Defective recruitment and activation of ZAP-70 in common variable immunodeficiency patients with T cell defects

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We have previously identified a subset of common variable immunodeficiency (CVID) patients with defective T cell function associated with impaired activation of the TCR-dependent tyrosine phosphorylation cascade. Here we have assessed the structural and functional integrity of the principal components involved in coupling the TCR/CD3 complex to intracellular tyrosine kinases in two of these patients. We show that ZAP-70 fails to bind the signaling-competent CD3ζ tyrosine phosphorylation isoform and to become activated following TCR engagement, suggesting that defective recruitment of ZAP-70 might underlie the TCR signaling dysfunction in these patients. Determination of the nucleotide sequences encoding the intracellular domains of the CD3/ζ subunits and ZAP-70 did not reveal any mutation. Furthermore, ZAP-70 from these patients could interact *in vitro* with recombinant phospho-ζ, ruling out genetic defects at the immunoreceptor tyrosine-based activation motif/SH2 domain interface responsible for ZAP-70 recruitment to the activated TCR. No defect was found in expression, activity or subcellular localization of Lck, which is thought to be primarily responsible for CD3ζ phosphorylation. Hence, while the T cell defect in these CVID patients can be pinpointed to the interaction between ZAP-70 and CD3ζ, the integrity in the components of the signaling machinery involved in this process suggests that additional components might be required for completion of this step.

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1 Introduction

Common variable immunodeficiency (CVID) is the most frequent primary dysfunction of the immune system. This pathology comprises a heterogeneous group of disorders characterized by low levels of circulating antibody, resulting in severe impairment in immune responses and increased susceptibility to infections [1]. Lack of characterization of the pathology has obscured, until recently, the genetic nature of the disease. It is now clear that, while B cell defects are responsible for a significant proportion of CVID, other cells involved in the orchestration of the immune response can be the target of the genetic lesion [2]. T cell defects in CVID include decreased proliferative responses to mitogens, impaired cytokine production and defective CD40 ligand expression [2]. A primary lesion in the initiation of the signaling cascade triggered by the TCR and leading to T cell activation could underlie a number of these defects. Impairment of early signaling events triggered by the TCR, such as calcium flux and phospholipid metabolism, has indeed been reported [3, 4]. Furthermore, we have recently shown in a subset of CVID patients a correlation between failure of T cells to proliferate in response to TCR engagement and defective triggering of the early tyrosine phosphorylation cascade, suggesting a defective coupling of the TCR to downstream protein tyrosine kinases (PTK) in these patients [5].

The TCR/CD3 complex is functionally coupled to Src and Syk family PTK. The Src family PTK Lck is primarily responsible for phosphorylation of the tyrosine residues

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Abbreviations: CVID: Common variable immunodeficiency **PTK:** Protein tyrosine kinase **ITAM:** Immunoreceptor tyrosine-based activation motif **CP:** Connecting peptide **GST:** Glutathione S-transferase

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within specific motifs, termed immunoreceptor tyrosine-based activation motifs (ITAM), in the cytoplasmic domains of the CD3/ζ subunits. Following ITAM phosphorylation, ZAP-70 is recruited to the phosphorylated ζ and ε chains of the CD3 complex [6]. This event is essential to T cell activation, as underlined by the correlation between defective ZAP-70 recruitment to the ζ chain and impaired T cell responses to anergizing peptide antigens [7, 8]. Here we present a structural and functional analysis of the principal components responsible for coupling the TCR/CD3 complex to the intracellular PTK in T cells from two CVID patients with defective triggering of the tyrosine phosphorylation cascade [5]. The results narrow down the genetic defect in these patients to the interaction between ZAP-70 and CD3ζ.

2 Results

2.1 Normal expression and structure of the TCR/CD3 complex in CVID patients with T cell dysfunctions

The TCR/CD3 complex is composed of a TCRαβ recognition module and a CD3/ζ signaling module, which includes the γε/δε dimers and a more loosely bound ζ/ζ dimers [9]. Following ligand binding, the TCR signals the encounter with antigen to the CD3/ζ complex. A key role in this process has been recently assigned to a specific motif within the connecting peptide (CP) in the extracellular membrane-proximal domain of the TCRαβ [10, 11]. The CD3/ζ complex in turn connects the TCR to the intracellular signaling machinery through the tyrosine-phosphorylated ITAM.

Flow cytometric analysis of PBL from the two CVID patients with T cell dysfunctions showed normal levels of CD3 surface expression (Table 1), indicating a correct assembly of the TCR/CD3 complex. To assess the possibility of point mutations in the TCR/CD3 complex subunits, we determined the nucleotide sequence of the cDNA encoding the CP and transmembrane regions of the TCR α and β chains, and the transmembrane and cytoplasmic domains of the γ, δ, ε and ζ chains. Neither non-conservative nor conservative substitutions were found in these patients as compared to disease or healthy controls (data not shown). Hence these patients have no intrinsic defect in the TCR/CD3 complex.

2.2 Defective interaction between ZAP-70 and phospho-ζ in CVID patients with T cell dysfunctions

The CD3ζ chain is primarily responsible for ZAP-70 recruitment to the activated TCR. In resting cells, CD3ζ is found both as an unphosphorylated 16 kDa molecule and as a 21 kDa isoform resulting from phosphorylation of two tyrosine residues localized within distinct ITAM [12, 13]. ZAP-70 binds weakly to p21ζ, however this interaction does not involve its SH2 domains and is not sufficient for activation [13]. TCR triggering results in full phosphorylation of the CD3ζ ITAM, with the generation of a p23 kDa isoform [12, 13] which can productively recruit ZAP-70 [7, 8]. To test the interaction between ZAP-70 and phospho-ζ in CVID T cells, ZAP-70 was immunoprecipitated from lysates of PBL either non-stimulated or activated by treatment with anti-CD3 mAb. The immunoprecipitates were subsequently probed with anti-phosphotyrosine antibodies. While ZAP-70 interac-

Table 1. Immunophenotyping of PBL from CVID patients

	CD3		CD4		CD8	
	%[a)]	MFI[b)]	%[a)]	MFI[b)]	%[a)]	MFI[b)]
Control	83.8±2	235.4±4.9	44.6±0.2	260.4±56.0	33.0±4.7	802.0±56.0
Patient 1	81.5	190.7	31.4	253.5	54.1	877.6
Patient 2	74.4	211.5	28.7	266.0	45.5	686.1
Patient 3	90.8	211.8	34.1	284.6	56.6	786.7
Patient 4	87.7	243.2	28.1	326.8	55.5	908.6

a) % of peripheral blood lymphocytes.
b) Mean fluorescence intensity obtained in a two-color flow cytometric analysis of PBL from two representative healthy donors and the four CVID patients.

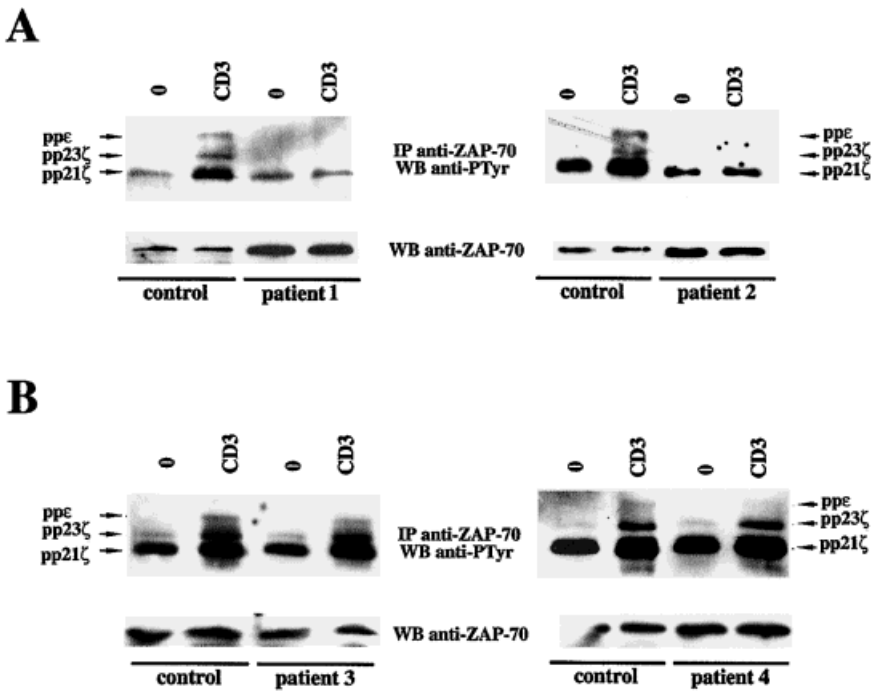

Fig. 1. Defective interaction between p23ζ and ZAP-70 in T cells from CVID patients with impaired T cell function. Anti-phosphotyrosine immunoblot of ZAP-70-specific immunoprecipitates from lymphocyte lysates of CVID patients and healthy donors, either resting or following activation with anti-CD3 mAb (top panels). After stripping, the filters were reprobed with anti-ZAP-70 mAb (bottom panels). The migration of molecular mass markers is shown. IP, immunoprecipitiation; WB, Western blot.

tion with p21ζ was unaffected, ZAP-70 failed to interact with p23ζ in T cells from the CVID patients with defective T cell function, as opposed to disease controls (Fig. 1). Interestingly, also ZAP-70 binding to tyrosine-phosphorylated CD3ε was abrogated in these patients (Fig. 1).

A defect either in phosphorylation of the CD3ζ ITAM or in the ZAP-70 SH2 domains could underlie the impairment in ZAP-70 recruitment to the activated TCR. Determination of the nucleotide sequence of the cDNA encoding the CD3ζ intracellular domain and ZAP-70 failed to reveal any mutation (data not shown), indicating the integrity of the interaction modules on these molecules. Furthermore, *in vitro* binding experiments on lysates of CVID lymphocytes using a glutathione S-transferase (GST)/CD3ζ fusion protein, phosphorylated using purified Lck, showed that ZAP-70 from the two CVID patients with T cell defects bound tyrosine-phosphorylated GST-CD3ζ (Fig. 2), but not GST or unphosphorylated GST-ζ (data not shown), demonstrating that the capacity of the ZAP-70 SH2 domains to interact with tyrosine-phosphorylated CD3ζ ITAM was unaffected in these patients.

2.3 Defective activation of ZAP-70 in CVID patients with T cell dysfunctions

Since ZAP-70 is activated only when bound to p23ζ [7, 8], no ZAP-70 activation should be detected in defective CVID T cells in response to TCR engagement. To test this point, the kinase activity of ZAP-70 was determined on ZAP-70-specific immunoprecipitates in *in vitro* kinase assays. No induction of ZAP-70 activity was detected in

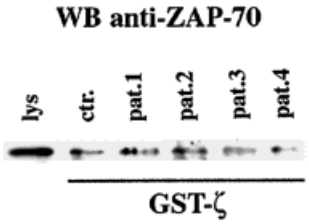

Fig. 2. ZAP-70 from CVID patients with T cell defects can bind phospho-ζ *in vitro*. Anti-ZAP-70 immunoblot of the proteins bound *in vitro* to a tyrosine-phosphorylated GST-ζ fusion protein. The migration of ZAP-70 in a total cell lysate separated on the same gel is shown.

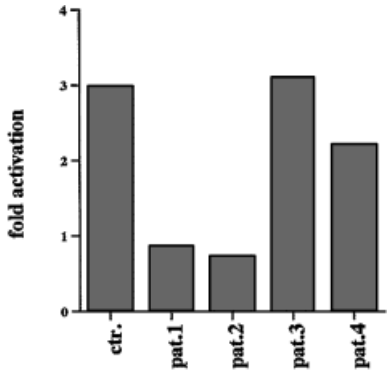

Fig. 3. Defective activation of ZAP-70 in CVID patients with impaired T cell function. Quantitation of ZAP-70 autophosphorylation in *in vitro* kinase assays of ZAP-70-specific immunoprecipitates from lymphocyte lysates of CVID patients and healthy donors, either resting or following activation with anti-CD3 mAb. After the kinase reaction, immunoprecipitates were subjected to SDS-PAGE, transferred to a nitrocellulose filter and exposed and analyzed using a Phosphorimager. The filter was subsequently probed with anti-ZAP-70 mAb. The levels of immunoprecipitated ZAP-70 were quantitated by laser densitometry. The levels of ^{32}P incorporation by ZAP-70 in each immunoprecipite were normalized to the respective levels of ZAP-70. The data show the ratio of ZAP-70 autophosphorylation in activated *vs.* resting cells.

defective CVID T cells (Fig. 3, pat. 1 and pat. 2), as opposed to disease control T cells (Fig. 3, pat. 3 and pat. 4). The basal activity of ZAP-70 was unaffected in these patients (data not shown), suggesting that defective ZAP-70 activation in CVID T cells is not due to an intrinsic defect in ZAP-70 kinase activity, in agreement with the lack of mutations in the cDNA encoding ZAP-70 in these patients (data not shown). Collectively, the data suggest that the defective T cell response to TCR triggering in the CVID patients with impaired T cell function is a consequence of defective recruitment and activation of ZAP-70.

2.4 Normal expression, activity and subcellular localization of Lck in CVID patients with T cell dysfunctions

The data presented suggest, albeit indirectly, that a defective phosphorylation of the ζ chain ITAM might underlie the defective recruitment and activation of ZAP-70; however, we have been unable to directly test this hypothesis because of the too large number of cells required. Current evidence strongly supports the notion that Lck is the primary kinase involved in phosphorylation of the CD3/ζ ITAM [6], making it a likely candidate for

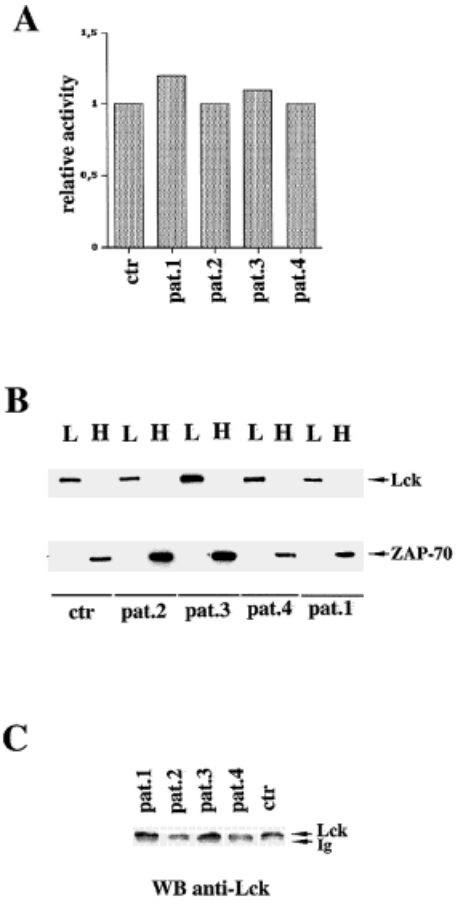

Fig. 4. Normal Lck activity, subcellular localization, and association with CD4 in CVID patients with impaired T cell function. (A) Basal Lck activity in CVID and and healthy control lymphocytes, as determined by quantitation of Lck autophosphorylation in *in vitro* kinase assays of Lck-specific immunoprecipitates from lymphocyte lysates of CVID patients and healthy donors. The data show the ratio of Lck autophosphorylation in CVID cells compared to control cells. (B) Anti-Lck (top) and anti-ZAP-70 (bottom) immunoblots of the proteins specifically enriched in the detergent-resistant fraction containing the lipid rafts (L, pooled light fractions) or in the detergent soluble fraction (H, pooled heavy fractions), following fractionation of total postnuclear cell lysates of CVID or healthy donor lymphocytes by sucrose gradient centrifugation. (C) Anti-Lck immunoblot of CD4-specific immunoprecipitates from lysates of CVID or healthy donor lymphocytes. The migration of Lck in a total T cell lysate separated on the same gel is shown.

the signaling defect in the two CVID patients with impaired T cell function. The activity of Lck was determined in *in vitro* kinase assays on Lck-specific immunoprecipitates. As shown in Fig. 4A, the activity of Lck was unimpaired. Furthermore, no mutation in the cDNA encoding Lck was detected (data not shown), suggest-

ing that the signaling defect does not result from any intrinsic defect in Lck.

Recent data have highlighted the importance of detergent-resistant membrane microdomains, or lipid rafts, in signal transduction. A variety of signaling molecules are constitutively localized in lipid rafts, including Src family kinases, small GTP-binding proteins such as Ras, and adaptors such as LAT. Furthermore, the TCR/CD3 complex, as well as its associated molecules, such as ZAP-70, inducibly localizes to the rafts following TCR stimulation [14, 15]. The subcellular localization of Lck in CVID T cells was determined. Rafts were purified from resting CVID lymphocyte lysates by sucrose gradient centrifugation and probed by immunoblot with anti-Lck or anti-ZAP-70 antibodies. As shown in Fig. 4B, Lck was found mostly localized in membrane rafts, as opposed to ZAP-70, indicating that it is correctly post-translationally modified in CVID T cells to reach the appropriate membrane localization.

Association of Lck with CD4 is an essential requirement for T cell activation [6]. As shown in Table 1, despite the inverted CD4/CD8 T cell ratio in most CVID patients, no difference could be detected in the levels of surface CD4 expression as compared to control T cells. Probing CD4-specific immunoprecipitates from CVID T cells with anti-Lck antibodies showed that similar levels of Lck were associated with CD4 in T cells from all CVID patients, ruling out a limited availability of CD4/Lck complexes (Fig. 4C). Furthermore, *in vitro* kinase assays on CD4-specific immunoprecipitates showed similar levels of CD4-associated kinase activity in all patients (data not shown).

3 Discussion

Recruitment of ZAP-70 to the p23ζ is a central feature in TCR signaling. The biochemical defect underlying the anergizing activity of altered peptide ligands has indeed been traced to a defective phosphorylation of the ζ chain, which results in dramatically reduced levels of p23ζ and lack of ZAP-70 recruitment [7, 8]. The data presented in this report show a clear correlation between T cell dysfunctions in CVID patients and failure of ZAP-70 to associate with p23ζ, which is reflected in a severe impairement in the TCR-inducible activation of ZAP-70. This signaling defect closely parallels the outcome of T cell treatment with anergizing peptides. Of note, p21ζ interaction with ZAP-70 in these patients was unaffected. Although ZAP-70 can interact with p21ζ, this interaction is not sufficient for ZAP-70 activation [13]. Hence our data reiterate a key role of p23ζ in ZAP-70 recruitment and activation, and suggest that a defect in that specific and initial step in TCR signaling might underlie the T cell dysfunction in these patients.

The integrity of ZAP-70 in the CVID patients with T cell defects points to a defect in ζ chain phosphorylation as the most likely cause of the impairment in ZAP-70 recruitment to the activated TCR. Although we have been unable to directly assess the state of ζ chain phosphorylation, due to the large number of T cells required, our data collectively support the notion that ζ fails to be correctly phosphorylated in defective CVID T cells. First, a dramatic reduction in TCR-inducible phosphoproteins was detected in these patients [5]. Secondly, together with the absence of p23ζ, a concomitant loss of tyrosine-phosphorylated CD3ε from ZAP-70-specific immunoprecipitates was observed. Thirdly, no mutation was detected either in the ζ chain cytoplasmic domain or in the intracellular domains of the CD3 subunits, ruling out intrinsic defects in the ITAM. Hence a defect in the PTK responsible for ζ chain phosphorylation is likely to be indirectly responsible for the defective interaction between p23ζ and ZAP-70 in CVID T cells.

ζ Chain phosphorylation is achieved by a Src family PTK. Although both Lck and Fyn can potentially phosphorylate CD3ζ [16], current evidence strongly supports a primary role for Lck in this process [6]. While the signaling defect in the two CVID patients with impaired T cell function is highly reminiscent of an Lck-defective phenotype, no intrinsic defect in Lck was found in these patients. Other factors implicated in the regulation of Lck, including its association to CD4 and its localization in lipid rafts, were unaffected. Another potential candidate is CD45, a tyrosine phosphatase which regulates Lck activity [17]. T cells from all CVID patients included in this study showed normal numbers of both $CD45RA^+$ and $CD45RO^+$ T cells, as well as high levels of CD45 expression (D'Elios and Baldari, unpublished data), however a defect in CD45 activity cannot be ruled out at this stage. Alternatively, the regulation of Lck might be fully operational, but the generation of p23ζ in normal human T cells might require the combined action of Lck and another PTK, such as Fyn. In this context, it should be noted that altered peptide ligands do not affect the generation of p21ζ, which is the precursor of p23ζ [13], suggesting that the regulated sequence of phosphorylation of the ζ chain ITAM might be achieved by sequential recruitment of distinct PTK. Furthermore, triggering of the early PTK cascade following TCR engagement was recently shown to occur in T cells from an infant with severe combined immunodeficiency, notwithstanding a significantly reduced expression of Lck [18], supporting the potential cooperation of PTK in phosphorylation of the CD3/ζ subunits.

4 Materials and methods

4.1 Patients

Patients were classified as CVID according to the WHO classification of primary immunodeficiencies [1]. The hematological and immunophenotypical characterization of the patients used in this study has been previously presented [5]. Four out of the six patients initially recruited (patients 1, 2, 4, 5) agreed to participate in the present study. Patients 3 and 4 correspond to patients 4 and 5 of the previous study [5]. Controls were sex- and age matched healthy volunteers. Peripheral blood was obtained after informed consent and sample size was kept small according to the guidelines of the ethical committee. No overt infectious disease was present at the time of blood sampling. To limit sampling from the patients, PHA T cell lines were derived from each patient following standard procedures. These lines were found to be heavily skewed towards the $CD8^+$ phenotype (data not shown) and were thus used only as a source of RNA. PBMC were isolated from whole blood by density centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech Italia, Milan) and subsequently depleted of macrophages by adherence. Immunophenotyping was carried out by two- or three-color flow cytometry using fluorochrome-conjugated mAb from Becton Dickinson (San Jose, CA).

4.2 Antibodies and GST fusion proteins

Polyclonal antisera against ZAP-70 and Lck and mAb against ZAP-70, phosphotyrosine and CD3ζ were purchased from Upstate Biotechnology Inc. (Boston, MA) and Santa Cruz Biotechnology (Santa Cruz, CA). An mAb suitable for immunoprecipitation of tyrosine-phosphorylated CD3ζ was kindly provided by M. Baniyash. Peroxidase-conjugated secondary antibodies were purchased from Amersham Pharmacia Biotech. Anti-CD3 (OKT3) and anti-CD4 (M55; a kind gift of F. Celada) mAb were affinity purified on protein G from hybridoma supernatants and titrated by flow cytometry. A mouse anti-Lck mAb was generated according to standard procedures using a GST fusion protein encoding amino acids 2–191 of human Lck.

4.3 Activations, immunoprecipitations, immunoblots and *in vitro* binding and kinase assays

Cell activations and lysis, immunoprecipitations, *in vitro* binding assays and immunoblots were carried out as described [19]. The GST-ζ fusion protein was phosphorylated *in vitro* using partially purified Lck (Upstate Biotechnology) in 10 μM Tris-HCl pH 7.6, 10 μM $MgCl_2$, 10 μM $MnCl_2$, 200 μM ATP for 20 min at room temperature, and washed extensively in PBS, 0.2 mg/ml sodium orthovanadate. Immune complex *in vitro* kinase assays on ZAP-70, Lck or CD4-specific immunoprecipitates were carried out as described, using [γ–^{32}P]ATP [19]. Immunoprecipitates were subsequently washed, separated by SDS-PAGE, transferred to nitrocellulose filters and exposed to a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The filters were then probed by immunoblot to check the levels of specific protein immunoprecipitated in each sample. Quantitation of the individual bands revealed by immunoblot was carried out by laser densitometry (Computing Densitometer 300S Molecular Dynamics). The levels of incorporated ^{32}P were normalized to the levels of specific protein in each immunoprecipitate.

4.4 Lipid raft purification

Lipid rafts were purified according to a modification of the protocol described in Montixi et al. [15]. Briefly, cells ($3x10^7$/sample) were washed twice with PBS and resuspended in 450 μl 25 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, in the presence of 0.2 mg/ml sodium orthovanadate, 1 μg/ml leupeptin, aprotinin and pepstatin and 10 mM PMSF (Buffer A). Cells were kept on ice for 30 min, disrupted with ten strokes of a Dounce homogenizer and 50 μl Brij-58 (10% in Buffer A) was added. Sucrose gradient centrifugation and protein concentration were carried out as described [15].

4.5 RT-PCR, cDNA sequencing and construction of chimeric cDNA encoding GST fusion proteins

Total RNA was extracted from either T cell lines or PBL using a kit from Qiagen (Germany). Reverse transcription (RT)-PCR was carried out using kits from Takara Shutzo Co. (Shiga, Japan) and Sigma (Saint Louis, MI). The primer for the first strand was oligo-dT, while pairs of specific primers were used for cDNA amplification. cDNA were amplified in a Perkin-Elmer 2400 Thermal Cycler (Norwalk, CT). PCR products were separated by agarose gel electrophoresis and recovered using a kit from Qiagen. Automatic sequencing was performed on both DNA strands on the RT-PCR products from three CVID patients (patients 1, 2 and 4) and two independent healthy controls. The nucleotide sequences of Cα, Cβ, CD3γ, CD3δ, CD3ε and ZAP-70 were identical to the published sequences. Both conservative and non-conservative substitutions as compared to published sequences were consistently found in the nucleotide sequences of CD3ζ and Lck cDNA. Specifically, when compared to the published sequences, the cDNA encoding Lck from the three patients and the two healthy controls all had four conservative (A160, G161, S162, Q172) and one non-conservative (P87Q) substitutions, while the cDNA encoding the CD3ζ precursor all had two non-conservative (E60D, A61P) substitutions. The cDNA sequences have been submitted to Genbank and are available under the accession numbers AF228312 (CD3ζ) and AF228313 (Lck). To engineer GST fusion proteins, the cDNA encoding amino acid residues 2–191 of Lck and residues 52–163 of CD3ζ were obtained by RT-PCR from Jurkat T cell total mRNA and cloned in the bacterial expression vector pGEX-2T (Amers-

ham Pharmacia Biotech). In both cases the primers were designed to provide the RT-PCR product with a BamHI site at the 5' end, and with an in-frame termination codon and an EcoRI site at the 3' end. GST fusion proteins were affinity purified from bacteria transformed with the pGEX constructs using Glutathione Sepharose™ 4B (Amersham Pharmacia Biotech).

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