 3A show that the TCR-induced p36 tyrosine phosphoprotein binds to wild-type GST-Grb2 and GST-Grb2\textsubscript{SH3}. The remainder of the TCR-induced Grb2-binding proteins do not bind to GST-Grb2\textsubscript{SH2}. Thus, as described previously (29), their interaction with Grb2 is SH3 domain-mediated. The 62-kDa B7-1-induced tyrosine phosphoprotein can bind to the intact GST-Grb2 fusion protein and to GST-Grb2\textsubscript{SH3}, suggesting that p62 binds to Grb2 SH2 domains. In contrast, mutation of Grb2 SH3 domains abrogated binding of the 120-kDa tyrosine phosphoprotein that is induced in response to both TCR and B7-1 stimulation.

Experiments with GST fusion proteins are valuable for studying protein/protein interactions, but it is important to establish whether the interactions between proteins that are identified in such in vitro studies actually occur in vivo. To study the proteins interacting with the SH2 domain of endogenous Grb2, we adopted a previously described technique that uses a GST fusion protein of the COOH-terminal proline-rich regions of mSos1 (27). This fusion protein binds effectively to Grb2 SH3 domains. It competes for any interactions between proteins that are studied in vitro and purified with endogenous Grb2 and associated SH2 domain-binding proteins. Fig. 3A shows the tyrosine-phosphorylated proteins that can bind to the SH2 domains of GST-Grb2 and GST-Grb2\textsubscript{SH3} or to the SH2 domains of endogenous Grb2 complexes isolated with GST-C-Sos. The TCR-induced tyrosine phosphoprotein of 36 kDa could bind to GST-Grb2 and GST-Grb2\textsubscript{SH3} and, more important, was copurified with endogenous Grb2. In contrast, the B7-1-induced 62-kDa tyrosine phosphoprotein could bind to the GST-Grb2 fusion proteins, but did not copurify with endogenous Grb2.

The GST-Grb2 binding experiments showed that tyrosine phosphorylation of the 120-kDa Grb2 SH3 domain-binding protein was induced by B7-1. This protein, which could correspond to the product of the c-cbl proto-oncogene (41), was also tyrosine-phosphorylated in the TCR-activated cells, as was an additional TCR unique molecule, p75. To determine whether the B7-1-induced p120 protein binds to the SH3 domains of endogenous Grb2, cellular Grb2 complexes were affinity-purified using a tyrosine-phosphorylated peptide from the cytoplas-
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A 62-kDa Protein Coimmunoprecipitating with GAP Is Tyrosine-Phosphorylated in Response to B7-1 or B7-2 Stimulation, but Not after TCR Stimulation—A 62-kDa protein, p62, has been reported to be highly tyrosine-phosphorylated and to associate with p120<sup>GAP</sup>, a p21<sup>GTPase-activating protein</sup> after signaling by tyrosine kinase growth factor receptors (43). p62, like Grb2, is now recognized as a multifunctional adapter protein (33). We therefore investigated whether p120<sup>GAP</sup> or associated proteins were tyrosine-phosphorylated in TCR- or CD28-activated cells. Jurkat cells were stimulated with mAb UCHT1 or CD28.2, with B7-1, B7-2, or B70 L cells, or with untransfected L cells for 5 min. The cell extracts were immunoprecipitated with anti-GAP mAb, and immunocomplexes were immunoblotted with phosphotyrosine or GAP antibodies. The data in Fig. 5 show that no tyrosine phosphoproteins were detected in quiescent or TCR-activated GAP immunoprecipitates, although GAP antibodies recognized a band with a molecular mass of 120 kDa corresponding to RasGAP. However, a tyrosine-phosphorylated GAP-associated 62-kDa protein was observed specifically after CD28 stimulation with mAb CD28.2 or the B7-1 or B7-2/B70 ligand. A tyrosine phosphoprotein of 55 kDa that could be a degradation product of the 62-kDa molecule was detected in GAP immunoprecipitates isolated from B7-1- or B7-2/B70-activated cells. A tyrosine-phosphorylated p190<sup>GAP</sup>-associated protein was detected in the GAP immunoprecipitates, but this did not appear to be a B7-1- or B7-2/B70-induced tyrosine phosphoprotein. Thus, p190 was observed in the GAP precipitates isolated from Jurkat cells exposed to B7-1 or B7-2 ligands. However, tyrosine phosphorylation of the p62<sup>GAP</sup>-associated protein induced via B7-1 or B7-2/B70 was inhibited by CTLA4-Ig preincubation of the transfected L cells. Moreover, tyrosine phosphorylation of the p62<sup>GAP</sup>-associated protein induced via B7-1 or B7-2/B70 was inhibited by CTLA4-Ig preincubation of the transfected L cells. In contrast, p190 tyrosine phosphorylation was not prevented by CTLA4-Ig.

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**Fig. 2.** B7-1 and B7-2/B70 ligation induces tyrosine phosphorylation of the vav and c-cbl proto-oncogene products in Jurkat cells. A. Jurkat cells (4 × 10<sup>6</sup>/point) were stimulated with UCHT1 (10 μg/ml); CD28.2 (10 μg/ml); B7-1, B7-2, or B70 L cells; or untransfected L cells (ata ratio of 1:2) for 5 min. The plus signs mean that the L cells were pretreated with CTLA4-Ig at 10 μg/ml for 5 min, and the minus signs mean that the L cells were used without CTLA4-Ig preincubation. The experiment shown is representative of two separate experiments. Vav immunoprecipitations were analyzed by Western blot analyses with anti-phosphotyrosine antibody 4G10 (upper panel) or anti-Vav antibodies (lower panel). B. Jurkat cells (4 × 10<sup>6</sup>/point) were stimulated with UCHT1 (10 μg/ml), CD28.2 (10 μg/ml), or B7-1 or B7-2 L cells (at a ratio of 1:2) for 5 min. The plus signs mean that the L cells were pretreated with CTLA4-Ig at 10 μg/ml for 5 min, and the minus signs mean that the L cells were used without CTLA4-Ig preincubation. The experiment shown is representative of two separate experiments. Anti-phosphotyrosine immunoprecipitations were analyzed by Western blot analyses with anti-p120<sup>GAP</sup> antibody.

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**Table 1.** The ability of tyrosine-phosphorylated proteins to interact with the Grb2 SH2 domain after B7-1 or B7-2 stimulation. Jurkat cells were incubated with B7-1 or B7-2 L cells or with untransfected L cells for 5 min (Fig. 3C). TCR-activated cells were included in these experiments as a comparison. The data show that B7-2, like B7-1, can induce tyrosine phosphorylation of a 62-kDa protein that is able to bind GST-Grb2<sub>SH2</sub>SH3 and interact with the Grb2 SH2 domain in vitro. p62 tyrosine phosphorylation induced by B7-1 or B7-2 was inhibited by CTLA4-Ig preincubation of B7 L cells.

In many cells, Grb2 via its SH2 domain can form a complex with receptors (26). To probe for the presence of CD28 in Grb2 complexes, Western blot analysis with CD28 antibodies was performed. The data in Fig. 4A indicate that CD28 molecules isolated from CD28-activated (but not quiescent or TCR-activated) T cell lysates could bind to the wild-type GST-Grb2 fusion protein and GST-Grb2<sub>SH2</sub>SH3, but not to GST-Grb2 N-SH3 or GST-Grb2 C-SH3. These fusion protein binding experiments suggest that the Grb2 SH2 domain is important for CD28/GST-Grb2 interactions.

The previous data show that CD28 induced tyrosine phosphorylation of a 62-kDa protein that could bind to the GST-Grb2 fusion proteins, but could not copurify with endogenous Grb2. A similar analysis was performed to see if CD28 could form a complex with endogenous Grb2. The data in Fig. 4B show that CD28 molecules from CD28-activated (but not quiescent or TCR-activated) cells could bind to GST-Grb2 and GST-Grb2<sub>SH2</sub>SH3, but not to the SH2 domain of endogenous Grb2. These data (Figs. 3 and 4) suggest that, after B7 ligation, at least two proteins (p62 and CD28 receptor) are able to interact with the Grb2 SH2 domain in vitro, but not in vivo.

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A nanomolar affinity to the SH2 domains of Grb2 and allows the interaction with the GST-Grb2 fusion protein and GST-Grb2<sub>SH2</sub>SH3, but not to GST-Grb2 N-SH3 or GST-Grb2 C-SH3. These fusion protein binding experiments suggest that the Grb2 SH2 domain is important for CD28/GST-Grb2 interactions.
In this study, we have compared the signal transduction pathways regulated by the CD28 ligands B7-1 and B7-2/B70. The data show that B7-1 and B7-2 both induce rapid tyrosine phosphorylation of cellular proteins in T cells. B7-1- and B7-2-induced patterns of tyrosine phosphorylation are different than the TCR stimulation pattern, which supports the hypothesis that CD28 and the TCR regulate different cellular PTKs. However, there were no discernible differences in the patterns of B7-1- and B7-2/B70-induced tyrosine phosphorylation, which indicates that B7-1 and B7-2/B70 activate similar PTKs and/or tyrosine phosphatases. One obvious difference between the TCR and CD28 is with regard to their ability to regulate adapter molecules. In particular, the present data reveal that the regulation of Grb2 can distinguish TCR and CD28 signaling: TCR ligation is associated with tyrosine phosphorylation of a 36-kDa Grb2 SH2 domain-binding protein and a 75-kDa Grb2 SH3-domain binding protein, whereas B7-1 and B7-2 have no
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A

B

Fig. 4. CD28 can bind to the Grb2 SH2 domain in vitro, but not in vivo in CD28-activated T cells via its ligand B7-1. In A, Jurkat cells (4 × 10⁶/point) were stimulated with UCHT1 (10 μg/ml), L cells expressing B7-1 (B7-L cells), or control L cells (at a ratio of 1:2) for 5 min. Proteins were precipitated from postnuclear cell lysates with GST-Grb2, double SH3 mutant GST-Grb2 49L/203R (GST-Grb2μSH3), GST-Grb2 N-SH3, or GST-Grb2 C-SH3 fusion protein immobilized on glutathione beads (A) or with GST-Grb2, double SH3 mutant GST-Grb2 49L/203R (GST-Grb2μSH3), or carboxyl-terminal GST-mSos1 (residues 1135–1336) (GST-C-Sos) fusion protein immobilized on glutathione beads (B). The presence of CD28 in the precipitates was analyzed by immunoblotting with anti-CD28 antibody CD28.6. The experiments shown are representative of three separate experiments.

discernible regulatory effect on endogenous Grb2 complexes. Accordingly, p36 and p75 seem to be selective substrates for TCR-activated PTKs rather than CD28-activated PTKs. In contrast, B7-1 and B7-2 induce tyrosine phosphorylation of a 62-kDa adapter molecule, p62, appears to have a selective function in CD28 signaling as it is not a substrate for TCR-regulated PTKs.

The Grb2-associated p36 molecule has also been described to associate with phospholipase C and may thus link the TCR to both p21ras and calcium signaling pathways (28). Previous work has established that the regulation of intracellular calcium and that of p21ras are TCR-regulated (but not CD28-regulated) responses. The failure of B7-1 or B7-2 to induce tyrosine phosphorylation of p36 may thus explain this divergence of TCR and CD28 signal transduction mechanisms. CD28 ligation with B7-1 or B7-2 induces identical tyrosine phosphorylation of 62-, 95-, and 120-kDa proteins that can bind to GST-Grb2 fusion proteins. The 120-kDa protein complex that binds to the SH3 domains of GST-Grb2 fusion proteins was shown recently to include the proto-oncogene c-cbl (41). The data in Fig. 2B indicate that c-cbl is a common substrate for TCR- and CD28-regulated PTKs.

The present data show that the proteins that are common targets for both TCR- and CD28-activated PTKs include p95vav. Vav function is essential for T cell development and activation, indicating that Vav has an important role in TCR function (44–46). However, if tyrosine phosphorylation of Vav is a marker for its functional regulation, then the present data suggest that Vav may also have a role in CD28 signal transduction. Interestingly, one of the many immune defects in Vav-deficient mice is a defect in cytokine production by T cells, which could occur as a consequence of the disruption of either the TCR or CD28 signaling pathway.

The 95-kDa tyrosine phosphoprotein seen in the GST-Grb2 protein complexes was shown by Western blot analyses to be p95vav (data not shown). GST fusion protein binding experiments are valuable for mapping protein/protein interactions, but can generate artifacts and show associations between proteins that are purely in vitro phenomena, i.e. that are not physiologically relevant because they do not occur in vivo under normal conditions. Vav/Grb2 association appears to fall in this latter category because Vav could not be detected in association with endogenous Grb2 complexes, nor could CD28 or the 62- and 120-kDa tyrosine phosphoproteins. With this approach, we are able to show a specific substrate of CD28-induced PTKs, p62 (Fig. 3). There are two tyrosine phosphoproteins, p36 and p75, in endogenous Grb2 complexes in TCR-stimulated T cells; however, no tyrosine phosphoprotein was detected binding to endogenous Grb2 after CD28 ligation by B7 molecules. Thus, tyrosine phosphorylation of proteins that bind to endogenous Grb2 is a TCR (but not CD28) response, which indicates that the adapter Grb2 is an important component of TCR signal transduction mechanisms, but is not similarly involved in the CD28 costimulatory pathways.

The TCR and CD28 clearly differ in their ability to induce tyrosine phosphorylation of a 62-kDa p120GAP-associated molecule. CD28-induced 62-kDa tyrosine phosphoproteins were also detected in Western blot analyses of total cell lysates. It is not yet clear whether these CD28-induced 62-kDa proteins seen in total cell lysates comprise solely the GAP-associated p62 molecule or whether there are multiple 62-kDa proteins that are substrates for CD28-induced (but not TCR-induced) PTKs. We show that B7-1 and B7-2/B7-0 both regulate tyrosine phosphorylation of p62. p62 has also been described as a Grb2-binding protein, but although p62 could bind to GST-Grb2 in vitro, no p62/Grb2 complexes in vivo could be detected in CD28-activated cells. The GAP-associated p62 protein is a substrate for CD28-activated (but not TCR-regulated) PTKs, which implies that p62 may have a selective function in CD28 (but not TCR) signal transduction. It has been shown previously that tyrosine phosphorylation of the p62 protein is triggered by the accessory receptor CD2 (47). These CD2 data, when coupled with the present results, raise the interesting possibility that, in T cells, p62 is involved selectively in accessory receptor
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signal transduction mechanisms.

The function of p62 is not known, although its association with p120RasGAP implies p62 function in the regulation of p21ras. This conclusion was premature and based entirely on initial observations that tyrosine phosphorylation of p62 correlates with p21ras activation in many cells (43, 48). However, in T cells, there is no obvious correlation between p62 tyrosine phosphorylation and p21ras activation. For example, TCR triggering stimulates p21ras, but does not induce p62 tyrosine phosphorylation, whereas B7-mediated activation of CD28 does not activate p21ras or p21ras-dependent kinases such as extracellular signal regulated kinase 2 (19) despite inducing a strong tyrosine phosphorylation of p62. Moreover, there is no direct evidence that p62 regulates RasGAPs. Rather, p62, like Grb2, is now recognized as a multifunctional adapter protein and can probably link receptor-coupled PTKs to several downstream signal transduction pathways (33). In this context, another protein, p190, is present in the p62-RasGAP complexes. This 190-kDa protein contains a domain with GTPase activity for the GTP-binding protein Rho and a second domain that has homology to RhogAP (49). Accordingly, p62 should be considered to have the potential to link receptor-activated PTKs to the Rho family of GTP-binding proteins. The role of Rho in T cells is not known, but Rho and a related protein, Rac, are essential for control of the actin cytoskeleton in fibroblasts (50). It has also been reported that Rho can regulate the activity of phosphatidylinositol 5'-kinase (51). CD28 is known to stimulate phosphatidylinositol metabolism via a mechanism attributed to the regulation of phosphatidylinositol 3'-kinase (24), and this regulation can occur in response to both B7-1 and B7-2 ligation of CD28 in human T cells (52). Future studies should thus explore whether CD28 regulation of other lipid kinases such as phosphatidylinositol 5'-kinase contributes to CD28 control of inositol lipid phosphorylation.

B7-1 and B7-2 can both costimulate T cells to produce interleukin-2 (13), but it was not previously assessed whether the different CD28 ligands use similar intracellular signaling mechanisms to costimulate human T cells. In this context, it has been reported that B7-1 and B7-2 may have different functions in T cell biology in vivo (15, 53), but this could reflect that the CD28 ligands are differentially expressed by antigen-presenting cells and would thus not be involved in equivalent stages of the T cell activation. It must also be considered that there is a second receptor for B7-1 and B7-2, CTLA4. This study thus provides the first comparison of the intracellular signals generated by B7-1 and B7-2/B70, and although the data reveal differences in TCR- and CD28 (using its ligands)-induced signaling pathways, no differences in B7-1 and B7-2 signal transduction responses could be detected. In particular, B7-1 and B7-2 can both induce tyrosine phosphorylation of similar cellular substrates. There is differential regulation of adapter proteins by the TCR and CD28 that probably explains the ability of these receptors to initiate divergent signal transduction responses during T cell activation.

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