Brief Definitive Report

Tyrosine Phosphorylation of CD6 by Stimulation of CD3: Augmentation by the CD4 and CD2 Coreceptors

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Summary

When T cells are activated via the T cell receptor (TCR) complex a number of cellular substrates, including some cell surface proteins, become phosphorylated on tyrosine (Tyr) residues. Phosphorylation of cytoplasmic Tyr renders these cell surface receptors competent to interact with proteins that link cell surface receptors to protein in the intracellular signaling pathways. Here we show that Tyr residues in the cytoplasmic domain of CD6 become phosphorylated upon T cell activation via the TCR complex. Tyr phosphorylation was observed when the T cells were activated by crosslinking CD3 or by cocrosslinking CD3 with CD2 or CD4, but not when the cells were stimulated by crosslinking CD2, CD4, or CD28 alone. Unlike other Tyr kinase substrates, such as the phospholipase Cγ1–associated pp35/36 protein, whose level of Tyr phosphorylation is highest when T cells are activated by cocrosslinking CD3 with CD2, the levels of CD6 Tyr phosphorylation are highest when T cells were activated by cocrosslinking CD3 with CD4.

The CD6 antigen is an ~130-kD cell surface glycoprotein expressed by peripheral blood T cells, thymocytes, a small subset of B cells, and some cells of the brain (1–6). We have recently reported the isolation and characterization of a cDNA clone encoding human CD6 (7). The predicted amino acid sequence of CD6 suggests that the mature protein, obtained after the removal of a 24-amino acid NH_2-terminal signal peptide, has a 374-amino acid extracellular domain, a 26-amino acid hydrophobic membrane-spanning domain, and a 44-amino acid cytoplasmic domain. Comparison of the predicted amino acid sequence of CD6 with those of other published protein sequences indicated that CD6 is a member of a large family of proteins that includes the lymphocyte cell surface glycoprotein CD5 (8) and the cysteine-rich domain of the type I macrophage scavenger receptor (9).

At present, the function of the CD6 is not known. However, a number of studies with anti-CD6 mAb suggest that CD6 plays an important role in T cell activation. The anti-CD6 mAb 2H1 has been shown to activate T cells in conjunction with PMA or with the anti-CD2 mAb $T11_3$ (2). Likewise, the anti-CD6 mAb T12 can activate T cells in a macrophage-dependent fashion (10). In addition, it has been shown that crosslinking of surface CD6 with an anti-CD6 mAb and polyclonal goat anti–mouse Ig or rat anti–κ chain can cause rapid mobilization of intracellular Ca^{2+} in T lymphocytes, and that cocrosslinking CD6 with CD3 enhances CD3-mediated signaling (11, 12).

MHC-restricted, antigen-specific activation of T cells via the TCR complex results in the rapid activation of a number of intracellular kinases, including Tyr kinases (13). These kinases phosphorylate a number of protein substrates rendering them competent to interact with proteins that form part of the intracellular signal-transduction pathway. Phosphorylation of these proteins initiates a cascade of events whereby signals initiated by receptor–ligand interactions at the cell surface are communicated to the cell interior where they modulate the effector function of the stimulated T cell. Antiphosphotyrosine (Anti-p-Tyr) blots of lysates obtained from activated T cells have shown that upon T cell activation, a discrete number of proteins become phosphorylated on Tyr residues (14). To date, only a few of these substrates have been identified. They include the ζ and ε chains of the CD3 complex, phospholipase C(PLC) γ1, CD5, ZAP-70, Raf-1, PI-3 kinase, $p95^{vav}$, $p60^{fyn}$, and $p56^{lck}$ (14–24). Here we show that upon T cell stimulation, CD6 is phosphorylated on Tyr residues, rendering it competent to interact with proteins in the intracellular signaling pathways. In addition, we show that when T lymphocytes are stimulated by crosslinking CD3 alone, CD6 is weakly phosphorylated on Tyr residues. The level of p-Tyr CD6 is slightly increased if the T cells are stimu-

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lated by cocrosslinking CD3 with CD2. However, when the T cells are stimulated by cocrosslinking CD3 with CD4, the level of p-Tyr CD6 is dramatically increased. We also found that the levels of Tyr phosphorylation of CD5 parallel those of CD6 after T cell stimulation.

Materials and Methods

Cells and Antibodies. Fresh peripheral blood lymphocytes from normal donors were isolated by density gradient centrifugation and used to generate PHA-P blasts. mAb 9.6 (anti-CD2), G19-4 (anti-CD3), G17-2 (anti-CD4), 10.2 (anti-CD5), 9.3 (anti-CD28) (25, 26), G3-6 (anti-CD6) (27), and an affinity-purified rabbit anti-p-Tyr antibody have been previously described (28, 29). The mAb against CD2, CD3, CD4, and CD28 were biotinylated as previously described (30).

T Cell Activation, Immunoprecipitation and Western Immunoblotting. Peripheral blood lymphocytes stimulated by PHA-P for 5 d (0.5 μg/ml, Wellcome Diagnostics, Dartford, UK) were activated by crosslinking CD2, CD3, CD4, CD28, or co-crosslinking CD3 and CD2, or CD3 and CD4 using 10 μg/ml of the appropriate biotin mAb and 5 μg avidin/μg mAb (11, 12). The cells were immediately pelleted and lysed in a modified RIPA buffer (31) supplemented with 1 mM PMSF, 1 mM sodium orthovanadate, and 20 μg/ml aprotinin. Cell nuclei and debris were cleared at 10,000 *g* for 10 min, and the cell lysates were incubated with either anti-CD5 or the anti-CD6 mAb for 2 h at 4°C. The immune complexes were incubated with protein A-Sepharose beads (Repligen Corp., Cambridge, MA) for 1 h. The immune complexes were washed twice in modified RIPA buffer and once in PBS, boiled in SDS sample buffer with dithiothreitol, separated by SDS-PAGE (8.5%), and subsequently electroblotted to PVDF membrane. After blocking the nonspecific binding sites with a buffer containing 6% BSA, the immunoblots were incubated with anti-p-Tyr (0.25 μg/ml) in the same buffer overnight at room temperature. The blots were rinsed twice in 10 mM Tris pH 7.4, 0.9% NaCl (rinse buffer), once in rinse buffer containing 0.05% NP-40, and twice in rinse buffer. The blots were incubated with 0.5 μCi/ml ^{125}I-protein A (ICN Radiochemicals, Costa Mesa, CA) in blocking buffer containing 6% BSA. The immunoblots were washed as described above and exposed to film (X-AR; Eastman Kodak Co., Rochester, NY) at −70°C with intensifying screen overnight.

Phosphoamino Acid Analysis. 5-d-old PHA-P blasts were washed twice with phosphate-free Earle's salts media containing 10% dialyzed fetal bovine serum. The cells were resuspended in 20 ml of media containing 10 mCi of [^{32}P]orthophosphate (PBS 13; Amersham Corp., Arlington Heights, IL) and incubated for 2 h at 37°C. The cells were then either rested or activated by crosslinking CD3 with CD4, lysed, and the anti-CD6 immunoreactive material was precipitated with protein A agarose beads (Repligen Corp.). The immunoprecipitates were resolved by SDS-PAGE (8.5%). The gel was dried on filter paper and autoradiographed for 1 h. Labeled proteins from both unstimulated and stimulated cells with a molecular mass of ~130 kD (CD6) were excised from the gel. Phosphoamino acid analysis was performed as described (32). The labeled phosphoamino acids were then resolved by 2-dimensional thin layer electrophoresis (32) on cellulose thin layer plates (VWR Scientific Corp., Philadelphia, PA), detected by autoradiography, and identified by comigration with ninhydrin-stained phosphoamino acid standards.

Preclearing of CD6 and CD5. Anti-CD6 and -CD5 were directly conjugated to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) and incubated with cell lysates from PHA-P blasts stimulated by cocrosslinking CD3 with CD4. The sequential immunoprecipitations were carried out as described above. After each immunoprecipitation, the resulting lysate was incubated with more Sepharose-conjugated mAb, allowing the progressive depletion of the anti-CD6 and anti-CD5 immunoreactive material from the lysates. The crude lysates and the precleared lysates were then subjected to anti-CD6 and anti-CD5 immunoprecipitation, followed by SDS-PAGE and anti-p-Tyr immunoblotting as described above.

Results and Discussion

To investigate which T lymphocyte cell surface glycoproteins become phosphorylated on tyrosine residues upon T cell activation, T cells were stimulated by crosslinking the T cell surface proteins CD2, CD3, CD4, and CD28, or by cocrosslinking CD3 with CD2 or CD4. After a 1-min stimulation period, the cells were lysed and treated with an anti-CD6 mAb and the immunoreactive material was precipitated with immobilized protein A. Crude cell lysates and the immunoprecipitated material were resolved by SDS-PAGE, transferred to a PVDF membrane, probed with the anti-p-Tyr antibody and ^{125}I-protein A, and exposed to film. The anti-CD6 mAb immunoprecipitated a protein of ~130 kD which bound to the anti-p-Tyr antibodies in material obtained from lysates of T cells activated by crosslinking CD3 alone or by cocrosslinking CD3 with CD2 or CD3 with CD4, but not from material obtained from lysates of unstimulated T cells

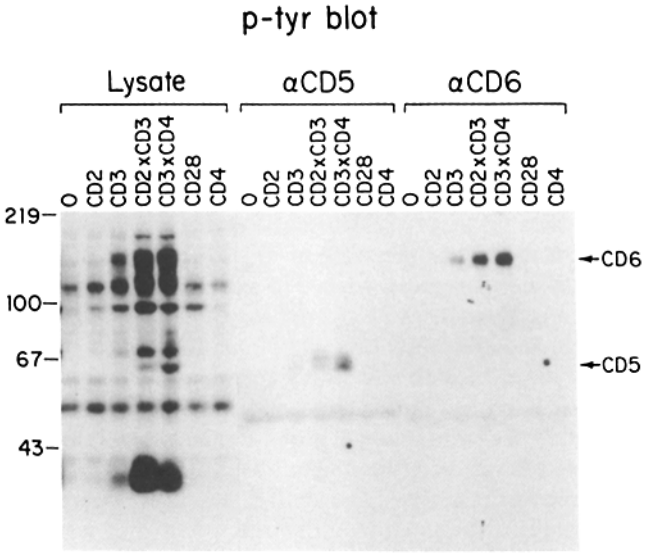

Figure 1. CD6 is phosphorylated on Tyr after T cell activation via the TCR. Lysates from unstimulated T cells (lane *0*), or T cells which were stimulated by crosslinking CD2, CD3, CD4, or CD28, or by co-crosslinking CD3 with either CD2 (*CD2 × CD3*) or CD4 (*CD3 × CD4*) were prepared. Crude lysates (*Lysate*) or lysates treated with an anti-CD5 (*αCD5*) or an anti-CD6 (*αCD6*) mAb followed by precipitation with immobilized protein A, were isolated by SDS-PAGE, blotted onto PVDF membrane, probed with an anti-p-Tyr antibody followed by ^{125}I-protein A, and exposed to film. (*Left*) Molecular weight standards.

or T cells stimulated by crosslinking CD2, CD4, or CD28 alone (Fig. 1). These results suggest that CD6 becomes Tyr phosphorylated after activating T cells via the TCR complex, but not upon crosslinking other surface receptors such as CD28 which induces the phosphorylation of an ~95-kD protein (Fig. 1).

Although crosslinking CD3 alone or cocrosslinking CD3 with CD2 resulted in CD6 Tyr phosphorylation, we observed that cocrosslinking CD3 with CD4 resulted in the highest levels of CD6 Tyr phosphorylation (Fig. 1). It is interesting that other Tyr kinase substrates such as the PLCγ1-associated pp35/36 protein are more effectively Tyr phosphorylated after cocrosslinking CD3 with CD2 (Fig. 1). These results suggest that differential receptor engagement during cellular interactions results in the phosphorylation of different Tyr kinase substrates.

To confirm the presence of p-Tyr residues on CD6, T cells were incubated for 2 h in the presence of [^{32}P]orthophosphate and either rested or stimulated by cocrosslinking CD3 with CD4. The anti-CD6 mAb immunoreactive material from these cells was precipitated with immobilized protein A, resolved by SDS-PAGE, hydrolyzed, and labeled phosphoamino acids were separated via two-dimensional thin-layer electrophoresis. As shown in Fig. 2, CD6 was phosphorylated on Ser and Thr on unstimulated T cells (Fig. 2 *A*) and became phosphorylated on Tyr (Fig. 2 *B*) after T cell stimulation. Previously, Swack et al. (10) showed that CD6 in Con A-activated T cells is phosphorylated on Ser residues. Our results indicate that T cell activation via the TCR complex results in CD6 Tyr phosphorylation and slightly higher levels of Ser phosphorylation (Fig. 2). The cytoplasmic domain of CD6 contains two Tyr residues (7) which could be phosphorylated. Comparison of the amino acid sequence surrounding these two Tyr and those surrounding the Tyr, which are autophosphorylation substrates in the *src* family kinases (33), did not provide information as to which of the two CD6 Tyr residues is the preferred substrate for Tyr kinases.

To investigate if CD6 accounted for the strong ~130-kD protein seen in anti-p-Tyr blots of whole cell lysates after T cell stimulation, lysates from stimulated T cells were progressively depleted of CD6 by multiple immunoprecipitations. The starting lysate and the precleared lysates and the anti-CD6 immunoreactive material from each immunoprecipitation were analyzed by SDS-PAGE followed by Western blotting using anti-p-Tyr antibodies and ^{125}I–protein A (Fig. 3). Our results indicate that within the detection limits of our experiment, CD6 is the major T cell protein of ~130 kD, which becomes phosphorylated on Tyr after T cell stimulation via the TCR complex.

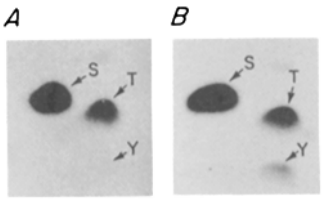

Figure 2. CD6 in unstimulated T cells is phosphorylated on Ser and Thr, T cell activation via the TCR complex results in Tyr phosphorylation. Lysates from unstimulated (*A*) or stimulated (*B*) T cells which had been incubated in the presence of [^{32}P]orthophosphate were resolved by SDS-PAGE. The phosphorylated protein was detected by autoradiography, excised from the gel, hydrolyzed, and resolved by 2-dimensional thin layer electrophoresis. Phosphorylated amino acids were detected by autoradiography. (*Arrows*) Phosphoserine (*S*), phosphothreonine (*T*), and phosphotyrosine (*Y*).

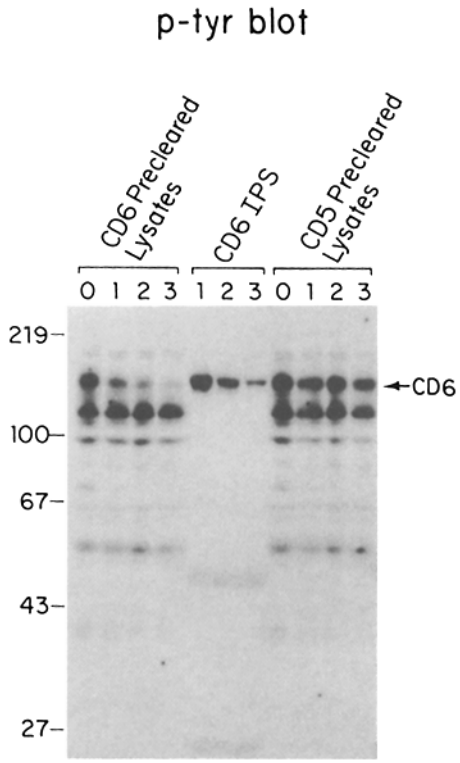

Figure 3. CD6 is the only ~130-kD protein to become Tyr phosphorylated after activating T cells via the TCR complex. Lysates from T cells that were stimulated by crosslinking CD3 and CD4 and treated with no antibody or sequentially treated with an anti-CD6 mAb followed by protein A agarose. The untreated lysate (lane *0*, *CD6 Precleared Lysates*), the anti-CD6 precleared lysates from each sequential immunoprecipitations (lanes *1–3*, *CD6 Precleared Lysates*), and the anti-CD6–reactive material from each sequential immunoprecipitation (lanes *1–3*, *CD6 IPS*), were resolved by SDS-PAGE, blotted, and treated with an anti-p-Tyr antibody followed by ^{125}I–protein A and exposed to film. Similarly, lysates from activated T cells were sequentially treated with an anti-CD5 mAb followed by protein A. The untreated lysate (lane *0*, *CD5 Precleared Lysate*) and the anti-CD5 precleared lysates from each sequential immunoprecipitation (lanes *1–3*, *CD5 Precleared Lysates*) were analyzed as described above.

Parallel CD5 immunoprecipitation and anti-p-Tyr blots were carried out with lysates prepared from unstimulated or stimulated T cells (Figs. 1 and 3, data not shown). CD5 Tyr phosphorylation was observed when T cells were stimulated by crosslinking CD3, or by cocrosslinking CD3 with CD2 or CD3 with CD4, but not from lysates from unactivated T cells or T cells activated by crosslinking CD2, CD4, and CD28 alone (Figs. 1 and 3). These results confirm and extend the results of Davies et al. (18) who showed that stimulation of T cells by crosslinking CD3 resulted in the Tyr phosphorylation of CD5. As was observed for CD6, CD5 is most effec-

tively Tyr phosphorylated after stimulating T cells by cocrosslinking CD3 with CD4. However, the Tyr phosphorylation level of CD6 was consistently higher than that of CD5 after T cell stimulation (Fig. 1). CD5 and CD6 are homologous proteins and their expression is tightly linked (7). The observation that the levels of Tyr phosphorylation of these two proteins parallel each other after T cell stimulation, suggests that the function of these two proteins may be similarly regulated.

The data presented herein show that activation of T cells via the TCR complex, but not by other receptors such as CD2 or CD28, results in different levels of Tyr phosphorylation on the T cell surface proteins CD6 and CD5. It is likely that Tyr phosphorylation may play an important role in the ability of these two proteins to mediate their functions as accessory molecules in T cell activation, and possibly affect their ability to signal to other cells by modulating their interaction with their counter receptors. Recently, Beyers et al. (34) described the formation of a loosely associated multimolecular complex between the TCR and the T cell surface proteins CD2, CD4, CD5, CD8, and the cytoplasmic Tyr kinases $p56^{lck}$ and $p60^{fyn}$. Two additional proteins of ~34 and ~105 kD were also observed to associate with this complex. Cardenas et al. (35) have shown that resting T cells express an ~105-kD form of CD6. This report leads us to postulate that the ~105-kD protein found to be associated with the TCR multimolecular complex may be CD6. The observation that stimulating T cells by cocrosslinking individual components of the TCR complex results in the differential phosphorylation of proteins loosely associated with the TCR complex may indicate that proteins within this multimolecular complex are spatially segregated and/or that a subset of the TCR-associated Tyr kinases may be preferentially activated depending on which cell surface proteins were engaged during activation. From a functional standpoint, these differences in Tyr phosphorylation may provide a molecular mechanism whereby the TCR complex is able to differentially engage the intracellular signaling pathways based upon which of its components participate in cellular contact.

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