A 68-kD GTP-binding Protein Associated with the T Cell Receptor Complex

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Summary

The identity of the guanine nucleotide-binding protein (G protein) involved in T cell activation pathways remains unclear. We identified a 68-kD GTP-binding protein associated with the T cell receptor (TCR)/CD3 complex using immunoprecipitation and GTP-affinity labeling techniques. Proteins coimmunoprecipitated with the TCR/CD3 complex in digitonin lysate of a human leukemic T cell line, MOLT 16, were incubated with α-[^{32}P]GTP and irradiated with ultraviolet rays to covalently link the labeled GTP to GTP-binding proteins. They were then analyzed by electrophoresis. The 68-kD protein exhibited nucleotide specificity for GTP-binding and was insensitive to cholera and pertussis toxins. The 68-kD GTP-binding protein could be coimmunoprecipitated with the TCR/CD3 complex but not with other surface molecules such as major histocompatibility complex class I and lymphocyte function associated-1, which do not cause rapid Ca^{2+} mobilization. These suggest that the 68-kD GTP-binding protein is specifically associated with the TCR/CD3 complex.

Triggering the TCR/CD3 complex induces phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis resulting in the activation of protein kinase C and the release of Ca^{2+} from intracellular store sites (1). It has been supposed that G proteins were involved in the TCR-mediated signal transduction because G protein stimulators, guanosine 5′-(3-O-thio) triphosphate (GTPγS), and fluoroaluminate (AlF_4^-), induced PIP_2 hydrolysis in permeabilized human T lymphocytes (2, 3) and because cholera toxin (CT), a well-known modulator of the Gs-α subunit, inhibited T cell proliferation induced by PHA stimulation (4). However, in light of the recent report that growth inhibition by CT was due to the loss of the TCR expressed on the T cell surface and not to the modulation of CT-sensitive G proteins (5), it remains unknown which G protein(s) participates in T cell activation pathways. Here, we demonstrate the association of a previously uncharacterized GTP-binding protein with the TCR/CD3 complex in human T cells.

Materials and Methods

Antibodies and Coupling mAb to Beads. CD3 mAb OKT3, CD11a mAb TS1/22.1.1.13, CD58 mAb TS2/9.1.4.3, and HLA-A,B,C mAb W6/32, were purchased from American Type Culture Collection (Rockville, MD) and purified with a protein A column. For coupling mAb to the beads, antibody (1.2 mg) was dissolved in 2 ml of a coupling buffer, pH 8.5, containing 0.2 M $NaHCO_3$ and 0.5 M NaCl, and added to 0.2 g of AF-Tresyl Toyopearl 650M (Tosoh Co., Tokyo, Japan). After the coupling was allowed to proceed for 10 h at 20°C, the gel was treated with 0.2 M Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl for 4 h at 20°C. The coupling yield was ~80% (i.e., 1 mg/ml wet gel).

Membrane Preparation, Cell Lysis, and Immunoprecipitation. Membranes of MOLT 16 cells, a human leukemic T cell line (6) generously provided by Dr. Jun Minowada (Hayashibara Biochemical Laboratories, Okayama, Japan), were prepared as described previously (7). The yield of membrane protein was ~1 mg/5 × 10^7 cells. Cells or membranes were lysed at 5 × 10^7 cells/ml or 1 mg/ml, respectively, in lysis buffer composed of 1% digitonin, 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4, 2 mM PMSF, 10 mM iodoacetamide, 5 μg/ml pepstatin A, and 5 μg/ml leupeptin at 4°C for 60 min. After centrifugation at 12,000 *g* for 15 min, lysates were precleared with mouse IgG-coupled beads at 4°C for 2 h. Immunoprecipitation was performed as described previously (8) by using antibody-coupled Toyopearl beads.

Photoaffinity Labeling of GTP-binding Proteins in Immunoprecipitate. α-[^{32}P]GTP-affinity labeling of GTP-binding proteins by UV irradiation was performed essentially as previously described (7). Briefly, immunoprecipitated beads (10–30 μl) were incubated in 50 μl of GTP exchange buffer containing 25 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.1 M NaCl, and 20 mM Tris-HCl, pH 8.0, with 1 MBq α-[^{32}P]GTP (29.6 TBq/mmol; New England Nuclear, Boston, MA) at 30°C for 90 min, and then irradiated on ice with an HP-115C UV lamp (254 nm, 230 W; ATTO Corp., Tokyo, Japan) at a distance of 5 cm for 15 min. Then the beads were washed twice with 20 mM Tris-HCl, pH 7.4, to remove free radioactivity. The proteins were eluted from the beads by incubating at 65°C for 10 min in 50 μl of Laemmli's reducing sample buffer, and analyzed by SDS/12% PAGE. Protein bands were visualized by autoradiography using Kodak X-Omat AR film (Eastman Kodak Co., Rochester, NY) with an exposure time of 3 d at −70°C.

ADP Ribosylation of Membrane and Immunoprecipitated Pro-

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teins. Membrane (100 μg) or immunoprecipitated (30 μl of beads) proteins were ADP ribosylated with 370 kBq [adenylate-^{32}P]NAD (29.6 TBq/mmol; New England Nuclear) and 20 μg/ml preactivated CT or pertussis toxin (PT) (List Biological Laboratories, Campbell, CA) as described previously (9) in 50 μl of reaction buffer, pH 7.4, containing 0.1 M sodium phosphate, 2.5 mM $MgCl_2$, 1 mM EDTA, 10 mM thymidine, 0.5 mM ATP, and 100 μM GTP at 37°C for 60 min.

Measurement of Intracellular Ca^{2+}. The measurement of cytoplasmic free calcium levels was performed with Fura-2 AM (Dojindo Laboratories, Kumamoto, Japan) as described previously (10). Briefly, Fura-2 AM-loaded MOLT 16 cells (2 × 10^6) were suspended in loading buffer, and mAbs (2 μg) to the surface molecules were added. Then, rabbit anti-mouse IgG Ab (8 μg) was added at the indicated time to crosslink the mAb. Fluorescence of the cell suspension was monitored with a Fluorescence Spectrophotometer F-3000 (Hitachi Ltd., Tokyo, Japan) at wavelengths of 340 nm for excitation, and 510 nm for emission with 10-nm slit widths for both.

^{35}S-internal and ^{125}I-surface Labeling. For internal labeling, the cells (10^7) were incubated in 0.5 ml of methionine-free RPMI 1640 medium (Cosmo Bio Co. Ltd., Tokyo, Japan) supplemented with 10% dialyzed FCS at 37°C for 30 min and then cultured with 3.7 MBq Tran^{35}S-label (37 TBq/mmol; ICN, Irvine, CA) for 2.5 h. After washing twice with PBS, the cells were lysed and the lysate was analyzed by SDS-PAGE. Fluorography was performed as described previously (11). ^{125}I surface labeling with Na^{125}I (3.7 GBq/ml; New England Nuclear) was performed by using Iodobeads (Pierce Chemical Co., Rockford, IL) according to the manufacturer's manual.

Results and Discussion

To search for GTP-binding proteins associated with the TCR/CD3 complex, we applied the techniques of immunoprecipitation and GTP photoaffinity labeling. Briefly, MOLT 16 cells were lysed in buffer containing 1% digitonin, and the TCR/CD3 complex was immunoprecipitated with an mAb against CD3, OKT3, coupled on beads. The proteins coimmunoprecipitated were incubated with α-[^{32}P]GTP, irradiated with UV to covalently link the labeled GTP to GTP-binding proteins (7), and then analyzed by SDS/12% PAGE. There were several proteins coimmunoprecipitated with OKT3 in the lysate of MOLT 16 cells labeled with [^{35}S]methionine in addition to those corresponding to TCR-α and β and to CD3 γ, δ, ε, and ζ chains, as shown in Fig. 1 *A*. Fig. 1 *B* shows GTP-binding proteins in membrane and OKT3-immunoprecipitated fractions. The membrane fraction contained several GTP-binding proteins including 68, 66, 45, and 40 kD, and smaller mass proteins which competed with cold GTP for α-[^{32}P]GTP-binding. In OKT3-immunoprecipitated fraction, a 68-kD GTP-binding protein was found. The 68-kD protein was also coimmunoprecipitated with the TCR/CD3 complex in the lysate of the membrane treated with α-[^{32}P]GTP (data not shown). No change in the size of the 68-kD GTP-binding protein was observed in SDS-PAGE under reducing and nonreducing conditions.

Since there are many nucleotide-binding proteins in the membranes and cytosols (12), the nucleotide specificity of this protein was examined. Fig. 2 shows the GTP-binding specificity of the 68-kD protein. ATP and UTP did not compete for α-[^{32}P]GTP, whereas cold GTP competed efficiently. These results indicated that the TCR/CD3 complex was associated with the 68-kD GTP-binding protein.

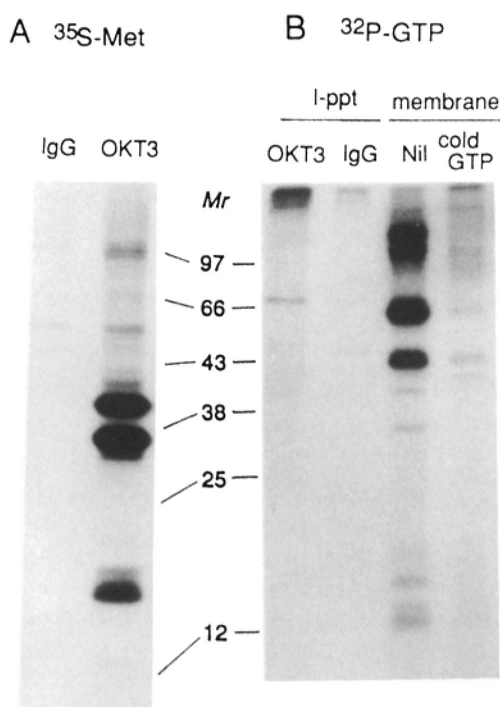

Figure 1. Proteins coimmunoprecipitated with the TCR/CD3 complex and GTP-binding proteins photoaffinity labeled with α-[^{32}P]GTP. (*A*) MOLT 16 cells (10^7) were incubated in methionine-free RPMI 1640 medium with [^{35}S]methionine for 2.5 h and then lysed in 1% digitonin solution. Precleared lysates were subjected to immunoprecipitation with mouse IgG or OKT3, and proteins in the immunoprecipitates were analyzed by SDS/12% PAGE under reducing conditions. (*B*) Proteins immunoprecipitated (*I-ppt*) with OKT3 (*OKT3*) or mouse IgG (*IgG*), and whole membrane proteins were treated with α-[^{32}P]GTP in the absence (*Nil*) or presence of 5-μM unlabeled GTP (*cold GTP*), and irradiated with UV as described in Materials and Methods. α-[^{32}P]GTP affinity-labeled proteins were analyzed by SDS/12% PAGE and autoradiography.

To characterize the 68-kD GTP-binding protein, the sensitivity for CT and PT was tested. CT and PT are well known ADP-ribosylation toxins and modify the function of Gs and Gi, respectively (13, 14). Fig. 3 shows the ADP ribosylation of proteins in membrane and OKT3-immunoprecipitated fractions treated with CT or PT. A 45- (faint) and a 40-kD ADP-ribosylated protein were seen in membrane fraction treated with CT and PT, respectively. The 38-kD band which appeared after treatment with either CT or PT was probably a proteolytic product of the Gi-α subunit as reported by Iiri et al. (9). A 40-kD ADP-ribosylated protein was observed with PT-treated OKT3 immunoprecipitate. The ADP-ribosylated Ig H chain appeared as a 50-kD band in both the CT- and PT-treated immunoprecipitates. It was noted that no ADP ribosylation of the 68-kD protein was observed in either the membrane or the OKT3-immunoprecipitated fraction. This is consistent with the result of a previous report that PIP_2 hydrolysis induced by triggering the TCR was insensitive for CT and PT (5).

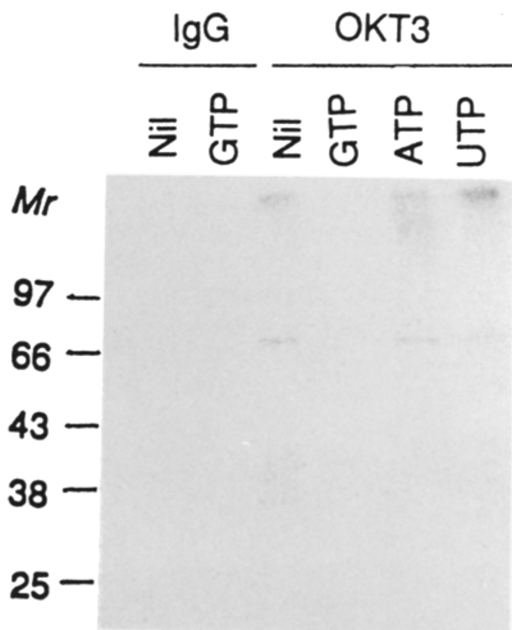

Figure 2. Specificity in nucleotide-binding of the proteins coimmunoprecipitated with the TCR/CD3 complex. MOLT 16 cells were lysed in 1% digitonin solution, and the lysates were subjected to immunoprecipitation with mouse IgG or OKT3. Immunoprecipitates were incubated with α-[^{32}P]GTP in the absence (*Nil*) or presence of 5-μM unlabeled GTP, ATP, or UTP, and proteins in immunoprecipitates were analyzed by SDS/12% PAGE.

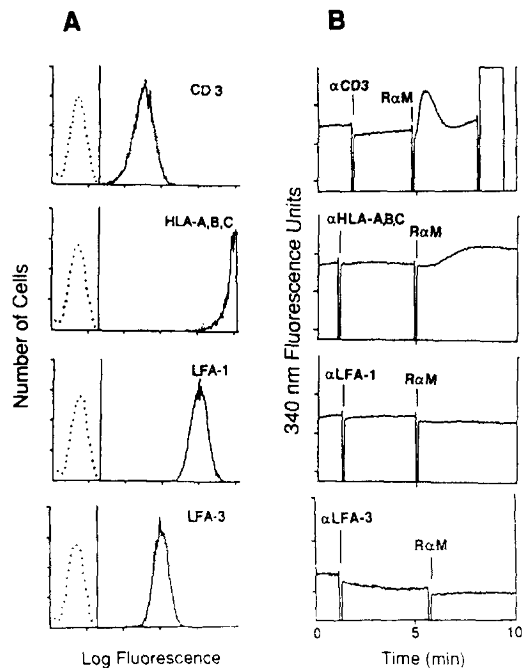

Figure 4. Expression of surface molecules on MOLT 16 cells and Ca^{2+} mobilization induced by crosslinking of the surface molecules. (*A*) MOLT 16 cells (5 × 10^5) were incubated with mouse IgG (*dashed line*) or mAbs against the indicated surface molecules (*solid line*) and stained with FITC-conjugated sheep anti–mouse IgG antibody. Flow cytometric analyses were performed by Epics profile (Coulter Corp., Hialeah, FL). (*B*) Cytoplasmic free calcium levels were measured with Fura-2 AM. We suspended Fura-2 AM-loaded MOLT 16 cells (2 × 10^6) in loading buffer and added mAb (2 μg) and then rabbit anti–mouse IgG antibody (RαM) (8 μg) to crosslink the mAb at the indicated times. Fluorescence of the cells was monitored at a wavelength of 340 nm as described in Materials and Methods. αCD3, αHLA-A,B,C αLFA-1, and αLFA-3 indicate OKT3, W6/32, TS1/22.1.1.13, and TS2/9.1.4 mAbs, respectively.

We next examined the association of GTP-binding proteins with other surface molecules related to T cell activation. MOLT 16 cells expressed CD3, MHC class I, LFA-1 (CD11a), and LFA-3 (CD58) molecules on their surface (Fig. 4 *A*). The ability of these surface molecules to mediate signaling for intracellular Ca^{2+} mobilization is shown in Fig. 4 *B*. The crosslinking of the TCR/CD3 complex caused biphasic Ca^{2+} mobilization, but MHC class I caused only the late phase mobilization. No increase was induced by the crosslinking of LFA-1 and LFA-3 molecules. We therefore examined the association of GTP-binding proteins with CD3, MHC class I, and LFA-1, which differently transduce Ca^{2+} signaling. As shown in Fig. 5 *A*, no definite GTP-binding protein was coimmunoprecipitated with MHC class I and LFA-1 molecules, although the immunoprecipitation of MHC class I α chain (45 kD), β_2 microglobulin (12 kD), and LFA-1 α (180 kD) and β (95 kD) chains was confirmed in each immunoprecipitated fraction from ^{125}I surface-labeled MOLT 16 cells (Fig. 5 *B*). These results suggested that the 68-kD GTP-binding protein was specifically associated with the TCR/CD3 complex which could mediate an early phase Ca^{2+} mobilization. The 40-kD GTP-binding protein observed in Fig. 5 *A* may be identified with that ADP ribosylated with PT (Fig. 3), although this needs to be confirmed. These 68- and 40-kD GTP-binding proteins were also coimmunoprecipitated efficiently with the TCR/CD3 complex in 1% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) lysate of MOLT 16 cells (data not shown).

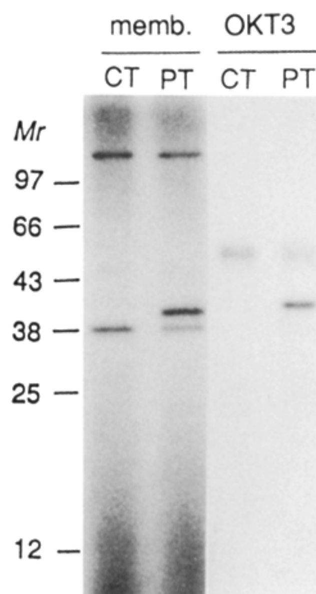

Figure 3. ADP ribosylation of membrane and OKT3-immunoprecipitated proteins with cholera and pertussis toxins. Membranes and OKT3 immunoprecipitates prepared from MOLT 16 cells were treated with preactivated cholera toxin (*CT*) or pertussis toxin (*PT*) in the reaction buffer containing [adenylate-^{32}P]NAD as described in Materials and Methods. ADP-ribosylated proteins were visualized by autoradiography.

We thus found the TCR-associated 68-kD GTP-binding

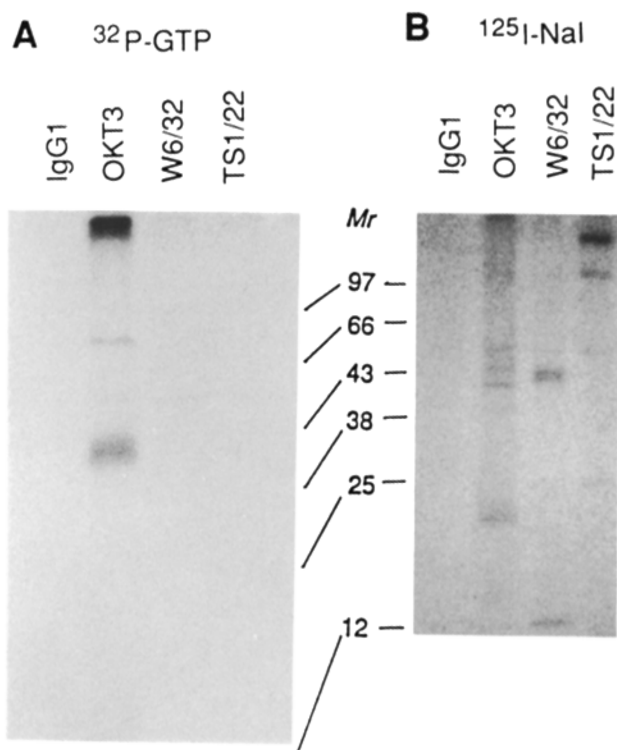

Figure 5. GTP-binding proteins coimmunoprecipitated with surface molecules. (*A*) Preparation of MOLT 16 cell lysate, immunoprecipitation, and α-[^{32}P]GTP affinity labeling were performed as described in Materials and Methods using indicated mAbs. (*B*) ^{125}I surface labeling was performed by using Iodo-beads (Pierce Chemical Co.) with [^{125}I]NaI according to the manufacturer's manual. Immunoprecipitated proteins with each mAb in lysates from ^{125}I-labeled MOLT 16 cells were analyzed by SDS/12% PAGE and autoradiography. W6/32 and TS1/22 are anti-HLA-A,B,C and anti-LFA-1 mAbs, respectively.

protein (termed TAGp68) in the human T cell line, MOLT 16. TAGp68 was also detected in peripheral blood T lymphocytes (data not shown). It has been reported that a 74-kD GTP-binding protein was associated with the α1-adrenergic receptor in rat liver membrane and was supposed to couple to phospholipase C (PLC) (15). Pessa-Morikawa et al. (7) reported that a 68-kD GTP-binding protein was weakly expressed in human thymocytes and increased in peripheral mature T lymphocytes. Our TAGp68 could be the same as those reported as above. Recently, a 70-kD protein was demonstrated to associate with the CD3 ζ chain, and its phosphorylation and association with the CD3 ζ chain were enhanced by TCR stimulation (16). TAGp68 may be distinct from the 70-kD protein because our preliminary data indicated that TAGp68 was partially dissociated from the TCR/CD3 complex by PHA stimulation.

We further demonstrated that TAGp68 was specifically associated with the TCR/CD3 complex which mediates an early phase Ca^{2+} mobilization. There are several lines of evidence that GTP-binding proteins contribute to signal transduction including Ca^{2+} mobilization. In addition to Gi, Gs, and $p21^{ras}$ (12), Gq and G11 have been reported to activate PLC-β1 (17). The role of TAGp68 in TCR signaling remains to be clarified.

Another GTP-binding protein of 40 kD associated with the TCR/CD3 complex which was ADP ribosylated with PT is probably a Gi-α subunit (18). Sommermeyer et al. (5) reported that PT did not inhibit PIP_2 hydrolysis in T cell membrane fraction by TCR stimulation. This PT-sensitive 40-kD protein, however, could be involved in TCR signaling indirectly because Gi decreases intracellular cAMP which inhibits PLC activity (19).

It was reported that a protein tyrosine kinase (PTK) $p59^{fyn}$ was associated with the TCR/CD3 complex (20), and cross-linking of the TCR induced tyrosine phosphorylation of PLC-γ1 (21). These support an involvement of the tyrosine-serine/threonine kinase network in T cell activation pathways. That is, the PTKs $p59^{fyn}$ and $p56^{lck}$ are activated by triggering the TCR and phosphorylate PLC, leading to the initiation of PIP_2 hydrolysis which results in the activation of protein kinase C, a serine/threonine kinase (22). Considering that the PTK inhibitors genistein and herbimycin A prevent the hydrolysis of PIP_2 induced by the TCR stimulation (23, 24), and that G protein stimulators GTPγS and AlF_4^- induce tyrosine phosphorylation in T and B lymphocytes (3, 25), GTP-binding proteins and PTKs seem to crosstalk in TCR-mediated signal transduction.

In this study, we provide direct evidence for the association of a 68-kD GTP-binding protein to the TCR/CD3 complex. Further investigation is necessary to identify target proteins and to make clear the relationship between the 68-kD GTP-binding protein and tyrosine kinases in T cell activation pathways.

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