Efficient CD28 signalling leads to increases in the kinase activities of the TEC family tyrosine kinase EMT/ITK/TSK and the SRC family tyrosine kinase LCK

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Optimal T cell activation requires crosslinking of the T cell receptor (TCR) concurrently with an accessory receptor, most efficiently CD28. Crosslinking of CD28 leads to increased interleukin 2 (IL2) production, inhibition of anergy and prevention of programmed cell death. Crosslinking of CD28 leads to rapid increases in tyrosine phosphorylation of specific intracellular substrates including CD28 itself. Since CD28 does not encode an intrinsic tyrosine kinase domain, CD28 must activate an intracellular tyrosine kinase(s). Indeed, crosslinking of CD28 increases the activity of the intracellular tyrosine kinases EMT/ITK and LCK. The phosphatidylinositol 3-kinase (PI3K) and GRB2 binding site in CD28 is dispensable for optimal IL2 production in Jurkat T cells. We demonstrate herein that murine Y170 (equivalent to human Y173) in CD28 is also dispensable

for activation of the SRC family tyrosine kinase LCK and the TEC family tyrosine kinase EMT/ITK. In contrast, the distal three tyrosines in CD28 are required for optimal IL2 production as well as for optimal activation of the LCK and EMT/ITK tyrosine kinases. The distal three tyrosines of CD28, however, are not required for recruitment of PI3K to CD28. Furthermore, PI3K is recruited to CD28 in JCaM1 cells which lack LCK and in which EMT/ITK is not activated by ligation of CD28. Thus optimal activation of LCK or EMT/ITK is not obligatory for recruitment of PI3K to CD28 and thus is also not required for tyrosine phosphorylation of the YNM motif in CD28. Taken together the data indicate that the distal three tyrosines in CD28 are integral to the activation of LCK and EMT/ITK and for subsequent IL2 production.

INTRODUCTION

Effective T lymphocyte activation requires the cooperation of at least two signals from antigen presenting cells. The first signal is delivered by the binding of the T cell receptor (TCR) on T cells to antigen presented by the major histocompatibility complex on antigen presenting cells [1–4]. The second costimulatory signal is most efficiently delivered by binding of CD28 to B7.1 or B7.2 on antigen presenting cells [3–5]. Preventing the interaction of CD28 with B7 results in prolonged allograft survival in rodents and reduces autoimmunity in murine lupus models [6–8]. Elimination of CD28 in knockout mice resulted in failure of peripheral T cells to undergo full activation following exposure to antigen [9,10]. Moreover, crosslinking CD28 enhances production of lymphokines such as interleukin 2 (IL2) which leads to T cell proliferation [1–4]. CD28 ligation also prevents anergy and programmed cell death in T cells [3,11,12]. Thus ligation of CD28 is essential for full immune activation.

CD28 crosslinking leads to a rapid and transient increase in tyrosine phosphorylation of a number of intracellular proteins including the intracellular domain of CD28 [13,14]. The observation that tyrosine kinase inhibitors decrease the ability of CD28 to enhance IL2 production provides additional support for a role for tyrosine phosphorylation in CD28 signalling [15]. Since CD28 does not contain an intrinsic kinase domain, CD28-induced increases in tyrosine phosphorylation are likely due to recruitment or activation of intracellular tyrosine kinases. The intracellular TEC family tyrosine kinase EMT/ITK [16–21] and the SRC family kinase LCK become activated following cross-

linking of CD28 [21,22]. Both LCK [23] and functional EMT/ITK [24] appear to be required for optimal IL2 production [23] and IL2 promoter activation [24]. However, as indicated by studies of 'knock out mice' [25], EMT/ITK may also play a role in feedback inhibition through CD28. However, EMT/ITK is clearly important for CD28-mediated IL2 promoter activation in some T cell lineages [24]. Thus EMT/ITK may play a dualistic role, both initiating signalling through CD28 [24] and then terminating the signal [25]. This is compatible with the delayed kinetics of EMT/ITK activation following ligation of CD28 [21,26,27]. Functional LCK is required for optimal EMT/ITK activation in T cells [26,27] suggesting that EMT/ITK may be located downstream of LCK in CD28 signalling. This contention is supported by the observation that cotransfection of LCK and EMT/ITK into Cos-7 cells leads to increased tyrosine phosphorylation of EMT/ITK [26]. LCK also phosphorylates EMT/ITK upon co-expression of these kinases in insect cells. This phosphorylation was mapped to the autophosphorylation site (Tyr 511) in EMT/ITK resulting in increased EMT/ITK kinase activity [28]. EMT/ITK and LCK have the capacity to tyrosine phosphorylate CD28 with LCK phosphorylation being restricted to the YNM motif, whereas EMT/ITK is capable of phosphorylating each of the four tyrosines in the cytoplasmic domain of CD28 [29]. In addition, EMT/ITK inducibly associates with CD28 following activation of CD28 [21]. Thus the EMT/ITK and LCK kinases have the potential to play an important role in CD28-induced tyrosine phosphorylation and both positive and negative signalling events.

The role that phosphorylation of specific tyrosines in the

Abbreviations used: F170, murine CD28 with a tyr to phe mutation at 170; F3, murine CD28 with a tyr to phe mutation at 188, 194 and 197; ALL F, murine CD28 with a tyr to phe mutation at all intracellular tyrosines; hCD28, human CD28; IL2, Interleukin 2; mCD28, murine CD28; PI3K, phosphatidylinositol 3-kinase; RAM, rabbit anti-mouse; TCR, T cell receptor; wt, wild-type.

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cytoplasmic tail of CD28 play in augmenting IL2 production remains controversial. In Jurkat T cells, the tyrosine in the YNM motif of CD28 which binds phosphatidylinositol 3-kinase (PI3K) and potentially an adapter protein grb2 [30–34] appears dispensable for optimal IL2 production [35–38]. In contrast, the distal three tyrosines in CD28 appear to be required for optimal IL2 production [36,37]. In other cell lines, however, the YNM motif as well as PI3K activation appears to be required for IL2 production [39,40]. The discrepancy between the cell systems has not yet been satisfactorily explained.

We have investigated the role that specific tyrosine residues in CD28 play in EMT/ITK and LCK activation, utilizing mutants of murine CD28 (mCD28) expressed in the Jurkat T cell line [36]. Mutation of tyrosine 170 (corresponds to Tyr 173 in human CD28) in CD28 to phenylalanine (F170) abolished PI3K association with CD28 but did not alter the ability of CD28 crosslinking to increase IL2 production. Similarly, tyrosine 170 in CD28 was dispensable for activation of the EMT/ITK and LCK kinases. In contrast, mutation of the distal three tyrosines in the cytoplasmic tail of CD28 resulted in a significant reduction in IL2 production as well as in the ability to increase kinase activity of EMT/ITK and LCK. Thus the distal three tyrosines of CD28 are required for optimal activation of EMT/ITK and LCK kinase activity which may be a prerequisite for CD28 to optimally augment IL2 production.

MATERIALS AND METHODS

Antibodies

The hamster monoclonal antibody 37.51 specific for mCD28 was provided by Dr. James Allison (University of California, Berkeley, U.S.A.). Monoclonal murine anti-human CD28 antibody 9.3 (IgG1) was provided by Dr. Jeffrey Ledbetter (Bristol–Meyers Squibb Pharmaceutical Research Institute, Seattle, WA, U.S.A.). Rabbit anti-murine antibodies were purchased from Western Blotting Inc. (Toronto, ON, Canada). Anti-phosphotyrosine antibodies (4G10, IgG1) and anti-p85 α subunit of phosphatidylinositol 3-kinase antibodies (rabbit, polyclonal) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.). Polyclonal anti-EMT/ITK and polyclonal anti-LCK used in these studies have been described previously [16,41].

Cell lines

The Jurkat T cell line E6.1 transfected with wild type or various mutants of mCD28 have been described previously [36]. mCD28 was mutated at tyrosine 170 to phenylalanine (F170), tyrosines 188, 194, 197 to phenylalanine (F3) or all tyrosines on mCD28 cytoplasmic tail to phenylalanine (All F) by polymerase chain reaction and mutations were confirmed by sequencing.

Cell culture, stimulation and lysis

Jurkat cells transfected with mCD28 were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.) containing 10 μ M gentamicin (Gibco), 2 mM L-glutamine (Gibco), 10% (v/v) fetal calf serum (Sigma, St. Louis, MO, U.S.A.), and 2 mg/ml G418 (Gibco) at 37 °C in a humidified atmosphere. Cells were changed to serum-free medium and incubated for 48 h prior to stimulation. Anti-CD28 (murine or human) antibodies were added at 10 μ g/ml 1 min before the addition of rabbit anti-mouse (RAM) antibodies (5 μ g/ml) for CD28 crosslinking. Cells were then pelleted, resuspended in lysis buffer (150 mM NaCl, 50 mM Hepes pH 7.4, 500 μ M sodium orthovanadate, 50 μ M

ZnCl₂, 50 μ M NaF, 50 μ M Na orthophosphate, 2 mM EDTA, 2 mM phenylmethylsulphonyl fluoride, and 1% NP40) and incubated at 4 °C for 15 min. Anti-CD28 antibody was added to each unstimulated cell lysate to ensure that equal amounts of anti-CD28 were present. Detergent cell lysates were collected after centrifugation at 14000 *g* for 10 min.

Immunoprecipitation and Western blotting

Detergent cell lysates were incubated with Protein A sepharose beads (Pharmacia, Piscataway, NJ, U.S.A.) and additional anti-CD28 antibodies (10 μ g/immunoprecipitate) for 1 h at 4 °C followed by three washes in lysis buffer. The beads were pelleted and supernatant was aspirated. The immunoprecipitated proteins were eluted from beads using Laemmli buffer and resolved by 10% SDS/PAGE. Proteins were transferred to Immobilon (Millipore, Bedford, MA, U.S.A.) and blocked in 5% milk. Membranes were incubated with the polyclonal antibodies against PI3K P85 α subunit (UBI) or polyclonal antibodies against EMT/ITK [16] for 1 h. Horseradish peroxidase-conjugated protein A (Amersham, Arlington Heights, IL, U.S.A.) was used as secondary reagent. After extensive washing, target proteins were detected by enhanced chemiluminescence.

In vitro kinase assay

Tyrosine kinase activity was assayed as described [21,26]. EMT and LCK immunoprecipitates were washed once in kinase wash buffer (150 mM NaCl, 10 mM Tris pH 7.2, 1 mM PMSF, and 1 mM sodium vanadate). The beads were then incubated in 45 μ l of kinase buffer (10 mM manganese chloride, 10 mM Hepes pH 7.0, 1 mM PMSF and 0.1 mM sodium orthovanadate) containing 5 μ Ci of [γ -³²P] ATP and 5 μ g of a peptide (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly) (Sigma Chemical Co., St. Louis, MO, U.S.A.) derived from the sequence surrounding the SRC tyrosine kinase autophosphorylation site [42]. This mixture was incubated for 15 min at room temperature which was found to be within the linear range of the assay. The mixture was then blotted onto phosphocellulose paper and washed six times with 1% phosphoric acid. The amount of [γ -³²P] incorporated into the peptide was determined by scintillation counting.

IL2 production assay

Jurkat cells (10⁶/ml) were incubated with PMA (5 $\times$ 10⁻⁸ M), in the presence or absence of anti-CD3 (antibodies against the T cell receptor complex, 10 μ g/ml), with or without anti-murine CD28 (10 μ g/ml), and RAM (5 μ g/ml) overnight at 37 °C. The cells were then pelleted. Supernatants were collected and IL2 production measured by ELISA (Biosource Int., Camarillo, CA, U.S.A.) as recommended by the manufacturer.

Flow cytometry

Transfected Jurkat cells (0.5 $\times$ 10⁶) were incubated with anti-murine CD28 (37.1, 80 μ g/ml) or anti-human CD28 (9.3, 80 μ g/ml) in staining buffer (phosphate buffered saline containing 5% foetal calf serum, and 0.001% sodium azide) for 45 min at 4 °C and washed. A secondary antibody, goat anti-hamster FITC (50 μ g/ml), was added and incubated for another 45 min. After washing with phosphate buffer saline, the cells were resuspended in 1% paraformaldehyde. Cell surface expression analysis was assessed with a FACSCAN (Beckton–Dickinson, San Jose, CA, U.S.A.).

RESULTS

Expression of murine CD28 in the Jurkat T cell lines alters IL2 production

To investigate the role of the intracellular tyrosine residues in the ability of CD28 to transmit specific intracellular signals and to regulate IL2 production, we have expressed murine wild type (wt) or mutant CD28 wherein tyrosine 170 is mutated to phenylalanine (F170), tyrosines 188, 194, and 197 to phenylalanine (F3) or all tyrosines to phenylalanine (ALL F) [36]. As expressed in Jurkat T cells, the murine and human CD28 molecules do not form mixed heterodimers [36], allowing independent interrogation of signalling through hCD28 or mCD28 by crosslinking with the anti-murine 37.51 or anti-human 9.3 species-specific anti-CD28 antibodies. As demonstrated by flow cytometry, each of the cell lines expressed similar levels of wt, F170, F3 and ALL F (Figure 1). Endogenous human CD28 expression is similar in each of the murine CD28 transfected cell lines and the level of endogenous human CD28 is comparable to the levels of transfected murine CD28 in the transfected Jurkat cells (Figure 1). As demonstrated herein, crosslinking of wt or F170 murine CD28 enhanced anti-CD3-induced IL2 production, whereas both F3 and ALL F mCD28 were significantly curtailed in their ability to augment anti-CD3-induced IL2 production

Figure 1 Expression of murine CD28 and human CD28 on Jurkat cell transfectants

Cells were incubated with either anti-murine CD28 (37.51) or anti-human CD28 (9.3) antibodies. A secondary fluorescein-conjugated goat anti-hamster Ig or fluorescein-conjugated goat anti-murine Ig antibody was then incubated with the appropriate cells. Secondary antibody alone is presented as a clear peak. Anti-CD28 staining is illustrated as a dark peak. Cell lines are indicated to the left of each histogram. In all histograms, the horizontal axis represents fluorescence and the vertical axis represents cell number.

Table 1 IL2 production in murine CD28 transfected Jurkat T cell lines

Cells (1×10^6 /ml) were incubated with PMA (5×10^{-8} M), PMA + anti-human CD3 (9.3, 10 μ g/ml), PMA + anti-murine CD28 (37.51, 10 μ g/ml), PMA + anti-human CD3 + anti-murine CD28, or PMA + anti-human CD3 + anti-human CD28 (10 μ g/ml) overnight. In all conditions, rabbit anti-murine antibodies (RAM, 5 μ g/ml) were added. RAM antibodies cross-react with hamster anti-murine CD28 antibodies. IL2 secretion was determined by an ELISA kit as recommended by the manufacturer. Amount of IL2 produced is presented as pg/ml. Each condition was assessed at least three times with similar results.

	PMA	PMA + CD3	PMA + mCD28	PMA + CD3 -mCD28	PMA + CD3 + hCD28
wt	15	60	90	500	680
F170	0	85	125	420	840
F3	0	40	15	125	350
ALL F	0	25	15	100	420

Table 2 Kinase activity of EMT/ITK following crosslinking of murine CD28 in Jurkat transfectants

Cells (10×10^6) were stimulated with anti-murine CD28 antibodies (37.51, 10 μ g/ml, mCD28XL) or anti-human CD28 antibodies (9.3, 10 μ g/ml) and RAM as indicated. Cells were lysed as described in 'Methods'. Additional anti-CD28 antibodies and RAM were added to lysates of unstimulated cells to ensure that equal amounts of antibodies were present. Lysates were immunoprecipitated with anti-EMT/ITK antibodies and assessed for kinase activity by measuring the extent of tyrosine phosphorylation of an exogenous substrate (SRC peptide) as described in Methods. Mean $\pm$ S.E.M. was determined from duplicates in three independent experiments. * represents statistically significant decreases in CD28-induced EMT/ITK kinase activity ($P < 0.006$, Student's *t* test) as compared to wild type CD28.

	Mouse CD28			Human CD28 (fold increase)
	Resting	mCD28XL	Fold increase	
wt	5195 $\pm$ 804	9798 $\pm$ 972	1.9 $\pm$ 0.2	1.9 $\pm$ 0.5
F170	5513 $\pm$ 522	8984 $\pm$ 532	1.6 $\pm$ 0.05	1.7 $\pm$ 0.15
F3	4363 $\pm$ 991	4876 $\pm$ 899	1.1 $\pm$ 0.05*	2.4 $\pm$ 0.9
ALL F	4867 $\pm$ 475	5861 $\pm$ 222	1.2 $\pm$ 0.06*	Not done.

(Table 1). Ligation of the endogenous human CD28 receptor augmented anti-CD3-induced IL2 production in all mCD28 transfected cell lines, suggesting that these transfected cells contain the necessary signalling machinery to produce IL2 (Table 1). However, the level of IL2 production induced by ligation of human CD28 was slightly but consistently decreased in cells expressing murine F3 and ALL F constructs. This may represent a small dominant negative effect of the murine F3 and ALL F constructs. Thus, at least as expressed in Jurkat T cells, the Y170 PI3K and grb-2 binding site is dispensable for CD28 crosslinking to augment CD3-induced IL2 production. In contrast, the distal three tyrosines of CD28 are obligatory for the optimal ability of CD28 ligation to synergize with CD3 ligation for IL2 production.

Y170 is dispensable whereas the distal three tyrosines are obligatory for optimal activation of the EMT/ITK and LCK tyrosine kinases

The tyrosine kinase activity of both EMT/ITK and LCK are increased following crosslinking of CD28 [21,22,26]. Further both LCK and EMT/ITK are required for optimal IL2 promoter activation [23,24]. As assessed by the ability to tyrosine phos-

Table 3 Kinase activity of LCK following crosslinking of murine CD28 in Jurkat transfectants

Cells (10×10^6) were stimulated with anti-murine CD28 antibodies (37.51, $10 \mu\text{g/ml}$, mCD28XL) or anti-human CD28 antibodies (9.3, $10 \mu\text{g/ml}$) and RAM as indicated. Cells were lysed as described in 'Methods'. Additional anti-CD28 antibodies and RAM were added to lysates of unstimulated cells to ensure that equal amounts of antibodies were present. Lysates were immunoprecipitated with anti-LCK antibodies and assessed for kinase activity by measuring the extent of tyrosine phosphorylation of an exogenous substrate (SRC peptide) as described in Methods. Mean $\pm$ S.E.M. was determined from duplicates in three independent experiments. * represents statistically significant decreases in CD28-induced EMT/ITK kinase activity ($P < 0.003$, Student's *t* test) as compared to wild type CD28.

	Mouse CD28			Human CD28 (fold increase)
	Resting	mCD28XL	Fold increase	
wt	553 $\pm$ 149	1046 $\pm$ 294	1.9 $\pm$ 0.07	2.4 $\pm$ 0.1
F170	523 $\pm$ 32	920 $\pm$ 134	1.8 $\pm$ 0.1	2.1 $\pm$ 0.3
F3	1172 $\pm$ 218	1405 $\pm$ 155	1.2 $\pm$ 0.1*	1.9 $\pm$ 0.1

phorylate an exogenous peptide containing the autophosphorylation site of the tyrosine kinase SRC [42], crosslinking of human CD28 in mCD28 transfected cells or crosslinking of wild type or F170 mCD28 with 37.51 antibodies specific to mCD28 and unreactive to human CD28 induced a consistent increase in EMT/ITK and LCK kinase activity (Tables 2 and 3). In contrast, ligation of F3 had a much lower effect on EMT/ITK and LCK kinase activity (Tables 2 and 3). ALL F was similarly unable to augment EMT/ITK kinase activity (Tables 2 and 3). Thus, concordant with the ability to augment IL2 secretion, the distal three tyrosines in the cytoplasmic tail of CD28, but not Y170, are required for optimal activation of EMT/ITK and LCK.

LCK and EMT/ITK activation are not required for recruitment of PI3K to CD28

The SH2 domain of PI3K binds to the phosphorylated but not the non-phosphorylated YNMN motif in CD28 [30,36]. Recruitment of PI3K to CD28 thus serves as a surrogate measure of tyrosine phosphorylation of the YNMN motif of CD28. To determine if PI3K associates with specific activated wild-type and mutant CD28, the transfected Jurkat cell lines were cross-linked with anti-CD28 antibodies. An equal amount of anti-CD28 antibodies was added to unstimulated cell lysates to control for the presence of the anti-CD28 antibodies used to stimulate the cells during the immunoprecipitation. As indicated by immunoprecipitation of CD28 and subsequent Western blotting with anti-PI3K p85 subunit antibodies, there was very little binding of PI3K to unstimulated CD28 but after activation of CD28 PI3K was recruited to the wild type and F3 receptors but not to the F170 receptor (Figure 2B). Previous studies using baculovirus expression and *in vitro* kinase assays have suggested that LCK and EMT/ITK [29,31] are capable of phosphorylating the YNMN motif and recruiting PI3K to CD28. We have previously demonstrated that EMT/ITK does not become activated following ligation of CD28 in JCaM1 cells which lack LCK [26,27]. Furthermore, EMT is not recruited to CD28 following ligation of CD28 in JCaM1 cells [26]. JCaM1 cells thus provide a model to assess whether activation of LCK or EMT/ITK are required for the recruitment of PI3K to CD28 and thus for the phosphorylation of the YNMN motif. As indicated in Figure 2A, crosslinking of CD28 in JCaM1 cells leads to the recruitment of PI3K to CD28, indicating that neither EMT/ITK activation nor LCK is required for the phosphorylation of the YNMN motif.

Figure 2 PI3K association with CD28 following crosslinking of CD28

Cells were stimulated with anti-murine CD28 (9.3, $10 \mu\text{g/ml}$) or anti-human CD28 (37.51, $10 \mu\text{g/ml}$) antibodies and crosslinked with RAM ($10 \mu\text{g/ml}$) that is cross-reactive with both mouse and hamster antibodies as indicated. The cells were then lysed in NP40 lysis buffer. Equal amounts of anti-CD28 antibodies were added directly to unstimulated cell lysates to ensure that equal amounts of antibody were present in each lysate. (A) Anti-human CD28 antibodies (9.3) were added as indicated to stimulate Jurkat and JCaM1 (which lacks LCK). Equal amounts of anti-CD28 antibodies were added directly to unstimulated cell lysates to ensure that equal amounts of antibody were present in each lysate. Cell lysates were immunoprecipitated with anti-CD28 antibodies and Western blotted with anti-p85 PI3K antibodies as described in 'Methods'. (B) mCD28 transfected Jurkat cells were stimulated with anti-murine CD28 antibodies (37.51). Equal amounts of anti-CD28 antibodies were added directly to unstimulated cell lysates to ensure that equal amounts of antibody were present in each lysate. Cell lysates were immunoprecipitated with anti-murine CD28 antibodies and Western blotted with anti-p85-PI3K antibodies. (C) F3 cells were stimulated with anti-murine CD28 antibodies (37.51). Equal amounts of anti-CD28 antibodies were added directly to cell lysates to ensure that equal amounts of antibody were present in each lysate. Cell lysates were immunoprecipitated with anti-murine CD28 antibodies and Western blotted with anti-EMT/ITK antibodies as described in 'Methods'. The arrow indicates the position of p85 PI3K or EMT/ITK. Each condition was assessed at least three times with similar results.

lation of the YNMN motif. This was further assessed with the F3 mutant in which EMT/ITK and LCK are not activated following ligation of CD28 (Table 2). In this cell line PI3K is recruited to CD28 to a similar extent as in wt cells (Figure 2B; [36]). As expected recruitment of PI3K to CD28 is greatly decreased or absent in F170 cells. EMT/ITK is also recruited to CD28 after crosslinking of CD28. In F3 cells, EMT/ITK is still recruited to stimulated F3 CD28, suggesting that decreased CD28-mediated EMT/ITK kinase activity is not due to a lack of recruitment of EMT/ITK to CD28 (Figure 2C). Thus, in as much as recruitment of PI3K to CD28 is a surrogate marker for the tyrosine phosphorylation of the YNMN motif of CD28, activation of EMT/ITK or LCK is not obligatory for the phosphorylation of the YNMN motif and the recruitment of PI3K.

DISCUSSION

Phosphorylated tyrosine residues but not non-phosphorylated tyrosine residues of cell surface receptors such as CD28 recruit intracellular signalling molecules containing SH2 and PTB motifs to form an activation nidus [43]. The intracellular domain of CD28 contains four tyrosines, the first of which is within a

consensus PI3K and grb2 binding motif (YMNM) and, indeed, is able to bind PI3K and grb2 [31,36,44]. Surprisingly, however, in at least some T cell lines, the YMNM motif is dispensable for the ability of CD28 to augment IL2 production [36,37]. CD28 mediated activation of PI3K or grb2 may be redundant due to activation of PI3K or grb2 following ligation of the T cell receptor. In contrast, as we and others have indicated [36,37], the distal three tyrosines of CD28 appear to be required for the ability of CD28 to optimally augment IL2 production.

Tyrosine phosphorylation plays an integral role in CD28 signalling [14,15]. However, CD28 does not contain an intracellular tyrosine kinase domain and thus must activate intracellular kinases. We and others have demonstrated that crosslinking of CD28 induces association of EMT/ITK with CD28 and as well increases EMT/ITK and LCK kinase activity [21,24,26,27,45]. In accord with the ability of CD28 to augment IL2 production (Table 1; [35,36,45]), we demonstrate herein that the YMNM motif of CD28 is dispensable for activation of both EMT/ITK and LCK by CD28 (Tables 2 and 3). Furthermore, the YMNM motif of CD28 is dispensable for the tyrosine phosphorylation of EMT/ITK (data not presented) which correlates with the activation of EMT/ITK [21,24,26,27,45]. In contrast, the distal three tyrosines of CD28, which are required for CD28 crosslinking to optimally augment anti-CD3 induced increases in IL2 production (Table 1), are also required for optimal CD28-mediated activation of EMT/ITK and LCK (Tables 2 and 3) but dispensable for EMT/ITK recruitment to CD28 (Figure 2). This suggests that unidentified molecules associate with the tyrosine phosphorylated distal three tyrosines in CD28, which allows for the optimal activation of EMT/ITK and LCK. The concordance between the ability of CD28 mutants to induce EMT/ITK and LCK activation and IL2 production further supports the contention that activation of EMT/ITK and LCK are critical to the ability of CD28 to augment IL2 production [21,24,26,37]. This is in apparent contrast to recent studies of 'knock out' EMT/ITK mice where the ability of CD28 to augment CD3-induced proliferation is increased [25]. The apparent discrepancy between these results may arise from studies of 'knock out' CD4 T cells [25] which may have undergone aberrant T cell selection [46], compared to studies of the Jurkat T cell line. Alternatively, as EMT/ITK plays a major role in CD3 signalling [25,46], CD3 induced T cell activation may be aberrant in the 'knockout' CD4 T cells.

In a recent study using a human CD8/CD8/CD28 (extracellular/transmembrane/intracellular) chimera [29], Dupont and colleagues demonstrated decreased EMT/ITK kinase activity following crosslinking of a F173 mutant (equivalent to the murine F170 mutant studied herein). This was in contradistinction to the retained ability of the F173 chimera to induce IL2 production [36,37]. In contrast, in our studies crosslinking wild type or F170 murine CD28 induced equivalent increases in EMT/ITK kinase activity (Tables 2 and 3) and IL2 production (Table 1). The reason for the discrepancy between the two studies is not immediately apparent as both constructs were expressed in Jurkat T cells. We have demonstrated previously that murine and human CD28 do not form mixed dimers [36], suggesting that the ability of the murine F170 mutant to activate EMT/ITK is not due to activation of endogenous human CD28 by murine CD28. Furthermore, as demonstrated in Tables 2 and 3, the F3 mutant fails to optimally activate both EMT/ITK and LCK further arguing against the crosslinking of murine CD28 activating human CD28. The likely reasons for the discrepancy between the two studies rest in either the antibodies used for crosslinking (anti-CD8 in the case of Dupont and colleagues and anti-CD28 in our studies) or in the CD8 extracellular and

transmembrane domain of the chimeras studied by Dupont and colleagues not recapitulating the structure or function of the extracellular and transmembrane domain of CD28. A final possibility is that the intracellular CD28 domain utilized herein was from murine CD28, whereas that studied by Dupont and colleagues was from human CD28. Given the high degree of sequence conservation [44] between the intracellular domains of murine and human CD28 this latter possibility seems unlikely. Nevertheless, the results presented herein are consistent with previous studies [23,24,26] indicating that the ability of CD28 to augment IL2 production is concordant with the ability to activate LCK and EMT/ITK.

EMT/ITK is tyrosine phosphorylated and activated following crosslinking of TCR, CD2, CD28 and Fc ϵ RI. EMT/ITK phosphorylation and kinase activity is either not [27] or only modestly [24] increased following coligation of TCR and CD28 and is thus unlikely to explain the synergistic ability of coligation of TCR and CD28 to increase IL2 production [1–4]. Decreased T cell proliferation in the EMT/ITK knock-out mouse [47] also does not distinguish between the relative requirements of EMT/ITK for signals downstream of the TCR or CD28 as engagement of these receptors is required for optimal T cell proliferation [3–5]. The studies presented herein suggest that activation of EMT/ITK by CD28 may be critical for CD28 signal transduction and that activation of EMT/ITK by the TCR is not sufficient to enable optimal IL2 production.

In summary, mutation of the last three tyrosine kinases in the cytoplasmic tail of CD28 substantially reduces the ability of CD28 to augment IL2 production and concordantly reduces the ability of CD28 to increase EMT/ITK and LCK kinase activity. In contrast, mutation of F170 neither alters the ability of CD28 to augment CD3-induced IL2 production nor the ability of CD28 to increase EMT/ITK and LCK kinase activity. In addition, a kinase(s) other than EMT/ITK and LCK appears capable of phosphorylating the YMNM motif in CD28 and recruiting PI3K to CD28. Taken together, these data provide further evidence supporting a role for the EMT/ITK and LCK kinases in CD28-mediated signal transduction leading to the ability to increase IL2 production.

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