Biochemical and Biophysical Research Communications **269,** 279–283 (2000)
doi:10.1006/bbrc.2000.2234, available online at http://www.idealibrary.com on IDEAL®

Tyrosine Phosphatase SHP-2 Binding to CTLA-4: Absence of Direct YVKM/YFIP Motif Recognition

Helga Schneider*,† and Christopher E. Rudd*,‡,[1]

**Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, Massachusetts; and †Department of Medicine and ‡Department of Pathology, Harvard University Medical School, Boston, Massachusetts, 02115*

Received January 3, 2000

CTLA-4 is well documented in its negative regulation of T-cell proliferation. However, little is known regarding the signaling mechanisms induced by CTLA-4. CTLA-4 associates with the phosphatidylinositol 3-kinase, the phosphatase SHP-2 and the clathrin adaptor complexes AP-1 and AP-2. SHP-2 SH2 domain binding to CTLA-4 is unusual given the absence of a I/VxYxxI/V/L motif. Here, we demonstrate that the phosphorylation of CTLA-4 tyrosines (YVKM and YFIP) fails to allow for single or tandem SHP-2 SH2 domain binding. This was observed using wild-type and inactive SHP-2 as well as a construct with the isolated two SH2 domains. The phosphorylated YVKM and YFIP motifs therefore do not appear to represent novel binding motifs for SHP-2 SH2 domains. At the same time, we could confirm that SHP-2 can associate with CTLA-4 in murine T-cells indicating that the interaction between the phosphatase and CTLA-4 is an indirect event, possibly mediated by PI 3-kinase/SHP-2 binding.

CD28 and CTLA-4 are differentially expressed on cells, bind to common ligands CD80 and CD86 (albeit with different aviditis), and are required for optimal T cell activation (1–3). CD28 is expressed on the surface of resting and activated T cells, while structurally related CTLA-4 is expressed only on activated T cells (4, 5). CD28 and CTLA-4 have different functions on the activation of T cells. CD28 can both lower the threshold needed for T cell activation and increase the longevity of the response, an effect that is linked to increased transcription and stability of mRNA for lymphokines, in particular, interleukin-2 and -4 (IL-2 and IL-4) (2, 3). CD28 deficient mice have markedly diminished T cell responses to certain antigens (6), while CD28 amplifies cytolytic responses in tumor and autoimmune models (7–10).

[1] To whom correspondence should be addressed. Fax: (617) 632-5259. E-mail: chris_rudd@macmailgw.dfci.harvard.edu.

CTLA-4 is well-documented in its ability to negatively regulate T-cell proliferation (1, 2, 11). Antibodies to the receptor inhibit TcR-CD28 signaling (12–14), and CTLA-4-negative mice show extensive lymphoadenopathy consistent with the loss of negative regulation (15, 16). In terms of molecular interactions, CTLA-4 has been reported to associate with the p85 subunit of PI 3-kinase, the tyrosine phosphatase SHP-2 and the clathrin adaptor complexes AP-1 and AP-2 (17–25). While PI 3-kinase and SHP-2 have been reported to recognize phosphorylated versions of the YVKM motif (17, 19), the AP-1 and AP-2 complexes bind to the nonphosphorylated version of the same motif in different cellular locales. AP-1/CTLA-4 complexes localize to the *trans*-Golgi network (TGN) and act as a homeostatic regulator of the density of intracellular CTLA-4 (25). The AP-2 complex binds to surface forms of the coreceptor and regulates receptor internalization (22–24).

In contrast to TcR and CD28-mediated signaling, little is known about the signaling mechanism induced by CTLA-4. One model proposes that CTLA-4 mediates negative signaling by binding to the protein-tyrosine phosphatase SHP-2 (19–21). In support of this, SHP-2 has been reported to bind directly to the CTLA-4 cytoplasmic GVYVKM motif and T-cells from CTLA-4 −/− mice show hyperphosphorylation of the TcRζ chain and other signaling proteins (15, 16). However, direct binding is surprising given that SHP-2 SH2 domain binds a canonical I/VxYxxI/V/L motif (26, 27). The suggestion that the YVKM motif may substitute for binding has suggested that an alternate motif may exist for the SHP-2 SH2 domains (27). Further, it would have implications to CTLA-4 signaling since SHP-2 binding would not only provide a phosphatase for CTLA-4 signaling, but would also displace the binding of the lipid kinase PI 3-kinase, an important factor in the production of D-3 lipids needed for membrane localization of PH domain carrying proteins such as Vav (28).

In this study, we confirm that although CTLA-4 can precipitate SHP-2 from detergent lysates derived from

0006-291X/00 $35.00

activated spleen/lymph node cells, the association of the phosphatase with the CTLA-4 pYVKM motif is indirect. Coexpression of $p56^{lck}$ induced the phosphorylation of CTLA-4 at both the YVKM and YFIP motifs, but failed to allow for detectable binding to either full length SHP-2 or the SH2 domains of SHP-2. Therefore, the phosphorylated YVKM and YFIP motifs do not appear to represent novel binding motifs for SHP-2 SH2 domains distinct from the canonical I/VxYxxI/V/L motif. The implications of indirect vs direct SHP-2 binding to CTLA-4 are discussed.

MATERIAL AND METHODS

Cells, reagents, and antibodies. COS cells were cultured in RPMI 1640 medium, supplemented with 5% (v/v) FCS, 100 U/ml penicillin and 100 mg/ml streptomycin. COS cells were transfected with cDNAs inserted into SRα2 expression vector (gift of Dr. M. Streuli, Dana-Farber Cancer Institute, Boston, MA). The transfections were conducted according to standard protocols. Anti-hCTLA-4 (11D4) has been previously described (29). Anti-mCTLA-4 (4F10-11) was a kind gift from Dr. J. Bluestone (University of Chicago, Chicago, IL). Anti-CD28 (4B10) was provided by Coulter (Hialeah, FL). The anti-p85 anti-serum was a gift from Dr. M. White (Joslin Diabetes Center, Boston, MA), the anti-phosphotyrosine mAb was a gift from Dr. T. Roberts (Dana-Farber Cancer Center, Boston, MA). GST-SHP-2 constructs were kindly provided by Dr. B. Neel (Harvard Medical School, Boston, MA). SHP-2 mAb was bought from Transduction Laboratories (Lexington, KY). Anti-murine CD3 (145-2C11) was purchased from American Type Culture Collection (Manassas, VA).

Immunoprecipitation and immunoblotting. For immunoprecipitation, cells were lysed in ice cold lysis buffer containing 1% TritonX-100 in 20 mM Tris-HCl, pH 8.3, 150 mM NaCl. The lysis buffer contained protease and phosphatase inhibitors. Postnuclear lysates were incubated for 1 hour with the indicated antibody. Protein A-Sepharose beads (30 μl, Pharmacia) were added and incubated for 1 hour at 4°C. The eluted proteins were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting. Bound antibody was revealed with the appropriate secondary antibody, and protein was visualized by enhanced chemiluminescence (ECL, Amersham).

Isolation of lymph node and spleen cells. Lymph node and spleen cells were isolated and stimulated as described (19). Following analysis of CTLA-4 surface expression by FACS, cells were lysed, immunoprecipitated with either anti-mCTLA-4 or anti-SHP-2 mAb and immunoblotted with anti-SHP-2 mAb and anti-p85 Ab.

RESULTS AND DISCUSSION

CTLA-4 has been reported to associate directly with the SH2 domain(s) of the protein tyrosine phosphatase SHP-2 in T-cells (19–21). To investigate this further, we attempted to confirm the finding that anti-CTLA-4 can precipitate SHP-2 from detergent lysates of activated spleen/lymph node cells (19, 21). Anti-CTLA-4 coprecipitated SHP-2, as detected by anti-SHP-2 immunoblotting (Fig. 1, left panel, lane 2). Anti-SHP-2 precipitates served as a positive control (lane 3). Reprobing of the same blot with anti-p85 of PI 3-kinase also showed the presence of associated p85 (right panel, lane 5). This was unusual given that neither the YVKM nor the YFIP sites conform to the canonical

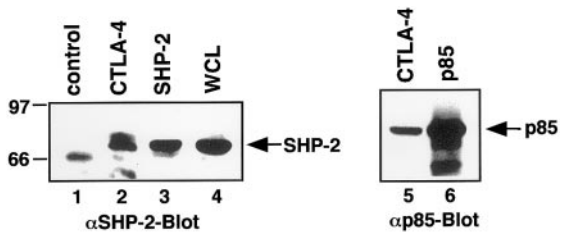

FIG. 1. Anti-CTLA-4 coprecipitates SHP-2 from anti-CD3-activated lymph node and spleen cells. Lymph node and spleen cells were stimulated as described by Marengère and co-workers (19), lysed and immunoprecipitated with CTLA-4 mAb (4F10-11) (lanes 2 and 5), SHP-2 mAb (lane 3) and p85 antiserum (lane 6). Precipitation with rabbit anti-mouse served as a negative control (lane 1). The precipitates were then subjected to immunoblotting with SHP-2 mAb (lanes 1–4) and p85 antiserum (lanes 5 and 6).

motif for SHP-2 SH2 domain binding (I/VxYxxI/V/L) (26, 27), but has lead to the suggestion that these motifs may constitute novel binding sites (19).

To address this issue further, phosphorylated versions of CTLA-4 were coexpressed in COS cells with versions of the SHP-2 protein and assessed for binding. Two tyrosine residues at Y201 and Y218 are located within the motifs YVKM and YFIP of CTLA-4, respectively. Peptides corresponding to the CTLA-4pYVKM motif bind to the SH2 domains of the p85 subunit of PI 3-kinase (17, 18). Further, we previously reported that Rlk/Txk (a member of the Tec family kinases) is capable of phosphorylating CTLA-4 at the YVKM motif (30). Other studies have demonstrated that src kinases $p56^{lck}$ and $p59^{fyn}$ are also capable of phosphorylating CTLA-4 (22, 23, 31), as previously observed for CD28 (32). As shown in Figure 2A, $p56^{lck}$ and $p59^{fyn}$ were coexpressed with wild-type CTLA-4 and the various mutants in COS cells and examined for the ability to phosphorylate the receptor in vivo as detected by antiphosphotyrosine immunoblotting. In the presence of $p56^{lck}$ or $p59^{fyn}$, CTLA-4 underwent significant levels of tyrosine phosphorylation (lanes 1, 6). Mutation of Y201F reduced some 80 percent of phosphorylation by $p56^{lck}$ (lane 2 vs 1). Mutation of Y218F had less of an effect on phosphorylation, but on average caused a 10–20 percent reduction in phosphorylation (lane 3). A complete loss of phosphorylation was observed with the mutation of both tyrosines (lane 4). As a positive control, coexpression of $p56^{lck}$ with CD28 showed significant phosphorylation of this receptor. Comparable levels of expression of the various mutants were obtained as measured by [^{35}S] methionine labeling (data not shown). Similar results were obtained with the coexpression of $p59^{fyn}$ and CTLA-4 (lanes 6–9).

To investigate the possibility of direct binding to SHP-2 to the cytoplasmic domain of CTLA-4, the receptor was coexpressed with the tyrosine kinase $p56^{lck}$ and assessed for an ability to bind the GST-SHP-2 SH2 domains, or coexpressed SHP-2 (Fig. 2B). Transfection

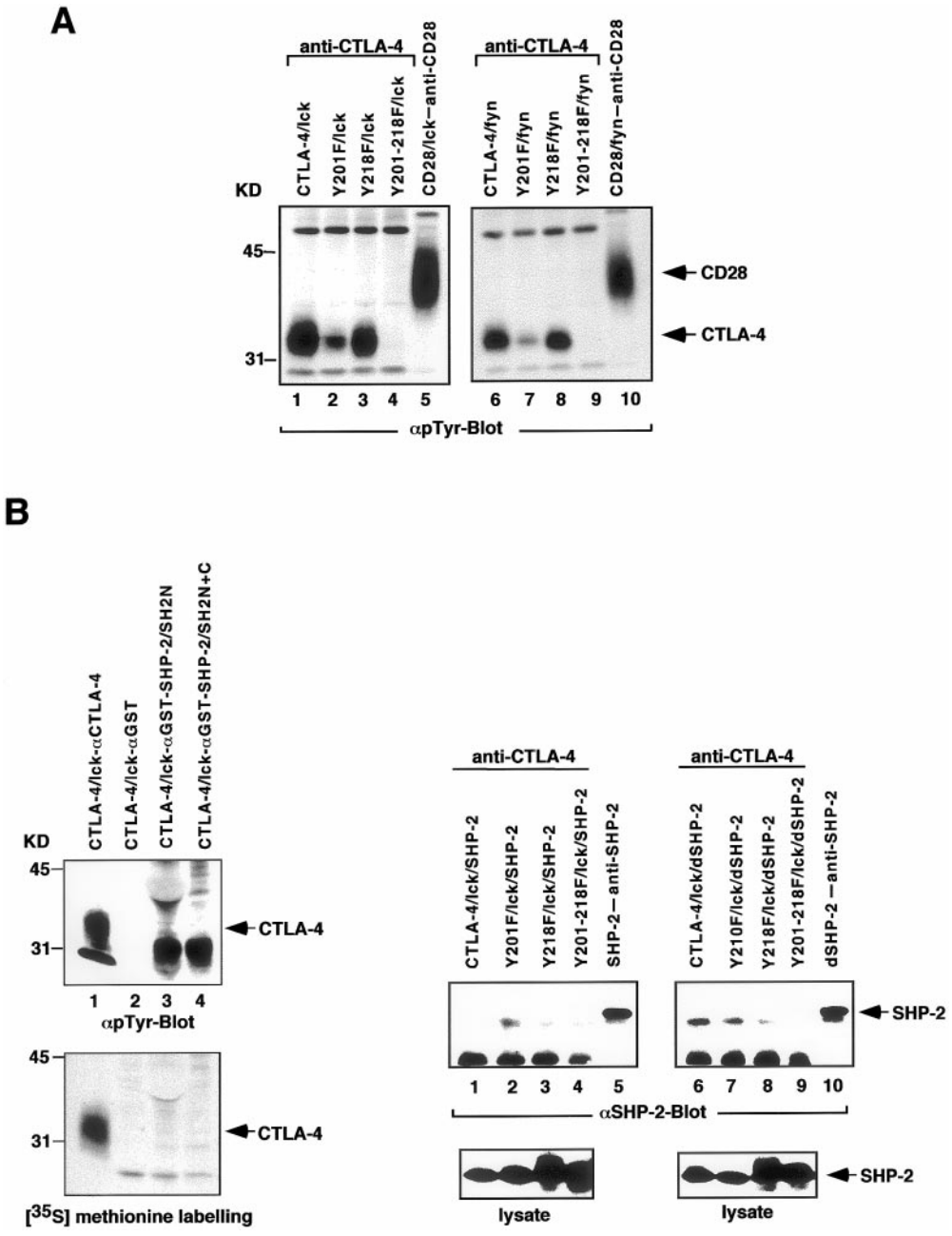

FIG. 2. (A) Phosphorylation of CTLA-4 by the protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$. Wild-type CTLA-4 (lanes 1, 6) or mutant CTLA-4 with Y201F (lanes 2, 7), Y218F (lanes 3, 8) and Y201–218F (lanes 4, 9) or CD28 (lanes 5, 10) were coexpressed with $p56^{lck}$ (lanes 1–5) or $p59^{fyn}$ (lanes 6–10) in COS cells, immunoprecipitated with anti-CTLA-4 mAb (lanes 1–4 and 6–9) or anti-CD28 mAb (lanes 5, 10) and immunoblotted with antiphosphotyrosine mAb. (B) Left panel: COS cells cotransfected with hCTLA-4 and $p56^{lck}$ were labeled with [^{35}S]methionine, lysed, and precipitated with CTLA-4 mAb (lane 1), anti-GST (lane 2), GST-SHP-2-SH2-N (lane 3), GST-SHP-2-SH2-N+C (lane 4). The precipitates were separated by 10% SDS–PAGE and transferred to nitrocellulose for immunoblotting with antiphosphotyrosine mAb (upper panel). Metabolically labeled CTLA-4 is shown in lane 1 (lower panel). Right panel: COS cells cotransfected with $p56^{lck}$, SHP-2 (lanes 1–5), SHP-2 defective phosphatase (dSHP-2) (lanes 6–10) and wild-type hCTLA-4 (lanes 1, 6), the mutants Y201F (lanes 2, 7), Y218F (lanes 3, 8) and Y201–218F (lanes 4, 9) were lysed, immunoprecipitated with anti-CTLA-4 mAb (lanes 1–4 and 6–9), and immunoblotted with anti-SHP-2 mAb (upper panel). Lanes 5 and 10 show anti-SHP-2 immunoprecipitates. The expression levels of SHP-2 and dSHP-2 were comparable in each transfectant (lower panel).

of heterologous cells such as COS cells allows for an analysis of lymphoid kinases and substrates in the absence of their lymphoid proteins. Although coexpressed $p56^{lck}$ induced phosphorylation of CTLA-4 at both tyrosine sites (Figs. 2A and 2B, left panel, lane 1), neither the N-terminal SH2 domain nor combination of

SH2 domains (lanes 3, 4) were found capable of precipitating CTLA-4 as detected by antiphosphotyrosine blotting of CTLA-4 (left upper panel, lanes 2–4 vs 1), or by [^{35}S] methionine labeling (left lower panel, lanes 2–4 vs 1).

In another approach, anti-CTLA-4 also failed to precipitate SHP-2 from COS cells transfected with wild-type receptor (Fig. 2B, right upper panel, lane 1), or with the receptor with substitutions at Y201 (lane 2), Y218 (lane 3) or at both residues (lane 4). This absence of binding occurred despite the expression of SHP-2 in cells, as detected in anti-SHP-2 immunoprecipitates (lane 5). Anti-SHP-2 blotting against cell lysates demonstrated significant levels of the phosphatase in each of the cell lysates from the individual transfectants (right lower panels). Concerned that SHP-2 might dephosphorylate the motif to which it might bind, we also used a phosphatase defective form of SHP-2 (dSHP-2). Again, dSHP-2 failed to bind to wild-type CTLA-4, or to the mutants (Fig. 2B, right upper panel, lanes 6–9).

These data indicate that neither SHP-2 nor the SH2 domains of SHP-2 bind directly to the tyrosine phosphorylated form of CTLA-4. SHP-2 binding to phosphorylated CTLA-4 is therefore most likely to be indirect, possibly mediated by an intermediate protein. This finding agrees with previous studies which have defined the I/VxYxxL/I/L consensus motif as the site for SHP-2 SH2 domain binding (26, 27). Therefore, the phosphorylated YVKM and YFIP motifs do not appear to represent novel binding motifs for SHP-2 SH2 domains distinct from the canonical I/VxYxxI/V/L motif.

Earlier studies showed that the addition of YVKM carrying peptide in competition studies disrupts SHP-2 binding to CTLA-4 (19). The findings in this study now suggest that this interaction occurs in an indirect manner, not necessitating the displacement of PI 3-kinase from the YVKM motif. In fact, our experiments showing CTLA-4 co-precipitated SHP-2 also showed coprecipitated PI 3-kinase (Fig. 1). The lipid kinase PI 3-kinase is an important factor in several receptor systems due to its production of D-3 lipids needed for membrane localization of PH domain carrying proteins such as Vav (28). Indirect SHP-2 binding to CTLA-4 would therefore necessitate an intermediate protein whose identity is unknown. This key component would control the association of SHP-2, and its proposed effect on TcRζ and LAT phosphorylation in cells (19–21). Whether the YVKM site (and by implication PI 3-kinase and/or SHP-2) is needed for CTLA-4 function is the subject of debate (19–21). SHP-2 is generally required as an essential component in positive signaling by growth factor receptors, interleukin-2 receptors and the TcRζ/CD3 complex (33, 34). Expression of a dominant negative phosphatase SHP-2 inhibited TcR activation in Jurkat cells (35). Recently, Nakaseko *et al.* reported that CTLA-4 could operate without cytoplasmic tyrosine motifs, instead requiring a membrane proximal region (36). Further studies will be needed to establish the role of SHP-2 and its bridging protein in CTLA-4-mediated suppression of T cell activation.

REFERENCES

1. Linsley, P. (1995) *J. Exp. Med.* **182,** 459–465.
2. Thompson, C. B. (1995) *Cell* **81,** 979–982.
3. Bluestone, J. (1995) *Immunity* **2,** 555–559.
4. Brunet, J. F., Denizot, F., Luciani, M.-F., Roux-Dosseto, M., Suzan, M., Mattei, M. G., and Golstein, P. (1987) *Nature* **328,** 267–270.
5. Lindsten, T., Lee, K. P., Harris, E. S., Petryniak, B., Craighead, N., Reynolds, P. J., Lombard, D. B., Freeman, G. J., Nadler, L. M., Gray, G. S., Thompson, C. B., and June, C. H. (1993) *J. Immunol.* **151,** 3489–3499.
6. Shahinian, A., Pfeffer, K., Lee, K. P., Kuendig, T. M., Kishihara, K., Wakeman, A., Kawai, K., Ohashi, P. S., Thompson, C. B., and Mak, T. W. (1993) *Science* **261,** 609–612.
7. Townsend, S. E., and Allison, J. P. (1993) *Science* **259,** 368–370.
8. Chen, L., Ashe, S., Brady, W. A., Hellstroem, I., Hellstroem, K. E., Ledbetter, J. A., McGowan, P., and Linsely, P. S. (1992) *Cell* **71,** 1093–1102.
9. Harlan, D. M., Hengarter, H., Huang, M. L., Kang, Y.-H., Abe, R., Moreadith, R. W., Pircher, H., Gray, G. S., Ohashi, P. S., Freeman, G. J., Nadler, L. M., June, C. H., and Aichele, P. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3137–3141.
10. Miller, S. D., Vanderlugt, C. L., Lenschow, D. J., Pope, J. G., Karandikar, N. J., Dal Canto, M. C., and Bluestone, J. A. (1995) *Immunity* **3,** 739–745.
11. Bluestone, J. (1995) *Immunity* **2,** 555–559.
12. Walunas, T. L., Lenschow, D. J., Bakker, C. Y., Linsley, P. S., Freeman, G. J., Green, J. M., Thompson, C. B., and Bluestone, J. A. (1994) *Immunity* **1,** 405–413.
13. Robey, E., and Allison, J. P. (1995) *Immunol. Today* **16,** 306–310.
14. Krummel, M. F., and Allison, J. P. (1995) *J. Exp. Med.* **182,** 459–465.
15. Waterhouse, P., Penninger, J. M., Timms, E., Wakeham, A., Shahinian, A., Lee, K. P., Thompson, C. B., Griesser, H., and Mak, T. W. (1995) *Science* **270,** 985–988.
16. Tivol, E. A., Borriello, F., Schweitzer, A. N., Lynch, W. P., Bluestone, J. A., and Sharpe, A. H. (1995) *Immunity* **3,** 541–547.
17. Schneider, H., Prasad, K. V. S., Shoelson, S. E., and Rudd, C. E. (1995) *J. Exp. Med.* **181,** 351–355.
18. Zhang, Y., and Allison, J. P. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 9273–9278.
19. Marengère, L. E., Waterhouse, P., Duncan, G. S., Mittrücker, H. W., Feng, G. S., and Mak, T. W. (1996) *Science* **272,** 1170–1173.
20. Lee, K. M., Chuang, E., Griffin, M., Khattri, R., Hong, D. K., Zhang, W., Straus, D., Samelson, L. E., Thompson, C. B., and Bluestone, J. A. (1998) *Science* **282,** 2263–2266.
21. Cilio, C. M., Daws, M. R., Malashichewa, A., Sentman, C. L., and Holmberg, D. (1998) *J. Exp. Med.* **188,** 1239–1246.
22. Bradshaw, J. D., Lu, P., Leytze, G., Rodgers, J., Schieven, L., Bennett, K. L., Linsley, P. S., and Kurtz, S. E. (1997) *Biochemistry* **36,** 15975–15982.
23. Chuang, E., Alegre, M.-L., Duckett, C. S., Noel, P. J., Vander Heiden, M. G., and Thompson, C. B. (1997) *J. Immunol.* **159,** 144–151.
24. Shiratori, T., Miyatake, S., Ohno, H., Nakaseko, C., Isono, K., Bonifacino, J. S., and Saito, T. (1997) *Immunity* **6,** 583–589.
25. Schneider, H., Martin, M., Agarraberes, F. A., Yin, L., Rapoport,

I., Kirchhausen, T., and Rudd, C. E. (1999) *J. Immunol.* **163,** 1868–1879.

26. Vivier, E., and Daeron, M. (1997) *Immunol. Today* **18,** 286–291.
27. Neel, B. G. (1997) *Curr. Opin. Immunol.* **9,** 405–420.
28. Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998) *Annu. Rev. Biochem.* **67,** 481–507.
29. Linsley, P. S., Greene, J. A. L., Tan, P., Bradshaw, J., Ledbetter, J. A., Anasettei, C., and Damle, N. K. (1992) *J. Exp. Med.* **176,** 1595–1604.
30. Schneider, H., Schwartzberg, P. L., and Rudd, C. E. (1998) *Biochem. Biophys. Res. Commun.* **252,** 14–19.
31. Miyatake, S., Nakaseko, C., Umemori, H., Yamamoto, T., and Saito, T. (1998) *Biochem. Biophys. Res. Commun.* **249,** 444–448.
32. Raab, M., Cai, Y.-C., Bunnell, S. C., Heyeck, S. D., Berg, L. J., and Rudd, C. E. (1995) *Proc. Natl. Acad. Sci USA* **92,** 8891–8895.
33. Hadari, Y. R., Kouhara, H., Lax, I., and Schlessinger, J. (1998) *Mol. Cell. Biol.* **18,** 3966–3973.
34. Gadina, M., Stancato, L. M., Bacon, C. M., Lamer, A. C., and O'Shea, J. J. (1998) *J. Immunol.* **160,** 4657–4661.
35. Frearson, J. A., and Alexander, D. R. (1998) *J. Exp. Med.* **187,** 1417–1426.
36. Nakaseko, C., Miyatake, S., Iida, T., Hara, S., Abe, R., Ohno, H., Saito, Y., and Saito, T. (1999) *J. Exp. Med.* **190,** 765–774.