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Specific Deficiency of p56\textsuperscript{\textit{lek}} Expression in T Lymphocytes from Type 1 Diabetic Patients\textsuperscript{1}

Solange Nervi,* Catherine Atlan-Gepner,* Brigitte Kahn-Perles,† Patrick Leclerc,† Bernard Viallettes,* Jean Imbert,† and Philippe Naquet\textsuperscript{2‡}

Peripheral T lymphocyte activation in response to TCR/CD3 stimulation is reduced in type 1 diabetic patients. To explore the basis of this deficiency, a comprehensive analysis of the signal transduction pathway downstream of the TCR/CD3 complex was performed for a cohort of patients ($n = 38$). The main result of the study shows that T cell hyporesponsiveness is positively correlated with a reduced amount of p56\textsuperscript{\textit{lek}} in resting T lymphocytes. Upon CD3-mediated activation, this defect leads to a hypophosphorylation of the CD3-\gamma chain and few other polypeptides without affecting the recruitment of ZAP70. Other downstream effectors of the TCR/CD3 transduction machinery, such as phosphatidylinositol 3-kinase p85\textsubscript{a}, p59\textsuperscript{fyn}, linker for activation of T cells (LAT), and phospholipase C-\gamma\textsubscript{1}, are not affected. In some patients, the severity of this phenotypic deficit could be linked to low levels of p56\textsuperscript{\textit{lek}} mRNA and resulted in the failure to efficiently induce the expression of the CD69 early activation marker. We propose that a primary deficiency in human type 1 diabetes is a defect in TCR/CD3-mediated T cell activation due to the abnormal expression of the p56\textsuperscript{\textit{lek}} tyrosine kinase. The Journal of Immunology, 2000, 165: 5874–5883.

1 Type 1 diabetes (1) is a T cell-mediated autoimmune disease leading to the destruction of pancreatic islet \beta cells. The severity of the disease is influenced by environmental and genetic factors, which may either delay or accelerate the onset of the overt disease (2, 3). Among the putative loci of predisposition identified, several genes regulating immune responses are candidates (4). The major susceptibility locus is linked to the MHC region, the expression of specific MHC alleles being necessary but not sufficient for the appearance of diabetes in rodents and humans (5). The involvement of other predisposition genes is suspected to explain the numerous immunological abnormalities shared in different models of type 1 diabetes. In humans, one of these immune phenotypes is characterized by low proliferation and poor cytokine production by T lymphocyte in response to TCR/CD3-mediated agonists in vitro (6, 7). The reduced T cell activation can be partially compensated by additional costimulatory signals, such as combinations of CD2/CD28 mAbs or exogenous cytokines. These results are in favor of a constitutive defect in the TCR/CD3 transduction pathway. A comparable phenotype has been described in the nonobese diabetic (NOD)\textsuperscript{3} mouse model for which thymocyte proliferation was used as a readout of T lymphocyte hyporesponsiveness (8). In this model, a normal in vitro proliferative response can be recovered by the addition of exogenous IL-4, and systemic IL-4 injection in disease-prone female NOD mice can prevent the development of diabetes (9, 10). The development of diabetes can also be abrogated by the injection of proinflammatory cytokines such as TNF (11–13) or by intercurrent infections (14). All these observations support the notion that a deficient rather than an exaggerated T cell response might predispose to the development of type 1 diabetes (15).

Several groups undertook a more refined exploration of the signal transduction pathway downstream of the TCR/CD3 complex in mice and humans. As for several receptors, early events in T cell activation require the recruitment of protein tyrosine kinases (PTK). In T lymphocytes, the coengagement of the TCR/CD3 complex with the CD4 or CD8 coreceptors brings the p56\textsuperscript{\textit{lek}} tyrosine kinase in the vicinity of the CD3 chains (16, 17). This kinase phosphorylates the CD3\gamma immunosyntyrosine activation motifs allowing the docking of the Syk-family tyrosine kinase ZAP70, which in turn phosphorylates neighboring linker proteins such as linker for activation of T cells (LAT). Adaptor proteins accumulate on this scaffold and recruit several key effector enzymes such as phospholipase C-\gamma\textsubscript{1} (PLC-\gamma\textsubscript{1}) or the p21rass GTPase. Further downstream events will trigger a \textit{Ca}\textsuperscript{2+} influx and activation of PKC and mitogen-activated protein kinases (MAPK). Whereas full T cell activation requires the simultaneous activation of all these pathways, several reports described that a partial activation signal can trigger a state of T cell unresponsiveness or skew the engagement of effector functions (18, 19). Although different signal transduction mechanisms are involved, incomplete activation signals can be due to a less efficient recognition of MHC-bound peptides, to a lack of engagement of costimulatory molecules such as CD28, or to the sequestration of the coreceptor as described for the CD4 molecule by the gp120 molecule in HIV-infected patients (20). In

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\textsuperscript{3} Abbreviations used in this paper: NOD, nonobese diabetic; LAT, linker for activation of T cells; MAPK, mitogen-activated protein kinase; PI3, phosphatidylinositol 3; PLC, phospholipase C; PTK, protein tyrosine kinase.

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the NOD mouse, the defective T cell activation is associated with a impairment of the p21V-RF/MAPK pathway due to the reduced recruitment of the Grb2-sos complex to the cell membrane (21). Although the mechanisms might not be directly related, the same authors described the enhanced engagement of the p59fyn and p56lck pathway and a sequestration of the CD4-associated p56lck from TCR/CD3 complexes (22). All these modifications of the TCR/CD3-dependent activation cascade seem to locate a putative defect during the early stages of NOD thymocyte activation. We have recently characterized a cohort of diabetic patients in whom a similar defect in TCR/CD3-mediated activation was documented (7).

In this study, a more systematic analysis of the early activation stages in peripheral T cells was performed. The main result of the study shows that the T cell hyporesponsiveness is positively correlated with a reduced amount of p56lck in some patients, this deficit could be attributed to reduced levels of p56lck mRNA.

Materials and Methods

Patient and control groups

A group of 38 patients fulfilling the American Association of Diabetes classification criteria for type I diabetes (4) (age 36 ± 10 years, range 15–56 years, 19 female and 19 male) was studied. They were all insulin treated, the mean diabetes duration was 12.9 ± 6.1 years, and the mean HbA1c level was 7.5 ± 0.9%. These patients did not receive any medication susceptible to influence lymphocyte function. A group of 13 type 2 diabetic patients (age 58.9 ± 8.5 years, range 44–69 years, 10 male and 3 female, mean diabetes duration 11.7 ± 6.1 years, mean HbA1c 7.3 ± 1.6%) was also included. Seven of them were treated with insulin. A group of 24 healthy volunteers (age 34.5 ± 10 years, range 17–53 years, 18 female and 6 male) without any familial history of autoimmunity was analyzed as controls. None of the diabetic patients or control subjects suffered at the time of the enrollment from any acute or chronic infectious disease. The local Ethics Committee approved the protocol of the study, and informed consent was obtained from the participating subjects. In the type 1 diabetic patients will be referred as patients.

Cells and Abs

PBMCs were obtained by Ficoll-Hypaque density centrifugation from heparinized peripheral venous blood (30 ml). Cells were rested overnight in RPMI 1640 supplemented with 4 mM glutamine and 10% heat-inactivated FBS, and the nonadherent cells were collected. This T cell-enriched population contained >85% CD3+, <3% CD14+, and <4% CD19+ cells, as determined by flow cytometric analysis (Becton Dickinson, Mountain View, CA), and the percentage of CD3+ cells was comparable in patient and control populations. Furthermore, in several cases, a more complete cytometric analysis was performed using activation or differentiation T cell markers defined by mAbs to CD25, CD26, CD62L, CD45RA, CD45RO epitopes (Immunotech, Luminy, France/Becton Dickinson). Results obtained from 10–20 patients confirmed the absence of preactivated T lymphocytes at the time of the assay, and no increase of memory vs naive T cell populations over controls (data not shown). The purified 289 anti-human CD3ε mAb used for cell stimulation was obtained from Dr. A. Moretta (Cancer Institute, Genova, Italy), mAbs or polyclonal Abs to p56lck (3A5), p59fyn (15), TCRγ (6B10.2), and phosphatidylinositol 3-kinase subunit of PI3-kinase (Tebu, France). Abs to LAT, PLC-γ1, and phosphatase inhibitors (Boehringer Mannheim, Indianapolis, IN). In some experiments, Triton X-100 or Brij 96 replaced Nonidet P-40. Lysates were clarified by centrifugation at 13,000 × g for 15 min at 4°C, and the protein concentration of cell lysate was evaluated using the Bradford assay (Bio-Rad Laboratories, Richmond, CA). Samples (corresponding to 25 μg of proteins) were boiled for 5 min in 2% reducing sample buffer. Alternatively, equivalent numbers of cells were directly boiled in SDS buffer (2% SDS, 0.125 M Tris-HCl, pH 6.8, 20% glycerol, 1% 2-ME) to allow a complete solubilization and to avoid in vitro proteolysis. Samples were resolved on 10% SDS-PAGE and transferred on polyvinylidene difluoride membranes (Millipore, Bedford, MA). After saturation in 0.1% Tween-20, 5% BSA, the membranes were blotted using relevant primary and secondary Abs for 1 h, and bound Abs were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

For immunoprecipitation, 500 μg of precleared Nonidet P-40 cell lysates were incubated with 5 μl of rabbit polyclonal Ab or with 10 μg of mAb at 4°C for 2–16 h, followed by addition of 100 μl of 40% slurry of protein A/G agarose (Amersham Pharma, Piscataway, NJ) for 1 h at 4°C. Agarose-bound immune complexes were washed three times in lysis buffer, solubilized for 5 min in sample buffer, loaded on 12.5% SDS-PAGE, and processed as above. In some experiments, membranes were stripped in 100 mM 2-ME, 2% SDS, 62.5 mM Tris-HCl (pH 6.7) for 30 min at 50°C, washed extensively, reblocked, and reprobed with appropriate control mAbs. Autoradiogram quantification was performed by densitometry with a BioImage analyzer (BioImage, Ann Arbor, MI). Because the amount of the p85α subunit of PI3-kinase was comparable between all assays, it was used in each experiment as the normalization standard to evaluate the amount of p56lck, p59fyn, LAT, and PLC-γ1.

In vitro proliferation assays and flow cytometry

For TCR/CD3 activation, PBMCs were seeded at 5 × 10⁵ cells/ml on anti-CD3 (10 μg/ml) or PBS-coated wells. For proliferation analysis, cells were pulsed on day 3 with 1 μCi of [3H]thymidine (Amersham) for the last 8 h, and harvested on glass fiber filters. Incorporation of radioactivity was measured using a Matrix Cell Counter (Packard, Zurich, Switzerland). For triplicate wells, the SEM was always <10% of the mean. For analysis of CD69 expression, cells were stimulated with anti-CD3 or with PMA (20 ng/ml) plus ionomycin (1 μg/ml), harvested at 24 h, and stained with an anti-CD69 PE mAb.

Confocal microscopy

Cells (10⁶/ml) were added at room temperature on polylysine-coated coverslips (30 min), saturated with 0.2% BSA (10 min), fixed in 3.7% paraformaldehyde in PBS (20 min), washed, incubated in 50 mM PBS/NH₄Cl (10 min), and permeabilized in Triton X-100 (5 min). Nonspecific staining was blocked by incubation in PBS/5% human serum (40 min). Samples were incubated with primary Ab diluted in PBS/5%/FCS (20 min), washed, and fluorescently labeled with the secondary Ab (20 min). Additionally, the nuclei were stained with 4′,6-diamidino-2-phenylindole D (diluted 1/40 in PBS; Sigma, St. Louis, MO) for 1 h. Cells were washed and mounted onto a glass slide. Optical sections (0.45 μm) were collected using a TCS 4D Leica laser-scanning confocal microscope (Heidelberg, Germany). Microscope settings were adjusted so that black level values were obtained with a mouse IgG1 isotypic control. Data are presented as individual optical sections.

Preparation of RNA, cDNA, and RT-PCR

RNA was isolated from 5 × 10⁶ cells using Trizol reagent (Life Technologies) and converted to cDNA using oligo(dT) primers (Promega, Madison, WI) and Moloney murine leukemia virus reverse transcriptase (Life Technologies). An aliquot of the resulting cDNA (1/250) was used as template and subjected to the PCR in a mixture containing 50 μM of each primer, 1 U of Taq DNA polymerase (Life Technologies), p56lck cDNA was amplified using three pairs of primers (Table I: 1F/1533R, 551/831R, and 1Oo fTaq primer,1Uo fPCR) was used in each experiment as the normalization standard to evaluate the relative levels in detergent-soluble fractions and PBMC proliferative response. The PCR products were separated by electrophoresis in 1% agarose gel and revealed by ethidium bromide staining.

Statistical analysis

The unpaired Student’s t test was used to analyze the differences between control and patient groups. Linear regression and correlation coefficient (r) were determined to analyze the relationship between p56lck relative levels in detergent-soluble fractions and in whole cell extracts, as well as between proliferative relative levels in detergent-soluble fractions and PBMC proliferative response. The χ² test was used to estimate the statistical significance of 2 × 2 contingency tables. Values of p < 0.05 were considered as statistically significant in all used tests.
Results

Altered pattern of protein tyrosine phosphorylation in PBMCs from type 1 diabetic patients

Early events in TCR/CD3 stimulation result in the activation of well-defined PTKs, which leads to the recruitment of downstream effector proteins (17). To investigate the molecular basis for the lymphocyte hyporesponsiveness from type 1 diabetic patients (6, 7, 23), PBMC were activated with an anti-CD3 mAb, and the global pattern of tyrosine phosphorylation was analyzed in cell lysates. Differences were observed both in resting and activation conditions. In resting state samples, phosphoproteins in the range of 30 and/or 70 kDa were detected in some type 1 diabetic patients (for the 30-kDa band, compare lanes 1 and 5 in Fig. 1A, lanes 1 and 4 in Fig. 1B; for the 70-kDa band, compare lanes 11 and 13 in Fig. 1C). These phosphoproteins, not necessarily associated, were observed in 47% of the patients tested (9/19) vs 7% of control subjects (1/11) (χ² = 6.18, p < 0.025). To test the link with type 1 diabetes, the basal phosphorylation pattern of type 2 diabetic patients was determined, and only one of seven individuals presented a hyperphosphorylated profile. We also tested whether insulin per se might be responsible for this result. No effect of insulin was observed in a nondiabetic volunteer who has received i.v. insulin as well as in type 2 diabetic patients treated by insulin (data not shown). Alternatively, the presence of these hyperphosphorylated polypeptides in resting samples from diabetic patients might be due to variations in the relative percentage of resting/activated memory T lymphocytes. A cytofluorometric analysis of most individuals performed at the time of the biochemical assay showed no significant difference between the percentage of activated (CD25+) or CD69+, or resting vs memory (CD62L+/CD45RA+/ CD45RO−) lymphocytes from diabetic and control individuals. However, we could not formally exclude that some of these hyperphosphorylated bands might be due to contaminating non-T cells in the assay.

After T cell activation, most control subjects showed enhanced protein phosphorylation (Fig. 1) in the range of 130, 95, 55–65, 36–40, and 20 kDa corresponding to previously described downstream effector proteins (24, 25). Similar modifications were also found in samples from patients precluding delineation of a global defect following TCR/CD3 ligation correlated with the T lymphocyte hyporesponsiveness. However, a survey involving 19 patients evidenced interindividual variations. Taken globally, a 15- to 20-kDa polypeptide corresponding to CD3ζ was less efficiently or less durably phosphorylated in 59% of the patients tested (10/17) vs 9% of control subjects (1/11) (χ² = 6.93, p < 0.01). Examples are shown in Fig. 1 for patients 2 (Fig. 1A, lanes 6–8), 3 (Fig. 1B, lanes 5 and 6), 4 (Fig. 1B, lanes 8 and 9), and 20 (Fig. 1C, lanes 13 and 14). In addition, three bands (33, 36, and 40–42 kDa) were less phosphorylated in 70% of the patients tested (12/17) vs none of control subjects (0/11) (χ² = 13.69, p < 0.0005). Examples are documented concerning hypophosphorylation (patient 18, Fig. 1C, lane 6; 22, Fig. 1C, lane 10; 20, Fig. 1C, lane 14), or transient phosphorylation (patient 3, Fig. 1B, lanes 5 and 6; 4, Fig. 1B, lanes 8 and 9). Because these polypeptides might correspond to CD3ζ, LAT, and MAPK, which are early targets of TCR/CD3-associated PTKs, this suggested a possible defect in PTK function and prompted us to analyze the CD3ζ-associated ZAP70 molecule.

Differential recruitment of phosphotyrosine proteins upon stimulation via TCR/CD3 in patients

Upon TCR/CD3 activation, the CD3ζ-chain is tyrosine phosphorylated on immunotyrosine activation motifs by the coreceptor-associated p56lck kinase (26) and recruits the cytosolic ZAP70 kinase (27–29), which then phosphorylates downstream targets such as the adaptor protein LAT (30). The total level of immunoprecipitable ZAP70 as well as the amount of its phosphorylated, activated form was comparable between control subjects, type 1 and type 2 diabetic patients (Fig. 2A). In contrast, the amount of phosphorylated CD3ζ-chain coprecipitated with ZAP70 appeared lower in

Table I. Specific PCR primers for the human lck and β2-microglobin genes

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences (5’ to 3’)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1F</td>
<td>ATGGGCTGTGGCTCAG6CATCACACCC</td>
<td>1–26a</td>
</tr>
<tr>
<td>1533R</td>
<td>TCAAGGCTGAAGCTGTA6CTGACTGCCCC</td>
<td>1509–1533a</td>
</tr>
<tr>
<td>551F</td>
<td>GTAACTGGAACAGCTTGGGC</td>
<td>551–570a</td>
</tr>
<tr>
<td>831R</td>
<td>CTTCAAGCTTCTTGCA6CCG</td>
<td>814–831a</td>
</tr>
<tr>
<td>1135F</td>
<td>TGCAAAGATTACACAGCTTT</td>
<td>1135–1152a</td>
</tr>
<tr>
<td>β2-MGF</td>
<td>CCAGCGAGGATGGAAAGTGC</td>
<td>900–919b</td>
</tr>
<tr>
<td>β2-MGR</td>
<td>GATGCTGCTTACATGTCTCG</td>
<td>1149–1168b</td>
</tr>
</tbody>
</table>

* Derived from the human lck gene coding sequence (GenBank AF072097).  
* Derived from the human β2-microglobin gene coding sequence (GenBank AF072097).
most of the tested type 1 diabetic patients (Fig. 2A, p < 0.05). To investigate the contribution of the TCR/CD3ζ-chain to this phenotype, we determined the amount of unphosphorylated and phosphorylated form of CD3ζ-chain under resting or activated conditions. We found no reduction in the total amount of TCR/CD3ζ-chain immunoprecipitated by an anti-CD3ζ mAb, although it was hypophosphorylated after activation in patients (Fig. 2B).

In most control (9/13) or type 2 diabetic (6/7) individuals, another phosphorylated polypeptide with an apparent molecular mass of 55–57 kDa was efficiently coimmunoprecipitated with ZAP70. In contrast, the amount of this phosphoprotein was significantly reduced in 16 of 25 patients (Fig. 2A, p < 0.001). These observations incite us to explore p56lck.

Specific reduction of p56lck protein level in resting T cells from patients

In resting lymphocytes, the p56lck molecule is predominantly localized at the cell membrane in interaction with the CD4 and CD8 coreceptors (31). Upon recognition of MHC/peptide complexes, the clustering of the TCR/CD3ζ and coreceptors in microdomains allows the p56lck-mediated phosphorylation of the CD3ζ-chain (32). Thus, the level of several transducing molecules of the TCR/CD3-associated activation pathway in unstimulated Nonidet P-40 cell lysates was investigated. These effectors included the p56lck and p59fyn src kinases, the LAT adaptor protein, and more downstream effector molecules such as the PLC-γ and PI3-kinase p85α. As shown in Fig. 3A, the amount of p56lck protein was specifically decreased among the tested molecules. This result was confirmed by a direct immunoprecipitation of the p56lck molecule (Fig. 3B). Quantification of the results obtained with a group of 10 patients and 9 control subjects showed a significant 2-fold reduction (p < 0.05) for the p56lck molecule (Fig. 3C). The detergent-soluble p56lck levels were then evaluated in a larger cohort of 38 patients and 24 control subjects. All patients showed a reduced level of p56lck (mean decrease 46.4 ± 26.2%), but ranged from less than 20% to subnormal levels (Fig. 3D). To evaluate the reproducibility of these results, five patients and three control subjects were tested at least twice at 1- to 4-mo time intervals and results were equivalent (data not shown).

An altered protein conformation might explain a less efficient immunoreactivity of the anti-p56lck used in our study. This possibility was excluded by testing two Abs to distinct p56lck epitopes, one within the SH3 domain and the other in the carboxyl-terminal domain (data not shown). The decreased p56lck detection in Nonidet P-40 lysates could be also due to an incomplete solubilization of the membrane. Similar results were obtained using other detergents, including those dissociating microdomains (data not shown). Alternatively, these mild conditions of extraction might enhance in vitro proteolysis of p56lck. To test this possibility, fresh PBMCs from nine patients and three control subjects were directly lysed and boiled in SDS buffer to obtain a complete solubilization of cellular p56lck, and the p56lck/PI3-kinase p85α ratios obtained under both experimental conditions were compared (Fig. 3E). A strong positive correlation was found between the two normalized values. Thus, the amount of detergent-extractable membrane-associated p56lck is representative of the available kinase in T cells.

The reduced p56lck level correlates with a lower proliferative response of PBMC from patients

The recruitment of p56lck is essential for optimal T lymphocyte activation (33, 34). As previously reported (7), the group of patients used in the present study showed a classical reduction of T-
cell responsiveness (Fig. 4A). This hyporesponsiveness was positively correlated with the p56\(^{\text{lk}}\) relative levels (Fig. 4B).

We then analyzed the induction of CD69 cell surface expression, an early marker of T cell activation (35). As illustrated in Fig. 5A, CD69 expression is undetectable on resting T lymphocytes and can be induced by the potent mitogenic combination PMA plus ionomycin in all individuals. However, the induction of CD69 expression in response to TCR/CD3 ligation was markedly reduced in a subset of patients (4/17) displaying weak p56\(^{\text{lk}}\) levels and proliferative responses. Fig. 5B illustrates the dramatically reduced CD69 expression on T cells from one representative patient, as already reported (6, 36). This result suggests a specific deficiency of the TCR/CD3-transducing module rather than a global defect in T cell activation. Furthermore, it allows the definition of at least two subsets of patients distinguishable by their relative level of T cell deficiency, as evidenced in Fig. 5A.

Altogether our observations support the hypothesis that a major cause of T cell hyporesponsiveness in type 1 diabetic patients is a reduced availability of membrane-bound, TCR/CD3-associated p56\(^{\text{lk}}\).

Normal cellular localization of p56\(^{\text{lk}}\) in T cells from patients

The failure to recruit p56\(^{\text{lk}}\) in the vicinity of the TCR/CD3 complex could be due to an abnormal subcellular localization of the PTK. This possibility was tested by confocal analysis of its distribution (Fig. 6). In these assays, the inhibitor 1XaB\(_{\alpha}\) was used as control of cytoplasmic distribution (37). In both patients (n = 10) and control (n = 8) subjects, p56\(^{\text{lk}}\) exhibited a preferential association to T cell plasma membrane (38, 39).

Reduced p56\(^{\text{lk}}\) mRNA level in T cells from some patients

To estimate the level of p56\(^{\text{lk}}\) mRNA, semiquantitative RT-PCR experiments were performed using total RNA extracted from unstimulated PBMC and an optimized pair of primers targeted to exons 10–12 (Fig. 7A). No significant variation was observed with all tested control individuals (n = 13). In contrast, among 28 tested patients, 9 showed a dramatically reduced level of p56\(^{\text{lk}}\) mRNA compared with a \(\beta_2\)-microglobulin standard (Fig. 7B). Results were confirmed several times for each sample, varying PCR cycle numbers (Fig. 7C) and using different pairs of primers (data not shown). For a limited number of randomly selected patient and control individuals, full-length cDNA were amplified by RT-PCR using a pair of 5\(^{\text{th}}\)- and 3\(^{\text{rd}}\)-end primers (Table I, 1F/1533R), hence confirming the expression of p56\(^{\text{lk}}\) mRNA with the expected size (data not shown). These results correlate with the low amount of p56\(^{\text{lk}}\) protein detected in the detergent-extractable fraction, because eight of those nine patients displayed less than 40% of normal levels and exhibited an impaired T cell proliferation (Fig. 4B).

An alternatively spliced p56\(^{\text{lk}}\) mRNA lacking exon 7 has been shown to be associated with a default in TCR/CD3 signal transduction (40). A RT-PCR analysis of p56\(^{\text{lk}}\) transcripts encompassing exon 7 (Fig. 7D) evidenced the presence of two transcripts in
all individuals tested. The sequence of the PCR amplification products confirmed the presence of transcripts with and without exon 7 (data not shown) (41). Densitometric analysis revealed no significant variation of the ratio of the two transcripts. Thus, our results indicate that this alternate form of p56\textsuperscript{lck} mRNA cannot explain by itself the general reduced T cell proliferation in patients.

Clinical features of the cohort of tested patients

The main clinical and molecular features of the patients as a function of p56\textsuperscript{lck} level are represented in Table II. The patients with the lowest p56\textsuperscript{lck} protein level did not differ from the others in terms of gender, age, duration of the disease, and metabolic control. This decrease was not associated with an earlier age of onset, as the proportion of diabetes occurring before and after puberty was similar in the two groups. The prevalence of autoantibodies directed to either glutamic acid decarboxylon or tyrosine phosphatase islet antigen 2 was also comparable. However, it is worth mentioning that the HLADQB1 0201/0302 genotype was essentially observed in the group with low p56\textsuperscript{lck} protein.

Discussion

In this study, we show that a defect in p56\textsuperscript{lck} expression is tightly associated with the lowered T lymphocyte proliferation observed in type 1 diabetic patients. This hyporesponsiveness, characterized by a reduced in vitro TCR/CD3-mediated T cell proliferation (6, 7, 36, 42), is correlated with an inefficient activation of PKC and/or p21\textsuperscript{ras} activation pathways, low expression of the early T cell activation marker CD69, and IL-2 secretion. The defective T cell response can be compensated by the addition of the PKC-activating drug PMA, and is not observed with a combination of agonistic anti-CD2/CD28 mAbs (7). In the NOD mouse model of type 1 diabetes (8), the deficiency in CD3-induced thymocyte proliferation (43), reversible by addition of IL-4 (10), is correlated with a reduced activation of the p21\textsuperscript{ras} pathway (15, 43) and to a sequestration of the CD4-associated p56\textsuperscript{lck} molecule (22). All these results suggested the existence of an early defect following engagement of the TCR/CD3 in type 1 diabetes. To test this hypothesis, we compared early PTK-mediated signaling events triggered by anti-CD3 mAb in PBMC from a panel of patients, nonautoimmune diabetic and healthy subjects. The initial observation consisted of a reduced phosphorylation of a few polypeptides upon TCR/CD3 engagement. This phenotype was variable in intensity and between patients. Similarly, in a few control or diabetic patients, a basal hyperphosphorylated pattern complicated the analysis of the results. This basal phosphorylation could not be correlated to other...
activation markers such as CD25 or CD69 expression or a variable ratio between naive and memory lymphocytes. However, despite these variations, an analysis performed on a large subset of patients suggested that CD3-induced phosphorylated polypeptides such as CD3ζ, LAT, and MAPK might be less efficiently activated in most diabetic patients. In a conventional model of T cell activation, p56<sup>lck</sup> phosphorylates CD3ζ, which recruits ZAP70, leading to the activation of downstream effectors (17, 44, 45). According to this model, we expect that a deficiency in p56<sup>lck</sup> function should lead to a reduced phosphorylation of CD3ζ, and consequently a diminished recruitment of ZAP70. In our study, we observed indeed a moderately but significantly reduced amount of phosphorylated CD3ζ, whereas the total amount of ZAP70 and of its phosphorylated form was comparable between control and diabetic individuals. Interestingly, the amount of the ZAP70-bound p56<sup>lck</sup> protein was lower in samples from diabetic patients. Several reports showed that p56<sup>lck</sup> acts as a downstream effector of ZAP70 (29, 46). It then activates the p21<sup>ras</sup>/MAPK pathway via its SH3 domain (47), leading to CD69 induction. Both CD69 expression (this study and 6) and the MAPK pathway (42) are affected in diabetes. Consequently, our results suggest that the predominant effect of a deficiency in p56<sup>lck</sup> function in diabetic individuals might affect late activation events rather the immediate recruitment of ZAP70. Furthermore, p56<sup>lck</sup> is also required for activation via CD2, CD28, and the IL-2R, and thus other pathways might also be affected and will require specific attention. In line with this hypothesis, a defect in IL-2 and soluble IL-2R secretion has already been reported in type 1 diabetic patients (23).

The mechanisms explaining p56<sup>lck</sup> reduction are most probably complex and heterogeneous. At the protein level among 38 tested patients, this deficit is experimentally defined by an at least 2-fold reduction in the amount of basal detergent-extractable p56<sup>lck</sup> for 66% of patients. Because p56<sup>lck</sup> is rapidly consumed after TCR/CD3 triggering (48), one can speculate that the deficit in type 1 diabetes could be consecutive to the apparent preactivated state observed in some patients. However, our survey (n = 14) excluded this hypothesis, as global hyperphosphorylation in resting state samples did not correlate with low p56<sup>lck</sup> protein level. Alternatively, in a model of T cell desensitization induced by soluble gp120 from HIV, a cytoskeletal sequestration has been documented that decreased the amount of membrane-bound p56<sup>lck</sup> (20).

Our confocal analysis confirmed the membrane localization of the kinase. Indeed, in resting T cells, most of the available p56<sup>lck</sup> is concentrated in microdomains, which gather several effector molecules of the TCR/CD3-transduction machinery (32, 38). A relative resistance to the solubilization by mild detergents characterizes these microdomains. All the results obtained with Nonidet P-40 lysates were confirmed with detergents that dissociate microdomains or whole cell extracts. Furthermore, the amount of the control p59<sup>fyn</sup> tyrosine kinase as well as that of several other effector molecules involved in the T cell activation pathway were not affected.

Another explanation of a low recovery of p56<sup>lck</sup> might be linked to modifications of the cell membrane induced by the metabolic disorder of diabetes. Enrichment of membrane with exogenous polyunsaturated fatty acids displaces p56<sup>lck</sup> from microdomains (49). In type 1 diabetes, the ω-6 and ω-3 polyunsaturated fatty acids present in the plasma membrane of erythrocytes and platelets have been shown to be in normal or slightly increased proportion (50). However, a metabolic cause for the decrease of p56<sup>lck</sup> is unlikely. First, there was no correlation between p56<sup>lck</sup> amount and the HbA1c value, representative of the quality of metabolic control. Second, p56<sup>lck</sup> levels observed in lymphocytes of type 2 diabetic patients were apparently normal despite hyperglycemia. Finally, the normality of the GPI-anchored protein p59<sup>fyn</sup> or LAT confirms that modifications of microdomains are not involved in the reduction of the amount of p56<sup>lck</sup>. Thus, one can conclude that the lowered expression of p56<sup>lck</sup> is specific of type 1 diabetes and correlated with the lower proliferative response.

Table II. Major characteristics of the type 1 diabetic patients relative to their low or subnormal p56<sup>lck</sup> protein levels

<table>
<thead>
<tr>
<th>Molecular characteristics</th>
<th>p56&lt;sup&gt;lck&lt;/sup&gt; &gt; 50% (n = 13)</th>
<th>p56&lt;sup&gt;lck&lt;/sup&gt; ≤ 50% (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low proliferation&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/5 (0%)</td>
<td>14/17 (82%)</td>
</tr>
<tr>
<td>Low mRNA level</td>
<td>1/8 (12.5%)</td>
<td>8/20 (40%)</td>
</tr>
<tr>
<td>Low CD69 expression</td>
<td>0/3 (0%)</td>
<td>4/13 (31%)</td>
</tr>
<tr>
<td>Clinical phenotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex ratio (F/M)</td>
<td>5/8</td>
<td>14/11</td>
</tr>
<tr>
<td>Age (years)</td>
<td>37.0 ± 9.0</td>
<td>35.5 ± 10.6</td>
</tr>
<tr>
<td>Prepubertal onset&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5/13</td>
<td>9/25</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>16.0 ± 11.3</td>
<td>14.8 ± 11.8</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.5 ± 0.8</td>
<td>7.8 ± 1.2</td>
</tr>
<tr>
<td>HLADQB1 0201/0302</td>
<td>1/9 (11%)</td>
<td>7/19 (37%)</td>
</tr>
<tr>
<td>GAD-Ab&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6/8 (75%)</td>
<td>12/19 (63%)</td>
</tr>
<tr>
<td>IA2-Ab&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2/8 (25%)</td>
<td>4/17 (24%)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Low CD3-mediated T cell proliferation.
<sup>b</sup> Prepubertal onset of diabetes; before 15 years old.
<sup>c</sup> Positive for glutamic acid decarboxylase Ab.
<sup>d</sup> Positive for islet tyrosine phosphatase Ab.

FIGURE 6. Confocal microscopy analysis of p56<sup>lck</sup> distribution in PBMC from one control subject and two patients. The green fluorescence (FITC) signal localizes p56<sup>lck</sup>, while the red fluorescence (7AAD) counterstains the nuclei. 1κBα was used as a control of cytoplasmic staining.
For 19 of the 28 patients tested for p56\(^{ck}\) mRNA expression, we found no obvious explanation for the reduced amount of p56\(^{ck}\). In contrast, in eight patients, this reduced level was associated with a strong diminution in the amount of p56\(^{ck}\) transcripts. The regulation of p56\(^{ck}\) transcription is controlled by several independent mechanisms. The p56\(^{ck}\) gene is transcribed from two widely separated promoters (51, 52). The type I (proximal) promoter is preferentially used in early fetal thymocytes and in nonlymphoid neoplasms, while the type II (distal) promoter is almost exclusively used in mature thymocytes and in normal mature T cells. In addition, an alternative splicing produces two type II (IIA and IIB) transcripts (53). The type IIA transcript is the most abundant p56\(^{ck}\) isoform in mature T cells, and the minor type IIB mRNA lacks exon 1 encoding for the N-terminal CD4 and CD8 interaction domain (54). Preliminary RT-PCR experiments designed to specifically amplify 5' untranslated regions showed the expected preferential usage of sequences derived from the distal promoter region in the tested control and type 1 diabetic individuals (data not shown). These results tend to exclude an obvious deficiency in promoter or exon usage.

Taken in the context of diabetes, the reduction of p56\(^{ck}\) level could not be related to any obvious clinical feature, but our cohort of patients is too small to allow any formal conclusion (Table II). Our results suggest that for a given patient, the pattern of p56\(^{ck}\) expression is time independent because patients studied several months after the initial evaluation presented the same response. The only putative correlation was found with the HLA haplotype of patients. From the available information, 7 of 19 patients with low p56\(^{ck}\) vs 1 of 9 patients with subnormal p56\(^{ck}\) level carried the HLA DQ\(^{b}\) 10201/0302 genotype that is highly associated with the appearance of diabetes (55). These preliminary results would suggest that the development of the autoimmune repertoire in the context of distinct self MHC molecules could be strongly dependent upon the amount of available p56\(^{ck}\) in thymocytes. Because the amount of p56\(^{ck}\) plays a significant role in the establishment of a functional T cell repertoire (34, 56), these results might suggest...
that the deficiency observed in peripheral T cells might also be detectable in early thymic development.

Alteration of p56\(^{lck}\) has also been found in other pathological situations, such as leprosy (57), cancer (58), rheumatoid arthritis (59), and lupus erythematosus (60). In cancer, this reduction was found to be secondary to the development of the tumors, partially reversible in vitro upon normal mitogenic stimulation and correlated with the clinical outcome (61). Nevertheless, in these pathologies, a more global reduction of molecules involved in signal transduction was observed, including the CD3 and ZAP70 molecules. This contrasts with the specific reduction observed in type 1 diabetes, and we favor the possibility that the deficiency in p56\(^{lck}\) is a primary event. Interestingly, we have observed this phenotypic in a patient before the onset of clinical type 1 diabetes. A systematic study among diabetes families and on larger cohorts might help to resolve these issues.

The functional consequences of this reduction in the amount of p56\(^{lck}\) are of potential interest. One possibility would be that the lowered p56\(^{lck}\) expression is preexisting before diabetes onset and is detectable among thymocytes. Several experimental arguments show that, depending upon the stage of maturation of the T lymphocyte studied, src kinases are differentially engaged in the T cell activation cascade. During intrathymic maturation, the preferential association of p56\(^{lck}\) with the TCR/CD3 is required for the appropriate adjustment of threshold responses to autoantigens (56). Another possible consequence of this deficit would be a skewing of T cell responses in the periphery. Indeed, differentiation toward the Th2 lineage requires high levels of recruited p56\(^{lck}\), and the introduction of a dominant-negative p56\(^{lck}\) transgene under the control of the distal promoter to target its expression toward mature T lymphocytes inhibits Th2 cell development (62, 63). It has been proposed that the progression toward diabetes in the NOD mouse model might be associated with a relative reduction of the Th2/Th1 cell ratio (5, 56, 64, 65). Thus, the lowered p56\(^{lck}\) expression could be the consequence of the Th1 bias in periphery, or more importantly a direct cause of the impaired Th2 lymphocyte differentiation.

More refined experiments are required to formally prove this putative link but, for the first time, the deficient expression in the amount of a src kinase correlated with an impairment in T cell activation can be directly associated to the development of a type 1 diabetes in human.

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