Interactions between the Protein-tyrosine Kinase ZAP-70, the Proto-oncoprotein Vav, and Tubulin in J urkat T Cells*

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Two molecules involved in signal transduction via the T cell antigen receptor, namely the protein-tyrosine kinase ZAP-70 and the proto-oncoprotein Vav, were found to be constitutively associated with tubulin in J urkat T cells. Both were able to bind to tubulin independently of one another, as determined by transient transfection into COS-7 cells. The ZAP-70 associated with tubulin was preferentially tyrosine-phosphorylated after T cell antigen receptor stimulation of J urkat T cells, suggesting that this interaction was functionally significant. Vav was also found to co-immunoprecipitate with ZAP-70 from cell extracts depleted of tubulin. This raised the possibility that Vav might be a substrate for ZAP-70 protein-tyrosine kinase activity. However, tyrosine phosphorylation of Vav preceded that of ZAP-70, indicating that Vav was unlikely to be a downstream target of ZAP-70. The association of ZAP-70 and Vav with tubulin implies that the microtubules may be involved in the signaling function of these two molecules, perhaps by targeting them to their appropriate intracellular location.

Stimulation of the T cell antigen receptor (TCR)1 initiates a cascade of signal transduction events, the most proximal of which is the induction of PTK activity, which is essential for the signaling process (1). This involves the cytoplasmic tails of the CD3 complex (γδε) and the ζ chain of the TCR becoming phosphorylated on specific tyrosine residues within ITAMs (1). Studies in mutant J urkat T cells and transfected COS cells have indicated that the Src family protein-tyrosine kinase, Lck, is required for tyrosine phosphorylation of the TCR immunoreceptor tyrosine-based activation motifs (2, 3). Biochemical and genetic experiments have also indicated a role for the Src family PTK, Fyn, in TCR signaling (4). However, its intracellular localization suggests that its function may be distinct from that of Lck (5).

ITAM phosphorylation results in recruitment of the Syk family PTKs, ZAP-70 and Syk, to the TCR via the binding of their two SH2 domains (6–9). ZAP-70 and Syk are then tyrosine-phosphorylated themselves, which for ZAP-70 has been shown to activate its kinase activity (10), and tyrosine phosphorylation of multiple intracellular proteins is induced (3, 6). An essential role for ZAP-70 in TCR signaling and T cell development has been revealed by genetic studies (11, 12).

TCR stimulation induces tyrosine phosphorylation of a number of other intracellular proteins besides TCR subunits and ZAP-70 (13). The identity of many of these proteins is not known, and the functional consequences of the majority of the tyrosine phosphorylations are unclear. However, several phosphotyrosyl proteins have been identified which appear to play an important role in the signaling process. These include phospholipase C-γ1, ERK mitogen-activated protein kinases, and Vav (14). Tyrosine phosphorylation of phospholipase C-γ1 increases its catalytic activity, resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (14, 15). These second messengers, in turn, induce the mobilization of cytoplasmic calcium and the activation of protein kinase C, respectively. The phosphorylation and activation of the ERK by the TCR induces the phosphorylation of a number of transcription factors that are important in the induction of gene transcription (16). The role of Vav is unknown. However, gene targeting experiments have indicated that it is required for optimal signaling via the TCR (17–19). Sequence homology suggests that Vav is a GDP/GTP exchange protein for a small G-protein of the Rho/Rac subfamily (20), but its identity is presently unclear.

This laboratory has demonstrated previously that α-tubulin is constitutively tyrosine-phosphorylated in human T cells (21). In this study, it is shown that TCR stimulation of J urkat T cells induced the tyrosine phosphorylation of two proteins that co-precipitated with tubulin. These proteins were identified as ZAP-70 and Vav and suggest a role for the microtubule cytoskeleton in the signaling functions of these two proteins.

EXPERIMENTAL PROCEDURES

CD8 and Antibodies—J 6 cells were cultured in RPMI containing 3% FCS. COS-7 cells (from the European Collection of Animal Cell Cultures, Porton Down, UK) were grown in DMEM containing 10% FCS. CD3 antibody, OKT3, was obtained from the American Type Tissue Collection (Rockville, MD), and (Fab′)2 fragments kindly prepared by A. Tutt and M. Glennie (Tenovus, Southampton, UK). Other antibodies were 4G10, anti-Tyr(P) monoclonal antibody (from Brian Duker, Oregon Health Sciences University, Portland, OR), TAT-1 antibody, α-tubulin (from Keith Gull, University of Manchester, Manchester, UK), KMX antibody, β-tubulin (Keith Gull, University of Manchester), and monodonal anti-adcent (Amersham International). Rabbit anti-Vav antisemur, Vav-1, was raised against a synthetic peptide corresponding to residues 575–590 of human proto-Vav, and was used for immunoprecipitation of Vav. A monoclonal anti-Vav antibody was used for immunoblotting (Upstate Biotechnology, Inc., Lake Placid, New York). Anti-ZAP-70 antisemur, ZAP-4, was raised against a synthetic peptide corresponding to residues 271–290 of human ZAP-70. For immunoblotting, the ZAP-4 antibody was affinity-purified using the immunizing peptide immobilized on SulfoLink coupling gel (Pierce).

DNA Constructs—To generate a GST fusion protein of the Vav SH2 domain, the polymerase chain reaction was used to generate a cDNA fragment of base pairs 2377-2669 of human Vav cDNA (from Shulamit Katzav, Jewish General Hospital, Montreal). This product was then subcloned into the EcoRI site of the PGEX 4T-3 vector (Pharmacia).
while maintaining the correct reading frame. The fidelity and orientation of the polymerase chain reaction product was confirmed by DNA sequencing. GST and the GST-Vav SH2 fusion protein were purified from lysates of transfected Escherichia coli DH5α cells using glutathione-Sepharose (Pharmacia) following a standard Pharmacia protocol. The purity of these proteins exceeded 80%. Purified proteins were dialyzed against 50 mM glycerol, 50 mM Tris, pH 8.

ZAP-70 cDNA (from Arthur Weiss, Howard Hughes Medical Institute, San Francisco, CA) and vav cDNA were subcloned into the eukaryotic expression vector pcDNA3neo (Invitrogen) for COS-7 transfection experiments.

Immunoprecipitation and Western Blotting Analysis—Jurkat T cells were stimulated for 2 min with OKT3 antibody (+) or left unstimulated (−). Cell extracts were then immunoprecipitated with anti-Tyr(P) (pTyr) or anti-α-tubulin (α-Tub) antibodies. Immunoprecipitated proteins were Western blotted and probed sequentially for Tyr(P), Vav, and ZAP-70, as indicated on the right of the panels. Cell extracts from α-tubulin-resistant Jurkat T cells were immunoprecipitated with anti-Vav (Vav) or anti-ZAP-70 (ZAP) antisera. The immunizing peptides to which the ZAP-70 and Vav antisera were raised were added to half of the lysates (+P), to confirm the specificity of immunoprecipitation. Western blots were probed sequentially for α-tubulin, β-tubulin, ZAP-70, and Vav, as indicated. COS-7 cells were transfected with the pcDNA3 expression vector containing ZAP-70 cDNA (ZAP), Vav cDNA or no insert (pC3). α-Tubulin was immunoprecipitated from extracts of the transfected COS-7 cells, Western blotted, and probed sequentially for ZAP-70, Vav, or α-tubulin, as indicated on the left-hand side of the panels.

RESULTS AND DISCUSSION

Tyrosine phosphorylation of two proteins which co-precipitated with α-tubulin was strongly induced after CD3 stimulation in Jurkat T cells (Fig. 1a). These proteins had relative molecular masses of 70 and 100 kDa. On longer exposure, constitutive tyrosine-phosphorylated α-tubulin (a doublet with an approximate relative molecular mass of 55 KDa) was detected, as reported previously (21). In some experiments, a weak Tyr(P) band was also detected at 75 kDa. Immunoprecipitation with other anti-α-tubulin antibodies and an anti-β-tubulin antibody produced qualitatively similar results (data not shown). Comparison of anti-α tubulin immunoprecipitates with anti-Tyr(P) immunoprecipitates indicated that the interaction between tubulin and the two Tyr(P) proteins was highly selective (Fig. 1a).

A panel of antibodies was used to investigate by immunoblotting whether the tubulin-associated proteins corresponded to previously identified Tyr(P) proteins. This analysis indicated that both ZAP-70 (9) and Vav (22) co-precipitated with tubulin in Jurkat T cells (Fig. 1a) and also in human T lymphoblasts (data not shown). These proteins had identical mobilities to the 70- and 100-kDa tubulin-associated Tyr(P) proteins, respectively. Several other proteins, which are tyrosine-phosphorylated in activated T cells, including the z chain (23), Lck (24), HS-1 (25), CD5 (26), and cbl (27), were not detected in anti-α tubulin immunoprecipitates (data not shown). Syk (6) was also undetectable in anti-α tubulin immunoprecipitates from J urkat T cells. However, this was probably due to its low expression levels in these cells as Syk was the major tubulin-associ-
lin, COS-7 cells, which do not express endogenous ZAP-70 or Vav, were transiently transfected with plasmids containing ZAP-70 or Vav cDNA. Both ZAP-70 and Vav were detected in anti-α-tubulin immunoprecipitates (Fig. 1c). Co-transfection of ZAP-70 and Vav did not alter the level of interaction of these proteins with tubulin (data not shown). Thus the interactions of ZAP-70 and Vav with tubulin were independent and did not require other proteins which were hematopoietic cell-specific.

A 100-kDa Tyr(P) band, with the same mobility as Vav, co-immunoprecipitated with ZAP-70 in CD3-stimulated Jurkat T cells (Fig. 2a, top panel). Immunoblotting with anti-Vav antibody demonstrated that Vav was constitutively co-purified in anti-ZAP-70 immunoprecipitates and probably corresponded to the 100-kDa Tyr(P) protein (Fig. 2a, middle panel). In some experiments, it was also possible to detect very low levels of ZAP-70 specifically co-purifying in anti-ZAP-70 immunoprecipitates and probably corresponded to the 100-kDa Tyr(P) protein (Fig. 2a, middle panel). Although co-immunoprecipitation of Vav with ZAP-70 was constitutive, a GST fusion protein of the Vav-SH2 domain precipitated ZAP-70 only after TCR stimulation (Fig. 2b), as has been reported previously by Katayama et al. (28). Our data suggest that a constitutive interaction between Vav and ZAP-70 existed which was not mediated via the Vav SH2 domain. One possible explanation for this is that TCR stimulation induces a conformational change allowing the SH2 domain of Vav, which is already complexed with ZAP-70, to bind to its tyrosine-phosphorylated target sequence on ZAP-70. However, it cannot be excluded that a small fraction of total Vav may be induced to associate with ZAP-70 after TCR stimulation via its SH2 domain, but this increase may be below the detection limit of the assay. The possibility that tubulin mediated the interaction between Vav and ZAP-70 is considered below.

In contrast to the present study, Katayama et al. (28) found that the interaction between ZAP-70 and Vav was induced by CD3 stimulation using an anti-Vav antibody for immunoprecipitation. This may reflect the use of an anti-Vav antibody which cannot recognize Vav that is constitutively associated with ZAP-70. Katayama et al. (28) also did not detect Vav in immunoprecipitates using the 1222 anti-ZAP-70 antibody. A direct comparison of 1222 anti-ZAP-70 antibody with ZAP-4 anti-ZAP-70 antibody, which is used in the present study, has demonstrated that only ZAP-4 antibody co-immunoprecipitated Vav (data not shown). These results suggest that there are qualitative differences between the two anti-ZAP-70 antibodies, such that Vav associated with ZAP-70 is only immunoprecipitated by ZAP-4 antibody and not by 1222 antibody in detectable quantities.

Microtubule-associated proteins can be operationally defined as proteins which co-purify with tubulin polymerized in vitro (29). The presence of ZAP-70 and Vav in anti-α-tubulin immunoprecipitates suggested that these proteins might also co-purify with polymerized tubulin. To test this hypothesis, taxol and GTP were added to Jurkat extracts to promote in vitro tubulin polymerization (30). Taxol/GTP treatment removed over 80–90% of tubulin from cytosolic extracts, and large amounts of tubulin were detected in the pellet, as expected (Fig. 3a). The pellet fraction contained only trace amounts of actin, and actin was not depleted from the cytosol by taxol/GTP treatment, confirming the specificity of this method for polymerization of tubulin (Fig. 3a). Immunoblotting demonstrated that 45% of Vav and 60% of ZAP-70 were depleted from cytosolic extracts by taxol/GTP treatment, and both of these molecules were detected in the pellet fraction (Fig. 3a). Two other cytosolic Tyr(P) proteins, HS-1 and cbl, were not depleted from the cytosol or detected in the pellet fraction after taxol/GTP treatment (data not shown), suggesting that the co-purification of ZAP-70 and Vav with polymerized tubulin was specific. The δ chain, Lck, and CD5 were not present in the cytosolic fraction, as they are membrane-associated. These data indicated that both ZAP-70 and Vav could interact with polymerized tubulin in vitro. Extraction of Jurkat T cells with PM2G buffer, which maintains the microtubule cytoskeleton intact (21, 31), indicated that both ZAP-70 and Vav were associated with soluble and polymerized tubulin in vivo (data not shown).
ZAP-70, Vav, and Tubulin Interactions

Similar amounts of ZAP-70 were immunoprecipitated from the control and taxol-treated cytotoxic extracts under conditions where ZAP-70 antibody was limiting (Fig. 3b). Under these conditions, taxol/GTP reduced the relative abundance of tyrosine-phosphorylated ZAP-70 in the cytoplasm by 70% (±5%; n = 4), as judged by anti-Tyr(P) immunoblotting (Fig. 3b). As expected, a tyrosine-phosphorylated band with identical mobility to ZAP-70 preferentially accumulated in the microtubule pellet from activated cell extracts (data not shown). These data indicate that ZAP-70 that was tyrosine-phosphorylated after TCR stimulation was preferentially associated with tubulin polymerized in vitro. This further suggests that the interaction of ZAP-70 with tubulin may be important for the activation of this kinase. In contrast, cytotoxic tubulin depletion had little effect on the amount of tyrosine-phosphorylated Vav (Fig. 3b) detected in anti-Vav immunoprecipitates (reduction of 13% ± 5% S.E.; n = 4).

The association of both ZAP-70 and Vav with tubulin (Fig. 2a) suggested that the presence of Vav in ZAP-70 immunoprecipitates might be mediated via tubulin. However, taxol/GTP depletion of the majority of cytotoxic tubulin only slightly reduced the level of Vav in ZAP-70 immunoprecipitates (Fig. 3c). These data indicated that Vav interaction with ZAP-70 was largely independent of tubulin.

The interactions of ZAP-70 and Vav with components of the microtubule cytoskeleton and with each other raised the possibility that these proteins might be functionally interlinked in TCR signal transduction. As Vav is tyrosine-phosphorylated after TCR stimulation (32, 33), this suggested that Vav might be a downstream substrate of ZAP-70 PTK. To investigate this, the kinetics of phosphorylation of ZAP-70 and Vav were analyzed after CD3 stimulation in J urkat T cells. Tyrosine phosphorylation of Vav was extremely rapid, reaching a maximum at 0.5 to 1 min and then falling to baseline levels by 10 min (Fig. 4). In contrast the tyrosine phosphorylation of ZAP-70 was slower, peaking at 5–10 min and falling to baseline levels by 30 min. The kinetics of tyrosine phosphorylation of ZAP-70 and Vav that co-immunoprecipitated with α-tubulin were very similar to that detected in anti-ZAP-70 and anti-Vav immunoprecipitates (Fig. 4, bottom panel). ZAP-70 PTK is activated by tyrosine phosphorylation (10). These data (Fig. 4) suggested that Vav was tyrosine-phosphorylated before ZAP-70 was activated and, therefore, that Vav was unlikely to be a downstream target of ZAP-70.

In conclusion, these data demonstrate that ZAP-70 and Vav interact with tubulin and each other in T lymphocytes. A functional link, therefore, is likely to exist between these signaling molecules and the tubulin cytoskeleton. The possibility that the intracellular targeting of ZAP-70 and Vav is dependent on interactions with tubulin is currently being investigated.

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REFERENCES

1. Chan, A., Desai, D. M., and Weiss, A. (1994) Annu. Rev. Immunol. 12, 555–592
2. Strauss, D. B., and Weiss, A. (1992) Cell 70, 585–593
3. Iwashima, M., Irving, B. A., Van Oers, N. S. C., Chan, A. C., and Weiss, A. (1994) Science 263, 1136–1139
4. Perlmuter, R. M., Levin, S. D., Appleby, M. W., Anderson, S. J., and Alberding-II, J. (1993) Annu. Rev. Immunol. 11, 451–499
5. Ley, S. C., Marsh, M., Bebbington, C. R., Proudfoot, K., and Jordan, P. (1994) J. Cell Biol. 125, 639–649
6. Chan, A. C., Van Oers, N. S. C., Tran, A., Turka, L. L., Law, C. L., Ryan, J. C., Clark, E. A., and Weiss, A. (1994) J. Immunol. 152, 4758–4766
7. Strauss, D. B., and Weiss, A. (1993) J. Exp. Med. 178, 1523–3150
8. Wange, R. L., Malek, S. N., Desiderio, S., and Samelson, L. E. (1993) J. Biol. Chem. 268, 19707–19805
9. Chan, A. C., Iwashima, M., Turck, C. W., and Weiss, A. (1992) Cell 71, 649–662
10. Chan, A. C., Dalton, M., Johnson, R., Kong, G.-H., Wang, T., Thoma, R., and Kuroskii, T. (1995) EMBO J. 14, 2499–2508
11. Negishi, I., Motoyama, N., Nakayama, K.-i., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A. C., and Loh, D. Y. (1995) Nature 376, 435–438
12. Hivroz, C., and Fischer, A. (1994) Curr. Biol. 4, 731–733
13. Ley, S. C., Davies, A. A., Druker, B., and Crompton, M. J. (1991) Eur. J. Immunol. 21, 2203–2209
14. Weiss, A., and Littman, D. R. (1994) Cell 76, 263–274
15. Rhee, S. G. (1991) Trends Biochem. Sci. 16, 297–301
16. Izquierdo Pastor, M., Reif, K., and Cantrell, D. (1995) Immunol. Today 16, 159–164
17. Fischer, K.-D., Zmuldzinas, A., Gardner, S., Barbadic, M., Bernstein, A., and Guides, C. (1995) Nature 374, 474–477
18. Tarakhovsky, A., Turner, M., Schiavinato, E., Mee, P. J., Duddy, L., Rajewsky, K., and Tybulewicz, V. (1995) Nature 374, 467–470
19. Zhang, R., Alt, W., Davidson, L., Orkin, S. H., and Swat, W. (1995) Nature 374, 470–473
20. Adams, J. M., Houston, H., Allen, J., Lints, T., and Harvey, R. (1992) Oncogene 7, 611–618
21. Ley, S. C., Verbi, W., Pappin, D. J., Druker, B., Davies, A. A., and Crompton, M. J. (1994) Eur. J. Immunol. 24, 99–106
22. Katzev, S., Martinez-Zanca, D., and Barbadic, M. (1989) EMBO J. 8, 2283–2290
23. Banisashvili, M., Garcia-morales, P., Luong, E., Samelson, L. E., and Klausner, R. D. (1988) J. Cell Biol. 106, 16225–16230
24. Danielian, S., Fagard, R., Alcover, A., Acuto, O., and Fischer, S. (1989) Eur. J. Immunol. 19, 2183–2189
25. Yamamishi, Y., Okada, M., Semba, T., Yamori, T., Umemori, H., Tsunasawa, S., Toyoshima, K., Kitamura, D., Watanabe, T., and Yamamoto, T. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3631–3635
26. Davies, A. A., Ley, S. C., and Crompton, M. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6368–6372
27. Donovan, J. A., Wange, R. L., Langdon, W. Y., and Samelson, L. E. (1994) J. Biol. Chem. 269, 22921–22924
28. Katzev, S., Sutherland, M., Packham, G., Yi, T., and Weiss, A. (1994) J. Biol. Chem. 269, 32579–32585
29. Olmedo, J. B. (1986) Annu. Rev. Cell Biol. 2, 421–457
30. Valle, R. B., and Collins, C. A. (1986) Methods Enzymol. 134, 116–127
31. Solomon, F. (1986) Methods Enzymol. 134, 139–147
32. Bustelo, X. R., Ledbetter, J. A., and Barbadic, M. (1992) Nature 356, 68–71
33. Marchis, B., Hu, P., Katzav, S., Li, W., Oliver, J. M., Ullrich, A., Weiss, A., and Schlessinger, J. (1992) Nature 356, 71–74
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