Ligand-induced association between the T-cell antigen receptor and two glycoproteins

(alkylation/conformational change/signal transduction mutants)

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ABSTRACT We have identified two cell surface glycoproteins of 34 and 38 kDa (gp34 and gp38) that associate with the T-cell antigen receptor (TCR). The coimmunoprecipitation of these proteins with the TCR is increased by treatment with monoclonal antibodies (mAbs) directed against the TCR prior to cell solubilization and immunoprecipitation. Treatment of T cells with mAbs directed against other cell surface molecules, CD2 or HLA, does not induce the association of these proteins with the TCR. The coimmunoprecipitation of gp34 and gp38 with the TCR requires solubilization in the presence of an alkylating agent, suggesting that subunit alkylation stabilizes the interaction. J.CaM1 and J.CaM2 are signal-transduction mutant cell lines derived from Jurkat. These cell lines fail to activate the inositol phospholipid second messenger pathway in response to anti-TCR mAbs. Treatment with mAb C305 (anti-TCR) induces the association of gp34 and gp38 with the TCR in J.CaM2 cells but not in J.CaM1. J.CaM1 modulates the TCR normally in response to anti-TCR antibody treatment. This observation suggests that gp34 and gp38 are involved in the signal-transduction pathway of the TCR complex rather than receptor internalization. Furthermore, since these proteins do associate with the TCR of J.CaM2, the induced association with the TCR is not a consequence of signal transduction.

The mechanism by which cell surface receptors communicate their ligand occupancy across the plasma membrane is largely unknown. The T-cell antigen receptor complex (TCR) contains a ligand-binding subunit, the immunoglobulin-like heterodimer (Ti), which has specificity for antigen bound to major histocompatibility complex molecules (1). Ti is non-covalently associated with a complex, CD3, comprised of at least five invariant proteins that are thought to be involved in signal transduction (2). Binding of the TCR to ligand, either foreign antigen and major histocompatibility complex or certain antibodies directed against the TCR, results in the activation of a tyrosine kinase and in phosphatidylinositol 4,5-bisphosphate hydrolysis (3, 4). Hydrolysis of phosphatidylinositol 4,5-bisphosphate results in increases in cytoplasmic free Ca²⁺ and activation of protein kinase C (5, 6), events that are thought to contribute to later T-cell activation (2). The mechanisms by which the TCR functionally interacts with intracellular signaling pathways are not known.

In numerous biological systems, cell surface receptors are linked to second messenger production through transient interactions with coupling molecules. In one model system, the functional association between the ligand-occupied β_2 -adrenergic receptor and a guanine nucleotide binding protein could be stably preserved by treatment with ligand in the presence of an alkylating agent (7–9). The TCR is thought to functionally interact with a number of other T-cell surface

molecules including CD2, CD4, CD8, Ly6, and Thy-1 (10–14). However, the structural basis for these functional interactions is unknown. Here we report the identification of two cell surface glycoproteins of 34 and 38 kDa (gp34 and gp38) whose association with the TCR is maintained by solubilization in the presence of alkylating agents. The association of gp34 and gp38 with the TCR is substantially augmented by TCR ligand binding. The functional significance of this ligand-induced association is suggested by the failure of these proteins to associate with the receptor in a mutant cell line with defective TCR-mediated signal transduction.

METHODS

Cell Lines. The human leukemic T-cell lines Jurkat, HPB-ALL, and HuT 78 were maintained as described (15). Lines J.CaM1 and J.CaM2 were isolated and maintained as described (16, 17).

Monoclonal Antibodies (mAbs). mAbs C305 and R140 recognize antigenic determinants expressed on the Jurkat Ti (18, 19). mAb WT31 recognizes an invariant determinant on the human TCR (20). mAb anti-Leu4 recognizes CD3 (Becton Dickinson). mAbs L41 and L42 recognize determinants expressed on HPB-ALL Ti (21). mAb W6/32 recognizes an invariant determinant expressed on human HLA class I antigens (22). mAbs 9-1 and 9.6 recognize human CD2 (23, 24).

Immunoprecipitations. Cells (2×10^7 cells per 0.5 ml of isotonic phosphate-buffered saline) were radioiodinated with 1 mCi (1 Ci = 37 GBq) of ¹²⁵I (Amersham) as described (18). The iodinated cells were washed three times with phosphate-buffered saline (4°C) and resuspended in RPMI 1640 medium (37°C) containing the indicated mAb for 10 min. The cells were pelleted by centrifugation and solubilized in lysis buffer consisting of 1% Nonidet P-40, 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, aprotinin (18 μ g/ml), 0.01% sodium azide, and 4 mM N-ethylmaleimide (NEM) unless otherwise indicated (at 10^7 cells per 0.1 ml). NEM, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and iodoacetamide were purchased from Sigma. After 30 min of incubation at 4°C, large debris was removed by centrifugation. Lysates were precleared with formalin-fixed *Staphylococcus aureus* (Calbiochem-Behring) and centrifuged at $100,000 \times g$ for 30 min. Cell surface molecules were immunoprecipitated using mAbs coupled to Affi-Gel beads (Bio-Rad). Isolated proteins were analyzed by electrophoresis on one-dimensional SDS/11% polyacrylamide gels and autoradiography. The N-Glycanase (Genzyme) digestion of the excised bands was performed as described (25).

RESULTS

To identify molecules that interact with the TCR upon ligand binding, immunoprecipitations of the TCR were performed in the presence of an alkylating agent after treatment with agonist mAbs directed against the Ti heterodimer of the human T-cell leukemic line, Jurkat. Jurkat cells were radioiodinated and then incubated with medium or with an agonist anti-Ti mAb, C305, for 10 min at 37°C. The cells were then solubilized in a 1% Nonidet P-40 lysis buffer containing the alkylating agent NEM and immunoprecipitation of the TCR complex was performed using the anti-CD3 mAb anti-Leu4. SDS/PAGE analysis of nonreduced immunoprecipitates revealed the 85- to 90-kDa Ti heterodimer, a 28-kDa CD3 γ chain, and a broad band of 22–24 kDa that consists of CD3 δ and ϵ chains (Fig. 1A) (26). Upon reduction, the Ti heterodimer dissociated into the individual α and β subunits and CD3 δ and ϵ chains became distinguishable (Fig. 1B). However, if the Jurkat cells were preincubated in the presence of mAb C305 prior to cell lysis, two additional molecules of 34 and 38 kDa were immunoprecipitated along with the TCR (Fig. 1A, lane 4). These molecules are not disulfide-linked, as reduction prior to SDS/PAGE had no effect on their apparent molecular mass (Fig. 1B, lane 4). In some cases, on reexamination of autoradiographs, faint 34- and 38-kDa bands could variably be detected in immunoprecipitates from untreated cells but the anti-Ti mAb treatment increased the amount of the 34- and 38-kDa bands that were coimmunoprecipitated with the TCR by 5- to 12-fold, as determined by densitometric analysis. Thus, treatment of Jurkat cells with an anti-Ti appears to increase or induce the association of these 34- and 38-kDa molecules with the TCR.

We examined whether anti-TCR antibodies can induce the coimmunoprecipitation of the 34- and 38-kDa molecules with

FIG. 1. Treatment of Jurkat cells with mAb C305 (anti-Ti of Jurkat) induces the coimmunoprecipitation of the TCR complex with two additional proteins of 34 and 38 kDa. Surface radioiodinated Jurkat cells were incubated in serum-free RPMI 1640 medium at 37°C for 10 min in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of mAb C305 (anti-Ti of Jurkat, 1:100 dilution of ascites fluid). The cells were solubilized and immunoprecipitation was performed with either mouse IgG (lanes 1 and 3) or anti-Leu4 (anti-CD3, lanes 2 and 4). Immunoprecipitates were analyzed by SDS/PAGE under nonreducing (A) or reducing (B) conditions. The relative mobilities of molecular mass standards in kDa are indicated.

the TCR in other human T-cell lines. Preincubation of HuT 78 cells with mAb WT31, which reacts with the TCR, prior to solubilization and anti-CD3 immunoprecipitation was found to induce the association of 34- and 38-kDa molecules with the TCR (Fig. 2, lane 4). Treatment of the leukemic line HPB-ALL with the agonist anti-Ti mAb L41 was also found to induce the coimmunoprecipitation of 34- and 38-kDa molecules with the TCR whereas treatment with the nonagonist anti-Ti mAb L42 did not (data not shown). These results, with three human T-cell lines, suggest that the induced association of these molecules with the TCR is a general response to agonist anti-receptor antibody treatment.

Preliminary experiments indicated that NEM in the solubilization buffer was necessary for preserving the association between the TCR complex and the 34- and 38-kDa molecules during immunoprecipitation (data not shown). To determine if the effect of NEM was indeed due to alkylation of -SH groups, other agents known to alkylate -SH groups were tested. Fig. 3 shows that the presence of each of the three alkylating agents, NEM, DTNB, or iodoacetamide, in the solubilization buffer after C305 treatment was sufficient for preserving the coimmunoprecipitation of the 34- and 38-kDa molecules with the TCR. This result suggests that subunit alkylation maintains a protein conformation necessary for the continued association of the TCR with the 34- and 38-kDa proteins during solubilization and immunoprecipitation.

To determine if these receptor-associated molecules were N-linked glycoproteins, TCR immunoprecipitates from C305-treated Jurkat cells were isolated and analyzed by SDS/PAGE. The individual 34-kDa, 38-kDa, and CD3 bands were excised and treated with N-Glycanase (Fig. 4). The N-Glycanase digestion of the 34- and 38-kDa molecules resulted in a change in their apparent molecular mass to 27 and 32 kDa, respectively, whereas the nonglycosylated CD3 ϵ chain was unaffected. These results indicate that both of these proteins contain N-linked oligosaccharides. Since the enzyme treatment resulted in essentially complete deglycosylation of CD3 γ and δ chains (data not shown), we presume that the 34- and 38-kDa molecules were likewise fully deglycosylated and that the 27- and 32-kDa chains are the resulting core proteins.

FIG. 2. Treatment of HuT 78 cells with WT31 (anti-TCR) induces the coimmunoprecipitation of the TCR complex with two proteins of 34 and 38 kDa. Surface radioiodinated HuT 78 cells were incubated in serum-free RPMI 1640 medium at 37°C for 10 min in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of WT31 (anti-TCR, 1:100 dilution of ascites fluid). The cells were solubilized and immunoprecipitation was performed with either mouse IgG (lanes 1 and 3) or anti-Leu4 (anti-CD3, lanes 2 and 4). Immunoprecipitates were analyzed by SDS/PAGE under nonreducing conditions.

FIG. 3. Coimmunoprecipitation of the 34- and 38-kDa molecules with CD3 requires alkylation. Radioiodinated Jurkat cells were incubated for 10 min in the presence (lanes 3–6) or absence (lanes 1 and 2) of mAb C305 (anti-Ti of Jurkat, 1:100 dilution of ascites fluid). The cells were then solubilized in 1% Nonidet P-40 lysis buffer containing no alkylating agents (lanes 1–3), 4 mM NEM (lane 4), 1 mM DTNB (lane 5), or 10 mM iodoacetamide (lane 6). Immunoprecipitation was performed with either mouse IgG (lane 1) or anti-Leu4 (lanes 2–6). Immunoprecipitates were analyzed under nonreducing conditions on SDS/11% polyacrylamide gels.

To investigate the specificity of the induced association, we treated Jurkat cells with several mAbs of differing specificity prior to cell lysis and anti-CD3 immunoprecipitation. As shown in Fig. 5, three mAbs, R140, C305, and WT31, directed against distinct epitopes of the TCR induced the coimmunoprecipitation of gp34 and gp38 with the TCR. mAbs directed against other cell surface molecules expressed on Jurkat, CD2 (9-1 or 9.6), or HLA (W6/32) failed to induce or augment the association. These results indicate that the association of the TCR with gp34 and gp38 is a specific response to the binding of antibody to the TCR.

FIG. 4. Endoglycosidase digestion of gp34 and gp38 reveals N-linked glycosylation. Radioiodinated Jurkat cells were incubated for 10 min in the presence of mAb C305 (anti-Ti of Jurkat, 1:100 dilution of ascites fluid). The cells were solubilized and immunoprecipitation was performed using anti-Leu4. The immunoprecipitates were subjected to one-dimensional SDS/PAGE and the CD3 ϵ chain, gp34, and gp38 were located by autoradiography and excised. The gel slices were rehydrated in N-Glycanase digestion buffer and were incubated for 16 hr at 37°C without or with 10 units of N-Glycanase. The digested samples were resolved on SDS/11% polyacrylamide gels.

In each case, the mAbs directed against the TCR that induce the coimmunoprecipitation of gp34 and gp38 with the TCR have been shown (19) to function as signal-transduction agonists. J.CaM1 and J.CaM2 are signal-transduction mutant cell lines independently derived from Jurkat (16, 17). Both of these cell lines possess surface levels of the TCR equivalent to that of Jurkat and the individual TCR subunits appear to be grossly normal. Despite their unaltered reactivity with anti-TCR mAbs, neither cell line responds to antibodies directed against the Ti heterodimer with phosphatidylinositol 4,5-bisphosphate hydrolysis and increases in intracellular Ca^{2+} that are observed in the wild-type Jurkat and other T-cell lines. When J.CaM1 and J.CaM2 were radioiodinated and treated with mAb C305 prior to anti-CD3 immunoprecipitation, we found that the antibody treatment induced the coimmunoprecipitation of the TCR with gp34 and gp38 in J.CaM2 but not in J.CaM1 (Fig. 6).

DISCUSSION

In these studies we have identified two cell surface glycoproteins gp34 and gp38 that exhibit an increased association with the TCR upon ligand binding. Both the 34- and 38-kDa molecules can occasionally be faintly detected in some anti-CD3 immunoprecipitations of untreated cells, indicating that these molecules may associate with the TCR in the absence of added stimulus. However, preincubation of the T cells with anti-TCR mAbs increases the coimmunoprecipitation of gp34 and gp38 by 5- to 12-fold, as determined by densitometric analysis of autoradiographs from experiments where gp34 and gp38 could be detected in immunoprecipitations of untreated cells. This indicates that the antibody treatment is either increasing the percentage of TCRs associated with these molecules or is increasing the affinity of that interaction.

The coimmunoprecipitation of gp34 and gp38 with the TCR requires the presence of an alkylating agent in the cell lysis buffer (Fig. 3). This suggests that alkylation of certain -SH groups preserves a conformational state in which gp34 and gp38 are "locked onto" the antigen receptor complex. Since NEM, DTNB, and iodoacetamide gave similar results, it is evident that the specific structure of the alkylating reagent is not a decisive factor. These results are similar to studies performed on the β_2 -adrenergic receptor where isoproterenol plus NEM was found to stably trap a hormone- β_2 -adrenergic receptor-guanine nucleotide binding protein complex (7–9). In that system it appears that hormone-receptor interaction with a guanine nucleotide binding protein causes a -SH group of the guanine nucleotide binding protein to be exposed (8). Hence, when this -SH group is alkylated, the normally transient interaction of hormone receptor with the guanine nucleotide binding protein is stabilized. The mechanism by which alkylation acts to stabilize the TCR-gp34/gp38 complex remains to be elucidated. However, the target of the alkylating agent is not likely to be either of the Ti chains as there are no predicted free -SH groups in the disulfide-linked heterodimer (1).

The association of gp34 and gp38 with the TCR in response to agonist anti-TCR mAbs was observed in all three human T-cell lines tested, suggesting that the expression of these proteins is broadly distributed among T cells and that the induced association with the TCR is a general response. The induced coimmunoprecipitation is also a specific response to anti-receptor antibodies since treatment of Jurkat with any of three anti-TCR mAbs induced the coimmunoprecipitation of gp34 and gp38 with the TCR whereas any of three mAbs directed against other T-cell surface molecules failed to induce the association. In addition, it was found that the agonist anti-TCR mAb L41 induces the association of gp34 and gp38 with the TCR of HPB-ALL whereas the nonagonist

FIG. 5. Coimmunoprecipitation of the 34- and 38-kDa molecules is a specific response to treatment with antibodies directed against the TCR. Radioiodinated Jurkat cells were preincubated for 10 min with no added antibody (lanes 1 and 2), mAb W6/32 (anti-HLA, 1:100 dilution of ascites fluid, lanes 3 and 4), mAb R140 (anti-Ti of Jurkat, 1:100 dilution of ascites fluid, lanes 5 and 6), mAb C305 (anti-Ti of Jurkat, 1:100 dilution of ascites fluid, lanes 7 and 8), mAb WT31 (anti-TCR, 1:100 dilution of ascites fluid, lanes 9 and 10), mAb 9-1 (anti-CD2, 10 μ g/ml, lanes 11 and 12), or mAb 9.6 (anti-CD2, 10 μ g/ml, lanes 13 and 14). Immunoprecipitation was performed with either mouse IgG (lanes 1, 3, 5, 7, 9, 11, and 13) or anti-Leu4 (anti-CD3, lanes 2, 4, 6, 8, 10, 12, and 14). Immunoprecipitates were analyzed under nonreducing conditions on SDS/11% polyacrylamide gels.

anti-TCR mAb L42 fails to do so, suggesting that only mAbs that are capable of acting as TCR signal-transduction agonists can induce the association. It will be of interest to determine the stage of T-cell ontogeny at which these molecules are expressed and the requirements for inducing the TCR-gp34/gp38 association.

To the best of our knowledge, the proteins reported here have not been identified on the surface of T cells, based on their relative mobilities by SDS/PAGE analysis. They are cell surface molecules based on their ability to be iodinated by lactoperoxidase labeling of intact cells and their content of N-linked carbohydrate groups. A membrane-immunoglobulin-associated 34-kDa protein has been described on the surface of a B-cell line (27). The relationship between this protein and those reported here is not known; however, it is

of interest that molecules of similar size appear to associate with the antigen receptors on T cells and B cells.

When the signal transduction mutant cell lines J.CaM1 and J.CaM2 were treated with mAb C305 and analyzed for the coimmunoprecipitation of gp34 and gp38 with the TCR, anti-TCR antibody treatment induced the association in J.CaM2 but not in J.CaM1 (Fig. 6). Whereas neither J.CaM1 nor J.CaM2 exhibit second messenger production in response to C305 treatment, both cell lines exhibit normal receptor internalization in response to C305 binding (16, 17). Therefore, the failure of C305 to induce the coimmunoprecipitation of gp34 and gp38 with the TCR of J.CaM1 suggests that the induced association of these proteins with the antigen receptor complex may relate to signal-transduction functions of the TCR rather than in the surface modulation of the TCR. Additionally, since these proteins do associate with the TCR of J.CaM2 after C305 treatment, the association of these molecules with the TCR is not a consequence of protein kinase C activation and increased cytoplasmic free Ca^{2+} . Two possible models that can relate the observed failure of gp34 and gp38 to associate with the TCR are (i) that one or both of these molecules may be defective in J.CaM1 or (ii) the TCR in J.CaM1 may not undergo appropriate allosteric changes after ligand binding, thus preventing its appropriate conformation-dependent association with gp34 and gp38. Consistent with either model, earlier studies suggested that the signal-transduction defect in J.CaM1 is located within a proximal component of the TCR signal-transduction apparatus whereas the defect in J.CaM2 is distal to this interaction (16, 17). Moreover, gp34 and gp38 fail to associate with the TCR after stimulation with anti-Ti antibody in a third signal-transduction mutant, J.CaM3 (data not shown). The signaling phenotype of J.CaM3 is similar to J.CaM1, but by complementation analyses J.CaM3 is defective in a distinct component (unpublished data). Therefore, gp34 and gp38 fail to associate with the TCR of two independently derived mutant T-cell lines that are defective in proximal components of the signal-transduction pathway.

It has been suggested (2, 17) that the CD3 complex serves to couple Ti binding to more distal components of the signal-transduction apparatus. One model consistent with the present results is that the ligand-induced association of the TCR with gp34 and gp38 functions to couple receptor occupancy to intracellular signal-transduction molecules. Recent studies with murine cell lines have linked expression of CD3 $\zeta\eta$ heterodimer to phosphatidylinositol hydrolysis (28). Since

FIG. 6. Treatment with mAb C305 fails to induce the coprecipitation of the 34- and 38-kDa molecules in the signal-transduction mutant J.CaM1. Jurkat cells and the signal-transduction mutant lines, J.CaM1 and J.CaM2, were surface radioiodinated. The cells were then incubated in the presence (lanes 3, 6, and 7) or absence (lanes 1, 2, 4, and 5) of mAb C305 (anti-Ti of Jurkat, 1:100 dilution of ascites fluid). Immunoprecipitation was performed with either mouse IgG (lanes 1 and 4) or anti-Leu4 (lanes 2, 3, 5, 6, and 7). Immunoprecipitates were analyzed under nonreducing conditions on SDS/11% polyacrylamide gels.

the CD3 $\zeta\eta$ heterodimer was not visualized in our studies, it will be important to determine which subunits of the TCR complex interact with gp34 and gp38 and whether that association is required for ligand-induced phosphatidylinositol 4,5-bisphosphate hydrolysis. Further characterization of these proteins should provide insight into the role of gp34 and gp38 in signal transduction and T-cell activation and to their possible relevance to other receptor systems.

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