REVIEW ARTICLE

CD28: a signalling perspective

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CD28 and the related molecule cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), together with their natural ligands B7.1 and B7.2, have been implicated in the differential regulation of several immune responses. CD28 provides signals during T cell activation which are required for the production of interleukin 2 and other cytokines and chemokines, and it has also been implicated in the regulation of T cell anergy and programmed T cell death. The biochemical signals provided by CD28 are cyclosporin A-resistant and complement those provided by the T cell antigen receptor to allow full activation of T cells. Multiple signalling cascades which may be independent of, or dependent on, protein tyrosine kinase activation have been demonstrated to be activated by CD28, including activation of phospholipase C, $p21^{ras}$, phosphoinositide 3-kinase, sphingo-

myelinase/ceramide and 5-lipoxygenase. The relative contributions of these cascades to overall CD28 signalling are still unknown, but probably depend on the state of activation of the T cell and the level of CD28 activation. The importance of these signalling cascades (in particular the phosphoinositide 3-kinase-mediated cascade) to functional indications of CD28 activation, such as interleukin 2 gene regulation, has been investigated using pharmacological and genetic manipulations. These approaches have demonstrated that CD28-activated signalling cascades regulate several transcription factors involved in interleukin 2 transcriptional activation. This review describes in detail the structure and expression of the CD28 and B7 families, the functional outcomes of CD28 ligation and the signalling events that are thought to mediate these functions.

INTRODUCTION

The term 'T cell activation' refers to events triggered by antigen stimulation of T cells that culminate in interleukin 2 (IL-2) production and IL-2 receptor expression (G_0 – G_1 cell cycle transition) [1]. A two-signal model of effective activation of resting T lymphocytes has been proposed [2]: Signal 1 is provided by engagement of the T cell antigen receptor (TCR)/CD3 complex with foreign antigen associated with MHC molecules; Signal 2 is provided by a co-stimulatory molecule(s) present on antigen presenting cells such as dendritic cells, monocytes and activated B cells [1,2]. Considerable attention has focused on the CD28 molecule as providing a potential 'second signal' required for T cell activation, since ligation of the CD28 T cell antigen by anti-CD28 monoclonal antibodies or the natural ligand B7.1 (also termed CD80) or B7.2 (also termed CD86) can lead to cyclosporin-resistant IL-2 production and cellular proliferation in the presence of an additional signal provided by the phorbol ester phorbol 12-myristate 13-acetate (PMA) [3]. Subsequently, the identification of additional family members has given the potential for at least four interactions in T cell co-stimulation which involve the proteins B7.1 [4,5], B7.2 [6,7], CD28 [3,8] and cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) [6,7,9]. The signalling events and functional outcomes that are elicited following B7–CD28 interaction are the best understood, and this review will describe the structure and expression of the CD28 and B7 families, the functional effects of CD28 ligation and the signalling events which mediate these functions.

STRUCTURE AND EXPRESSION OF CD28 AND B7 RECEPTOR FAMILIES

CD28 and CTLA-4

The known members of this family of receptors have now been cloned and analysed in some detail at both the DNA and protein level [3,10,11]. The genes for CD28 and CTLA-4 are closely linked on human chromosome 2 (2q33-34) and share the same genomic organization, suggesting a common evolutionary origin [10]. However, the overall amino acid conservation between the genes is as little as 30%. CD28 is a homodimeric cell surface glycoprotein expressed on 95% of $CD4^+$ T cells and approx. 50% of $CD8^+$ T cells [3]. Although the extracellular domains have limited sequence identity, mapping of the ligand binding sites of CD28 and CTLA-4 has localized the site of interaction with B7.1 and B7.2 to a conserved sequence Met-Tyr-Pro-Pro-Tyr in the complementary determining region 3-like region (CDR3)-like region of both CD28 and CTLA-4 [2,3].

CD28 is composed of two glycosylated 44 kDa chains which are members of the immunoglobulin superfamily, each containing a single disulphide-linked extracellular Ig variable-like (V) domain. The mature CD28 polypeptide contains 202 amino acids giving a molecular mass of 23 kDa, which is then glycosylated at five sites to give the molecular mass of the mature protein. The extracellular domain is linked via a single transmembrane region to a 41-amino-acid cytoplasmic domain which is presumed to be responsible for initiating co-stimulatory signals [3]. Sequence

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free calcium concentration; CTLA-4, cytotoxic T lymphocyte-associated molecule-4; ERK, extracellular-signal-regulated kinase; FRAP, FK506 binding protein/rapamycin-associated protein; GAP, GTPase-activating protein; GM-CSF, granulocyte/macrophage colony-stimulating factor; Grb, growth-factor-receptor binding protein; ICAM, intracellular adhesion molecule; $I\kappa B$, inhibitor of NF κB ; Ig V and Ig C domains, Ig variable-like and constant-like domains respectively; IL-2, interleukin 2; JNK, c-Jun N-terminal kinase; mAb, monoclonal antibody; MAP kinase, mitogen-activated protein kinase; NFAT, nuclear factor of activated T cells; NF κB , nuclear transcription factor κB ; PI 3-kinase, phosphoinositide 3-hydroxy kinase; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; $p70^{S6K}$, p70 ribosomal protein-S6 kinase; PTK, protein tyrosine kinase; RANTES, regulated on activation, normal T cell expressed and secreted; SAP kinase, stress-activated protein kinase; SH domain, src-homology domain; Sos, mammalian Son of sevenless; TCR, T cell antigen receptor; TOR, target of rapamycin.

Figure 1 Schematic diagram showing specific regions of the intracytoplasmic domains of CD28 and CTLA-4

comparison between human, rat, mouse and chicken CD28 cytoplasmic domains demonstrates high interspecies conservation, suggesting a crucial role for this domain in signal transduction. The cytoplasmic domain of CD28 lacks any direct enzymic activity and is therefore presumed to signal via the recruitment of cellular enzymes. Of specific interest, there is a consensus sequence motif within the cytoplasmic domain of CD28 [(p)Tyr¹⁷³-Met-Asn-Met] which forms a potential binding site for interaction with signalling proteins, including the *src*-homology 2 (SH2) domains of the p85 subunit of phosphoinositide 3-hydroxy kinase (PI 3-kinase) and growth-factor-receptor binding protein (Grb-2), a ubiquitous adaptor protein [3,12] (Figure 1). Moreover, CD28 also contains two proline-rich motifs (Pro¹⁷⁸-Arg-Arg-Pro and Pro¹⁹⁰-Tyr-Ala-Pro) which conform to the Pro-Xaa-Xaa-Pro SH3 binding consensus sequence [13], and these regions of the CD28 tail may mediate interactions with signalling proteins.

CTLA-4 is also a disulphide-linked homodimer of predicted molecular mass 20 kDa, with only a single glycosylation site. CD28 and CTLA-4 are expressed on the cell surface as either monomeric or homodimeric forms [3,8]. Both CD28 and CTLA-4 can bind to the same physiological ligands, namely the 60 kDa B7.1 and the 70 kDa B7.2 [3,9], but CD28 is a low-avidity receptor for both B7.1 and B7.2, whereas CTLA-4 is a high-avidity receptor [3]. CTLA-4 was originally identified in a screen for genes involved in T-cell-mediated cytotoxicity [14], but CTLA-

4 transcripts have been detected in both CD4⁺ and CD8⁺ T cell clones [15]. CTLA-4 contains a single disulphide-linked extracellular Ig V domain, a transmembrane region and a cytoplasmic domain of 36 amino acids. Interestingly, there is 100 % conservation between the cytoplasmic domains of human and mouse CTLA-4, suggesting a strongly conserved function [3]. In contrast, there is only limited sequence conservation (approx. 30 % amino acid similarity) between CD28 and CTLA-4 in the cytoplasmic domain, perhaps suggesting an ability to generate different signals. Although the biochemical signals generated by CTLA-4 are poorly defined, CTLA-4 does contain a consensus binding site (Tyr¹⁶⁴-Val-Lys-Met) for the SH2 domains of the p85 subunit of PI 3-kinase. Indeed, anti-CTLA-4 monoclonal antibodies (mAbs) trigger binding of the p85 subunit of PI 3-kinase to this site in the CTLA-4 cytoplasmic domain [16]. To date, tyrosine phosphorylation of CTLA-4 has not been demonstrated, but phosphopeptide binding analysis has confirmed that CTLA-4 can interact with PI 3-kinase via the Tyr¹⁶⁴-Val-Lys-Met motif [16]. There is evidence that these sites may not be functionally equivalent in CD28 and CTLA-4, since the CTLA-4 motif (but not the CD28 motif) is located within an intracellular localization motif (Thr¹⁶⁰-Thr-Gly-Val-Tyr-Val-Lys-Met-Pro-Pro-Thr; Figure 1) that restricts CTLA-4 expression to intracellular membranes such as the perinuclear Golgi or post-Golgi compartment [17].

B7.1 and B7.2

B7.1 and B7.2 are also members of the immunoglobulin superfamily with two Ig-like domains [Ig V domain and Ig constant-like (C) domain], and share approx. 25% amino acid conservation [3]. Unlike their counter-receptors CD28 and CTLA-4, B7.1 and B7.2 are not disulphide-linked and are not expressed as homodimers. There is also evidence that an additional B7 molecule may exist which has been provisionally termed B7.3 [18]. B7.1 is a 60 kDa glycoprotein (30 kDa polypeptide) which consists of two extracellular Ig-like domains with a transmembrane region and a short 19-amino-acid cytoplasmic domain. In contrast, B7.2 is a 70 kDa glycoprotein (34 kDa polypeptide) and also consists of two Ig-like domains, but its major feature is an extended cytoplasmic domain which contains phosphorylation sites for protein kinase C (PKC), possibly indicating a signalling function in antigen presenting cells. B7.0 is an alternate splice variant of B7.2 that is identical to B7.2, except for six additional amino acids at the N-terminus of the B7.2 sequence [7]. B7.1 and B7.2 bind CTLA-4 with 20–100-fold higher affinity than they bind CD28 [3–8], with B7.2 being at most 2–3-fold less active than B7.1 [19]. Site-directed mutagenesis of the B7.1 and B7.2 Ig V and Ig C domains has revealed that full CD28 and CTLA-4 binding to B7 molecules is dependent upon residues in the GFCC'C' β -sheet face of the Ig V domain and the ABED β sheet face of the Ig C domain [20]. Another study reported two hydrophobic amino acid residues, Tyr⁸⁷ and Trp⁸⁴, in the Ig V domain which are critical for binding to CD28 and CTLA-4. These residues lie in the sequence Trp-Pro-Xaa-Tyr-Lys-Asn-Arg-Thr, which is conserved across species [21].

FUNCTIONAL OUTCOME OF B7–CD28 INTERACTIONS

Up-regulation of lymphokines and chemokines

Ligation of CD28 alone has little effect on resting T cell proliferation, but CD28 ligation in the presence of limited concentrations of anti-CD3 or antigen promotes T cell proliferation and IL-2 production (Figure 2) in both CD4⁺ and CD8⁺ T cell subsets, by regulating IL-2 mRNA at the level of both transcription and translation [4,5]. Co-stimulation via CD28 also mediates strong up-regulation of IL-4, IL-5, IL-13, γ -interferon, tumour necrosis factor α and granulocyte/macrophage colony-stimulating factor (GM-CSF) [22–25], up-regulation of the IL-2 receptor α [26] and β [27] chains, and up-regulation of CD40 ligand expression [24,28]. CD28 also induces up-regulation of the chemokine IL-8 [29] and the regulated on activation, normal T cell expressed and secreted (RANTES) chemokine (L.Turner, S. Ward and J. Westwick, unpublished work). The gene promoters of IL-2, GM-CSF, IL-3, γ -interferon [30], IL-8 [29] and RANTES [31] have conserved sequences which are responsive to CD28 signal transduction.

CD28 can prevent anergy

Studies using soluble antagonists such as CTLA-4-Ig fusion proteins or antibodies to block CD28–B7.1 or –B7.2 interactions *in vitro* have shown that T cells which are deprived of CD28-mediated co-stimulatory signals enter into a state of proliferative hyporesponsiveness [32–35]. This long-term antigen-specific unresponsiveness is termed anergy *in vitro* and tolerance *in vivo*.

Figure 2 Model of potential outcomes of CD28/CTLA-4 ligation

+ denotes activation; – denotes inhibition; ? denotes unknown intermediates. The broken line represents a putative interaction.

Anergic T cells could be rescued from this non-functional state by the addition of exogenous IL-2 or by the delivery of a co-stimulatory signal via CD28, indicating that the anergized cells were functionally intact and that unresponsiveness was limited to a specific antigen [32]. The B7-family-mediated co-stimulation appears to be unique in its ability to prevent T cells from entering the anergic state. Co-stimulation induced by intracellular adhesion molecule (ICAM), which produced equivalent levels of proliferation to those induced by B7, was sensitive to cyclosporin A and did not result in IL-2 production; furthermore, upon subsequent antigen-specific rechallenge, T cells were anergic when ICAM had delivered the initial co-stimulatory signal [32]. These data, along with *in vivo* CD28^{-/-} experiments [36], provide ample evidence of a critical role for CD28 signals in controlling T cell responsiveness. Moreover, blockade of CD28 signalling can result in suppression of humoral responses, delay allograft and block xenograft rejection, and inhibit the progression of autoimmune diseases [37–39]. In addition, a number of *in vivo* experimental systems have suggested that B7–CD28 interactions generate anti-tumour immune responses (reviewed in [8]).

CD28 can signal for T cell survival

Although much attention has focused on the role of CD28 in the prevention of anergy, it may also play a role in the modulation of programmed cell death (also termed apoptosis). During T cell development in the thymus, activation of immature thymocytes results in apoptosis, which may be a mechanism for negative selection of immature T cells [40–42]. In contrast, mature naive T cells usually produce cytokines and proliferate upon activation by the TCR and co-stimulatory molecules such as CD28. The

level of CD28 expression is regulated during T cell development as well as during T cell activation and proliferation, suggesting that CD28 may play a role in determining the effect of TCR activation during T cell ontogeny. Indeed, anti-CD28 mAbs prevent apoptosis in immature thymocytes and peripheral blood T cells induced by TCR mAbs [43,44]. There are, however, conflicting studies regarding the effect of CD28 on activation-induced apoptosis in mature and immature T cells ranging from no effect [45] to induction of apoptosis [46]. Nevertheless, CD28 co-stimulation of human T cells has been demonstrated to augment the expression of the intrinsic cell survival factor Bcl-x_L and IL-2 (Figure 2), which correlates with enhanced resistance to apoptosis induced by γ -irradiation, antibodies to Fas or CD3, or IL-2 withdrawal [47]. The expression of Bcl-x_L induced in T cells co-stimulated through CD28 could not be mimicked by treatment of cells with lymphokines such as IL-2, suggesting that CD28-induced expression of Bcl-x_L may be through a direct signalling mechanism and not via the enhanced production of lymphokines [47].

DIFFERENTIAL REGULATION OF IMMUNE RESPONSES

There is considerable evidence that the interactions involving CD28 and B7 family members function to differentially regulate the immune response (Figure 2). For instance, CTLA-4, unlike CD28, is not expressed constitutively and is only expressed maximally 2–3 days after T cell activation by TCR/CD3 and CD28 ligation [48]. The biological consequences of CTLA-4–B7.1/B7.2 interactions are only now being clearly defined, but CTLA-4 cannot replace CD28 in providing co-stimulation in

Table 1 Biochemical signals elicited by anti-CD28 mAbs, B7.1 and B7.2 in T cells

ND, not determined; +, activated; –, no detectable effect; * denotes that cross-linking is required.

Response	mAb	B7.1	B7.2	Remarks	References
1. Early signals					
Tyrosine phosphorylation	+	+	+	Phosphorylation of unidentified substrates in activated T cells and cell lines	[3,82,83,87,166]
PLC γ phosphorylation	+	+	ND	Activated T cells, Jurkat and murine hybridomas	[3,87]
[Ca ²⁺] _i elevation	+	+	–	Cross-linking is a prerequisite in resting T cells, activated T cells and murine hybridomas but not necessarily Jurkat	[3,46,83–89]
Ins(1,4,5)P ₃ elevation	+	ND	ND	Jurkat or activated T cells; B7.1 elicits no detectable PtdIns(4,5)P ₂ degradation	[3,85,86,91]
PI 3-kinase association	+	+	+	CD28 associates with the p85 α subunit	[70,92,114–117]
PI 3-kinase activation	+	+	+	Occurs in resting T cells, activated T cells, Jurkat and murine hybridomas	[70,91,92,114]
Association with Grb-2	+	ND	ND	Resting T cells, Jurkat and HPB-ALL	[173]
p21 ^{ras} activation	+	–	–	Jurkat	[96]
Sphingomyelinase activation	+	+	+	Acidic isoform activated in resting T cells, activated T cells and Jurkat	[125,126]†
5-Lipoxygenase	+	ND	ND	Resting T cells	[128]
2. Intermediate events					
Vav	+	+	+	Tyrosine phosphorylated in Jurkat	[96]
p36	+	–	ND	Tyrosine phosphorylated in Jurkat	[96]
p62 (p120 ^{GAP} -associated)	+	+	+	Tyrosine phosphorylated in Jurkat	[96]
c-cbl	+	+	+	Tyrosine phosphorylated in Jurkat	[166]
Raf-1 activation	+	–	ND	Jurkat	[96]
ERK activation	+	–	–	Jurkat	[96,97]
JNK activation	+	+	+	Synergizes with anti-CD3 mAb in Jurkat cells; cyclosporin A-sensitive	[215]
				Synergizes with PMA/ionomycin; inactive alone	[97]
p70 ^{S6k} activation	+	ND	ND	Resting T cells; rapamycin-sensitive and cyclosporin A-insensitive	[197]
3. Nuclear events					
I κ B α down-regulation	+	ND	ND	Jurkat and resting T cells; requires PMA; rapamycin-sensitive and cyclosporin A-insensitive	[193,194]
AP-1 regulation	+	+	+	Requires TCR signal in murine T cells; down-regulated during anergy	[211,212]
4. Co-stimulation (e.g. IL-2 secretion)	+	+	+	Resting T cells and Jurkat	[70,92,97,114]

† Also D. Sansom, personal communication.

CD28 mutant mice, ruling out functional redundancy [36]. Antibodies to CTLA-4 can enhance T cell proliferation, suggesting that CTLA-4 may synergize with CD28 and sustain co-stimulation [49]. However, cross-linked antibodies to CTLA-4 inhibit proliferation of naive T cells [50,51]. These data suggest that blockade of CTLA-4 removes, whereas aggregation of CTLA-4 provides, inhibitory signals that down-regulate T cell responses [49–51]. Recent evidence from CTLA-4 knock-out mice further suggests that CTLA-4 mediates immune response suppression [52,53]. In addition, anti-CTLA-4 mAbs mediate antigen-specific apoptosis [54] and can allow for, and potentiate, effective immune responses against tumour cells [55]. Furthermore, it is interesting to note that CD28 is transiently down-regulated upon binding to its ligand, and may become hyporesponsive to subsequent stimulation that occurs at approximately the same time that CTLA-4 is normally expressed [56].

A number of differences between B7.1 and B7.2 have also been reported that suggest differential immune regulation by these molecules. (i) Whereas both B7.1 and B7.2 are expressed on activated monocytes, activated B and T cells and activated natural killer cells, only B7.2 is constitutively expressed on dendritic cells and resting monocytes [3,6–8,18,57,58]. (ii) B7.2 is rapidly expressed on B cells following activation, whereas maximum B7.1 expression appears significantly later [6,7,18,57,59]. (iii) In contrast to anti-B7.1 mAbs, anti-B7.2 mAbs are potent inhibitors of T cell proliferation and cytokine production *in vitro* [58,60,61], and *in vivo* B7.2 is the major CD28 ligand active in clonal expansion systems [62], development of diabetes in non-obese diabetic mice [63] and in the stimulation of T-cell-dependent antibody responses [59]. (iv) B7.1 is superior to B7.2 co-stimulation in the induction and maintenance of T-cell-mediated anti-leukaemia immunity [64]. (v) The B7.2–CTLA-4–Ig complex dissociates much faster than the B7.1–CTLA-4–Ig complex [19]. Thus the fast on–off nature of B7.2–CTLA-4 interactions may function to limit an immune response by allowing more efficient disengagement of B7.2 from the high-avidity CTLA-4 receptor. In support of the hypothesis that B7.1 and B7.2 can regulate different functions, several groups have presented *in vivo* evidence to show that B7.1 and B7.2 may play distinct roles in the differentiation of CD4⁺ Th cells. CD4⁺ Th0 cells can differentiate into either Th1 cells (which produce IL-2, γ -interferon and tumour necrosis factor α , and mediate cell-mediated immune responses) or Th2 cells (which produce IL-4, IL-5 and IL-10, and which regulate humoral immune responses). It has been proposed that Th2 immune responses are dependent on high doses of initial antigen and CD28 co-stimulation, whereas Th1 immune responses are less dependent on CD28 co-stimulation (reviewed in [65]). Hence recent reports have shown that B7.1 preferentially acts as a co-stimulator of Th1 cells, whereas B7.2 co-stimulates and induces Th2 cells [66,67]. The molecular basis for the distinct regulation of differentiation remains obscure, since there are no significant intrinsic differences between B7.1 and B7.2 (Table 1) in their ability to activate second messengers and to co-stimulate proliferation, cytokine secretion *in vitro* from CD4⁺ T cells and cell-mediated cytotoxicity [68,69].

CD28-ACTIVATED SIGNALLING CASCADES

The two-signal concept

Individually, neither the TCR nor CD28 can induce levels of IL-2 production that are comparable with those resulting from the combination of TCR and CD28 receptor ligation, unless second messenger agonists such as phorbol esters are also added [70]. A hallmark of CD28-mediated signal transduction is the production

of IL-2 that is resistant to cyclosporin A and FK506 [3]. Accordingly, CD28 has been postulated to provide the co-stimulatory Signal 2 to T cells, which is thought to consist, at least in part, of a unique biochemical signal that is distinct from that provided by the TCR-induced cyclosporin A-sensitive Signal 1 [2,3]. Thus, in order to determine the signals generated by CD28, it is first necessary to understand the signals generated by the TCR.

Signal transduction pathways elicited by the TCR have been reviewed extensively elsewhere [71,72] and will only be described in brief detail here. Triggering of the TCR/CD3 complex initiates a cascade of biochemical events, the earliest of which is the activation of intracellular protein tyrosine kinases (PTKs) [71,72]. This activation of PTKs appears to be absolutely required for all subsequent T cell responses [73] and couples the TCR to an array of signal transduction and adaptor molecules [72]. The TCR does not possess intrinsic enzymic activity, and a specific motif, Glu-Xaa₂-Tyr-Xaa₂-Leu/Ile-Xaa₇-Tyr-Xaa₂-Leu/Ile, termed an immunoglobulin receptor family tyrosine-based activation motif, is crucial for TCR coupling to PTKs and hence subsequent TCR signalling processes [72]. This motif, after phosphorylation by *src* kinases, is responsible for coupling the TCR to non-receptor tyrosine kinases such as ZAP-70 [71]. Several cellular substrates have been identified as substrates for TCR-activated PTKs [72], including components of at least two signalling pathways believed to be important pivotal links in the control of cell activation. The first of these is phospholipase C γ (PLC) [74], which regulates the hydrolysis of phosphatidylinositol lipids, releasing diacylglycerols which activate the serine/threonine kinase PKC, and Ins(1,4,5)P₃ which regulates intracellular calcium release [75]. Secondly, TCR activation of the guanine nucleotide binding protein p21^{ras} is PTK-dependent and is essential for the coupling of the TCR/CD3 complex to mitogen-activated protein kinase (MAP kinase) [also known as extracellular-signal-regulated kinase 1/2 (ERK1/2)] [76]. The calcium signalling system co-operates, via the calcium phosphatase calcineurin, with p21^{ras} for induction of the transcription factor nuclear factor of activated T cells (NFAT) which, along with AP-1, nuclear transcription factor κ B (NF κ B) and Oct-1, is involved in IL-2 gene expression [76]. A third signalling pathway, which is putatively mediated by PI 3-kinase, has also been implicated in cell growth and activation [77]. This may also be controlled by TCR-activated PTKs, since the p85 subunit of PI 3-kinase binds to the TCR ζ chain [78], although this has not been consistently observed to correlate with PI 3-kinase activation [79].

The use of distinct signal transduction pathways by CD28 would lead to a complementation between the TCR and CD28 to allow full activation of T cells, which would fit well with the idea of co-stimulation. However, it is possible that CD28 may enhance the amplitude or duration of a TCR-triggered signal, thereby crossing a threshold to activate downstream signalling cascades, or it may provide signals similar to those provided by the TCR, but at different time points, in different cellular compartments. Although the CD28 co-signal appears to be insensitive to inhibition by cyclosporin A and thus independent of Ca²⁺ and calcineurin [80,81], CD28 signal transduction does appear to be regulated by PTKs [82,83]. The tyrosine phosphorylation pattern induced by CD28 ligation is similar to that induced by TCR ligation, but unique CD28-induced tyrosine phosphorylation of 62 kDa, 75 kDa and 100 kDa proteins is also observed [82,83], suggesting that unique pathways may be coupled to CD28. Until recently, studies addressing CD28-mediated signal transduction events were based on the use of specific anti-CD28 mAbs as stimulators, but the advent of specific ligands (B7.1 and B7.2) has provided interesting results and has highlighted the fact that

the ability of CD28 to activate certain signalling pathways depends on whether the activating stimulus is provided by an anti-CD28 mAb or by ligand stimulation (summarized in Table 1). Several signalling cascades which may be dependent on PTK activation have been demonstrated to be activated by CD28, and these will be reviewed here.

The PLC pathway

It has been described that CD28 regulates the tyrosine phosphorylation of PLC γ , PtdIns hydrolysis and the cytosolic free calcium concentration ($[Ca^{2+}]_i$) [84–87]. There appears to be a difference, however, depending on the type of T cell model used, in the relative ability of CD28 to elicit these responses (summarized in Table 1). For instance, in Jurkat cells, PtdIns hydrolysis, increases in $[Ca^{2+}]_i$ and diacylglycerol generation can be elicited in the absence of cross-linking [88]. However, it has been noted that the ability of anti-CD28 mAbs to elicit these signals is dependent on the epitope recognized by the mAb [88]. Moreover, other groups have reported that CD28 cross-linking is a prerequisite for eliciting PLC activity in purified resting T cells, activated T cells and human CD28-transfected murine T cell hybridoma [46,84–86]. Interestingly, ligation of CD28 by B7.1, but not B7.2, has been shown to result in elevation of $[Ca^{2+}]_i$ in activated T cells [89]. Human CD28 intracytoplasmic tail deletion mutants (Figure 1), which have been stably transfected into a murine hybridoma cell line, have been used to assess the regions of the CD28 cytoplasmic domain required for coupling to PLC. Thus deletion of the last 10 amino acid residues disrupts the distal proline-rich region and Tyr²⁰⁰, but has no effect on the anti-CD28-mAb-induced elevation of $[Ca^{2+}]_i$ (D. Olive, personal communication). In contrast, deletion of the last 30 amino acids additionally disrupts the SH2 binding motif, the proximal proline-rich region, Tyr¹⁸⁸ and Tyr¹⁹¹, and prevents the CD28-induced elevation of $[Ca^{2+}]_i$. This implies that the proximal proline-rich region and/or the Tyr¹⁸⁸ and Tyr¹⁹¹ residues are involved in regulating the coupling of CD28 to PLC (Figure 1). It is possible that the SH2 binding motif is also involved in coupling CD28 to PLC, although this is unlikely since CD28 does not form complexes with SH2 domains derived from PLC γ [90].

In marked contrast to anti-CD28 mAbs, B7.1 failed to elicit any detectable activation of PLC as assessed by PtdIns(4,5) P_2 degradation and phosphatidic acid generation [91] (Table 1), although B7.1 has been reported to induce PLC γ phosphorylation [87] and IL-2 production [70,92]. This suggests that PLC activation following mAb activation of CD28 may be functionally redundant. There is evidence to suggest, however, that there are both cyclosporin A-sensitive and -resistant components of the CD28 signalling responses, depending on the activation state of the T cell, such that the cyclosporin A-sensitive pathway is dominant in activated T cells and tumour cells [3,46], whereas the cyclosporin A-resistant pathway is dominant in resting T cells [80]. Recent studies have shown that the CD28-induced calcium signal was mainly limited to T cells of the CD4⁺ subset [93], indicating that the co-stimulatory signal delivered by CD28 may have different biochemical properties in CD4⁺ and CD8⁺ T cell subsets and therefore that the role of CD28 may differ in these cells. The role of PKC activation in CD28 signalling is also unclear, since CD28 activation of Jurkat cells is not associated with PKC translocation [85], although levels of diacylglycerol are elevated following CD28 ligation by mAbs [88]. Moreover, PKC inhibitors have been reported to either inhibit [88] or have no effect on [94] CD28-dependent IL-2 production.

The p21^{ras} pathway

The intracellular proteins linking receptor-associated PTKs to the regulation of p21^{ras} have been defined: the adaptor molecule Grb-2, which is composed of two SH3 domains that bind to mammalian Son of sevenless (Sos), a guanine nucleotide exchange protein that activates p21^{ras}, and an SH2 domain that interacts with tyrosine phosphoproteins including Shc and a membrane-located tyrosine phosphoprotein p36 [76,95]. Two PTK substrates, Vav (a guanine nucleotide exchange factor) and a 36 kDa membrane protein, are potential mediators of the TCR/p21^{ras} link [76,95]. Interestingly, at least one 100 kDa substrate for CD28-induced tyrosine phosphorylation has been identified as Vav [96], suggesting that CD28 may also activate p21^{ras}. Indeed, an anti-CD28 mAb (CD28.2) can activate the p21^{ras} pathway, since it induced an increase in Ras-GTP complexes and in the phosphorylation of Vav and of the Grb-2/Sos-associated protein p36 [96]. Moreover, the MAP kinase cascade is also activated by CD28.2, resulting in hyperphosphorylation of Raf-1 and activation of ERK2 [96]. In marked contrast (Table 1), B7.1 stimulation of CD28 is unable to induce tyrosine phosphorylation of p36, although it can tyrosine phosphorylate Vav to a greater extent than CD28.2 or CD3 ligation [96]. Another anti-CD28 mAb (CD28.5), which binds CD28 but is unable to induce Ca²⁺ mobilization or IL-2 production, is devoid of an effect on p21^{ras} [96]. Thus CD28 does have the capacity to couple to the p21^{ras} pathway, as demonstrated by the effect of the CD28.2 mAb, although CD28 does not detectably activate p21^{ras} following ligation by B7.1 [96]. Accordingly, the physiological relevance of the anti-CD28-mAb-induced activation of ERK2 is not resolved, but it is probably not essential for CD28 co-stimulation of IL-2 production, since B7.1 and B7.2 can function equally in this context [97].

PI 3-kinase

PI 3-kinase controls an alternative inositol phospholipid metabolic pathway which phosphorylates the membrane phosphatidylinositol lipids at the 3-position on the inositol ring, resulting in the generation of PtdIns3 P , PtdIns(3,4) P_2 and PtdIns(3,4,5) P_3 [77]. At present, at least three biochemically distinct PI 3-kinases have been described in mammalian cells: (i) a p85/p110 heterodimer, which associates with tyrosine-phosphorylated proteins through SH2 domains in the p85 subunit [77]; (ii) PI 3-kinase γ , which is activated by G-protein $\beta\gamma$ subunits [98]; and (iii) a mammalian PtdIns-specific 3-kinase [99]. Use of the term PI 3-kinase should be taken to mean the PTK/SH2-coupled form, unless otherwise stated. This form is the best characterized and comprises a heterodimer of a 85 kDa regulatory subunit (α , β and γ isoforms) containing two SH2 domains, one SH3 domain and two proline-rich sequences that mediate interactions with other signalling molecules [100]. The p85 subunit is tightly associated with a 110 kDa catalytic subunit (α and β isoforms) [101], which is the first reported incidence of a dual-specificity kinase that possesses both protein serine and lipid kinase activities [102].

The D-3 phosphoinositide lipids represent only a minor fraction of cellular phosphatidylinositol lipids and are not substrates for PLC [77,103]. However, considerable importance has been attached to this pathway, particularly concerning the putative role of these lipids as regulatory molecules [77,103]. Kinetic evidence has suggested that PtdIns(3,4,5) P_3 formed by the PtdIns(4,5) P_2 -specific 3-kinase is the probable physiological intracellular mediator of downstream signalling cascades, and this is subsequently degraded and inactivated by sequential dephosphorylation to PtdIns(3,4) P_2 and PtdIns3 P [103]. The

downstream effector molecules of PI 3-kinase-activated signalling cascades in T cells remain relatively obscure, although several proteins such as PKC ϵ , δ , η , λ and ζ isoforms [104–107], protein kinase B/Akt [108,109], p70 ribosomal protein S6 kinase (p70^{s6k}) [110,111] and Rac [112] and SH2 domains [113] have been demonstrated to lie downstream of PI 3-kinase in other systems. These proteins may be direct targets for PI 3-kinase or its lipid metabolites, or there may be intermediate target proteins involved in the activation of these proteins. PtdIns3P may have another specialized function, since it is the major product of mammalian PtdIns-specific 3-kinase, which shares extensive sequence conservation and substrate specificity with the yeast lipid kinase Vps34p [77,99]. Vps34p shares sequence conservation with mammalian p110 [101], and mutagenesis experiments have demonstrated that the PtdIns 3-kinase activity of Vps34p is required for protein sorting and vesicular trafficking [77,103]. Accordingly, it has been postulated that PtdIns3P may have a distinct function related to the trafficking of cell surface receptors [77,103], rather than acting as a signalling molecule. This function of PtdIns3P may be relevant to the mechanisms which regulate CTLA-4 expression, since the CTLA-4 p85 SH2 binding motif [16] is located in an intracellular localization motif within the CTLA-4 cytoplasmic domain [17].

An interaction between PI 3-kinase and CD28 was suggested by the presence of the p85 SH2 domain binding motif Tyr¹⁷³-Xaa-Xaa-Met around Tyr¹⁷³ [3] (Figure 1). The first indication that CD28 could indeed couple to PI 3-kinase was the demonstration that ligation of CD28 by B7.1 induced the accumulation of *D*-3 phosphoinositide lipids, the products of PI 3-kinase activation, in the leukaemic T cell line Jurkat [91]. Later studies revealed that B7.2 or mAb ligation of CD28 also induced formation of *D*-3 phosphoinositide lipids in Jurkat cells [70] (Table 1) and that the association of PI 3-kinase with CD28 occurred in CD4⁺ but not CD8⁺ cells following CD28 ligation [114]. The accumulation of PtdIns(3,4,5)P₃ following CD28 ligation occurs with delayed kinetics, greater magnitude and prolonged duration when compared with the accumulation in response to ligation of the TCR alone [70]. The p85 α subunit of PI 3-kinase can associate with the cytosolic domain of CD28 via the (p)Tyr¹⁷³-Met-Asn-Met motif [90,115,116], as predicted. The interaction of the (p)Tyr¹⁷³-Met-Asn-Met motif with the SH2 domains of the p85 α subunit depends on phosphorylation of the tyrosine residue within the motif, and CD28 is indeed phosphorylated on tyrosine following ligation [90,117]. Phosphopeptide binding studies have confirmed that PI 3-kinase binds directly to CD28 via the (p)Tyr¹⁷³-Met-Asn-Met motif [115,116]. Interestingly, CD28 binds to the C-terminal SH2 domain of p85 α with 10-fold greater affinity than to the N-terminal domain [116], but the relevance of this observation is not presently known.

The sphingomyelinase/ceramide signalling pathway

The sphingomyelinase pathway is a ubiquitous signalling pathway coupled to receptors for tumour necrosis factor α , γ -interferon, IL-1, nerve growth factor and Fas [118,119], and is initiated by hydrolysis of the phospholipid sphingomyelin by the specific PLC sphingomyelinase, resulting in ceramide production. Ceramide serves as a second messenger, and may activate downstream targets such as PKC ζ [120], serine/threonine-specific protein kinases [121] and phosphatases [122]. Sphingomyelinase exists in two forms: a Mg²⁺-dependent membrane-bound enzyme with a neutral pH optimum, and a lysosomal acidic form [118]. The acidic form has been shown to be activated by Fas [123] and the tumour necrosis factor receptor [124]. Since ligation of Fas and the tumour necrosis factor receptor generally results in cell

death, ceramide has been suggested to mediate death signals. Recent reports have shown that anti-CD28 mAbs induce the activation of acidic sphingomyelinase [125,126]; this was prevented by agents shown to be inhibitory to the activation of acidic sphingomyelinase, such as chloroquine. Moreover, this pathway appears to be required for co-stimulation, since the cell-permeable ceramide analogue C-6-ceramide mimicked the CD28 signal by inducing T cell proliferation and IL-2 gene transcription [126]. The natural ligands B7.1 and B7.2 also appear to activate sphingomyelinase (D. Sansom, personal communication). The ability of CD28 to potentially activate both the PI 3-kinase and sphingomyelinase pathways (Table 1) may be a key distinguishing feature between the signals provided by CD28 and the so-called 'death' receptors such as Fas and the tumour necrosis factor receptor [127], which lack any known PI 3-kinase consensus binding motif but activate the acidic sphingomyelinase signalling pathway. Thus there may be key synergy between the CD28-activated PI 3-kinase and ceramide-mediated signalling pathways in protecting the cell from apoptosis and promoting cell survival and/or IL-2 production.

5-Lipoxygenase

It has been reported recently that NF κ B activation and IL-2 gene expression following addition of anti-CD28 mAbs requires leukotriene B4 production by 5-lipoxygenase [128]. In addition, it was demonstrated that leukotriene B4, when added in combination with anti-CD3 mAbs, could mimic anti-CD28 effects [128]. However, these observations have not been confirmed and the effects of the natural ligands B7.1 and B7.2 on this pathway (Table 1) have yet to be determined.

CD28 interactions with PTKs

The primary structure of CD28 indicates that the cytoplasmic tail has no obvious enzymic activity (Figure 1), but CD28 clearly couples to PTKs, as indicated by the tyrosine phosphorylation of a number of substrates [3,8,82,83]. In addition, CD28 must also act as a substrate for PTKs, since it is tyrosine phosphorylated [90,117] and this tyrosine phosphorylation appears critical for its interaction with PI 3-kinase [90]. From analogy with the TCR, CD4 and CD8 T cell surface molecules, which couple to *src*-family non-receptor tyrosine kinases such as p59^{lyn} and p56^{lck} [129], it was postulated that CD28 also couples to similar non-receptor PTK(s). Deletion of the last 30 amino acids (but not the last 10 amino acids) of the CD28 cytoplasmic domain disrupts the tyrosine phosphorylation of substrates by CD28 ligation (D. Olive, personal communication). This implies that the proximal proline-rich region and/or the Tyr¹⁸⁸ and Tyr¹⁹¹ residues are involved in regulating the coupling of CD28 to PTK(s) as well as coupling to PLC, as described earlier (Figure 1). It is unlikely that the SH2 binding motif is involved in coupling CD28 to PTKs, since CD28-induced tyrosine phosphorylation of substrates is still observed in mutants where the motif has been disrupted by site-specific mutagenesis of Tyr¹⁷³ (D. Olive, personal communication).

The involvement of PTK(s) in CD28 signalling events has been investigated using the tyrosine kinase inhibitor herbimycin A, which prevents tyrosine phosphorylation of substrates [82,83], elevation of [Ca²⁺]_i [83,89], PI 3-kinase activation (S. Ward and C. June, unpublished work), leukotriene B4 release [128] and IL-2 production [82,83,128] induced by anti-CD28 mAbs. However, recent evidence indicates that herbimycin A induces non-specific covalent modification of thiol groups [130], and results using herbimycin should therefore be interpreted with extreme caution. Nevertheless, there is other evidence that suggests the involve-

ment of PTK(s) in CD28 signalling. For instance, p56^{lck} has been demonstrated to be activated following cross-linking of anti-CD28 mAbs, but not by anti-CD28 mAbs alone [131], and anti-CD28 mAbs have been demonstrated to induce the association of CD28 with p56^{lck} and p59^{fun} in Jurkat cells as detected by *in vitro* kinase assays [132]. Anti-CD28 mAbs also induce the association with, and immediate tyrosine phosphorylation and activation of, the Tec family kinase p72^{ITK/EMT} in the Jurkat T cell line [133]. Accordingly, it was postulated that p72^{ITK/EMT} activation by CD28 may result in tyrosine phosphorylation of CD28 and the recruitment of signalling proteins such as PI 3-kinase. A novel member of the Tec/itk/Btk family of PTKs has been reported to exist in resting T cells and has appropriately been termed resting lymphocyte kinase [134]. This newly identified protein kinase may prove to play an important role in T cell co-stimulation, although this has yet to be verified. Recent evidence from a transfected insect cell model (*Spodoptera frugiperda*) has clearly demonstrated that p56^{lck} and p59^{fun} (but not ZAP-70 or ITK/EMT) both phosphorylate CD28 at Tyr¹⁷³ and induce PI 3-kinase, Grb-2 and ITK/EMT binding to CD28 [135]. In the human Jurkat leukaemic cell line, however, although functional p56^{lck} is required for optimal CD28-mediated activation of ITK/EMT [136], the association of CD28 with PI 3-kinase and the subsequent activation of PI 3-kinase can still occur in p56^{lck}-deficient cells [137,138]. In contrast, anti-CD28 mAbs are unable to elicit Ca²⁺ mobilization and tyrosine phosphorylation of certain substrates in these p56^{lck}-deficient cell lines [137]. Taken together, the data from human T cell models implies that there are at least two PTK pathways coupled to CD28: p56^{lck}-dependent event(s) (e.g. ITK/EMT phosphorylation and activation, as well as Ca²⁺ mobilization) and p56^{lck}-independent event(s) (e.g. PI 3-kinase association and activation) [137]. However, given the specificity problems associated with the use of herbimycin A [130], it is unwise to speculate that the herbimycin A-sensitive and a herbimycin A-insensitive pathway correlate with the p56^{lck}-dependent and a p56^{lck}-independent pathways respectively.

ROLE OF PI 3-KINASE IN CD28-MEDIATED CO-STIMULATION

In contrast to the sphingomyelinase/ceramide pathway, the PI 3-kinase pathway has been studied extensively with a view to defining its role in CD28-mediated IL-2 production, using a combination of pharmacological and genetic manipulations, which have yielded controversial results and conclusions.

Effect of inhibition of PI 3-kinase on IL-2 production

The fungal metabolite wortmannin at concentrations up to 100 nM irreversibly inhibits the lipid and serine kinase activity of PI 3-kinase, by covalent interaction with the p110 catalytic site [139]. Wortmannin is cell permeable and commercially available, and has been widely used to assess the biological role of PI 3-kinase (reviewed in [140]). The highly electrophilic C-20 position of the furan ring of wortmannin is known to be sensitive to amines and is thought to be the alkylating part of the molecule [141]. Since site-directed mutagenesis of p110 α revealed that a Lys⁸⁰²-to-Arg mutation abolished wortmannin binding to p110, it seems that nucleophilic attack of Lys⁸⁰² results in the formation of an enamine on the furan ring (at C-20) of wortmannin, leading to alkylation and irreversible inhibition of PI 3-kinase [142]. Equivalent residues to Lys⁸⁰² are present in all lipid kinases, such as p110 β , PI 3-kinase γ , PtdIns 3-kinase, Vps34 and human and yeast PtdIns 4-kinases, as well as in protein kinases such as myosin light chain kinase, protein kinase A, DNA-dependent protein kinase catalytic subunit and the target of rapamycin

(TOR)-related proteins [142]. Wortmannin can be predicted, therefore, to react with other known or not yet identified protein and lipid kinases if they show identity within the PI 3-kinase Lys⁸⁰² region. The concentration of wortmannin required to inhibit these enzymes varies (reviewed in [143]), and may depend on the level and efficiency of initial non-covalent interactions with the substrate binding sites of individual kinases and/or the presence of amino acid residues such as Glu⁸²¹ in p110, which may increase the nucleophilicity of Lys⁸⁰² [142]. Although PI 3-kinase is the only high-affinity target for wortmannin in mammalian cells (K_i 1–10 nM), with the possible exception of a soluble PtdIns 4-kinase [144] and phospholipase A₂ [145], non-specific interactions are to be expected at pH values above 8, when the nucleophilicity of lysine is increased by deprotonation. Indeed, wortmannin at concentrations above 1 μ M has been reported to inhibit other enzymes such as PKC and phospholipase D (reviewed in [140,143]). Hence information gained from the use of wortmannin to assess the biological implications of PI 3-kinase may have limited value, at least under some circumstances.

Wortmannin has been used to assess the functional relevance of PI 3-kinase activation with respect to CD28-mediated co-stimulation of IL-2 production, but it has yielded controversial results. Hence in the leukaemic T cell line Jurkat, wortmannin inhibits the CD28-stimulated accumulation of D-3 phosphoinositide lipids with an IC₅₀ of less than 10 nM [70,92]. However, similar concentrations of wortmannin (10–100 nM) actually potentiate the CD28-mediated co-stimulation of IL-2 production by these cells [70]. In addition, nanomolar concentrations of wortmannin can potentiate NFAT induction (D. Cantrell, personal communication) and induce low amounts of IL-2 production from Jurkat cells [70] in the absence of any other stimulus, implying that PI 3-kinase may be constitutively active in Jurkat cells. In marked contrast, other groups were unable to detect any effect of wortmannin (at concentrations up to 10 μ M) on CD28-induced IL-2 production by Jurkat cells [146,147]. The apparent resistance of CD28-mediated co-stimulation to wortmannin under certain conditions is not unique to Jurkat cells, since similar observations were made using freshly isolated murine CD4⁺ cells [148]. These findings have led to much confusion and considerable speculation that PI 3-kinase has no functional role in the induction of IL-2 production by CD28 [146–148]. The use of Jurkat cells to study the CD28-mediated co-stimulation of IL-2 production could be misleading, however, since transformed cell lines may be poor models of Signal 2 because they have been selected to be independent of co-stimulatory signals. Of major importance, therefore, is the observation that nanomolar concentrations of wortmannin (< 100 nM) inhibit the CD28-mediated co-stimulation of IL-2 production in purified resting T cells and human T lymphoblasts [70,92,114]. In these cells, optimal IL-2 production is usually much more dependent on the provision of Signal 2, thus making them much more relevant models in which to study the co-stimulatory signalling cascades regulating IL-2 production.

The quercetin derivative LY294002 has been reported to competitively inhibit PI 3-kinase at micromolar concentrations, and is without effect on PtdIns 4-kinase and several other protein and lipid kinases at concentrations up to 50 μ M [149]. This compound is structurally unrelated to wortmannin and has no site for nucleophilic attack. Thus LY294002 is unlikely to have the same non-specific actions as wortmannin and provides an alternative to wortmannin in the pharmacological assessment of the cellular role of PI 3-kinase. Interestingly, micromolar concentrations of LY294002 (< 3 μ M) also potentiate the CD28-mediated co-stimulation of IL-2 production in Jurkat cells, mimicking the effect of wortmannin [70]. The effects of wort-

mannin on resting T cells and T cell lymphoblasts argue strongly in favour of PI 3-kinase having an important role in regulating CD28-induced IL-2 production. It is possible, however, that non-specific actions of wortmannin may contribute to its observed effects on IL-2 production. Moreover, the effect of LY294002 on the CD28-mediated co-stimulation of IL-2 production in purified resting T cells or T lymphoblasts has yet to be reported. Despite the obvious limitations of using wortmannin as a PI 3-kinase inhibitor, the effects of this agent on IL-2 production in normal T cells [70,92,114], as well as in Jurkat cells under certain conditions [70], imply that PI 3-kinase may deliver either positive (normal T cells) or negative/suppressive (Jurkat) signals for IL-2 production. The apparent ability of PI 3-kinase to transmit either a negative or a positive signal may also be important in understanding (i) the ability of CTLA-4 to co-operate with CD28 in the induction of production of IL-2 [49] but to generate negative signals under other circumstances [50,51,55], and (ii) the contrasting effects of CD28 on apoptosis [43–47].

Effect of mutagenesis of the SH2 domain binding motif on IL-2 production

Other controversial observations concerning the role of PI 3-kinase in CD28-mediated co-stimulation have been obtained from site-specific mutagenesis of the CD28 cytoplasmic tail. For instance, mutation of the human CD28 cytoplasmic tail residue Tyr¹⁷³ to Phe¹⁷³ and subsequent expression in a murine T cell hybridoma prevents CD28–PI 3-kinase association, activation of PI 3-kinase [90,150] and production of IL-2 in response to CD28 ligation. It should be noted that the murine T cell hybridoma is unusual in that CD28 ligation alone is sufficient to elicit IL-2 production in these cells. PI 3-kinase and Grb-2/Sos bind the same CD28 cytoplasmic (p)Tyr¹⁷³-Met-Asn-Met motif, and mutation of Tyr¹⁷³ disrupts both PI 3-kinase binding and Grb-2 binding [150]. Thus this mutation does not distinguish the roles played by PI 3-kinase and Grb-2/Sos in CD28 signalling. In contrast, mutation of Met¹⁷⁶ to Cys¹⁷⁶ disrupts only PI 3-kinase binding, and this mutant is also unable to support CD28-driven IL-2 production [150].

The use of Jurkat cells to study the effect of site-specific mutagenesis of murine CD28 on IL-2 production has produced contrasting data. Thus mutation of Tyr¹⁷⁰ of murine CD28 (which corresponds to Tyr¹⁷³ of human CD28) to Phe¹⁷⁰ prevented the ligation-induced association of CD28 with PI 3-kinase, but CD28 ligation still resulted in IL-2 production in these models [148,151]. These studies also identified different regions which were required for CD28 co-stimulatory activity. (i) The minimal elements required for co-stimulatory activity were suggested to be the Asp¹⁶⁹ and Asn¹⁷² residues (corresponding to Asp¹⁷² and Asn¹⁷⁵ respectively of human CD28) which reside in or near the consensus p85 binding motif. The Asp¹⁶⁹ mutant failed to bind PI 3-kinase and did not co-stimulate IL-2 production, whereas the Asn¹⁷² mutant did bind PI 3-kinase but failed to co-stimulate IL-2 production [151]. (ii) CD28 co-stimulatory activity requires the integrity of one or more of the other three C-terminal tyrosine residues at Tyr¹⁸⁵, Tyr¹⁸⁸ and Tyr¹⁹⁷ (which correspond to Tyr¹⁸⁸, Tyr¹⁹¹ and Tyr²⁰⁰ respectively of human CD28; Figure 1), since mutation of these residues (while preserving Tyr¹⁷⁰) was sufficient to inhibit CD28-induced IL-2 production in Jurkat cells, but had no effect on PI 3-kinase activation by CD28 [148]. The effect of mutation of the three C-terminal tyrosine residues and Asp¹⁷² or Asn¹⁷⁵ on other signalling pathways activated by CD28 (e.g. p21^{ras} activation, PLC activation, sphingomyelinase activation and PTK activation) has

yet to be elucidated. However, at least one of these C-terminal tyrosine residues is involved in modulating the coupling of PI 3-kinase to human CD28 in the murine hybridoma model, since: (i) site-specific mutation of human CD28 Tyr²⁰⁰ (while preserving Tyr¹⁷³) is sufficient to induce an approx. 90 % decrease in CD28-induced PI 3-kinase activation and also reduced CD28-mediated IL-2 production [152]; and (ii) phosphopeptides corresponding to residues 186–202 of CD28 with Tyr²⁰⁰ phosphorylated interact directly with the SH2 and SH3 domains of p85 *in vitro* [152]. Hence, in some models at least, other tyrosine residues within the CD28 cytoplasmic tail may be involved in the recruitment and/or activation of PI 3-kinase [152] (Figure 1).

Although IL-2 gene expression has traditionally been used as a functional indication of CD28-mediated signal transduction, it has been suggested that CD28 function may also be assessed by changes in β_1 -integrin-mediated adhesion, which is up-regulated following CD28 ligation in the absence of any signal from the TCR [153]. Similarly, ligation of a chimaeric construct containing the CD28 cytoplasmic domain fused to the extracellular and transmembrane domains of CD2 results in increased β_1 -integrin-dependent adhesion of HL60 transfectants to fibronectin, which was completely inhibited by wortmannin [154]. Furthermore, cross-linking of a CD2/CD28 chimaera containing a Tyr to Phe substitution corresponding to position 173 of the CD28 cytoplasmic tail prevented both ligation-induced association of the chimaera with PI 3-kinase and up-regulation of β_1 -integrin function, implying a critical role for PI 3-kinase in the regulation of β_1 -integrin activity by CD28 [154]. It is interesting to note that wortmannin does not inhibit the CD28-mediated up-regulation of integrin adhesiveness on Jurkat cells [154]. These data further emphasize the differences that have been observed in the role of PI 3-kinase in CD28-mediated stimulation in Jurkat cells, and indicates that PI 3-kinase may mediate multiple functions of CD28.

Is PI 3-kinase a specific CD28 signal?

The accumulation of *D*-3 phosphoinositide lipids has been reported to occur in response to ligation of several other T cell surface molecules, such as (i) the TCR [155], CTLA-4 [16], CD2 [155] and CD4 [156,157], which modulate the early events (G_0 to G_1 transition) in T cell activation; (ii) CD7 [158], which has a relatively undefined role; and (iii) in response to interactions between IL-2 and the IL-2 receptor [159–161], which control later events (e.g. the G_1 to S transition) in T cell growth. The *D*-3 phosphoinositide lipids generated by molecules such as the TCR [155] may target the same proteins as the *D*-3 phosphoinositides generated by CD28, but may be generated at insufficient levels or at inappropriate times to elicit full-scale activation events independently of the CD28 signal. Interestingly, TCR ligation alone or in combination with phorbol esters can induce small but significant levels of wortmannin-sensitive IL-2 production [70,114]. The significance of PI 3-kinase activation in the events leading to T cell growth mediated by the IL-2 receptor [159–161] is also unclear, since wortmannin has no effect on the IL-2-induced proliferation of normal T lymphoblasts [92] but does inhibit the IL-2-induced proliferation of tumour cells [161].

Several notable features of the enzyme may allow the participation of PI 3-kinase in diverse and specific cellular responses. These include the following. (i) Several biochemically distinct PI 3-kinases exist that exhibit different substrate specificities and wortmannin sensitivities [98,99,140,143]. (ii) The different lipid products PtdIns3P, PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ may mediate different functions, such as trafficking of cell-surface receptors (PtdIns3P) and cell signalling [PtdIns(3,4)P₂ and

PtdIns(3,4,5) P_3] [77,103]. (iii) Heterogeneity of each distinct PI 3-kinase, similar to the heterogeneity observed for p85 and p110 subunits [77,100,101], would allow differential and specific regulation of this enzyme by different receptors. At least two forms of p85 (α and β) have been shown to be expressed in T cells and to be differentially regulated by the TCR [162]. (iv) The D-3 phosphoinositides, particularly PtdIns(3,4,5) P_3 , are generally restricted to the membrane compartments in which they are made [163], giving rise to a high degree of compartmentalization and possibly different functional consequences. (v) The additional wortmannin-sensitive protein kinase activity of PI 3-kinase [102,164] also allows for diversity in PI 3-kinase function and regulation. The role of the protein serine kinase function of PI 3-kinase, as well as the involvement of PtdIns 3-kinase and PI 3-kinase γ in T cell activation, have yet to be investigated.

REGULATION OF ADAPTOR PROTEINS BY CD28

It is becoming clear that a growing number of proteins that do not exhibit catalytic functions can act as specialized adaptor molecules which facilitate the coupling of receptors to downstream signalling cascades. Generally, these proteins feature domains that are important for protein–protein interactions such as SH2/SH3 domains and/or proline-rich regions. Recent evidence suggests that the multi-functional SH2 and SH3 domain binding protein p62 [165], complexed with p120-Ras-GTPase-activating protein (GAP), is a substrate for CD28- but not TCR-activated PTK(s) [166]. In addition, tyrosine phosphorylation of p62 is also triggered by the accessory receptor CD2 [167]. This raises the possibility that, in T cells at least, p62 is involved selectively in accessory receptor signal transduction mechanisms. Although the role of p62 in T cells has yet to be fully determined, another tyrosine phosphorylated protein, p190, is present in the p62-Ras-GAP complexes, which contains a domain with GTPase activity for the GTP binding protein Rho and a second domain that has identity to Rho-GAP [168]. 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 the related protein Rac are essential for the control of the actin cytoskeleton in fibroblasts [169], and it is recognized that Rho may be equally as important as p21^{ras} and Rac in the regulation of protein kinase cascades that culminate in transcription factor phosphorylation [170]. Moreover, Rho can regulate the activity of PtdIns 5-kinase [171] and can activate PI 3-kinase [172]. Thus Rho may be involved in modulating the established effect of CD28 on PI 3-kinase and/or possible effects of CD28 ligation on PtdIns 5-kinase.

The PI 3-kinase binding motif (p)Tyr¹⁷³-Met-Asn-Met present in the CD28 cytoplasmic tail also corresponds to the consensus binding motif [(p)Tyr-Xaa-Asn-Xaa] for Grb-2 [12]. Indeed, a recent report demonstrated that CD28 binds to the Grb-2/Sos complex by means of the cytoplasmic (p)Tyr¹⁷³-Met-Asn-Met motif, although the Grb-2 binding has much lower affinity (up to 100-fold lower) than CD28/PI 3-kinase binding [173]. It should also be noted that the CD28/Grb-2 association was observed using 1.5×10^8 cells (resting T cells, Jurkat or HPB-ALL) in the presence of extensively cross-linked CD28 mAbs [173], and has not so far been reported following ligation of CD28 by either of the natural ligands. The relevance of this association has not yet been determined, but it may be responsible for mediating CD28 coupling to the p21^{ras} pathway and/or PLC γ [76] in response to anti-CD28 mAbs. The demonstration that CD28 ligation induces tyrosine phosphorylation of p62 provides an alternative mechanism by which CD28 may be coupled to p21^{ras}, since p62 co-associates with p120-Ras-GAP [166]. This, therefore, implicates p62 in the regulation of p21^{ras}, although there is no obvious correlation between p62 tyrosine phosphorylation and p21^{ras} activation. The functional importance of p21^{ras} activation by anti-CD28 mAbs has yet to be demonstrated, although it is interesting to note that certain anti-CD28 mAbs which can induce p21^{ras} activation can also induce small but significant levels of IL-2 production [96,174] in the absence of any detectable association with PI 3-kinase; this is resistant to wortmannin [114]. Thus the p21^{ras} signalling pathway may be a relatively minor, but

Figure 3 Putative interactions of CD28 with adaptor molecules

The p85 subunit of PI 3-kinase is bound via the (p)Tyr¹⁷³-Met-Asn-Met motif to the CD28 cytoplasmic tail upon ligation. Grb-2, p62 and/or unknown proteins, together with their associated proteins (e.g. *cbl*, Ras-GAP, Rho), are recruited to p85 via SH2/SH3 interactions (denoted by coloured arrows). These p85-bound proteins facilitate coupling of CD28 with other proteins and signalling cascades as shown. The possible direct interaction of Grb-2 with CD28 via the (p)Tyr¹⁷³-Met-Asn-Met motif is not shown. Refer to the text for further details.

nonetheless significant, contributing pathway in the CD28-derived signals required for IL-2 production, at least in some T cell models. Another putative role for p21^{ras} activation by CD28 may be to enhance or facilitate the activation of PI 3-kinase induced by CD28, in the light of recent reports that show that p21^{ras} can stimulate PI 3-kinase [175–177]. Such p21^{ras} stimulation of PI 3-kinase may also be an important function, at least in part, of TCR-induced p21^{ras} activation, mediating a kind of ‘cross-talk’ between the TCR and CD28. Conversely, CD28 may also influence TCR activation of p21^{ras} in a reciprocal manner, since some cellular responses to constitutively active PI 3-kinase are Ras-dependent [178], and PI 3-kinase has been demonstrated to inhibit GAP activation in adipocytes, allowing insulin to fully activate p21^{ras} [179].

It is becoming clear that Grb-2 SH3 domains can bind molecules other than Sos and may therefore regulate multiple effector pathways. In particular, recent studies have identified at least three other potential Grb-2 SH3 binding effector proteins in T cells: a proline-rich and SH2-domain-containing 75 kDa protein [180], a 116 kDa protein [181], PI 3-kinase [182] and the proto-oncogene *c-cbl* [183]. The functions of p75, p116 and *c-cbl* are not known, but p75 and p116 are both substrates for TCR-activated PTKs [180,181], while *c-cbl* is a substrate for CD28- and TCR-activated PTKs [166,183]. TCR ligation has recently been observed to modulate the interaction of p85 with the putative SH3 binding molecule *cbl*, as well as with Grb-2 [182]. The interaction of PI 3-kinase with Grb-2 has been observed in other systems [184] and may provide an additional mechanism for coupling PI 3-kinase to receptors, such as the TCR, which lack the ability to bind PI 3-kinase directly via p85 SH2 binding motifs.

The SH2, SH3 and proline-rich regions of p85 can putatively mediate a plethora of protein–protein interactions [77], such that the p85 subunit may play an important adaptor-like role in other signal transduction cascades quite unrelated to the lipid or serine kinase activities of p110; these adaptor functions would thus be expected to be resistant to inhibition by wortmannin (Figure 3). In this respect, p85 has been reported to function as an adaptor molecule linking the activated insulin receptor to the multifunctional SH2 and SH3 domain binding protein p62, and with Ras-GAP [185]. PI 3-kinase, by virtue of its adaptor molecule potential, may therefore participate in coupling CD28 to other signalling cascades such as the activation of p21^{ras}.

DOWNSTREAM EFFECTOR TARGETS FOR CD28-ACTIVATED SIGNALLING CASCADES

The 5′ upstream region of the IL-2 promoter between position – 325 and the transcriptional start site contains a number of sites for the binding of transcription factor complexes including AP-1, NFκB, NFAT and Oct for transcriptional activation [30]. Since IL-2 gene expression cannot be induced by a single signalling pathway, studies of transcriptional regulation utilize a combination of potent signals such as those induced by cross-linked mAbs, PMA with ionomycin or mitogens to mimic activation signals. Moreover, much of the information on IL-2 transcription has been obtained from studies on tumour cell lines such as Jurkat and EL-4, since it is difficult to measure the low transcriptional rate of the IL-2 gene in normal T cells by conventional assays, and normal T cells are generally resistant to transfection by reporter genes. However, a recent study has succeeded in optimizing conditions for transient transfection of normal human peripheral blood T cells with mutagenized promoters [186]. This approach has revealed the interesting observation that the important IL-2 *cis*-regulatory elements in

normal T cells are the proximal AP-1 site and the NFκB site [186], while the NFAT sites are much less important in normal T cells than in Jurkat cells. Thus IL-2 transcriptional regulation differs in tumour cell lines compared with normal T cells [186], further highlighting the problems of using transformed cell lines as models of T cell activation. To date, few data are available relating to the effects of natural CD28 ligands on transcriptional regulation, and there is little direct evidence concerning the effects of CD28 on the known effector targets of the PI 3-kinase and sphingomyelinase/ceramide pathways. However, it is possible to gauge the involvement of these putative downstream targets on transcriptional regulation by studying the IL-2 transcriptional events modulated by CD28 in the presence of pharmacological and/or genetic manipulations of PI 3 kinase or sphingomyelinase/ceramide, or by studying the putative effector molecules themselves.

The CD28 response complex and NFκB

Anti-CD28 mAbs induce the formation of a CD28-responsive complex that binds to a site on the IL-2 gene distinct from previously described binding sites [30,187]. This binding site has been termed the CD28 response element, and is located between positions – 160 and – 152 relative to the transcription start site. The IL-8 [29], RANTES [31], IL-3 and GM-CSF [30,188] promoters also contain distinct elements of similar sequence which specifically bind a CD28-induced nuclear complex. Mutation of the CD28 response element results in the loss of CD28-induced activity without affecting TCR-induced IL-2 promoter activity [187]. The CD28 response element is distinct from, but related to, the NFκB element [30]. The NFκB/Rel family of transcription factors consists of five family members: NFκB1 (p50), NFκB2 (p52), c-Rel, p65 RelA and RelB [189]. The Rel family proteins p50, p65 and c-Rel are among the components of the CD28 response complex [190], and the recruitment of these proteins was shown to be resistant to cyclosporin A [190]. Evidence for the importance of p50 in the immune response can be seen from knock-out mice which show multiple B cell and T cell defects, including defective CD28 responses [191]. Activation of NFκB can be brought about by a variety of agents, including PMA and ionomycin, which result in the rapid and transient degradation of the cytoplasmic inhibitor of NFκB (IκBα) and dissociation of active NFκB from the inhibitory NFκB–IκBα complex [189,192]. Interestingly, anti-CD28 mAbs, in the presence of PMA, can induce sustained, cyclosporin A-resistant, down-regulation of IκBα which is associated with an increase in the nuclear translocation of c-Rel [193,194].

Regulation of NFκB by CD28-activated signalling cascades

The signalling mechanisms by which CD28 regulates NFκB have still to be fully elucidated. However, there is evidence that the signalling cascades mediated by both the sphingomyelinase and PI 3-kinase pathways are involved, at least to some degree. For instance, ceramide has been shown to modulate the activity of several intracellular proteins including NFκB [195], which may be mediated via ceramide-activated PKCζ [119,120], ceramide-activated protein kinase [121] or a ceramide-activated protein phosphatase [122]. It is particularly interesting to note that overexpression of sphingomyelinase substitutes for CD28 with regard to NFκB activation [125].

The signalling cascade(s) regulated by PI 3-kinase may also be involved in regulating NFκB activation. PI 3-kinase may exert regulation of NFκB via the activation of the serine/threonine protein kinase p70^{s6k} [196]. There is considerable evidence that PI 3-kinase and its *D*-3 phosphoinositide lipid products may mediate

p70^{s6k} activation, since the PI 3-kinase inhibitors wortmannin and LY294002 block platelet-derived growth factor- and insulin-mediated p70^{s6k} activation [110]. Moreover, PI 3-kinase mediates activation of p70^{s6k} *in situ* through site-specific threonine phosphorylation [111]. Given that CD28 elicits marked activation of PI 3-kinase [70,91,92,114], several groups have investigated whether p70^{s6k} is a target for CD28-activated signalling cascades. Accordingly, cross-linked CD28 mAbs (but not soluble CD28 mAbs) have been found to induce activation of p70^{s6k} in resting purified T lymphocytes [197]. Similar to the CD28-induced elevation of D-3 phosphoinositides [70], the activation of p70^{s6k} occurs with slower kinetics than the kinetics observed for anti-CD3-mAb-induced activation of p70^{s6k} [197]. In addition, both TCR- and CD28-induced effects are inhibited by the macrocyclic compound rapamycin (but not by FK506 or cyclosporin A) which inhibits activation of p70^{s6k} [197]. Since the activation of p70^{s6k} by the TCR and CD28 appears to be additive [197], this dual stimulation of a common pathway may be sufficient to cross critical thresholds that are not crossed upon ligation of individual molecules, and this pathway may be a necessary component of growth regulation via either cell surface receptor.

While not sufficient to drive proliferation, the induction of p70^{s6k} activity may be necessary for the initiation of cell cycle progression in resting T cells. Indeed, a requirement for p70^{s6k} activity has already been demonstrated for the G₁ to S progression triggered by IL-2 [198]. Rapamycin inhibits anti-CD28-mAb-induced I κ B α down-regulation and c-Rel translocation [194], but it has no effect on CD28-induced PI 3-kinase activation [199]. In contrast, wortmannin has been demonstrated to inhibit the activation of p70^{s6k} induced by anti-CD28 mAbs [199]. Thus there may be upstream regulation of I κ B α and/or c-Rel by a signalling cascade involving the lipid products of PI 3-kinase and p70^{s6k}. However, experiments assessing whether PI 3-kinase inhibitors prevent CD28-induced activation of NF κ B and/or c-Rel translocation have not yet been reported. Blocking of p70^{s6k} either by injection of antibodies or by the use of rapamycin has suggested a role for p70^{s6k} in G₁-S phase transition [200,201]. Given the importance of CD28-derived signals in regulating the proliferation of resting T cells, a role for CD28-induced signals in p70^{s6k} activation therefore seems appropriate.

It should be noted that the activation of p70^{s6k} by CD28 has only been observed in response to anti-CD28 mAbs [197] and, to date, there are no reports concerning the effect of ligand activation of CD28 on p70^{s6k} activity. Moreover, there is some doubt as to whether CD28-activated p70^{s6k} alone is sufficient to mediate I κ B α down-regulation, since the signals generated by PMA alone (which include p70^{s6k} activation [110]) induced rapamycin-resistant, transient, down-regulation of I κ B (as opposed to rapamycin-sensitive, sustained, down-regulation in the presence of anti-CD28 mAbs [193,194]). This discrepancy could be due, in part, to the activation by CD28 of a phorbol ester-insensitive PKC isoform such as PKC ζ , which is activated *in vitro* by D-3 phosphoinositides [105]. PKC ζ has also been suggested as a direct target for ceramide and thus may play a critical role in sphingomyelinase signalling [119,120]. Hence PKC ζ could be a potential target for integration of the CD28-induced PI 3-kinase signals and sphingomyelinase signals. It should be noted, however, that active mutants of PKC ζ have no effect on p70^{s6k} activity when transfected into COS cells [202].

The PKC responsive element in the IL-2 gene enhancer contains sites for the transcription factors NF κ B, AP-1 and NFAT [76]. The role of specific PKC isoenzymes is poorly understood, although PKC ϵ and PKC θ can regulate the transcription factors AP-1 and NFAT in leukaemic T cells [203,204], while PKC ζ modulates NF κ B induction in fibroblasts [120]. In

addition, PKC has been implicated in the control of many signalling pathways, including p21^{ras} and the MAP kinases such as ERK1/2, and c-Jun N-terminal kinase (JNK-1/2) [76,97,205]. JNK1/2, along with p38, belongs to the stress-activated protein (SAP) kinase subfamily of MAP kinases, which are subject to distinct upstream regulatory kinases, such as SAP kinase/JNK(s) rather than MAP kinase kinase(s) [205,206]. Whereas the MAP kinase cascade can be activated by anti-CD28 mAbs in the absence of PMA [96], CD28 ligation (by anti-CD28 mAbs or natural ligands) only activates the SAP kinase/JNK cascade in the presence of PMA [97]. While both of these cascades appear to be important for the activation of κ B-dependent promoters [202], active mutants of PKC ζ have been demonstrated to activate the MAP kinase cascade but not the SAP kinase/JNK cascade [202]. Thus the effects of CD28 on NF κ B probably require the integration of PKC ζ -dependent and -independent signalling cascades. The PKC ζ -independent cascades may be regulated by other phorbol ester-insensitive PKC isoforms or by undefined targets of CD28-activated PI 3-kinase and/or sphingomyelinase.

The intermediate signals that mediate the effect of CD28 on NF κ B may also involve the mammalian equivalent of the TOR protein, which is the site of action of rapamycin in yeast [207]. One such protein, FK506 binding protein/rapamycin-associated protein (FRAP), is a member of the PI 3-kinase family of proteins that is expressed in T cells [208]. FRAP, like PI 3-kinase, exhibits serine kinase activity that results in autophosphorylation. This kinase activity is rapamycin-sensitive and is required for regulation of p70^{s6k} [209]. Although the kinase activity of FRAP is insensitive to wortmannin [209], the possibility remains that another wortmannin-sensitive TOR-like protein(s) lies upstream of p70^{s6k}. Thus it will be interesting to determine the effect of CD28 ligation on TOR-like protein kinases such as FRAP.

Regulation of AP-1 and JNK by CD28-activated signalling kinases

In addition to binding a functionally important AP-1 site in the IL-2 promoter, AP-1 proteins participate in the formation of NFAT and NF-IL-2 complexes [210]. Interestingly, AP-1 transcriptional activity requires both TCR- and CD28-mediated signals [211], and a lack of AP-1 induction has been suggested as one of the abnormalities found in anergic murine T cells [212]. Given that CD28 is the proposed co-stimulus required to prevent anergy, this suggestion appears to be somewhat at odds with a recent observation that CD28 stimulation also causes down-regulation of AP-1 [213]. AP-1 activity is regulated both at the level of *jun* and *fos* gene transcription and by post-translational modification of their products [210]. A key event in *c-fos* induction is phosphorylation of the transcription factor Elk-1 by the MAP kinases ERK1 and ERK2 [210]. Accordingly, the role of p21^{ras} in coupling the TCR to ERK2 and the ability of ERK2 to translocate to the nucleus, where it can directly regulate transcriptional factors such as Elk-1, could explain the role of p21^{ras} in TCR signal transduction [76]. In contrast, induction and regulation of c-Jun appears to be mediated by the SAP kinases JNK-1/2 and p38 [214].

JNK, unlike ERK, is not activated by phorbol ester alone, but rather requires phorbol ester in combination with calcium ionophore [97,215]. Triggering of CD3 and CD28 also results in cyclosporin A-sensitive activation of JNK, whereas each stimulus alone results in little or no activation [215]. This observation prompted speculation that the integration of signals that lead to T cell activation and IL-2 induction occurs at the level of JNK, although subsequent studies have failed to detect synergistic activation of JNK after CD3 and CD28 ligation ([97]; S. Ward,

Figure 4 Schematic representation of signal transduction by CD28 at different states of T cell activation

Coloured arrows represent the proposed importance of the major signalling cascades in resting and activated T cells. Broken arrows represent the transition from the resting state to the activated state of the T cell. The major target of PI 3-kinase in resting T cells is $p70^{S6K}$. This wortmannin-sensitive activation of $p70^{S6K}$ by CD28 (and subsequently by IL-2, produced in response to the synergistic action of Signal 1 and wortmannin-resistant components of Signal 2a such as sphingomyelinase) facilitates cell cycle progression. In activated T cells, the wortmannin-sensitive activation of $p70^{S6K}$ by CD28 is non-essential. Instead, $p70^{S6K}$ activity is maintained by IL-2, produced in response to Signal 1, Signal 2b and possibly wortmannin-resistant components of Signal 2a. These wortmannin-resistant components may comprise sphingomyelinase-dependent signals and/or adaptor functions of p85 which facilitate coupling of CD28 to $p21^{ras}$ via Grb-2 and/or p62-associated Ras-GAP. Other signalling pathways (e.g. PLC) may also be coupled to CD28 via Grb-2 or unknown adaptor-protein-protein interactions. Abbreviation: SMase, sphingomyelinase. Refer to the text for further details.

unpublished work). Rather, the combination of CD28 ligation (following B7.1, B7.2 or anti-CD28 mAb stimulation) plus PMA and ionomycin was found to markedly increase JNK activity above the levels observed in response to PMA and ionomycin alone [97]. Taken together, these observations suggest that CD28-activated signals co-operate with calcium ionophores and phorbol esters to regulate JNK. Calcium signals mediated by calcineurin are important for JNK activation in T cells [215]. The TCR controls calcium homeostasis during T cell activation, and this is one mechanism whereby the TCR may contribute to JNK regulation [215]. However, calcium signals and CD28 signals are not sufficient for JNK activation in T cells, implying that there is a minimal requirement for the convergence of three signals for JNK activation. The third signal can be provided by phorbol esters, and may thus be mediated by $p21^{ras}$ and/or PKC.

The TCR- and CD28-activated signals that contribute to JNK activation have not so far been fully elucidated. There are at least two distinct MAP kinase cascades in mammalian cells which control ERK and JNK activation [205,206], and the point of convergence of calcium/TCR/CD28 signals in the JNK regulatory cascade is at present unknown. It is interesting to note, however, that the PI 3-kinase and sphingomyelinase signalling pathways, which are potently activated by CD28, have been demonstrated to modulate both the ERK and JNK cascades in other systems and may therefore be involved in the regulation of *c-fos* or *c-jun*. For instance, a constitutively active p110 mutant

activates *fos* transcription in a Ras-dependent manner [178], implying that PI 3-kinase can directly modulate the $p21^{ras}$ pathway and thus the eventual modulation of ERKs that are thought to be involved in the transmission of signals from $p21^{ras}$ to the nucleus. Moreover, the Ras-related small GTP binding protein Rac, which has been implicated as an upstream regulator of the kinase cascades involved in the activation of JNK [214,216], appears to be a downstream effector for PI 3-kinase [112]. Furthermore, the sphingomyelinase/ceramide signalling pathway is also known to contribute to the regulation of ERK and JNK, since ceramide has been demonstrated to activate components of the ERK cascade such as Raf-1 [217], MAP kinase [218] and JNK [219–221].

CONCLUDING REMARKS

An updated view of Signal 2

It has previously been suggested that CD28-activated Signal 2 consists of two distinct signals: Signal 2a is cyclosporin A-resistant and occurs in naive T cells after low-level CD28 activation (subsaturating bivalent anti-CD28 mAbs or low-affinity natural ligands), whereas Signal 2b is cyclosporin A-sensitive, is transmitted in activated T cells and requires saturating CD28 activation (cross-linked anti-CD28 mAbs and high-affinity natural ligands) [3]. It is clear that both Signal 2a and Signal 2b may actually consist of several components. For instance, the

components of Signal 2a defined to date probably consist of the signals mediated by PI 3-kinase and sphingomyelinase (Figure 4), given that the TCR triggers accumulation of *D*-3 phosphoinositide lipids with different stoichiometries and kinetics in comparison with CD28 and does not activate sphingomyelinase. The PI 3-kinase- and sphingomyelinase-mediated signals may act synergistically, although the level at which this putative synergy occurs has yet to be elucidated. Hence cyclosporin A-resistant Signal 2a is, at least in part, wortmannin-sensitive. In contrast, Signal 2b is likely to consist predominantly of the p21^{ras} and PLC signalling pathways, and these appear to be shared signals that can be activated by both the TCR and CD28 (Figure 4). Thus Signal 2b is, at least in part, cyclosporin A-sensitive and wortmannin-resistant. One of the major targets of components (e.g. PI 3-kinase) of Signal 2a may be p70^{s6k}, which has been implicated in regulating G₁-S phase transition [200,201]. Therefore it is likely that, in activated T cells, p70^{s6k} is a non-essential downstream target of PI 3-kinase, since the activity of p70^{s6k} is sufficiently maintained by IL-2, produced as a result of T cell activation. In the activated state only, Signal 1 and Signal 2b may be identical, additive and sufficient to drive further IL-2 production. Alternatively, the wortmannin-resistant components of Signal 2a (e.g. sphingomyelinase and adaptor functions mediated by the p85 subunit of PI 3-kinase) may still be required for activation of other downstream targets in synergy with Signal 2b and/or Signal 1. For instance, p85 may be required for recruitment of Signal 1 or Signal 2b signalling proteins such as Grb-2 [182,184] and p120-Ras-GAP associated with p62 [166]. In addition, sphingomyelinase has been shown to modulate the ERK cascade [217,218] as well as the JNK cascade [219–221].

Recent evidence has demonstrated that the JNK signalling pathway contributes to apoptosis, whereas the ERK signalling pathway promotes cell survival in neuronal cells [222]. As described above, the relative contributions of PI 3-kinase and sphingomyelinase to CD28 signalling probably depend on the state of activation of the T cell and the level of CD28 activation. This will determine the effect of CD28 ligation on the overall balance of the ERK and JNK signalling pathways, which in turn will direct the cell to apoptosis or cell survival. This may explain why CD28 has been reported to protect from [47], have no effect on [45] or induce [46] apoptosis. Since several signalling cascades, including those regulated by PI 3-kinase [178], p21^{ras} [76] and sphingomyelinase [217–221], contribute to the regulation of ERK and/or JNK, the occurrence of cell survival or apoptosis probably requires complex integration of multiple signals from several receptors and cascades.

Future prospects

The known downstream effector targets of the PI 3-kinase- and sphingomyelinase-mediated signalling cascades have yet to be fully evaluated in terms of their regulation by CD28 and their functional importance with respect to cytokine gene regulation. Thus it will be important in the future to determine the effect of CD28 ligation on the downstream PI 3-kinase targets (protein kinase B, PKC isoforms and Rac) and ceramide-activated targets (PKC ζ , phosphatases and kinases), and the effects of mutation of these targets on CD28-mediated co-stimulation. Other outstanding questions concern the role of p62 and p85 as specialized adaptor molecules in CD28 signalling cascades and the possible role of Rho in CD28-activated protein kinase cascades. Given the large number of cytokines/chemokines and cell surface molecules reportedly influenced by CD28, it is perhaps not surprising that CD28 couples to multiple signalling cascades, as this multiple signal capacity provides CD28 with the mechanistic

potential to regulate diverse events. It will therefore be particularly interesting to determine how multiple CD28-activated signalling cascades are integrated and whether distinct signalling cascades are responsible for regulating distinct transcriptional regulation of cytokine and chemokine genes.

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