Functional LCK Is Required for Optimal CD28-mediated Activation of the TEC Family Tyrosine Kinase EMT/ITK*

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Activation of CD28 on T lymphocytes initiates a cascade of intracellular events, which in concert with activation of the T cell receptor, culminates in production of cytokines and a functional immune response. One of the earliest biochemical changes observed following stimulation of CD28 is tyrosine phosphorylation. We have demonstrated that both the LCK and the EMT/ITK/TSK (EMT) intracellular tyrosine kinases are activated following cross-linking of CD28. Utilizing somatic cell mutants lacking LCK, we demonstrate that functional LCK is required for CD28-induced activation of EMT as evidenced by increased tyrosine phosphorylation and kinase activity. In support of a role for LCK in EMT activation, reconstitution of a LCK-negative Jurkat T cell line by transfection with wild type LCK recreates CD28-mediated EMT activation. Furthermore, co-transfection of LCK and EMT into COS-7 cells showed that EMT becomes phosphorylated in the presence of LCK. In addition, increases in EMT association with CD28 were eliminated in a LCK-negative Jurkat cell line, but were restored following transfection of wild type LCK. The data are most compatible with a model in which LCK, either directly or indirectly, initiates EMT activation and association with CD28 following ligation of CD28.

Co-stimulation of T lymphocytes requires the cooperation of two signals delivered by antigen presenting cells: one stimulatory signal derived from interaction of the T cell receptor (TCR) complex with antigen in the context of the major histocompatibility complex and a second co-stimulatory signal from the ligation of accessory molecules (1). In the absence of the co-stimulatory signal, T cells fail to undergo clonal expansion and instead ultimately enter an abortive pathway characterized by antigen desensitization, anergy, or programmed cell death (1–4). The interaction of CD28 on T lymphocytes with B7.1 (CD80) or B7.2 (CD86) on antigen-presenting cells is the most potent identified co-stimulatory signal. Indeed, cross-linking of CD28 can prevent activation-induced desensitization, anergy, and programmed cell death (4–8).

Upon activation of CD28, there is a rapid and immediate increase in tyrosine phosphorylation of a number of specific substrates (8–12). However, because CD28 does not contain an intrinsic kinase domain, it must activate intracellular tyrosine kinases. In addition, cross-linking of CD28 leads to the induction of a number of early signaling events, including increases in cytosolic free calcium (7,8), activation of RAS (13), activation of mitogen-activated protein kinase (13), activation of phosphotyidylinositol 3’-kinase (14–18), activation of JNK kinase (also known as stress-activated kinase) (19) and activation of RAF kinase (20). Although it is clear that cross-linking of CD28 can induce a number of early signals, the role that activation of these biochemical changes plays in the ability of CD28 to synergize with the TCR to induce a functional T cell response remains unclear. Furthermore, the mechanisms leading to activation of these enzymes by CD28 and in particular the order of activation of each of these enzymes is unknown.

We have demonstrated recently that EMT/ITK/TSK (EMT), a TEC family protein tyrosine kinase, becomes activated after CD28 cross-linking, as evidenced by a transient increase in tyrosine phosphorylation and kinase activity. In addition, stimulation of CD28 results in a rapid increase in the association of EMT with CD28 (21). Thus EMT has the potential to play a role in CD28 signal transduction. LCK is also activated after stimulation of CD28, suggesting that this kinase may have a signaling role through CD28 in addition to its dual role downstream of both the TCR and CD40 (21, 22).

The TEC family of intracellular kinases currently consists of members that contain SH2 and SH3 SRC homology (SH) domains but lack the negative regulatory tyrosine present at the carboxyl terminus and the myristoylation site found at the amino terminus of SRC family members. Thus the TEC family of tyrosine kinases must be regulated in a different manner from the SRC family of tyrosine kinases. BTK and EMT contain, in addition to the SH2 and SH3 domains, a pleckstrin homology (PH) domain. The exact function of this domain is currently unknown, but it may play a role in the ability of BTK and EMT to associate with other molecules such as protein kinase C (23, 24). Both BTK and EMT have restricted patterns of expression; BTK is expressed mainly in mast cells and B cells and EMT is expressed primarily in mast cells and T cells (25, 26). BTK is involved in B cell signal transduction; mutations in BTK have been causally linked to X-linked agammaglobulinemia, a severe human B cell immunodeficiency (27). In addition, since cross-linking mouse FcR1 leads to activation of BTK (28), this kinase may also play a role in mast cell activation.
Jurkat T cells were stimulated by cross-linking CD28 with 10 μg/ml anti-CD28 (9.3) antibody and 10 μg/ml RAM for the indicated times. Similar results were observed in the absence of RAM (see Ref. 18 and data not presented). The cells were lysed as described under "Materials and Methods." The precipitates were loaded on a 10% SDS-PAGE gel, transferred to Immobilon, and Western-blotted with anti-phosphotyrosine antibody (4G10, 1:2500 dilution) or EMT antibodies (1:1000) were added for 1 h. Membranes were washed, incubated with the appropriate secondary antibody, and protein was detected by enhanced chemiluminescence (ECL). Where indicated, the blots were stripped with 1% SDS, reprobed with anti-EMT antibodies (1:1000 dilution), and visualized by ECL.

In Vitro Kinase Assay—Tyrosine kinase activity of EMT was assayed by immunoprecipitation of lysates as described above. The immunoprecipitates were then washed in Kinase Buffer (10 mM Mops, pH 7.2, 30% glycerol, 1 mM dithiothreitol, 0.05 mM EDTA, 0.05 mM EGTA, 0.5 mM sodium orthovanadate, 0.1 mg/ml BSA) and resuspended in kinase buffer containing 50 mM Tris, pH 7.2, 10 mM MgCl₂, 10 mM MnCl₂, 0.1 μM ATP, and 0.1 μM α-[γ-32P]ATP. A 1:1000 dilution of rabbit anti-phosphotyrosine antibody (Upstate Biotechnology, Inc. [Lake Placid, NY]) was incubated with the immunoprecipitates for 1 h at 4°C, and immunocomplexes were collected on microcentrifuge tubes and washed once in wash buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 0.02% Tween 20). Tyrosine kinase activity was determined by the incorporation of [γ-32P]ATP into protein. Immune precipitation was carried out by the addition of 2 μg of polyclonal anti-phosphotyrosine antibody (Upstate Biotechnology, Inc., Lake Placid, NY) to 50 μl of the immunoprecipitates, which were incubated overnight at 4°C.

Herein, we demonstrate that CD28-mediated EMT activation and EMT association with CD28 is greatly decreased in cells lacking functional LCK. Reconstitution of LCK kinase activity by enforced expression of the normal human LCK ligand-induced EMT activation and increased EMT association with CD28, confirming a role for LCK in EMT activation. In addition, co-expression of EMT and LCK in COS-7 cells lead to tyrosine phosphorylation of EMT. Thus, EMT appears to be located downstream of LCK in the signaling pathways activated by CD28.

**MATERIALS AND METHODS**

Antibodies—Monoclonal anti-CD28 antibody 9.3 (IgG2a) was a kind gift of J. Ledbetter (Bristol-Myers Squibb Research Institute, Seattle). Anti-phosphotyrosine antibody (4G10, IgG1) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The production and specificity of the anti-EMT serum used in these studies was described previously (26).

Cell Lines—COS-7 cells, the parental human Jurkat leukemic cell line E6.1 and the J CaM1.6 Jurkat clone (which is LCK-negative, Ref. 29) were from the American Type Culture Collection (ATCC, Rockville, MD). J CaM 1.6 transfected with LCK (J CaM 1.6-LCK) was obtained from Art Weiss (University of California, San Francisco, Ref. 40). By Western blotting the protein level of LCK in J CaM1.6-LCK was comparable with the parental Jurkat cell line (data not shown) and was not detected in the J CaM1.6 cell line. Similar levels of EMT were present, as assessed by Western blotting, in all the Jurkat T cell lines studied (data not shown). CD28 expression was the same for all Jurkat cell lines studied (data not shown). Both Jurkat E6.1 and J CaM1.6 have been demonstrated to lack functional SYK (30).

Cell Culture, Stimulation, Transfection, and Lysis—Jurkat cells were cultured and starved as described previously (21, 26). Anti-CD28 antibodies were added at 1 μg/ml × 10⁶ cells at 37°C as indicated. Rabbit anti-mouse (RAM) antibodies (10 μg/ml) were added 1 min after addition of anti-CD28 antibodies, in order to induce cross-linking. After activation, the cells were pelleted and immediately incubated with lysis buffer (26) for 15 min at 4°C. COS-7 cells were transfected with plasmids pA1068 (hck in pMEVneo) and/or pCMVEMTneo (hEMT in pCMVneo) by calcium phosphate precipitation and lysed 2 days later.

Immunoprecipitation and Western Blotting—Cell lysates were centrifuged at 14,000 × g for 15 min at 4°C. After centrifugation the supernatant was immunoprecipitated and Western-blotted as described previously (21, 26). The blots were blocked overnight in either 5% bovine serum albumin for the detection of phosphotyrosine residues or 5% non-fat milk for detection of EMT. Anti-phosphotyrosine antibodies (4G10, 1:2500 dilution) or EMT antibodies (1:1000) were added for 1 h. Membranes were washed, incubated with the appropriate secondary antibody, and protein was detected by enhanced chemiluminescence (ECL). Where indicated, the blots were stripped with 1% SDS, reprobed with anti-EMT antibodies (1:1000 dilution), and visualized by ECL.
Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly (Sigma) derived from the sequence surrounding the SRC tyrosine kinase autophosphorylation site (31). This mixture was incubated for 15 min at room temperature which was found to be within linear range of the assay. The mixture was then blotted onto phosphocellulose paper and washed six times with phosphoric acid. The amount of $^{32}$P incorporated was determined by scintillation counting.

**RESULTS**

Optimal CD28-induced EMT Activation Requires LCK—Activation of CD28 initiates an intracellular kinase cascade that involves the LCK kinase (21, 22). To test whether LCK expression is required for optimal activation of EMT by CD28, we measured the level of EMT activation, as assessed by tyrosine phosphorylation and kinase activity, after CD28 cross-linking in the J CaM 1.6 Jurkat T cell line that does not express LCK. In contrast to results in the parental J urkat T cell line, both basal and CD28-induced total tyrosine phosphorylation was markedly decreased in the J CaM 1.6 line in at least three similar experiments (Fig. 1, panels b and d, and data not presented). Strikingly, although CD28-induced tyrosine phosphorylation of EMT was readily detectable in parental J urkat cells (Fig. 1, panels a and d) (21), no CD28-induced increase in EMT phosphorylation was detected in J CaM 1.6 cells (Fig. 1, panels b and d). In parallel with the tyrosine phosphorylation data, incubation of J CaM 1.6 with anti-CD28 antibodies did not induce EMT kinase activity (Fig. 2). Furthermore, increased EMT association with CD28 was not detected in the J CaM 1.6 cell line as compared with the parental J urkat cell line (Fig. 3 a).

To confirm that the decreased activation of EMT and reduced association of EMT with CD28 in the J CaM 1.6 line was due to a lack of functional LCK, we determined whether the response to CD28 cross-linking was restored in a J CaM 1.6 cell line transfected to express normal human LCK. Previous studies had demonstrated that expression of LCK in J CaM 1.6 restores anti-TCR-mediated signaling and interleukin-2 production (29). In J CaM 1.6 LCK transfectants, CD28-induced tyrosine phosphorylation, and specifically, tyrosine phosphorylation of EMT induced by cross-linking CD28 was completely restored (Fig. 1, panels c and d). Similarly, anti-CD28 mediated increases in EMT kinase activity were restored in the J CaM 1.6 LCK transfectants (Fig. 2). In addition, the increase in association of EMT with CD28 was restored (Fig. 3b). The ability of transfected LCK to reconstitute CD28-mediated EMT activation confirms that the defect in EMT activation in J CaM 1.6 was indeed a consequence of lack of functional LCK.

Co-transfection of LCK and EMT into COS-7 Cells Leads to EMT Phosphorylation—CD28-mediated EMT activation seems to require functional LCK in J urkat cells. This suggests that EMT could be a target for tyrosine phosphorylation by LCK. In support of this possibility, both EMT and LCK were transfected in COS-7 cell either alone or together and the extent of EMT phosphorylation determined. As shown in Fig. 4, transfection of vector alone or EMT alone did not result in detectable tyrosine phosphorylation of EMT. However, concurrent transfection of both LCK and EMT in COS-7 cells resulted in tyrosine phosphorylation of a unique 72-kDa band consistent with LCK phosphorylating EMT.

**DISCUSSION**

The data presented are most consistent with a model wherein CD28-induced activation of the LCK tyrosine kinase is proximal in a cascade leading to CD28-induced activation of EMT. In support of this possibility, we have demonstrated that LCK kinase activity is increased following stimulation of CD28 (21, 22) and that LCK can lead to tyrosine phosphorylation of EMT in COS-7 cells (Fig. 4). Whether LCK directly phosphorylates EMT or EMT activation is a consequence of LCK-mediated phosphorylation of an intermediary molecule following CD28 activation is currently unknown. As J urkat cells express...
both SRC and FYN (32), these Src family tyrosine kinases, in contrast to LCK, are either not sufficient for CD28-mediated EMT activation or are not stimulated following CD28 ligation.

Regulation of EMT kinase activity may well be at the level of tyrosine phosphorylation, since tyrosine phosphorylation of EMT and EMT kinase activity demonstrated concurrent changes following cross-linking of CD28 (Figs. 1d and 2). EMT contains a tyrosine in a conserved internal site which becomes autophosphorylated in Src family tyrosine kinases and likely positively regulates kinase activity (26). However, kinase assays revealed that EMT is very inefficient at autophosphorylation (21) and LCK has been demonstrated previously (21), EMT binds to CD28 constitutively and after activation the association is up-regulated presumably by SH2 domain interactions. This increase in association was greatly reduced in the JCaM 1.6 cell line and restored in the JCaM 1.6 cell line transfected with LCK. This suggests that functional LCK is required for CD28 cross-linking induced increases in EMT association with CD28. This may be a consequence of LCK either directly or indirectly tyrosine phosphorylating CD28. Recent results, using transfection into Spodoptera cells, supports LCK as the primary mediator of CD28 phosphorylation (38). Furthermore, EMT, phosphatidylinositol 3’-kinase, and GRB2 association with CD28 in transfected Spodoptera cells required co-expression of LCK (38).

Although the PH domain of both BTK and EMT associate with protein kinase C isozymes (24), limited data are present to date on the interactions between SRC family kinases and other tyrosine kinases. Although EMT and BTK appear to associate with SRC family kinases (including LCK and FYN) in the yeast two-hybrid system and when expressed as bacterial fusion proteins (40–42), it has been difficult to demonstrate interactions in intact cells (40–42). Indeed, under conditions (using Nonidet P-40 buffers) in which we can readily demonstrate association of EMT with LCK, FYN, SRC, or TTK (all of which are expressed by J urkat T cells; 10, 32, 43, 44) as indicated by co-immunoprecipitation (data not shown).

In summary, we have demonstrated that the ability of CD28 to optimally activate EMT is dependent on the presence of functional LCK. The data are most compatible with a model of CD28 signaling in which activated LCK mediates phosphorylation and activation of EMT and mediates EMT association with CD28. Thus EMT activation seems to be located downstream of LCK in a kinase cascade stimulated by CD28.

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REFERENCES

1. Janeway, C., and Bottomly, K. (1994) Cell 76, 275–285
2. Schwartz, R. (1992) Cell 71, 1065–1068
3. Weiss, A., and Littman, D. (1994) Cell 76, 263–274
4. Harding, F., McArther, J., Gross, J., Raulet, D., and Allison, J. (1992) J. Nature 356, 607–609
5. Shi, Y., Radvanyi, L., Shaw, P., Miller, R., and Mills, G. B. (1995) J. Immunol. 155, 1829–1837
6. Shahnazian, A., Preffer, K., Lee, K., Kundig, T., Kishihara, K., Wakeham, A., Kawai, K., Ohashi, P., Thompson, C., and Maks, T. (1993) Science 261, 609–612
7. J. Immunol. 153, 24–29
8. Vandenbergh, P., Freeman, G. J., Riedler, M. L., Fletter, M. C., Kamoun, M., Turka, L. A., Ledbetter, J. A., Thompson, C. B., and June, C. H. (1992) J. Exp. Med. 175, 951–960
9. Nunes, J., A., Collette, Y., Truneh, A., Olive, D., and Cantrell, D. (1994) J. Exp. Med. 180, 1067–1076
10. Trifurt, K., Nilles, C., and Imboden, J. (1994) J. Exp. Med. 179, 1071–1076
11. August, A., and Dupont, B. (1994) Immuno. Med. 6, 769–774
12. Prasad, K. V., Cai, Y. C., Raab, M., Duckworth, B., Cantley, L., Schoel, S. E., and Rudd, C. E. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 2834–2838
13. Siegel, J. N., Rapp, U. R., and Samelson, L. R. (1993) J. Immunol. 151, 4126–4127
14. August, A., Gibson, S., Kawakami, Y., Kawakami, T., Mills, G. B., and Dupont, B. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 9347–9351
15. August, A., and Dupont, B. (1994) Biochem. Biophys. Res. Commun. 199, 1466–1473
16. Yao, L., Kawakami, Y., and Kawakami, T. (1994) Proc. Natl. Acad. Sci. U.S.A.


24. Kawakami, Y., Yao, L., Tashiro, M., Gibson, S., Mills, G. B., and Kawakami, T. (1995) J. Immunol. 155, 3556–3562
25. Yamada, N., Kawakami, Y., Kimura, H., Fukamachi, H., Baier, G., Altman, A., Kato, T., Inagaki, Y., and Kawakami, T. (1993) Biochem. Biophys. Res. Commun. 192, 231–240
26. Gibson, S., Leung, B., Squires, J., Hill, M., Arima, N., Goss, P., Hogg, D., and Mills, G. B. (1993) Blood 82, 1561–1572
27. Tsukada, S., Safran, D., Rawlings, D., Parolini, O., Allen, R. C., Klisak, I., Sparks, R., Kubagawa, H., Mohandas, T., Quan, S., Belmont, J., Cooper, M., Conley, M., and Wittes, O. (1993) Cell 72, 279–290
28. Kawakami, Y., Yao, L., Miura, T., Tsukada, S., Witte, O., and Kawakami, T. (1994) Mol. Cell. Biol. 14, 5108–5113
29. Straus, D., and Weiss, A. (1992) Cell 70, 585–593
30. Fargnol, J., Burkhartd, A., Laverty, M., Kut, S., van Oers, N., Weiss, A., and Bolen, J. (1993) J. Biol. Chem. 268, 26533–26537
31. Piket, L., Eakes, A., and Krebs, E. G. (1986) J. Biol. Chem. 261, 3782–3789
32. Branch, D. R., and Mills, G. B. (1995) J. Immunol. 154, 3678–3685
33. Chow, L., Fournel, M., Davidson, D., and Veillette, A. (1993) Nature 365, 156–160
34. Hurley, T., Hyman, R., and Setton, B. (1993) Mol. Cell. Biol. 13, 1651–1656
35. Kurosaki, T., Takata, M., Yamansahi, Y., Inazu, T., Taniguchi, T., Yamamoto, T., and Yamamura, H. (1994) J. Exp. Med. 179, 1725–1729
36. Schlaepfer, D. D., Hank, S. K., Hunter, T., and van der Geer, P. (1994) Nature 372, 786–791
37. Twamley-Stein, G. M., Pepperkok, R., Ansorge, W., and Courtneidge, S. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7696–7700
38. Raab, M., Cai, Y. C., Bunnell, S., Heyeck, S., Berg, L., and Rudd, C. E. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8891–8895
39. Schneider, H., Cai, Y. C., Prasad, K. V. S., Shoelson, S. E., and Rudd, C. E. (1995) Eur. J. Immunol. 25, 1044–105
40. Cheng, G., Ye, Z., and Baltimore, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8152–8155
41. Alexandropoulos, K., Cheng, G., and Baltimore, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3110–3114
42. Yang, W., Malek, S., and Desiderio, S. (1995) J. Biol. Chem. 270, 20832–20840
43. Schmandt, R., Hill, M., Amendola, A., Mills, G. B., and Hogg, D. (1994) J. Immunol. 152, 96–105
44. Mills, G. B., Schmandt, R., McGill, M., Amendola, A., Hill, M., Jacobs, K., May, C., Rodricks, A., Campbell, S., and Hogg, D. (1992) J. Biol. Chem. 267, 16000–16006
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