International Immunology, Vol. 11, No. 9, pp. 1411–1422

Defective TCR signaling events in glycosylphosphatidylinositol-deficient T cells derived from paroxysmal nocturnal hemoglobinuria patients

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Keywords: glycosylphosphatidylinositol, paroxysmal nocturnal hemoglobinuria, TCR signaling

Abstract

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic disorder characterized by the presence of abnormal cells of various hematopoietic cell lineages deficient in surface expression of glycosylphosphatidylinositol (GPI)-anchored molecules. By analyzing T cells isolated from patients affected with PNH, it was found that *ex vivo* GPI-deficient $CD4^+$ and $CD8^+$ peripheral T cells display a more naive phenotype as compared to wild-type cells. In addition, *in vitro* proliferative responses to allogeneic antigen-presenting cells were shown to be reduced in mutant T cells. To investigate the molecular basis responsible for defective T cell activation in GPI-deficient T cells, T cell lines and T cell clones were generated from patients affected with PNH. When stimulated with anti-CD3ε mAb, mutant cells displayed a significantly decreased activation of protein tyrosine kinase $p56^{lck}$. The decreased kinase activity was accompanied by a delayed TCR capping and internalization. Interestingly, protein tyrosine phosphorylation is not only quantitatively but also qualitatively affected, with one substrate being more intensively phosphorylated in mutant than in wild-type cells. These observations suggest that a defective activation of $p56^{lck}$ contributes to the depressed immune responses observed in GPI-deficient T cells derived from PNH patients.

Introduction

Clonal T lymphocyte activation is controlled by the interaction of the TCR with its ligand, a peptide–MHC molecule complex. The initial membrane proximal event triggered by TCR engagement is the activation of non-receptor protein tyrosine kinases (PTK) (1–3). This event is essential to couple the TCR, which does not have any intrinsic kinase activity, to downstream signaling pathways which lead to proliferation and cytokine production. Two PTK of the Src family, $p56^{lck}$ and $p59^{fyn}$ have been implicated in the phosphorylation of tyrosine residues present in the cytoplasmic tail of the TCRζ chain and CD3 subunits of the TCR complex.

The function and membrane localization of $p56^{lck}$ and $p59^{fyn}$ are regulated by dual acylation (4). $p56^{lck}$ mutants that lack either myristate or palmitate moieties do not localize to the plasma membrane and do not function properly (5). Post-translational addition of lipids has been shown to target $p56^{lck}$ and $p59^{fyn}$ to a glycolipid-enriched membrane (GEM) compartment or detergent-insoluble glycolipid-enriched domain (6) or detergent-resistant membrane domain (7). This membrane compartment contains glycolipids, sphingolipids, cholesterol, glycosylphosphatidylinositol (GPI)-anchored proteins and signal transducing molecules, such as trimeric G protein, Ras and phosphoinositides (4). Interestingly, LAT, a recently identified critical substrate of PTK activated upon TCR engagement also localizes to this compartment (8). The specific enrichment in signaling components suggests that the GEM fraction represents a specialized signaling domain on the cell membrane. In agreement with this hypothesis, it has been reported that antibody cross-linking of GPI-anchored molecules leads to T cell proliferation and cytokine production. Interestingly, signaling through GPI-anchored molecules requires surface expression of a functional TCRζ chain (9,10)

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Transmitting editor: L. Moretta

Received 8 February 1999, *accepted* 10 May 1999

and expression of p59^{fyn} kinase (11,12). Furthermore, in addition to p59^{fyn}, also p56^{lck} is also found in GPI-anchored molecule immunoprecipitates of T cells (13,14).

Collectively these results show that GPI-anchored molecules and the TCR complex share proximal components of the T cell signaling machinery (p56^{lck}, p59^{fyn} and TCRζ), and when engaged are able to elicit similar responses. It is thus of interest to study the relationship and cross-talk between these two signaling complexes.

Functional studies using T cells deficient in GPI biosynthesis suggest that GPI-anchored molecules may play a role in TCR-mediated activation. In patients affected with paroxysmal nocturnal hemoglobinuria (PNH), an acquired hemolytic disorder characterized by the presence of GPI-anchor deficient (GPI-deficient) hematopoietic cells, GPI-deficient peripheral T cells display a more naive phenotype as compared to wild-type controls (15). Furthermore, proliferative responses to allogeneic antigen-presenting cells (APC) are defective in mutant T cells (16). Similarly, ovalbumin-specific T cell hybridomas, lacking surface expression of several GPI-anchored molecules, were shown to have a decreased capacity to respond to antigen, concanavalin A and anti-CD3ε ligation (17). In this regard we have recently shown that TCR engagement in five murine mutant cell lines with deficiencies in GPI biosynthesis fails to induce tyrosine phosphorylation of the TCRζ chain and ZAP-70 (18). These data suggest that the connection between signaling through the TCR and GPI-anchored molecules may reside in the modulation of Src kinases, p56^{lck} and p59^{fyn}, known to be involved in tyrosine phosphorylation of the TCRζ chain and ZAP-70.

To further investigate the role of GPI-anchored molecules in TCR-mediated T cell activation, TCR signaling events were analyzed in human T cells derived from two patients affected with PNH. PNH is caused by somatic mutations in the X-linked gene encoding a protein termed phosphatidylinositol glycan class A (PIG-A) necessary for the synthesis of the very early intermediates of GPI-anchor (19). The fact that only part of bone marrow-derived cells is affected allows for the isolation of wild-type and mutant (GPI-deficient) T cells from the same individual. Here we report that TCR engagement fails to optimally activate p56^{lck} in GPI-deficient T cells, ultimately resulting in a defective calcium flux and proliferative response. Interestingly, decreased activation of p56^{lck} in mutant T cells induces not only a quantitatively but also a qualitatively different pattern of tyrosine phosphorylated substrates. These findings could explain the high proportion of naive GPI-deficient T cells found in the peripheral blood of PNH patients and the depressed proliferative responses to allogeneic APC.

Methods

Patients/cells

Blood (10 ml) was obtained from one healthy donor and two patients affected with PNH. The clinical diagnosis of PNH was established by a positive Ham test or sucrose test. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Hypaque density centrifugation. PBMC were then stained with anti-decay accelerating factor (DAF) mAb (Bric 216) followed by FITC-labeled goat anti-mouse antiserum. DAF$^+$ and DAF$^-$ cells were sorted by flow cytometry using a FACStar Plus flow cytometer (Becton Dickinson, Mountain View, CA). DAF$^+$ and DAF$^-$ PHA T cell lines were expanded in 2 μg/ml phytohemagglutinin (PHA-P; Wellcome, Beckenham, UK) and IL-2 containing medium. HuIL-2-t8 supernatant, kindly provided by Drs S. Demotz (Insitute of Biochemistry, University of Lausanne, Switzerland) and A. Lanzavecchia (Basel Institute for Immunology, Basel, Switzerland), was used as IL-2 source. The culture medium was RPMI 1640 supplemented with L-glutamine (2 mM), non-essential amino acids, 1 mM sodium pyruvate, 1mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin (Gibco/BRL, Basel, Switzerland) and 10% heat-inactivated FCS. DAF$^+$ and DAF$^-$ alloreactive T cell lines were generated by co-culturing sorted PBMC with irradiated PBMC (5000 rad) of a healthy donor in normal medium. After 1 week the cell lines were expanded in IL-2-containing medium for 7 days. T cell clones (TLC) were obtained by limiting dilution in the presence of 2 μg/ml PHA-P and IL-2 containing medium and allogeneic irradiated PBMC (5000 rad). The clones were expanded and maintained in culture by periodic re-stimulation (3–4 weeks) with allogeneic irradiated PBMC, 2 μg/ml PHA-P and IL-2 containing medium. The T cell lines and TLC were used for the experiments 10–15 days after re-stimulation.

Antibodies and antisera

The following antibodies were used for immunoprecipitations, immunoblotting, FACS analysis and confocal microscopy: anti-CD3ε mAb OKT3 (ATCC, Rockville, MD); anti-TCRζ mAb H146-968 (20) and 6B10.2 (Santa Cruz Biotechnology, Santa Cruz, NM); anti-DAF mAb Bric 216 (International Blood Group Reference Laboratory, Bristol, UK); phycoerythrin (PE)-conjugated anti-CD4 mAb, PE-conjugated anti-CD8 mAb, FITC-conjugated anti-CD45 RO mAb and FITC-conjugated anti-CD3ε mAb (Becton Dickinson, San Jose, CA); anti-CD4 mAb 101.69 (21), kindly provided by Dr D. Rimoldi, Ludwig Institute for Cancer Research, Lausanne, Switzerland; anti-CD45 mAb, anti-phospholipase C-γ1 antiserum (Santa Cruz Biotechnology); anti-p56^{lck} antisera p56-1 and 2166 (5) (kindly provided by Dr S. C. Ley, Division of Cellular Immunology, National Institute for Medical Research, London, UK); anti-p59^{fyn} antiserum (kindly provided by M. F. White, Harvard Medical School, Boston, MA); anti-PY mAb 4G10 (unlabeled and biotin-conjugated; Upstate Biotechnology, Lake placid, NY). Horseradish peroxidase-conjugated goat anti-mouse, goat anti-rat and goat anti-rabbit antisera were purchased from Sigma (Buchs, Switzerland). Streptavidin–FITC, FITC-conjugated and Texas Red-conjugated donkey F(ab′)$_2$ fragment anti-mouse IgG and FITC-conjugated hamster F(ab′)$_2$ fragment anti-rabbit IgG were purchased from Jackson ImmunoResearch (West Groove, PA).

Proliferation assay

Round-bottom microplates were coated with different concentrations of anti-CD3ε mAb (OKT3) and 5×10^3 T cells/well were added. Cells were stimulated for 2 days at 37°C and then pulsed with 1 μCi of [^{3}H]thymidine. Incorporation of radiolabeled nucleotide was determined 16 h later. PHA responses were assessed by culturing 5×10^3 cells/well T cells with 5×10^3 irradiated allogeneic PBMC in IL-2 containing

medium in flat-bottomed microplates. After 2 days cells were pulsed with 1 μCi of [^{3}H]thymidine and harvested 16 h later. Alloreactivity was assessed by culturing 2×10^4 alloreactive T cells with increasing concentration of an irradiated Epstein–Barr virus-transformed B cell line derived from PBMC of the healthy donor in flat-bottomed microplates for 48 h; 1 μCi of [^{3}H]thymidine was added during the last 16 h of incubation.

Surface staining

Cells were washed twice in PBS containing 2.5% FCS and 0.02% NaN_3, and incubated on ice for 20 min with antibody at saturating concentration. Hybridoma culture supernatants or directly labeled antibodies were used. Where applicable, after two washes with PBS containing 2.5% FCS and 0.02% NaN_3, cells were incubated with the indicated FITC-conjugated second antibodies for 20 min on ice (see figure legends). The stained cells were analyzed with a FACScan using Lysys II software (Becton Dickinson). Dead cells were excluded from analysis by appropriate gating on forward and side scatter.

Stimulation, precipitation and immunoblotting

Cells were washed twice in PBS and resuspended at a concentration of 2×10^7/ml. They were incubated for 20 min at 37°C and subsequently stimulated for the indicated amount of time by addition of 10 μg/ml (final concentration) of anti-CD3ε mAb OKT3. After stimulation cells were rapidly washed with ice cold PBS containing 1 mM Na_3VO_4, and lysed in lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM Na_3VO_4, 5 μg/ml leupeptin, 10 μg/ml aprotinin and 2 mM PMSF) containing 0.5–1% Triton X-100 (as indicated in the figure legends). Lysates were immunoprecipitated using mAb prebound to Protein A–Sepharose beads or rabbit anti-mouse IgG-coated agarose beads. Eluted samples were resolved by SDS–PAGE in reducing conditions, transferred to PVDF membranes and immunoblotted with the indicated antibody (see figure legends).

Immunofluorescence microscopy

Cells were washed twice in PBS and resuspended in PBS containing 2.5% FCS at a concentration of 5×10^6 cells/ml. Cells were subsequently incubated for 15 min on ice with either anti-CD3ε (OKT3) or anti-CD4 mAb. After a quick wash, cells were incubated for 15 min on ice with Texas Red-conjugated donkey F(ab′)$_2$ fragment anti-mouse IgG. Cells were washed and either left on ice or incubated for indicated amount of time at 37°C. After incubation cells were rapidly washed with cold PBS containing 2% BSA. They were then fixed in 3% paraformaldehyde for 30 min at room temperature, and permeabilized for 4 min with PBS containing 0.1% Triton, 2.5% FCS and 10 mM Na_3VO_4. Non-specific staining was blocked by incubation in PBS containing 5% FCS and 10 mM Na_3VO_4. Cells were subsequently incubated with anti-p56^{lck} rabbit antiserum p561 (1:400 diluted) or with biotinylated anti-phosphotyrosine mAb 4G10 (2 μg/ml) at room temperature for 45 min. After three washes with PBS, cells were incubated with either FITC-conjugated hamster F(ab′)$_2$ fragment anti-rabbit IgG or with streptavidin–FITC for 45 min at room temperature, washed and mounted on slides with Fluor Save reagent (Calbiochem Novabiochem, La Jolla, CA). Cells were analyzed using a Zeiss Confocal Laser Scanning System attachment LSM (Zeiss, New York, NY) with the Axiovert 100 photomicroscope system.

In vitro kinase assays

Precipitated immune complexes were washed 3 times in lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1 mM Na_3VO_4, 5 μg/ml leupeptin, 10 μg/ml aprotinin and 2 mM PMSF) containing 1% Brij 96 and once in lysis buffer without detergent. Complexes were resuspended in 15 μl of kinase buffer (25 mM HEPES, pH 7, 10 mM $MnCl_2$, 5 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 mM Na_3VO_4 and 10 μCi [γ-^{32}P]ATP), and kinase reactions performed for 15 min at room temperature and subsequently terminated by the addition of sample buffer. Proteins were resolved by 10% SDS–PAGE in reducing conditions. Gels were treated with 1 M KOH for 1 h at 58° C to remove [^{32}P]Ser and [^{32}P]Thr, washed, and [^{32}P]Tyr content assayed by autoradiography.

Results

Wild-type and GPI-deficient T cells were isolated from PBMC of PNH patients by cell sorting based on the expression of the GPI-anchored molecule DAF. Wild-type and GPI-deficient PHA and alloreactive T cell lines and clones were subsequently established and used in this study. DAF surface expression was regularly checked on the T cell lines and clones by flow cytometry before each experiment.

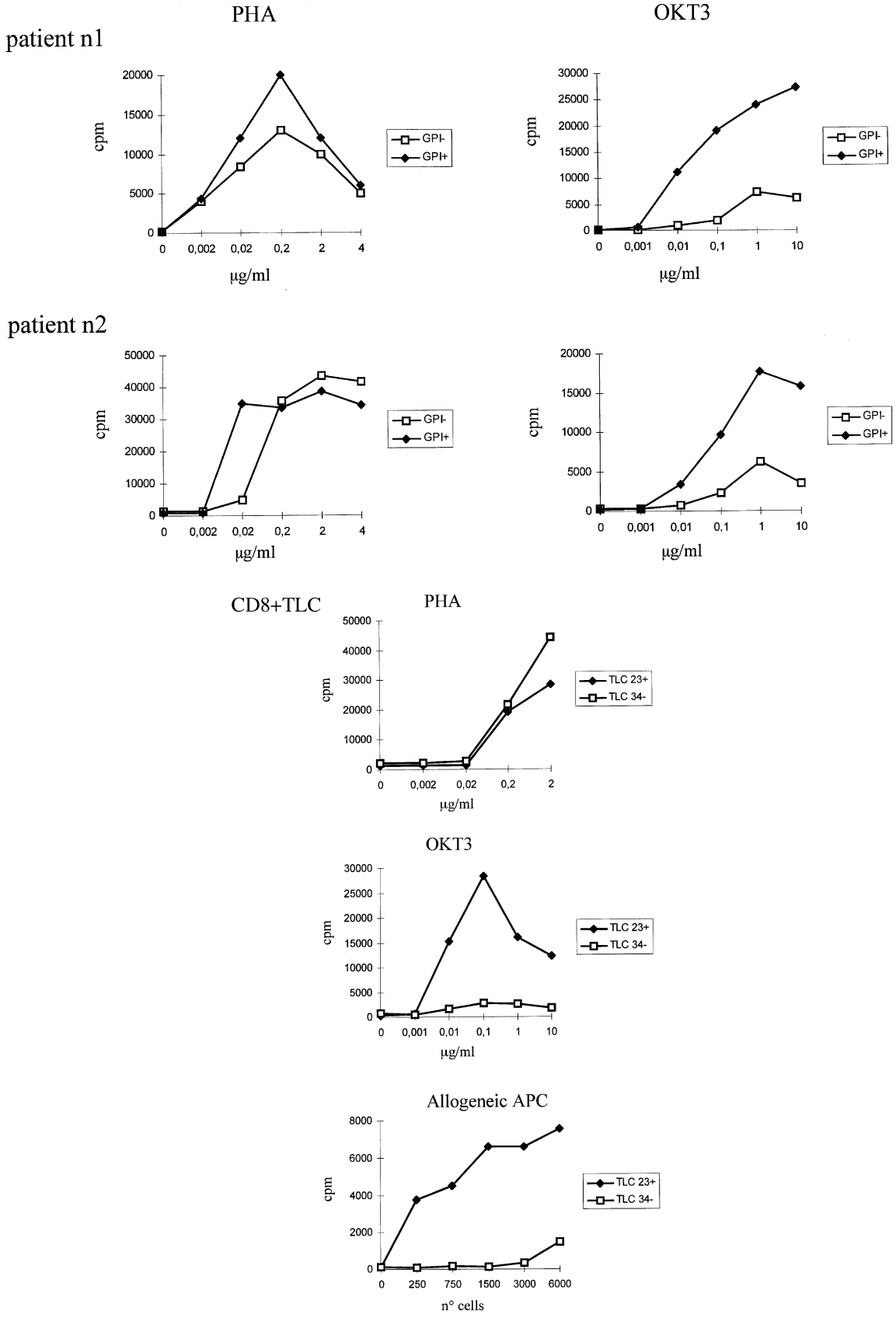
Decreased proliferation in response to TCR engagement in GPI-deficient T cell lines and TLC

Dose-dependent responses to anti-CD3ε mAb (OKT3) and to PHA were analyzed in wild-type and GPI-deficient T cell lines obtained from two PNH patients. A strongly reduced proliferative response to TCR ligation was observed in GPI-deficient T cell lines as compared to wild-type controls, while PHA responses were only marginally affected (Fig. 1a).

Alloreactive CD8$^+$ TLC were cloned from alloreactive T cell lines of patient no. 1 and their proliferation was assessed by [^{3}H]thymidine incorporation. Depressed proliferative responses to anti-CD3ε mAb and to allogeneic APC were found in GPI-deficient CD8$^+$ TLC as compared to wild-type controls, while PHA responses were comparable in all the TLC (Fig. 1b). Interestingly cytotoxic activity was not affected in GPI-deficient TLC (data not shown).

CD4$^+$ TLC were isolated from PHA T cell lines of patient no. 1. Proliferative responses to anti-CD3ε mAb of GPI-deficient CD4$^+$ TLC were strongly impaired as compared to wild-type controls, while PHA responses where only marginally decreased (Fig. 1c).

Collectively these experiments show that proliferative responses to TCR ligation are affected in GPI-deficient T cells. These results confirm and extend the previously reported weaker responses of GPI-deficient T cells to allogeneic B cells blasts (16). However, they are not in agreement with the reported similar proliferation to stimulation via CD3 in wild-type and GPI-deficient T cells (15). The discrepancy between our and the previously published results can be explained by the different experimental set-up. In previous experiments T cells were stimulated with a fixed concentration of antibody (15). In our study a dose-dependent response was analyzed,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TLC 23+
TLC 34-
Allogeneic APC
8000
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TLC 23+
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allowing us to identify the proliferative defect, that is most striking at low antibody concentrations. In contrast to anti-CD3ε mAb-mediated proliferation, PHA-induced proliferation was not affected in GPI-deficient TLC, suggesting quantitative and/or qualitative (22) differences in the signaling pathways following anti-CD3ε mAb or PHA stimulation.

We then proceeded to characterize the molecular basis of defective TCR signal transduction in GPI-deficient T cells, mainly using wild-type and GPI-deficient $CD4^+$ TLC.

Wild-type and GPI-deficient TLC derived from a PNH patient express similar levels of proteins involved in TCR-proximal signaling events

First, we assessed the level of surface expression of the TCR complex on wild-type and GPI-deficient TLC. CD3ε was found to be expressed at comparable levels in wild-type and GPI-deficient TLC (Fig. 2a). TCRζ chain is known to be involved in TCR signaling and its expression has been shown to be regulated independently from that of the rest of the TCR complex (20,23,24). Western blot analysis revealed that wild-type and GPI-deficient TLC expressed comparable amounts of TCRζ (Fig. 2b).

Upon TCR-mediated activation CD4 or CD8 co-receptors are thought to recruit the Src kinase $p56^{lck}$ to the TCR complex (25,26) and variations in their expression-level are known to affect T cell activation (27,28). We therefore analyzed CD4 expression by wild-type and mutant TLC, and observed no significant differences (Fig. 2a). Similar results were obtained when analyzing GPI-deficient and wild-type T cell lines (data not shown).

Altered TCR-mediated signaling could result from differences in the expression of CD45, a phosphatase known to activate $p56^{lck}$ (29). However, we have observed no difference in the cell surface expression of the CD45 isoform expressed by antigen-experienced T lymphocytes (CD45RO) between wild-type and mutant TLC (Fig. 2a). Similar results were obtained analyzing GPI-deficient and wild-type T cell lines (data not shown).

In addition, Western blot analysis of the expression level of the Src kinases $p56^{lck}$ and $p59^{fyn}$ (both involved in the earliest intracellular signaling events following TCR-mediated activation), and of phospholipase C-γ1 (initiating the essential phosphoinositide pathway) did not reveal any difference between wild-type and GPI-mutant cells (Fig. 2b).

Collectively these data indicate that impaired TCR-mediated responses of GPI-deficient cells are not caused by a reduced

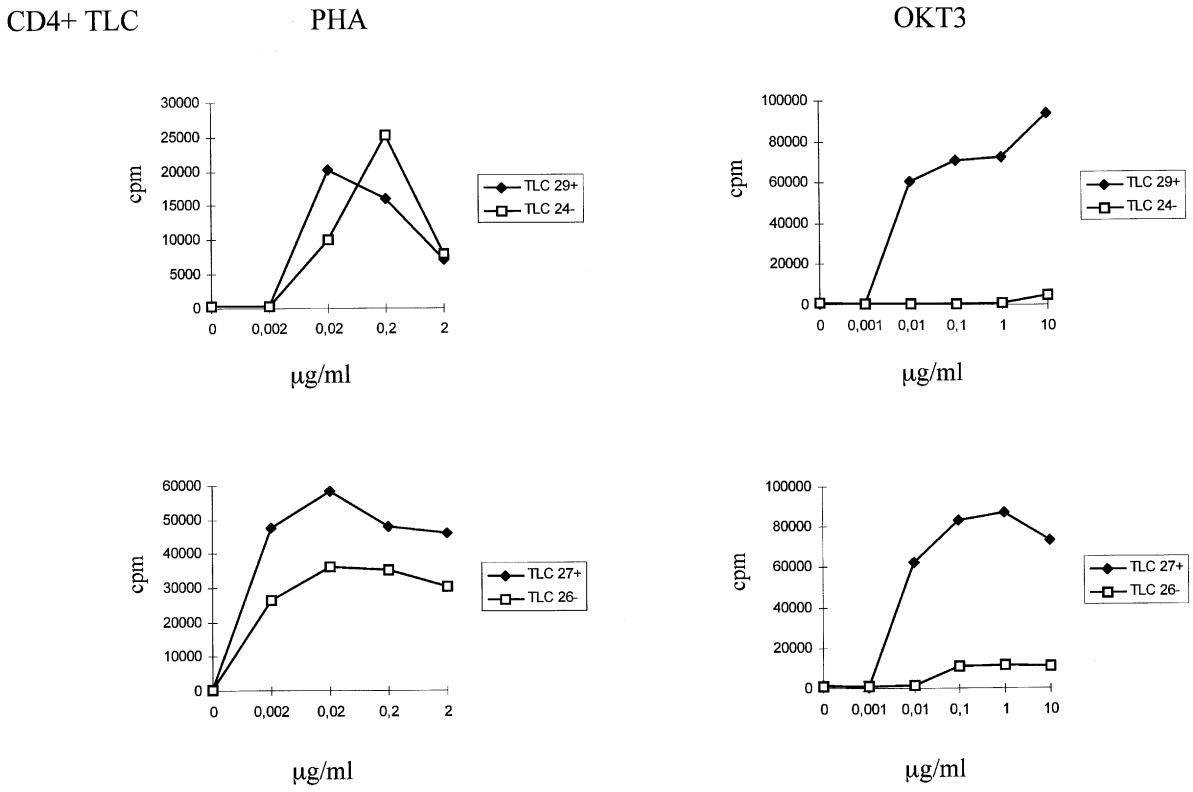

Fig. 1. Proliferative responses to PHA and anti-CD3ε mAb in wild-type and GPI-deficient T cell lines and $CD4^+$ and $CD8^+$ TLC. For PHA stimulation T cells were co-cultured with irradiated allogeneic PBMC in duplicates in the presence of increasing concentration of PHA. For anti-CD3ε mAb stimulation, OKT3 was coated at increasing concentration on round-bottom 96-well plates and T cells were subsequently added. For the alloreaction, T cells were co-cultured with increasing concentration of irradiated allogeneic Epstein–Barr virus-transformed B cells. [^{3}H]Thymidine incorporation was measured 48 h later. Results are mean values of c.p.m. Experiments were repeated 2–4 times with similar results.

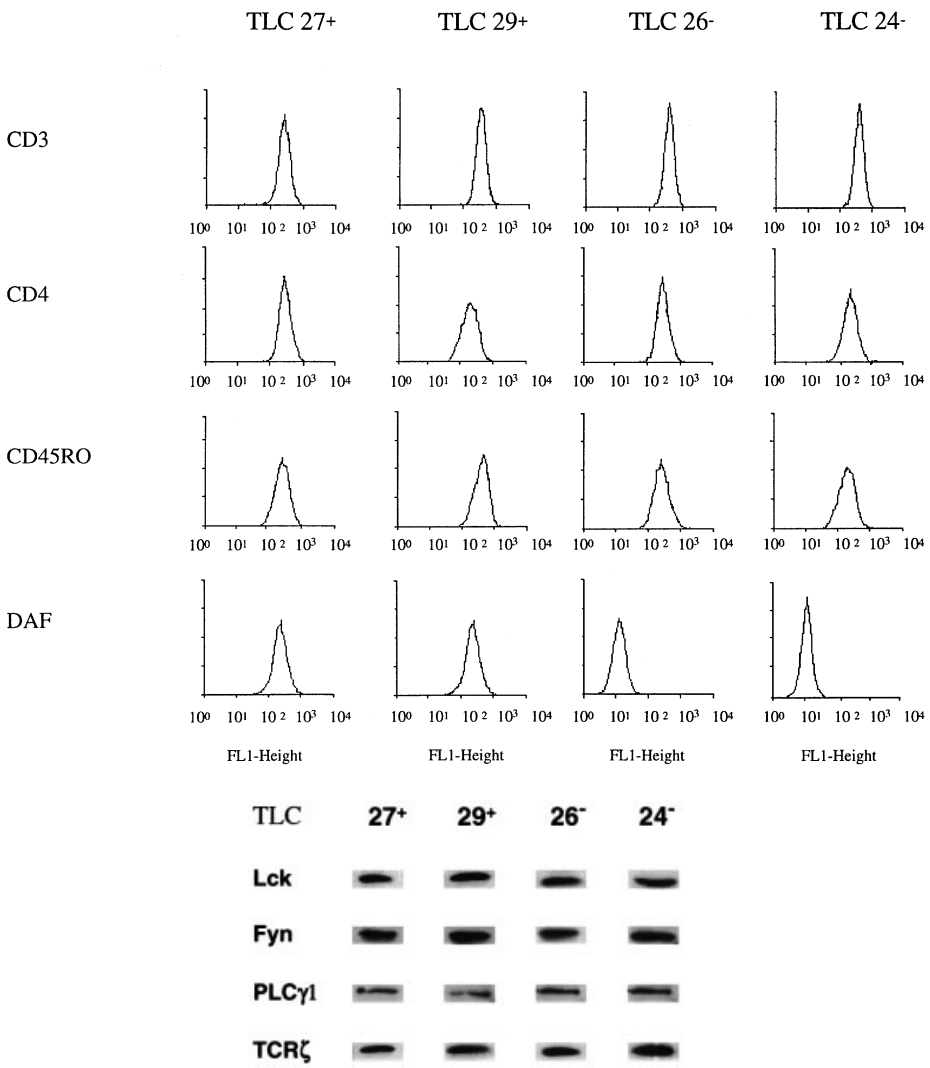

Fig. 2. Wild-type and GPI-deficient TLC express similar levels of proteins involved in TCR proximal signaling events. Surface levels of CD3ε, CD4, CD45RO and DAF was analyzed as follows: cells were washed with PBS containing 2.5% FCS and 0.02% NaN_3, and incubated on ice for 20 min with directly labeled anti-CD3ε, anti-CD4, anti-CD45RO mAb (Becton Dickinson) and anti-DAF mAb (Bric 216). After washing, cells were analyzed by flow cytometry. Life cells were gated by forward and side light scatter (A). The levels of TCRζ chain, $p59^{fyn}$, $p56^{lck}$ and phospholipase C-γ1 were analyzed by Western blot in total lysates of wild-type and GPI-deficient TLC obtained from the same number of cells (B).

expression level of proteins implicated in early TCR signaling events.

Impaired calcium mobilization upon TCR ligation in GPI-deficient T cell lines and TLC

To examine TCR-mediated signaling events, changes in $[Ca^{2+}]_i$ were analyzed in wild-type and GPI-deficient T cell lines (periodically re-stimulated with PHA) and clones by flow cytometry. After stimulation with anti-CD3ε mAb, a rapid and sustained increase in $[Ca^{2+}]_i$ was found in wild-type T cell lines and TLC, while a delayed and quantitatively decreased mobilization of $[Ca^{2+}]_i$ was observed in wild-type GPI-deficient T cell lines and TLC (Fig. 3).

These data indicate that TCR signal transduction is defective in GPI-deficient T cells and that the signaling defect probably resides upstream of calcium mobilization.

Quantitative and qualitative differences in the induction of phosphorylated substrates in wild-type and GPI-deficient TLC

To examine TCR proximal events in the signal transduction pathway, the induction of tyrosine phosphorylation was analyzed in total lysates of wild-type and mutant TLC by Western blot. Upon stimulation, an increase in tyrosine phosphorylation of low mol. wt proteins of 20, 28 and 36–38 kDa was observed in wild-type TLC, which was not appreciable in GPI-deficient TLC (Fig. 4). These data reveal that the decreased responsiveness of GPI-deficient T cells upon anti-CD3ε stimulation correlates with the reduction in CD3ε-induced tyrosine phosphorylation.

Intriguingly, tyrosine phosphorylation of a protein of 110 kDa was significantly increased in GPI-deficient TLC as compared to wild-type TLC with a maximum reached after 2 min of TCR-mediated stimulation. Initial attempts made to identify this

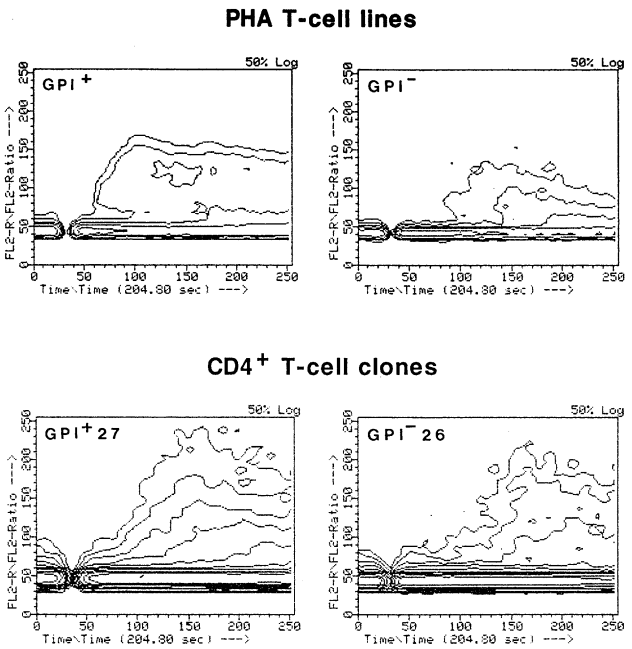

Fig. 3. Impaired calcium mobilization upon TCR engagement in GPI-deficient T cell lines and TLC. Wild-type and GPI-deficient T cell lines and TLC were loaded with Indo-1 for 30 min at 37°C, washed and resuspended in calcium buffer at a concentration of 0.2×10^6 cells/ml. The cells were then stimulated with an anti-CD3ε mAb (OKT3) and changes in $[Ca^{2+}]_i$ were measured over time.

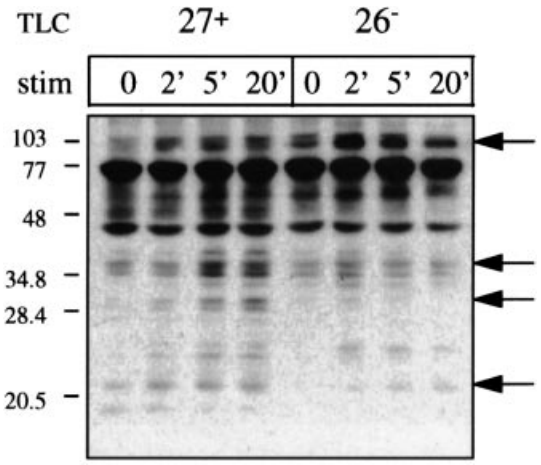

Fig. 4. TCR-mediated induction of phosphorylated substrates in wild-type and GPI-deficient TLC. Wild-type and GPI-deficient TLC were stimulated with an anti-CD3ε mAb (OKT3) for the indicated time and the induced tyrosine phosphorylated substrates were analyzed by Western blot of total lysates. Arrows indicate differences in the tyrosine phosphorylation pattern of wild-type and mutant TLC.

protein have not been successful. p110 does not co-precipitate with CD3ε, $p56^{lck}$, $p59^{fyn}$, Grb-2 and phospholipase C-γ1 (data not shown). An altered pattern of TCR-induced tyrosine phosphorylated substrates was also observed in TLC 24^- (data not shown).

In conclusion, both quantitative and qualitative differences

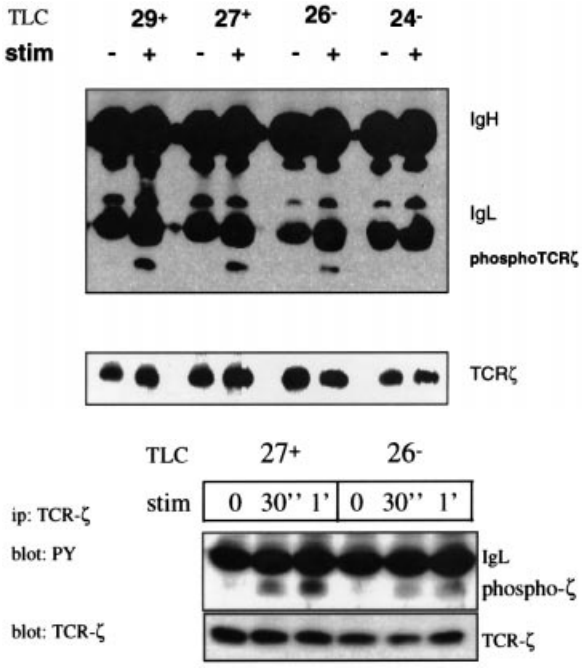

Fig. 5. Impaired induction of TCRζ chain tyrosine phosphorylation upon TCR triggering in GPI-deficient TLC. Inducible phospho-ζ was analyzed as follows: cells were washed twice with PBS, resuspended in PBS at the concentration of 10^7/ml and incubated for 30 min at 37°C. To trigger the TCR, 10 μg/ml of anti-CD3ε mAb (OKT3) was added for the indicated time at 37°C. Cells were subsequently rapidly washed with ice-cold PBS containing 1 mM Na_3VO_4, and lysed in lysis buffer containing 0.5% Triton X-100, 1 mM Na_3VO_4 and protease inhibitors. After centrifugation, lysates were immunoprecipitated with anti-TCRζ mAb prebound to Protein A–Sepharose beads. Precipitates were resolved on 12.5% SDS–PAGE and blotted with anti-phosphotyrosine mAb (4G10). The region <18 kDa was blotted with anti-TCR-ζ mAb (6B10.2). The ratio of intensity of tyrosine phosphorylated versus non-phosphorylated TCRζ, as determined by densitometry, for the TCR activated TLC, was: (A) 2.9, TLC 29^+; 2.75, TLC 27^+; 1.4, TLC 26^-; 0.6, TLC 24^-; (B) TLC 27^+: 1.3, stim 30 s; 2.3, stim 1 min; TLC 26^- : 0.7, stim 30 s; 1.2, stim 1 min.

in the induction of phosphorylated substrates were observed between wild-type and GPI-deficient TLC.

Decreased tyrosine phosphorylation of the TCRζ chain upon TCR engagement in GPI-deficient TLC

One of the earliest events of TCR-mediated signaling is tyrosine phosphorylation of the TCRζ chain. To examine whether this early activation event was affected in human GPI-deficient TLC, lysates from unstimulated and anti-CD3ε antibody stimulated T cells were immunoprecipitated with a mAb specific for the TCRζ chain. Precipitates were resolved by SDS–PAGE and immunoblotted with anti-phosphotyrosine or anti-TCRζ mAb. In wild-type TLC, after 2 min of TCR-mediated stimulation an increase in TCRζ chain phosphorylation was observed (Fig. 5a). The GPI-deficient TLC showed a significant reduction in the level of TCR ζ chain phosphorylation as compared to wild-type cells. Normalization of the level of TCR ζ chain phosphorylation to the total amount of precipitated TCRζ from wild-type and mutant cells (as detected by anti-TCRζ chain mAb) reveals a 2- to 5-fold

reduction in phospho-ζ. A quantitatively similar reduction in the induction of phospho-ζ was already detectable at earlier time points after TCR stimulation indicating that phosphorylation is quantitatively decreased and not simply delayed in mutant TLC (Fig. 5b).

These results support the notion that the activation of a Src PTK may be defective in GPI-deficient TLC derived from PNH patients, and they are in agreement with the failure to induce tyrosine phosphorylation of the TCRζ chain in murine GPI-deficient T cell lymphomas and thymomas that we recently reported (18).

Localization of p56^{lck} in wild-type and GPI-deficient TLC

None of the components of the TCR contains intrinsic kinase activity. Rather, kinases are recruited to the TCR complex early upon activation. To test whether kinase recruitment to the TCR is affected by the PNH mutation, TCR capping was induced by anti-CD3ε antibody cross-linking and p56^{lck} redistribution was analyzed by confocal microscopy. Intracellular distribution of p56^{lck} in unstimulated wild-type and mutant TLC was found to be similar, with a predominant membrane localization, as previously described for normal human T cells (30) (Fig. 6). This result was confirmed by immunoblot analysis of cytosolic and membrane fractions isolated from wild-type and mutant TLC (data not shown).

Upon TCR ligation, p56^{lck} rapidly redistributed to the TCR cap in wild-type clones (Fig. 6a). Interestingly, while TCR cap formation, internalization and p56^{lck} redistribution was preserved in GPI-deficient TLC, these events occurred with delayed kinetics when compared to wild-type TLC (Fig. 6). In wild-type TLC after 1 min of activation the TCR was already partially internalized, as highlighted by the intracellular punctuated staining, and the internalization was complete by 2 min of stimulation. In contrast, in GPI-deficient TLC, the TCR caps persisted until after 5 min of stimulation. The TCR was completely internalized in mutant TLC by 10 min of stimulation (data not shown). A more detailed time course analysis of TCR capping and internalization revealed that in wild-type TLC caps were observed after 30 s of stimulation, while in GPI-deficient TLC only rare patches were seen at this time point (data not shown). Similar results were obtained when TLC 29^{+} and TLC 24^{-} were compared (data not shown).

To investigate whether the delayed kinetics of capping and internalization of the TCR reflected a general defect in surface receptor mobilization of GPI-deficient TLC, the co-receptor CD4, was induced to cap using anti-CD4 mAb. CD4 caps formed with indistinguishable kinetics in GPI-deficient and wild-type TLC (Fig. 7).

Taken together these results indicate that receptor and kinase mobilization is not generally defective in GPI-deficient T cells.

Activation of p56^{lck} in wild-type and GPI-deficient TLC

Given the predominant role of p56^{lck} in TCRζ phosphorylation (31), we next investigated whether an altered activation of p56^{lck} could explain the signaling defect observed in GPI-deficient TLC. It has previously been demonstrated that upon TCR engagement a new form of p56^{lck} appears with an apparent mol. wt of 60 kDa (p60^{lck}) (32). To analyze p56^{lck} activation in wild-type and mutant TLC we performed Western blot analysis on cell lysates using anti-p56^{lck} antibodies. Upon TCR-mediated activation, p60^{lck} induction is reproducibly decreased in TLC 26^{-} as compared to wild-type cells (Fig. 8a), indicating that activation of p56^{lck} is impaired in GPI-deficient cells. Normalization of the level of p60^{lck} to p56^{lck} in wild-type and mutant cells (as detected by anti-Lck mAb) reveals a 1.6-fold reduction in p60^{lck} induction in GPI-deficient TLC. An altered activation of p56^{lck} was also observed in TLC 24^{-} when compared to wild-type TLC (data not shown). *In vitro* kinase assay of Lck precipitates did not reveal any reproducible differences between wild-type and GPI-deficient TLC (Fig. 8b), indicating that Lck is not defective in GPI-deficient cells.

It has recently been reported that anergic T cells, which do not proliferate when stimulated by antigen because of defective ZAP-70 and Ras activation, have a constitutively increased p59^{fyn} kinase activity (33). p59^{fyn} *in vitro* kinase activity was measured in wild-type and GPI-deficient TLC, and was found comparable (Fig. 8b), suggesting that this kinase is not responsible for the hyporeactivity of GPI-deficient TLC.

Discussion

PNH is a disorder caused by somatic mutations in hematopoietic stem cells, characterized by the presence of abnormal cells of various cell lineages deficient in surface expression of GPI-anchored molecules (19). GPI-deficient T cells isolated from PNH patients display a naive phenotype and show depressed immune responses *in vitro*, suggesting that T cell activation is defective in these cells (15,16). Understanding the molecular basis of this signaling defect could give new insight in the role that GPI-anchored molecules play in TCR signal transduction. In this regard, we have recently shown that deficiencies in GPI-biosynthesis in murine T cell lymphomas and thymomas resulted in a reduced induction of the TCRζ chain and ZAP-70 phosphorylation (18). As compared to wild-type cells, TCR engagement in GPI-deficient cells leads to a significantly decreased TCR-associated kinase activity, suggesting that the activation of Src PTK may be defective.

In this report, we now provide evidence that TCR-mediated activation of p56^{lck} is defective in GPI-deficient human TLC. The reduction in p56^{lck} activation is accompanied by the induction of a quantitatively and qualitatively altered pattern of phosphorylated substrates in mutant cells, ultimately resulting in a strongly reduced proliferative response to TCR ligation. Interestingly although the extent of TCR cap formation and internalization is not dramatically affected in GPI-deficient TLC, they occur with delayed kinetics. These observations are consistent with the previously reported importance of the rate of TCR triggering in the onset of the response (34).

The defect in TCR signaling observed in GPI-deficient TLC probably reflects the importance of GPI-anchored proteins in TCR signal transduction. However, since in our system TCR ligation is achieved with soluble anti-CD3ε mAb without engagement of GPI-anchored molecules, a specific role of their ectodomain seems unlikely.

p56^{lck} plays an important role in signaling events mediated by both TCR (31) and GPI-anchored molecules (14). While

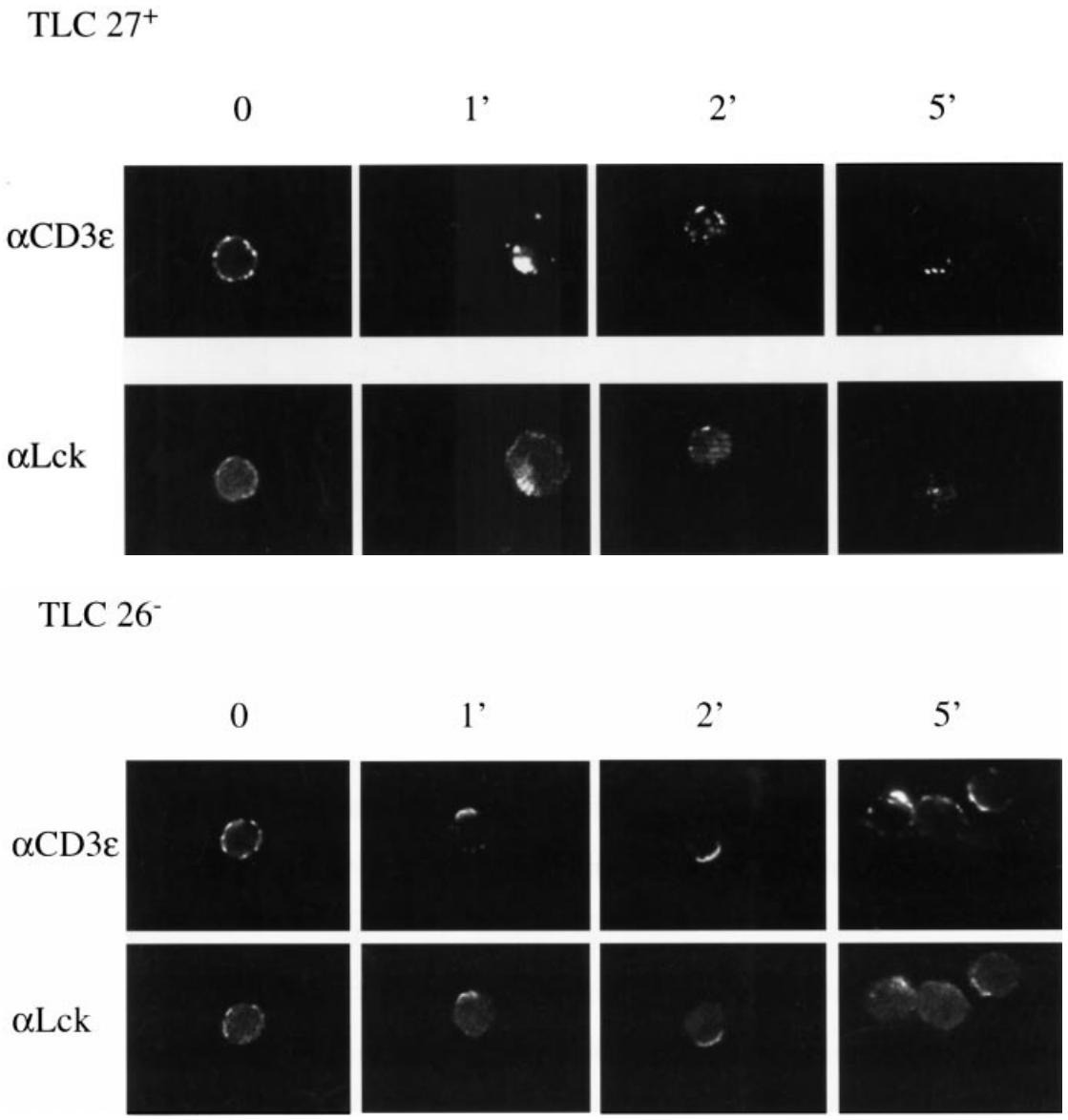

Fig. 6. p56^{lck} redistribution in wild-type (A) and GPI-deficient (B) TLC upon the formation of TCR caps. TCR capping was induced by staining the cells with anti-CD3ε mAb (OKT3) and incubating them at 37°C for the indicated time in the presence of Texas Red-conjugated donkey F(ab′)$_2$ fragment anti-mouse IgG. At each time point, cells were washed, fixed, permeabilized and subsequently stained with anti-p56^{lck} rabbit sera (p56-1). As second reagent, FITC-conjugated hamster F(ab′)$_2$ fragment anti-rabbit IgG were used.

p56^{lck} does not associate directly with the TCR, it co-precipitates with GPI-anchored molecules (14,35). Intriguingly, p56^{lck} (5) and GPI-anchored molecules (36) localize in GEM microdomains recently implicated in TCR signaling (37,38). It has been proposed that self-association of sphingolipids and cholesterol induces the formation of microdomains that separate from the more abundant glycerophospholipids (7). Strikingly, the majority of the proteins found in these domains are anchored in the membrane by lipid moieties. These include both GPI-anchored molecules and acylated signaling molecules such as p56^{lck}, p59^{fyn} (39), Ras (40) and LAT (8). Extensive mutagenesis studies have shown that the presence of fatty acids is very critical in GEM targeting and signaling function of all these molecules (4,8,41).

One of the proteins excluded from these domains is CD45 (42). CD45 exclusion from GEM may selectively regulate p56^{lck}, by constituting a reservoir of enzyme hyperphosphorylated on tyrosine that can be readily activated. The importance of membrane compartmentalization in T cell activation may thus resides in concentrating certain signaling molecules, allowing the initiation of an effective TCR-mediated signaling cascade.

It has been recently reported that several other components of the T cell signaling machinery such as Cbl, Syk, Vav, ZAP-70, phospholipase C-γ1 and TCRζ chain translocate to GEM upon TCR engagement (37,38). Interestingly, in murine thymocytes, it has been shown that Lck, ZAP-70 and TCRζ chain are present in the same GEM vesicles as GPI-anchored

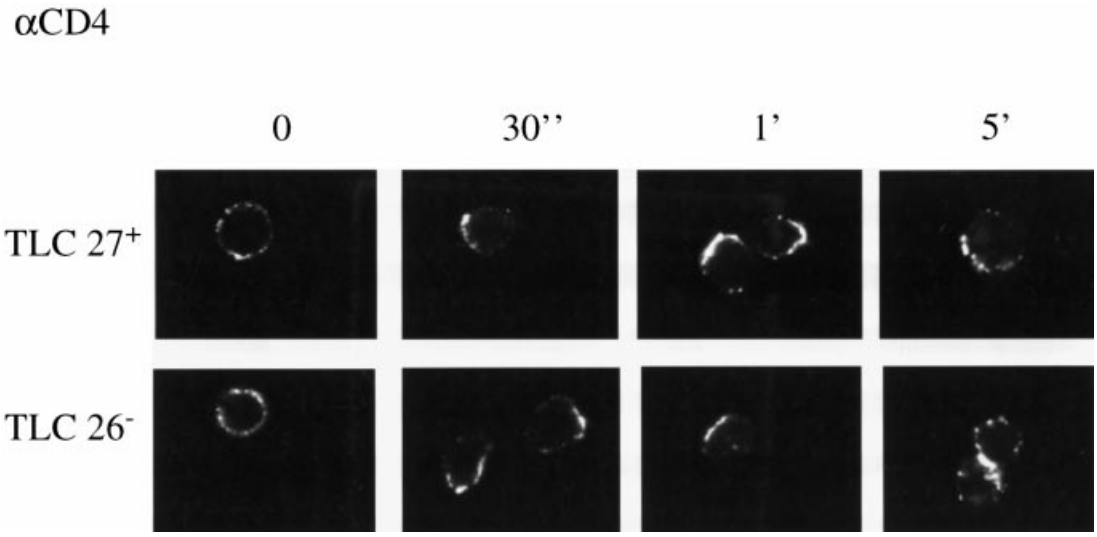

Fig. 7. CD4 capping and internalization in wild-type and GPI-deficient TLC. CD4 was induced to cap as described in the legend to Fig. 3 for the TCR using 101.69 mAb.

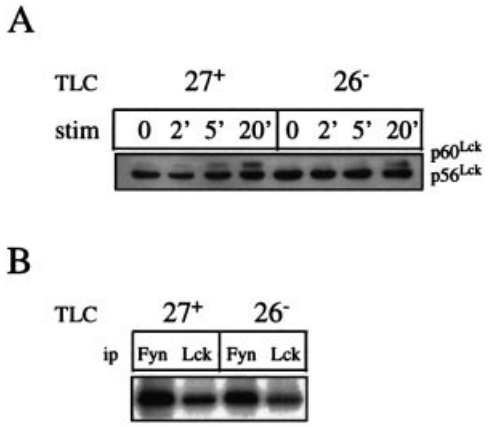

Fig. 8. p56^{lck} and p59^{fyn} activity in wild-type and GPI-deficient TLC. The appearance of p60^{lck} was monitored, in a time course experiment, by Western blot analysis of total lysates of wild-type and GPI-deficient TLC upon TCR stimulation (A). The ratio of intensity of p60^{lck} versus p56^{lck}, determined by densitometry and calculated as percentage, for TLC 27$^+$ and TLC 26$^-$, was: TLC 27$^+$: 4, unstim; 7, stim 2 min; 14, stim 5 min; 40, stim 20 min; TLC 26$^-$: 0, unstim; 0, stim 2 min; 6, stim 5 min; 24, stim 20 min. The activity of p56^{lck} and p59^{fyn} was measured by performing an *in vitro* kinase assay on p56^{lck} and p59^{fyn} precipitates from wild-type and GPI-deficient TLC (B).

molecules (38). A deficiency in GPI biosynthesis may affect this membrane microdomain and consequently TCR signal transduction. The large quantity of cells required to separate such domains has hampered so far their isolation from wild-type and GPI-deficient TLC.

At least two mechanisms can be envisaged on how surface expression of GPI-anchored molecules could affect TCR-mediated p56^{lck} activation. GEM formation might be defective in GPI-deficient TLC, leading to an alteration in p56^{lck}. The fact that CD4 cap formation is not affected in GPI-deficient TLC as compared to wild-type cells, argues against this possibility. CD4 localizes in GEM (35,43) and is directly associated with Lck (44). Taken together these results suggest that a deficiency in GPI biosynthesis does not compromise receptor activation and mobilization in GEM, suggesting that these domains are not grossly altered.

Alternatively, the interactions between proteins that localize in the glycerophospholipid membrane domains and proteins that reside in GEM could be affected in GPI-deficient T cells. Consistent with this hypothesis we observed a decreased activation of p56^{lck}, and a delay in TCR cap formation and p56^{lck} redistribution in mutant cells, suggesting that upon TCR engagement events leading to p56^{lck} activation are impaired. In this regard it is important to remember that in epithelial cells two subcompartments of GEM have been identified: caveolae, enriched in signaling molecules such as Yes, Lyn and the α subunit of G proteins, and a GPI-anchored molecule rich domain located proximally to the caveolae neck (45). If a similar structural organization is conserved in caveolin-negative cells, such as T cells, GPI-anchored molecules could play an important structural or functional role in connecting TCR and p56^{lck}.

The data presented in this paper provide evidence for a role of GPI-anchored molecules in enhancing TCR-mediated Lck activation and indicate that expression of GPI-anchored molecules is required for optimal TCR signal transduction. These findings may explain the unusually large fraction of naive T cells found among GPI-deficient T cells in PNH patients (15), as well as their decreased response to alloantigens (16).

Acknowledgements

We thank P. Schmidt for kindly providing us with blood sample from PNH patients, J. van Meerwijk and S. Valitutti for their careful reading and helpful suggestions during the preparation of this manuscript, and M. Rosseaux and F. Dubugnon for technical assistance. This work was supported by grant 3100-050713.97 from the Swiss National Foundation for scientific research to C. B.

Abbreviations

APC antigen-presenting cell

DAF	decay accelerating factor
GEM	glycolipid-enriched membrane compartment
GPI	glycosylphosphatidylinositol
HRP	horseradish peroxidase
PBMC	peripheral blood mononuclear cell
PE	phycoerythrin
PHA	phytohemagglutinin
PNH	paroxysmal nocturnal hemoglobinuria
PTK	protein tyrosine kinase
TLC	T cell clone

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