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Immunology

CD28 is associated with and induces the immediate tyrosine phosphorylation and activation of the Tec family kinase ITK/EMT in the human Jurkat leukemic T-cell line

(costimulation/signal transduction/lymphocyte activation/tyrosine kinase/vav)

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ABSTRACT **T lymphocytes require two signals to be activated. The antigen-specific T-cell receptor can deliver the first signal, while ligation of the T-cell surface molecule CD28 by antibodies or its cognate ligands B7-1 (CD80) or B7-2 has been demonstrated to be sufficient for the delivery of the second signal. Signaling via CD28 and the T-cell receptor results (*i*) in their costimulation of T cells to produce numerous lymphokines including interleukin 2 and (*ii*) in the prevention of anergy induction. Little is known about the pathway by which CD28 mediates its signals except that protein-tyrosine phosphorylation is involved. We show here in human Jurkat cells that the Tec-family protein-tyrosine kinase ITK/EMT ($p72^{ITK/EMT}$) is associated with CD28 and becomes tyrosine-phosphorylated and activated within seconds of CD28 ligation. This tyrosine phosphorylation of $p72^{ITK/EMT}$ is rapid (within 30 sec), occurs in the absence of LCK activation, and precedes tyrosine phosphorylation of the guanine nucleotide exchange factor VAV. Secondary crosslinking of CD28 is unnecessary for the induced tyrosine phosphorylation of $p72^{ITK/EMT}$. Thus, tyrosine phosphorylation of $p72^{ITK/EMT}$ may represent one of the earliest events in CD28 signaling. This demonstrates that a member of the Tec family of protein tyrosine kinases, similar to members of the Src and Syk families, plays a role in the activation of T cells. Furthermore, the data demonstrate that $p72^{ITK/EMT}$, and by analogy other members of the Tec family, responds to extracellularly generated signals.**

Antigen-specific activation of T lymphocytes is under stringent control, which is achieved by the requirement for an antigen-specific signal and the delivery of a costimulatory signal (the two-signal hypothesis) (1). The antigen specificity is conferred by the antigen-specific T-cell receptor (TcR), which recognizes antigenic peptides in the context of the major histocompatibility complex class I and class II gene products (2). The recognition of antigen by the TcR results in the activation of a number of tyrosine kinases including members of the Src and Syk family, leading to downstream effects, including lymphokine production (3, 4).

Since the concentration of antigen is usually limiting, and as an additional mechanism to prevent aberrant activation, T cells require costimulation. This costimulation can be delivered by the binding of T-cell surface protein CD28 to its cognate ligands B7-1 (CD80) (5) and B7-2 (6–8) on antigen-presenting cells. This second signal serves to enhance production of key lymphokines required for the growth of activated T cells (5, 9) and also can prevent anergy induction (10) and human immunodeficiency virus-primed apoptosis (11). The primary structure of CD28 indicates that this molecule has a short cytoplasmic tail (41 residues) with no obvious enzymatic activity (12). While there is an outline of the pathway by which the TcR signals, very little is known about signal transduction resulting from ligation of the costimulatory molecule CD28. However, it is clear that the signal delivered by CD28 differs from that given by the TcR in a number of ways, including sensitivity to cyclosporin A and rapamycin (5, 9). Since both pathways result in protein-tyrosine phosphorylation (3, 4, 13, 14), there may be subtle differences that are manifest in the differences in drug sensitivity.

The Tec family of protein tyrosine kinases is a newly emerging family that includes the prototypical members mouse Tec I and Tec II and the products of the X-linked agammaglobulinemia gene *BTK* (*Btk* in the mouse), *Drosophila* Dsrc28 gene, and mouse *Itk* (formerly called *Tsk* and *Emt*), the human homologue of which has been assigned the name *ITK* (15–23). All the members of this family contain Src homology (SH) domains SH2 and SH3 but lack the negative regulatory tyrosine found in the Src family members. They also contain extensive N-terminal regions, which differ from that seen in Src family members (15–23). While the product of the *BTK* gene has been shown genetically to be essential for proper B-cell development (19, 20), no description of function has been reported for any other member of this family. We now demonstrate that the 72-kDa product of human *ITK*, which we call $p72^{ITK/EMT}$, is associated with CD28 and is rapidly tyrosine-phosphorylated upon CD28 ligation. This occurred whether CD28 was crosslinked with secondary antibody or not and preceded all other known tyrosine-phosphorylation events induced by CD28 crosslinking. Furthermore, the association of $p72^{ITK/EMT}$ with CD28 increases after crosslinking. We suggest that tyrosine phosphorylation of $p72^{ITK/EMT}$ is a very early step in CD28 signaling and that the activity of this kinase may be key to the biological function of CD28 after binding to its ligands, CD80 (B7) and B70/B7-2.

MATERIALS AND METHODS

Antibodies. Monoclonal antibodies (mAbs) 9.3 (IgG2a, anti-CD28) and NE-51 (IgG2b, anti-CD28) were gifts of J. Ledbetter (Bristol-Myers Squibb Research Institute, Seattle) and S. M. Fu (University of Virginia, Charlottesville). OKT3 (IgG1, anti-CD3ε) and Leu-3a (IgG1, anti-CD4) were supernatants of hybridoma cells from the American Type Culture

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Abbreviations: PI3, phosphatidylinositol 3; PMA, phorbol 12-myristate 13-acetate; GAM and RAM, goat and rabbit anti-mouse immunoglobulins; TcR, T-cell receptor; mAb, monoclonal antibody; SH, Src homology.

Collection. W6/32 (IgG2, anti-class I) was from S. Y. Yang (Sloan–Kettering Institute for Cancer Research). Rabbit antiserum to VAV was from Santa Cruz Biotechnology (Santa Cruz, CA), to LCK was from Upstate Biotechnology (Lake Placid, NY), and to phosphotyrosine (RC20, anti-phosphotyrosine antibody conjugated to horseradish peroxidase) was from Transduction Laboratories (Lexington, KY); anti-EMT (anti-ITK/EMT), which detects $p72^{ITK/EMT}$, has been described (23).

Cell Culture and Stimulation. Human Jurkat leukemic T cells, clone E6-1, were cultured in RPMI 1640 medium containing antibiotics and 10% (vol/vol) fetal calf serum at 37°C in a humidified atmosphere. Cells were serum-starved as described (24). These cells are referred to as "resting" cells because of their low levels of phosphotyrosine. These "rested" cells were then treated on ice with saturating concentrations of antibodies (anti-CD28, anti-CD3, or anti-HLA class I at 1 μg of antibody per 10^6 cells) for 15 min. This treatment does not result in any activation. After one wash with ice-cold phosphate-buffered saline (PBS), the cells were resuspended in room-temperature PBS, and where indicated (all but Fig. 4), rabbit anti-mouse immunoglobulin (RAM) or goat anti-mouse immunoglobulin (GAM) was added to 40 μg/ml (for secondary crosslinking). The cells were then immediately placed at 37°C for the indicated time periods (these cells are referred to as "activated" cells). Following the desired time point, the cells were immediately lysed in 2× lysis buffer (1× = 150 mM NaCl/10 mM Tris, pH 7.5/1% Nonidet P-40/1 mM phenylmethylsulfonyl fluoride/2 mM sodium orthovanadate/1 mM EDTA) on ice. Inclusion of NaF in the lysis buffers did not change tyrosine phosphorylation patterns.

Immunoprecipitation and Immunoblotting. Lysates were clarified by centrifugation at 14,000×*g* for 15 min at 4°C. After centrifugation, the lysates were precleared with Sepharose beads; then heterologous receptors were removed with protein A-agarose or protein G-Sepharose. Immunoprecipitates were then formed with the respective antibodies, including antibodies to HLA class I, CD28, VAV, LCK, or $p72^{ITK/EMT}$; washed with lysis buffer; and loaded onto 10% polyacrylamide gels. Gels were blotted to poly(vinylidene difluoride) membranes (Millipore) and blocked overnight with 10% fetal calf serum in TBS/Tween 20 (24). Anti-phosphotyrosine antibody RC20 was added to 100 ng/ml for 1 hr, washed, and detected by enhanced chemiluminescence (ECL). Some blots were stripped with Western blot stripping buffer (100 mM 2-mercaptoethanol/2% SDS/62.5 mM Tris, pH 6.8), washed, and reblocked with 5% milk in TBS/Tween 20 before probing with anti-ITK/EMT antibodies (at 1:1000) or anti-VAV antibodies (at 1 μg/ml) and detecting with protein A conjugated to horseradish peroxidase and ECL (Amersham).

***In Vitro* Kinase Assay.** To assay for protein-tyrosine kinase activity of LCK, immunoprecipitates were washed as described above and then washed once in kinase wash buffer (25 mM Hepes, pH 7.5/0.1% Nonidet P-40/2 mM sodium orthovanadate). The precipitates were then incubated in 45 μl of kinase buffer (10 mM manganese chloride/25 mM Hepes/2 mM sodium orthovanadate) containing 1 μM cold ATP, 10–20 μCi (1 μCi = 37 kBq) of [γ-^{32}P]ATP, and 5 μg of acid-denatured enolase (Sigma) at 30°C for 15 min. The reaction was stopped by adding 2× SDS/PAGE buffer (24).

To assay for tyrosine kinase activity of $p72^{ITK/EMT}$, the immunoprecipitates were washed as described above for the LCK assay, resuspended in kinase buffer containing 5 μCi of [γ-^{32}P]ATP and 5 μg of Src peptide (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly) (Sigma), and then incubated for 15 min at room temperature (found to be within the linear range of the assay).

RESULTS AND DISCUSSION

Lu *et al.* (13) and Vandenberghe *et al.* (14) have reported that after crosslinking of CD28, increases in tyrosine phosphorylation could be observed on at least two major substrates of ≈70 kDa and ≈100 kDa. In addition, Lu *et al.* (13) reported that inhibitors of protein-tyrosine kinases inhibited the CD28-induced appearance of the nuclear factor(s) binding to the CD28 responsive element within the interleukin 2 promoter region. Since the tyrosine kinase $p72^{ITK/EMT}$ is expressed primarily in T cells and has a molecular mass of around 72 kDa (16–18, 21–23), this could represent one of the tyrosine-phosphorylated molecules of ≈70 kDa previously observed (13, 14). Thus, the activation of $p72^{ITK/EMT}$ would represent an early event in CD28 signaling.

To demonstrate the effect of CD28 crosslinking on tyrosine phosphorylation, Jurkat cells were stimulated with CD28 and GAM for 30 sec as described, and whole cell lysates were blotted for the presence of phosphotyrosine. Fig. 1 shows that a molecule of ≈70 kDa is tyrosine-phosphorylated, in addition to one at ≈100 kDa (Fig. 1, lane 2). To test whether $p72^{ITK/EMT}$ was tyrosine-phosphorylated after CD28 crosslinking, Jurkat T cells were stimulated with antibodies to CD28 and RAM for 2 min. Fig. 2*A Left* shows that $p72^{ITK/EMT}$ becomes tyrosine-phosphorylated after CD28 crosslinking. This CD28-induced tyrosine phosphorylation was rapid (within 30 sec of CD28 crosslinking), peaked at ≈2 min after CD28 crosslinking, and was prolonged, persisting for at least 10 min (Fig. 3*A*). CD3 crosslinking (Fig. 2*A Middle*) also induces tyrosine phosphorylation of $p72^{ITK/EMT}$. Crosslinking of HLA class I molecules (Fig. 2*A Right*) or CD4 (data not shown) did not induce tyrosine phosphorylation of $p72^{ITK/EMT}$.

We have reported (25) that crosslinking CD28 results in a small but reproducible increase in $p56^{LCK}$ activity. To determine if tyrosine phosphorylation of $p72^{ITK/EMT}$ induced by CD28 preceded or followed activation of LCK, we tested these lysates for activation of LCK in comparison with activation of $p72^{ITK/EMT}$. Fig. 3*B* shows that the activity of LCK drops at 30 sec, then becomes weakly activated (of four separate experiments, average of 1.5- to 2-fold), peaking 1 min after CD28 crosslinking, followed by a down-regulation of kinase activity at 10 min (Fig. 3*B*, lane 7) coinciding with a shift to $p60^{LCK}$ (25). This was after the observed tyrosine phosphorylation of $p72^{ITK/EMT}$ (compare Fig. 3 *A* and *B*). This suggests that $p72^{ITK/EMT}$ becomes tyrosine-phosphorylated before the activation of LCK.

In the experiments described above and those reported by Vandenberghe *et al.* (14), activation of the CD28 pathway was

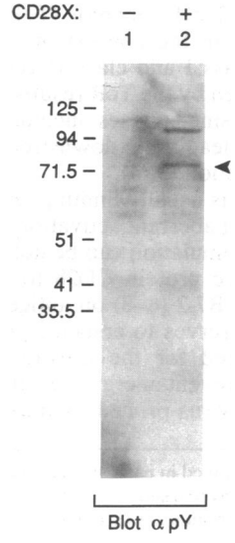

FIG. 1. Tyrosine phosphorylation of putative p72 and putative p100 after CD28 crosslinking (CD28X). Jurkat leukemic cells were stimulated with anti-CD28 and GAM for 30 sec and then lysed. The lysates were separated by SDS/PAGE and blotted with anti-phosphotyrosine (αpY). Lanes: 1, unstimulated cells; 2, stimulated cells. Arrowhead points to putative p72. Sizes are shown in kDa.

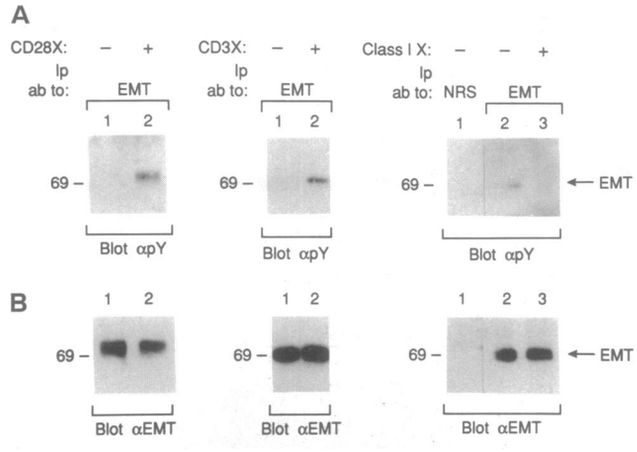

FIG. 2. Tyrosine phosphorylation of p72$^{ITK/EMT}$ after CD28, CD3, and HLA class I crosslinking (X). (*A*) Jurkat cells were stimulated with anti-CD28 and RAM (*Left*), anti-CD3 and RAM (*Middle*), or anti-HLA class I and RAM (*Right*), and p72$^{ITK/EMT}$ was immunoprecipitated (Ip). The precipitates were blotted and probed with anti-phosphotyrosine (αpY). Lanes: 1, unstimulated cells; 2, cells antibody-stimulated for 2 min. In some experiments, the control cells had low levels of p72$^{ITK/EMT}$ tyrosine phosphorylation (see *Right* and Fig. 3*A*, lane 1, for examples), which did not increase upon class I crosslinking. Arrows point to tyrosine-phosphorylated p72$^{ITK/EMT}$; 69 kDa is indicated. (*B*) Blots in *A* were stripped and probed for p72$^{ITK/EMT}$ with anti-ITK/EMT (αEMT), demonstrating equal loading.

achieved by secondary crosslinking of the CD28 molecules (i.e., supercrosslinking by RAM as opposed to crosslinking by the bivalent antibody). However, in the experimental conditions used to activate T cells by the CD28 pathway, secondary crosslinking is unnecessary for its functional effects on T-cell anergy and interleukin 2 secretion (9, 26). If indeed p72$^{ITK/EMT}$ were involved in CD28 signaling, secondary crosslinking of the receptors should be unnecessary for it to have its effects on p72$^{ITK/EMT}$. To test this, Jurkat cells were stimulated with CD28 antibodies without crosslinking with RAM, and p72$^{ITK/EMT}$ tyrosine phosphorylation was evaluated. Fig. 4 shows that, indeed, secondary crosslinking of CD28 was not necessary for the induced tyrosine phosphorylation of p72$^{ITK/EMT}$, although the kinetics of tyrosine phosphorylation was slower (Fig. 4*A*, lanes 1–7). This tyrosine phosphorylation occurred in the absence of any detectable LCK activation (Fig. 4*B*) or any other detectable tyrosine phosphorylation of other proteins in whole-cell lysates (data not shown). These results show that ligation of CD28 (as opposed to supercrosslinking with RAM) is sufficient to cause tyrosine phosphorylation of p72$^{ITK/EMT}$ in the absence of detectable activation of LCK. We have also observed that after binding of a murine B7Ig (CD80Ig) fusion protein to murine CD28, p72$^{ITK/EMT}$ became tyrosine phosphorylated (S.G. and G.B.M., unpublished data). However, although crosslinking of CD28 antibodies is not required to induce tyrosine phosphorylation of p72$^{ITK/EMT}$, this response was stronger and more prolonged when the CD28 antibodies were crosslinked with secondary antibodies.

The kinetics of tyrosine phosphorylation of p72$^{ITK/EMT}$ suggests a direct linkage between p72$^{ITK/EMT}$ and CD28. To test this further, CD28 was immunoprecipitated from control or CD28-activated Jurkat cells and analyzed for the presence of p72$^{ITK/EMT}$ by Western blotting. Fig. 5 demonstrates that p72$^{ITK/EMT}$ could be coimmunoprecipitated with CD28 in both control and CD28-crosslinked cells but was not observed in HLA class I precipitates (Fig. 5*A*) or in CD4 precipitates (data not shown). The amount of p72$^{ITK/EMT}$ coimmunoprecipitating with CD28 increases after crosslinking. We estimate by densitometry that ≈1% of ITK/EMT molecules in the cell was associated with CD28 after crosslinking. This increase in the association of p72$^{ITK/EMT}$ with CD28 peaked at 2 min (Fig. 5*B*). These results demonstrate that p72$^{ITK/EMT}$ associates with CD28, an association that increases upon crosslinking. Reciprocal experiments—i.e., immunoprecipitation of p72$^{ITK/EMT}$ and blotting for CD28—could not be performed because of the lack of availability of antibodies that detect CD28 on Western blots.

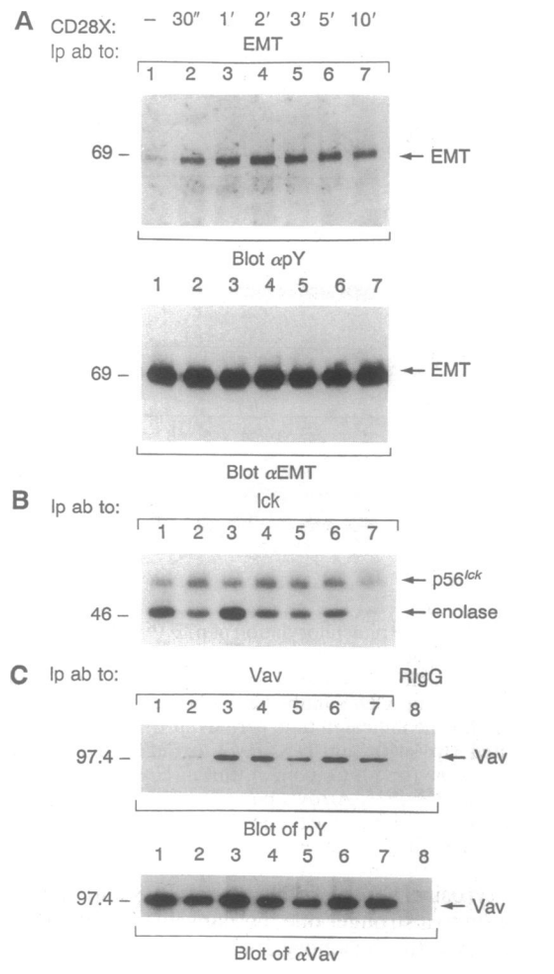

FIG. 3. Time course of CD28-induced tyrosine phosphorylation of p72$^{ITK/EMT}$. (*A Upper*) Jurkat cells were stimulated with anti-CD28 antibody and RAM for the indicated time periods, and p72$^{ITK/EMT}$ was immunoprecipitated (IP) with anti-ITK/EMT (αEMT) and analyzed for phosphotyrosine (pY). (*A Lower*) Blot in *Upper* was stripped and probed for p72$^{ITK/EMT}$. Arrows point to p72$^{ITK/EMT}$. (*B*) Lysates used in *A* were used to immunoprecipitate LCK, and an *in vitro* kinase reaction was performed. The activation of LCK was reproducibly small (1.5- to 2-fold over four experiments), with a concomitant shift to p60^{LCK} and down-regulation of kinase activity (lane 7). (*C Upper*) Vav was immunoprecipitated and probed for phosphotyrosine. (*C Lower*) Blot in *Upper* was stripped and probed for Vav. All lanes are as in *A*. Sizes are shown in kDa.

While p72$^{ITK/EMT}$ can be found in CD28 immunoprecipitates before crosslinking, the amount increases upon CD28 crosslinking. In addition, we have recently reported that CD28 becomes tyrosine-phosphorylated upon crosslinking (27). These results suggest that tyrosine phosphorylation of CD28 is a signal for its increased association with p72$^{ITK/EMT}$. We cannot rule out the possibility that CD28 contains low levels of phosphotyrosine before crosslinking, accounting for the association seen before crosslinking. Alternatively, a specific binding site for p72$^{ITK/EMT}$ in the cytoplasmic tail of CD28 may account for the association before crosslinking,

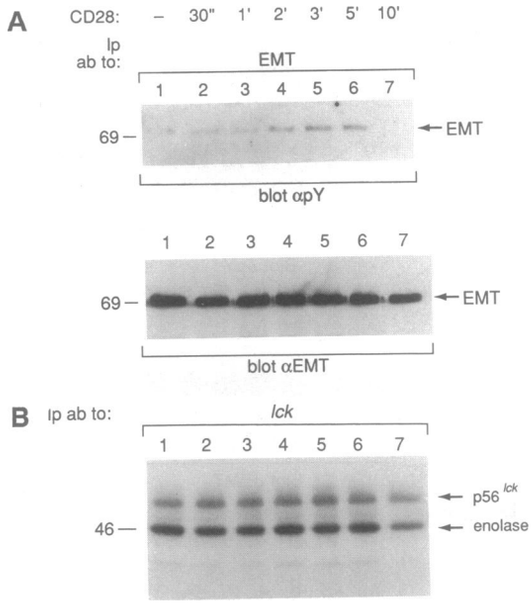

FIG. 4. Tyrosine phosphorylation of p72$^{ITK/EMT}$ does not require CD28 crosslinking with secondary antibody and occurs in the absence of detectable activation of LCK. (*A Upper*) Jurkat cells were stimulated with anti-CD28 antibodies without secondary crosslinking with RAM for the indicated time periods. p72$^{ITK/EMT}$ was immunoprecipitated (Ip) with anti-ITK/EMT (αEMT) and analyzed for phosphotyrosine (αpY). (*A Lower*) Blot in *Upper* was stripped and probed for p72$^{ITK/EMT}$. Arrows point to p72$^{ITK/EMT}$. (*B*) LCK was immunoprecipitated from the lysate shown in *A* and an *in vitro* kinase reaction was performed. Lanes are as in *A*. Sizes are shown in kDa.

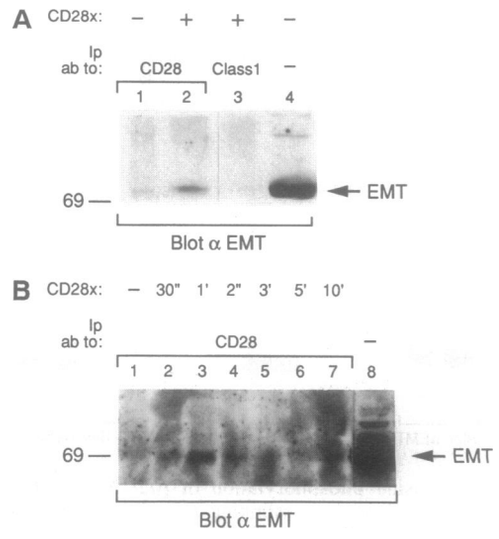

FIG. 5. CD28 is associated with p72$^{ITK/EMT}$. (*A*) Jurkat cells were stimulated with anti-CD28 antibodies and RAM for 2 min, and the activated receptors were isolated by immunoprecipitation (Ip) with anti-CD28 mAb. CD28 was also immunoprecipitated from unstimulated cells. Lanes: 1, unstimulated cells, CD28-precipitated; 2, CD28-crosslinked cells, CD28-precipitated; 3, CD28-crosslinked cells, HLA class I-precipitated; 4, whole-cell lysate of Jurkat cells. The blot was probed for p72$^{ITK/EMT}$ with anti-ITK/EMT (αEMT). Arrow indicates p72$^{ITK/EMT}$. (*B*) Jurkat cells were stimulated with CD28 for the indicated periods of time, and the activated receptors were isolated by immunoprecipitation with anti-CD28 mAb. Lanes: 1, CD28-precipitated receptors from nontreated cells, 2–7, CD28-crosslinked cells for the indicated time periods; 8, a whole-cell lysate of Jurkat cells. The blot was probed for p72$^{ITK/EMT}$. Arrow indicates p72$^{ITK/EMT}$; 69 kDa is indicated.

and the tyrosine phosphorylation of CD28 may convert this association to a stronger one, perhaps due to ITK/EMT SH2 interactions with phosphorylated tyrosines within the cytoplasmic tail of CD28, as has been suggested for the Src family members and the T-cell receptor (28). These results demonstrate, however, that p72$^{ITK/EMT}$ associates with CD28.

It has been reported that CD28 crosslinking results in early tyrosine phosphorylation of two major substrates of ≈70 kDa and ≈100 kDa (refs. 13 and 14 and Fig. 1). The molecular weight of the guanine nucleotide exchange factor VAV is ≈98 kDa. Jurkat cells were therefore crosslinked with anti-CD28 and RAM. After immunoprecipitation with anti-VAV, the precipitates were blotted for phosphotyrosine. It is shown in Fig. 3*C* that VAV rapidly becomes tyrosine-phosphorylated (30 sec to 1 min). This tyrosine phosphorylation of VAV is, however, later than the tyrosine phosphorylation of p72$^{ITK/EMT}$ (compare Fig. 3 *A* with *C*). These data demonstrate that at least one 100-kDa substrate for tyrosine phosphorylation observed after CD28 crosslinking is VAV, although other 100-kDa protein tyrosine kinase substrates may also be present.

Tyrosine phosphorylation of a number of tyrosine kinases seems to be a general signal for activation of their kinase activity. We therefore tested p72$^{ITK/EMT}$ precipitates for kinase activity after CD28 crosslinking. Very little phosphorylation of enolase by p72$^{ITK/EMT}$ could be detected. However, when the SRC peptide was used as a substrate, we observed a rapid and dramatic increase in trans-phosphorylating activity after CD28 crosslinking (Fig. 6). This increase was 3.5-fold over unstimulated cells. Similarly, we detected trans-phosphorylating activity of p72$^{ITK/EMT}$, using a peptide representing the phosphatidylinositol 3 (PI3) kinase SH2 binding site within the cytoplasmic tail of CD28 (data not shown). Fig. 6 also demonstrates that CD28 crosslinking results in minimal increase in the autokinase activity of p72$^{ITK/EMT}$. Stimulation of CD3 resulted in smaller increases (2- to 2.5-fold) in trans-phosphorylating activity, also involving primarily the trans-phosphorylation activity of p72$^{ITK/EMT}$ (unpublished data). Thus, tyrosine phosphorylation of p72$^{ITK/EMT}$ after CD28 crosslinking results in an increase in its kinase activity.

CD28 has no intrinsic tyrosine kinase activity (12), but crosslinking CD28 has been reported to have effects on the activation of the protein-tyrosine kinase pathway and the generation of novel lipids that differ from those generated by the TcR (29). We and others have also reported that crosslinking CD28 induces the association of the lipid PI3 kinase to its cytoplasmic tail (27, 30–32). This recruitment appears to be due to tyrosine phosphorylation of a PI3 kinase-binding motif within the cytoplasmic tail. It has been speculated that the Src family member LCK may be responsible for the tyrosine phosphorylation of CD28 as well as its downstream effects. Although we have observed that LCK is weakly activated as a consequence of supercrosslinking of CD28 with secondary antibody (ref. 25; also Fig. 3*B*), LCK was not activated by addition of CD28 mAb alone (Fig. 4*B*). p72$^{ITK/EMT}$ is, however, tyrosine-phosphorylated under both of these conditions (Fig. 3*A* and Fig. 4*A*). Therefore, LCK does not appear to be solely responsible for the downstream effects of CD28. Furthermore, crosslinking of CD28 results in markedly different effects than crosslinking CD4 or CD2, two receptors that also activate LCK (9). These findings suggest that the p72$^{ITK/EMT}$ may be the kinase that tyrosine-phosphorylates CD28, leading to its recruitment of PI3 kinase, and its downstream effects. Although we have not detected any effect of CD28 crosslinking on p72$^{ITK/EMT}$ in resting peripheral blood T cells, consistent with the findings that treatment with phorbol 12-myristate 13-acetate (PMA) is necessary for such cells to activate the protein tyrosine kinase pathway via CD28 (13, 14), we have been able to detect activation of p72$^{ITK/EMT}$ after CD28 crosslinking in 5-day phytohemagglutinin-stimulated blasts (data not shown).

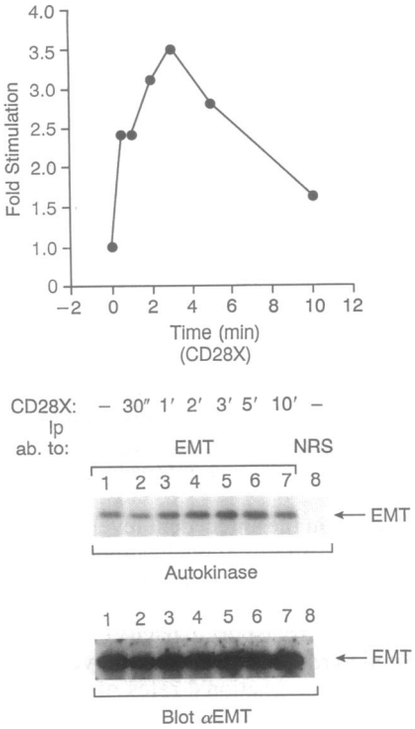

FIG. 6. CD28 crosslinking (CD28X) activates the tyrosine kinase activity of $p72^{ITK/EMT}$. Jurkat cells were stimulated with anti-CD28 antibodies and RAM for the indicated time periods, and $p72^{ITK/EMT}$ was immunoprecipitated (Ip) with antibody to ITK/EMT. (*Top*) Immunoprecipitates were tested for kinase activity as described with the SRC peptide. Activity is presented as fold increase over unstimulated cells. (*Middle*) Immunoprecipitates used in *Top* were separated by SDS/PAGE, blotted to poly(vinyledine fluoride) membrane, and exposed to x-ray film to detect the autokinase activity of $p72^{ITK/EMT}$. (*Bottom*) Blot in *Middle* was probed for $p72^{ITK/EMT}$, demonstrating equal levels of $p72^{ITK/EMT}$ in all lanes. Arrow points to $p72^{ITK/EMT}$. NRS, normal rabbit serum.

The observed tyrosine phosphorylation of $p72^{ITK/EMT}$ upon CD3 crosslinking suggests that $p72^{ITK/EMT}$ may also play a role in CD3 signaling. CD3 crosslinking does not cause tyrosine phosphorylation of CD28 (A.A. and B.D., unpublished data), suggesting that there may be at least two pools of $p72^{ITK/EMT}$ and that CD28 is not in proximity to the CD3 complex, preventing tyrosine phosphorylation by $p72^{ITK/EMT}$. Regardless, these results suggest a role for Tec family tyrosine kinases in both TcR-mediated signaling (the first signal) and CD28 signaling (second signal).

CD28 ligation along with suboptimal doses of PMA and ionomycin causes the appearance of a CD28-responsive complex (CD28RC), which binds to a CD28-responsive element (CD28RE) upstream in the interleukin 2 promoter. This binding of the CD28RC to the CD28RE can increase the enhancer activity 5-fold (4). The tyrosine phosphorylation of $p72^{ITK/EMT}$ as a consequence of CD28 ligation, in combination with PMA and ionomycin, may serve to signal the generation of this complex. The results obtained above suggest a model for CD28 signaling where the $p72^{ITK/EMT}$ tyrosine kinase becomes tyrosine-phosphorylated as a consequence of CD28 ligation, leading to the tyrosine phosphorylation of CD28 and the recruitment of PI3 kinase (27, 30–32) followed by or concurrent with tyrosine phosphorylation of other substrates. The net result of these signals ($p72^{ITK/EMT}$ tyrosine phosphorylation, PI3-kinase activation, and either signals by the TcR or PMA and ionomycin) is the generation of signals that increases the half-life of lymphokine mRNAs and the transcriptional activity of lymphokine genes (5, 9).

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