Gain-of-function polymorphism in mouse and human Ltk: implications for the pathogenesis of systemic lupus erythematosus

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Systemic lupus erythematosus (SLE), a complex multigenic disease, is a typical antibody-mediated autoimmune disease characterized by production of autoantibodies against a variety of autoantigens and immune complex-type tissue inflammation, most prominently in the kidney. Evidence suggests that genetic factors predisposing to aberrant proliferation/maturation of self-reactive B cells initiate and propagate the disease. In SLE-prone New Zealand Black (NZB) mice and their F1 cross with New Zealand White (NZW) mice, B cell abnormalities can be ascribed mainly to self-reactive CD5+ B1 cells. Our genome-wide scans to search for susceptibility genes for aberrant activation of B1 cells in these mice showed evidence that the gene, Ltk, encoding leukocyte tyrosine kinase (LTK), is a possible candidate. LTK is a receptor-type protein tyrosine kinase, belonging to the insulin receptor superfamily, and is mainly expressed in B lymphocyte precursors and neuronal tissues. Sequence and functional analyses of the gene revealed that NZB has a gain-of-function polymorphism in the LTK kinase domain near YXXM, a binding motif of the p85 subunit of phosphatidylinositol 3-kinase (PI3K). SLE patients also had this type of Ltk polymorphism with a significantly higher frequency compared with the healthy controls. Our findings suggest that these polymorphic LTKs cause upregulation of the PI3K pathway and possibly form one genetic component of susceptibility to abnormal proliferation of self-reactive B cells in SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex multigenic disease (1). Clinical manifestations are extremely diverse and variable, mainly because of variable combinations of contributing genes at multiple loci in individual patients. Genes that predispose to SLE are undoubtedly related to key events in pathogenesis and involve a variety of genes in the immune system. Identification of such susceptibility alleles is hampered mainly because of difficulties in precisely determining SLE-associated immunological abnormalities in genetic studies. In this respect, related studies on animal models are valuable and may contribute to studies on identification of genes underlying basic immunoregulatory abnormalities of autoreactive lymphocytes in the pathogenesis of SLE (2).

New Zealand Black (NZB) mice spontaneously develop a mild form of immune complex-type glomerulonephritis later in life, in association with IgM hypergammaglobulinemia, including anti-DNA antibodies. Like NZB mice, the F1 hybrid of the NZB and the non-autoimmune New Zealand White...
(NZW) mice have IgM hypergammaglobulinemia early in life. Unlike NZB, however, (NZB × NZW) F1 mice develop florid SLE with a much earlier onset and with a higher incidence of renal disease, in parallel with antibody class switch from IgM to IgG when the animals are about 6 months of age (3). Thus, NZB genes determine disease phenotype and NZW genes augment the disease severity.

Much evidence supports the notion that the majority of early B cell abnormalities found in young NZB and (NZB × NZW) F1 mice can be ascribed to CD5⁺ B1 cells (4–6). Currently, at least two subpopulations, B1 and B2 cells, can be divided on the basis of differences in phenotype, physiology and antibody repertoire (7). Compared with conventional B2 cells that mainly participate in acquired immunity, B1 cells mainly participate in innate immunity, and produce IgM natural antibodies of a low-avidity nature, which are polyreactive and cross-react with a variety of self-antigens (8,9).

To determine the genes responsible for the aberrant activation of B1 cells, we did a genome-wide quantitative trait loci (QTL) analysis in (NZB × NZW) F1 × NZB back-cross mice. One major susceptibility allele was mapped to the close vicinity of Ltk, the gene for leukocyte tyrosine kinase (LTK) on chromosome 2. The sequence analysis revealed that there is an amino acid substitution in the kinase domain of LTK in NZB mice. This type of polymorphism was also detected in an amino acid substitution in the kinase domain of LTK in chromosome 2. The sequence analysis revealed that there is significant linkage by permutation test (over 1517. The deduced amino acid substitution is glutamic acid for glycine at position 746 in the kinase domain (Fig. 2B). With genotyping of back-cross mice for this SNP using PCR-SSCP (Fig. 2C), the LOD score for the increased frequency of B1 cells reached 4.80 (over 4.17, the threshold for highly significant linkage by permutation test) at the Ltk locus, thereby suggesting that Ltk is a strong candidate (Fig. 2A). Linkage of the Ltk locus with serum IgM levels was also significant (LOD 3.13), based on the permutation test (over 3.11, the threshold for significant linkage).

Among those, we focused on Ltk, because LTK was shown to be mainly expressed in B lineage cells (10). To examine Ltk as a candidate gene, the nucleotide sequence of Ltk was compared between NZB and NZW mice. The NZW sequence was the same as the reported one (10) and the BALB/c sequence (data not shown); however, there was a single nucleotide polymorphism (SNP) in NZB mice with A, instead of G, at position 1517. The deduced amino acid substitution is glutamic acid for glycine at position 746 in the kinase domain (Fig. 2B). With genotyping of back-cross mice for this SNP using PCR-SSCP (Fig. 2C), the LOD score for the increased frequency of B1 cells reached 4.80 (over 4.17, the threshold for highly significant linkage by permutation test) at the Ltk locus, thereby suggesting that Ltk is a strong candidate (Fig. 2A). Linkage of the Ltk locus with serum IgM levels was also significant (LOD 3.13), based on the permutation test (over 3.11, the threshold for significant linkage).

Sequence polymorphism in the Ltk gene in human SLE

To determine if the Ltk polymorphism is also associated with human SLE, PCR-SSCP was done using genomic DNA extracted from SLE patients and from healthy controls with primers covering the human LTK kinase domain corresponding to the mouse polymorphic region. Eleven of 151 patients (7.3%) and 16 of 575 healthy individuals (2.8%) were heterozygous for amplified region (Fig. 3A), and the heterozygous genotype frequency was significantly higher in the SLE patients compared to the case in healthy controls (χ² = 6.77, P = 0.009). The nucleotide sequence revealed that individuals with homozygous alleles have G at position 9465, corresponding with glutamic acid at position 763 in the kinase domain. Those with heterozygous polymorphic alleles have both G and A at position 9465, corresponding with glutamic acid and lysine, respectively, at position 763 (Fig. 3B). Allele frequencies of 763K were 3.6% and 1.4% in SLE patients and controls, respectively, and the difference was statistically significant (χ² = 6.64, P = 0.01). The odds ratio for the development of
SLE with the 763E/K genotype versus the 763E/E genotype was 2.75 (95% CI 1.24–6.04).

**Effects of polymorphic Ltk allele on kinase activity and p85 binding**

Functional analyses of human LTK revealed that LTK is involved in the process of cell proliferation and survival, in which tyrosine 485, 753 and 862 contribute to specific binding with insulin receptor substrate-1 (IRS-1), p85 regulatory subunit of PI3K and Shc, respectively (11–13) (Fig. 4A). The observed amino acid substitution in NZB Ltk and human Ltk9465A allele is situated three amino acids upstream and 10 amino acids downstream from the tyrosine residue of p85-binding YXXM motif, respectively. Considering the importance of this region for the function of LTK, we examined the effect of the polymorphic alleles on the LTK activity. As the deduced protein sequence of LTK kinase domain shows 90% homology between mouse and human (14), we introduced NZB-type (750E) or human Ltk9465A-type (763K) substitution onto the Ltk9465G-type human Ltk allele (Fig. 4A), and the efficiency of autophosphorylation and the amount of p85 binding were examined, using COS7 cells transfected with these polymorphic Ltk alleles. Consistent with reported data (15), the capacity to catalyze autophosphorylation could still be observed in the Ltk9465G-type LTK in the absence of ligands. The extent of autophosphorylation was increased in both NZB-type and Ltk9465A-type LTK. The effect was more prominent in NZB-type substitution (Fig. 4B). These findings paralleled to extent of p85 binding (Fig. 4C), suggesting that both 750E and 763K variants enhance recruitment of PI3K to the cell membrane, resulting in activation of the PI3K pathway.

**Effects of polymorphic Ltk allele on cell proliferation and survival**

We then examined effects of 750E and 763K LTK variants on cell proliferation and survival. As no specific ligands for LTK have yet been identified, we took advantage of a chimeric receptor EL3 (hEGFR-hLTK) composed of an extracellular domain of human epidermal growth factor receptor (hEGFR) and transmembrane/cytoplasmic domains of human Ltk9465G-type LTK (hLTK) to analyze the function. The IL-3-dependent mouse pro-B cell line, Ba/F3 (16), was transfected with genes for the EL3 chimeric receptor (EL3 cells), or chimeric receptors with NZB-type (EL3-750E cells) or human Ltk9465A-type substitution (EL3-763K cells). Cells with the same expression levels of transfected genes were selected, using flow cytometry, and proliferative responses were compared with medium containing EGF in the absence of IL-3. The results of H3-thymidine uptake showed a significant increase in both EL3-750E and EL3-763K compared with findings in EL3 cells. This increase was probably due to the up-regulated PI3K pathway, since the effect was abolished in the presence of a specific PI3K inhibitor, Ly294002 (Fig. 5A). Cell cycle analysis revealed a higher proportion of cells in the S/M phase and a lower proportion of apoptotic cells in EL3-750E than in EL3 cells (Fig. 5B), suggesting that the up-regulated PI3K pathway in EL3-750E cells contributes not only to cell proliferation but also to anti-apoptosis.

**DISCUSSION**

In the present studies, we carried out a genome-wide QTL analysis to search for susceptibility loci to the activation of self-reactive B1 cells seen in SLE-prone NZB and (NZB × NZW)
compared with controls (zygous genotype frequency was significantly higher in SLE patients). Sixteen of 575 healthy controls were also heterozygous, but the heterozygous SLE patients were heterozygous (shown by the arrow) for amplified polymorphism. The results for 10 SLE patients are shown. Eleven of 151 kinase domain (Fig. 4A).

at position 9465, corresponding to glutamic acid and lysine at codon 763 in the vicinity of C24.

Figure 3. Polymorphism in human Ltk. (A) PCR–SSCP for detection of Ltk polymorphism. The results for 10 SLE patients are shown. Eleven of 151 SLE patients were heterozygous (shown by the arrow) for amplified Ltk fragment. Sixteen of 575 healthy controls were also heterozygous, but the heterozygous genotype frequency was significantly higher in SLE patients compared with controls ($\chi^2 = 6.77, P = 0.009$). Primers for PCR were designed to amplify the ~200 bp fragment corresponding to the mouse polymorphic region. (B) Direct sequence analysis of homozygous (Ltk9465G/G) and heterozygous (Ltk9465A/G) Ltk alleles. Heterozygous individuals had both G and A at position 9465, corresponding to glutamic acid and lysine at codon 763 in the kinase domain (Fig. 4A).

F1 mice. We found that one NZB-derived allele located in the vicinity of Ltk on chromosome 2 was significantly linked to the increased frequency of B1 cells in conjunction with IgM hypergammaglobulinemia. Among several genes located in the vicinity of Ltk, such as Pch2, Tyro3 and B2m (MGI database), Ltk itself was thought to be a possible candidate for aberrant activation of self-reactive B cells, because LTK is preferentially expressed on B lineage cells, and because NZB has a gain-of-function polymorphism in LTK kinase domain.

LTK is a receptor-type protein tyrosine kinase, belonging to the insulin receptor family (10,17,18), and is mainly expressed in pre-B cells and brain (10). In previous studies, we found that human LTK utilizes two major signaling molecules, Shc, which binds to tyrosine 862 at the carboxyl-terminal domain, and IRS-1, which binds to tyrosine 485 at the juxtamembrane domain, and that both molecules stimulate growth signals transmitted through the Ras pathway (11,12). It was also noted that the p85 regulatory subunit of PI3K directly binds to tyrosine 753, which is located within a YXXM motif, a consensus binding amino acid sequence for the SH2 domain of p85, in the kinase domain of human LTK (13). The deduced amino acid sequence of mouse LTK kinase domain shows 90% homology to human LTK (14). The observed NZB-type amino acid substitution of glutamic acid for glycine is situated three amino acids upstream from the tyrosine residue in the YXXM motif. Our data suggest that NZB-type substitution enhances ligand-independent autophosphorylation of tyrosine residue in YXXM motif and recruitment of PI3K to the cell membrane through the up-regulated p85-binding, the result being activation of the PI3K pathway. It seems that an amino acid substitution of cationic lysine for anionic glutamic acid in human Ltk9464A-type allele can also increase autophosphorylation and p85 binding to a given extent.

The PI3K pathway is an important signaling cascade that regulates various cellular events, including cell proliferation and survival (19). Several recent studies using transgenic or gene knock out mice have linked the PI3K pathway with autoimmune susceptibility (20–22). Transgenic mice with T cell-specific expression of p65<sup>PI3K</sup>, an active form of PI3K, or of protein kinase B (PKB), a downstream effector of PI3K, developed splenomegaly and lymphadenopathy, in association with increased serum immunoglobulin levels and widespread inflammation including autoimmune kidney disease (21,22). Thus, T cell-specific up-regulation of the PI3K/PKB pathway results in activation of not only T cells but also B cell populations. Analysis of T cells from these mice showed that they were resistant to Fas-mediated apoptosis. Furthermore, mice with heterozygous deletion of the phosphatase and tensin homologue (PTEN) gene (PTEN<sup>+/−</sup>) mice developed lymphoid hyperplasia composed of both T and B cells with hypergammaglobulinemia (20). PTEN was shown to negatively regulate PKB (23,24). Taken collectively, these studies show that enhanced activation of the PI3K/PKB pathway alters lymphocyte homeostasis and predisposes mice to autoimmunity (25). This is consistent with our idea that a gain-of-function polymorphism of LTK could form one aspect of the genetic susceptibility to SLE with altered autoreactive B cell homeostasis, through PI3K pathway activation.

Since LTK is expressed in immature B cells (10), one can speculate that frequencies of not only B1 cells but also B2 cells per total lymphocytes are linked to LTK gene polymorphism in our QTL analysis. However this was not the case, perhaps due to characteristic features of B1 cells. In contrast to conventional B2 cells, B1 cells have a self-replenishing capacity (7) and are constitutively stimulated by self-antigens via their self-reactive B cell receptors (26). Furthermore, B1, but not B2, cells in NZB and (NZB × NZW) F1 mice are resistant to Fas-mediated activation-induced cell death (27). Thus, it seems likely that B1 cells in these mice are more sensitive to the altered activation levels of the PI3K pathway than are B2 cells. This notion is consistent with the finding that mice lacking the p110<sup>δ</sup> catalytic subunit of PI3K have a reduced number of B1 cells (28).

The occurrence of multigenic diseases such as SLE has been explained in a threshold liability model, in which individuals will develop disease when the effects of total numbers of disease susceptibility genes exceed a given threshold (29). Such multigenic control of SLE involves genetic heterogeneity, in which several independently segregating susceptibility loci control the same disease phenotype (30). In the present studies, 7.3% of SLE patients had the gain-of-function type polymorphic Ltk allele. It is possible that many other unexplored molecules may be involved in abnormal activation of
autoreactive B cells. Notably, 2.8% of healthy controls also had the same polymorphic Ltk allele, undoubtedly indicating that this type of LTK variant per se is insufficient to develop SLE.

In this context, NZB mice only develop a mild form of SLE, and NZW-derived genetic factors, one of which is linked to NZW major histocompatibility complex (MHC) (reviewed in 2), are essential for the severe form of SLE associated with IgM to IgG class switch of autoantibodies observed in (NZB × NZW) F1 mice. The association between human SLE and HLA-DR2 and DR3 has also been shown in many studies in Caucasian populations. In addition to MHC, cumulative evidence supports several gene variants conferring predisposition to SLE (reviewed in 1), such as genes encoding classical complement components, low affinity receptors for IgG and PDCD1 (31). In SLE mouse models, several chromosomal segments have shown the association with disease phenotypes (reviewed in 32), and possible involvement of several gene variants, such as Cd22 (33), Ccr2 (34), Ifi202 (35), C1q (36) and Fcgr2b (37), has been described. Different combinations of these and not yet identified genetic variants may contribute to SLE susceptibility in different cohorts.

The human LTK gene lies in 15q15.1–15q21.1 (LocusLink database, Locus ID 4058). A genome-wide search for susceptibility genes in SLE sib-pair analysis showed suggestive linkages between SLE and microsatellite markers located at 15q15.1 on chromosome 15 (38). There is a large variation among studied samples in size, ethnic composition and family structure (1). Thus, further genetic studies in different cohorts and different races will be needed to confirm the linkage with marker loci in 15q15.1–15q21.1 and LTK polymorphism. Based on studies in both murine models and human SLE, a wider knowledge and a more thorough understanding of the genetic mechanisms of SLE are expected to provide clues as to the pathogenesis, then prophylactic and therapeutic clinical approaches can be better designed.

MATERIALS AND METHODS

Mice and subjects

NZB, NZW and (NZB × NZW) F1 mice obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan) were maintained in our laboratory. Back-cross mice were obtained by crossing female (NZB × NZW) F1 mice with male NZB mice. In human studies, 19 autopsy cases and 132 patients with SLE were analyzed. The patients were diagnosed according to the American College of Rheumatology criteria for SLE (39). The SLE group consisted of 18 male patients and 133 female patients at ages ranging from 22 to 81 years (mean ± SD = 42.7 ± 12.5). The apparently healthy control group consisted of researchers, laboratory workers, and students (183 men and 392 women) at ages ranging from 11 to 98 years (mean ± SD = 47.8 ± 21.8). All subjects were of the Japanese race, and relatively homozygous with respect to genetic background, permitting the case–control approach used in this study. The study was reviewed and approved by the research ethics committee.
Peripheral blood was obtained from periorbital sinuses of mice, followed by lysis of red blood cells using ammonium chloride. Cells were then incubated with FITC-labeled rat anti-mouse CD5 monoclonal antibody (mAb; clone 53-7.3) and biotinylated rat anti-mouse CD45R (B220) mAb (clone RA3-6B2), followed by phycoerythrin (PE)–avidin (Becton-Dickinson, Mountain View, CA, USA), and examined using FACStar (Becton Dickinson).

Genotyping and PCR-SSCP

DNA was extracted from the mouse-tail. Genotyping was done using microsatellite markers (Research Genetics, Huntsville, AL, USA) distributed approximately every 10 cM over the entire genome, except for the sex chromosome. PCR was done in a three-temperature protocol (94, 55 and 72°C) for 45 cycles using a GeneAmp 9600 Thermal Cycler (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). PCR products were run on 18% polyacrylamide gels and visualized after ethidium bromide staining. For genotyping of Ltk SNP, PCR-SSCP was done using primers designed to amplify fragments encompassing nucleotide substitution at position 1517. The 5’ and 3’ primers used were 5’-TCATTGCCACAGGGAACAGG-3’ (1472–1491) and 5’-TCAGGGTCCTGAGTGCAGTA-3’ (1603–1622), respectively. After the initial denaturation at 95°C for 5 min, PCR was done in a three-temperature protocol (95, 55 and 72°C) for 40 cycles, using GeneAmp reagents and AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems). PCR products were denatured at 98°C for 10 min, immediately cooled on ice, and run on a 10% polyacrylamide gel with 10% glycerol in 0.5X TBE buffer overnight in 5 W at 4°C. Autoradiograms were obtained using the BAS 2500 (Bio Imaging System, Fuji Film, Tokyo, Japan).

Flow cytometry for peripheral B cell subset

Peripheral blood was obtained from periorbital sinuses of mice, followed by lysis of red blood cells using ammonium chloride. Cells were then incubated with FITC-labeled rat anti-mouse CD5 monoclonal antibody (mAb; clone 53-7.3) and biotinylated rat anti-mouse CD45R (B220) mAb (clone RA3-6B2), followed by phycoerythrin (PE)–avidin (Becton-Dickinson, Mountain View, CA, USA), and examined using FACStar (Becton Dickinson).

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For PCR–SSCP analysis for human samples, genomic DNA was extracted from peripheral blood leukocytes from patients with SLE and control subjects, or kidney samples of autopsied patients, using a standard method. Primers were designed to amplify fragments encompassing nucleotide substitution at position 9465. PCR was done with radiolabeled forward primer 5’-TGGACTTCGTCGTTGGAGGA-3’ (9301–9320) and unlabeled reverse primer 5’-CAAGATGCTGGCAAAGCTAG-3’ (9481–9500), and amplified products were electrophoresed on a 5% polyacrylamide gel with 10% glycerol in 1X TBE buffer overnight in 5 W at 4°C. Autoradiograms were obtained using the BAS 2500 (Bio Imaging System, Fuji Film, Tokyo, Japan).
Nucleotide and amino acid sequences and positions are based on the following database. Mouse Ltk cDNA, GenBank X52621; mouse LTK, swissprot P08923; human Ltk genomic DNA, GenBank NM_002344; human LTK, swissprot P29376.

Statistics
The linkage of a particular locus with peripheral B1 cell frequency in total B cells was examined in 261 (NZB × NZW) F1 × NZB back-cross mice, using a computer package program of Map Manager/QTL. LOD score over 1.9 and 3.3 was used as thresholds for statistically suggestive and significant linkage, respectively, according to Lander and Kruglyak (40). Permutation test was also done for estimating the statistical significance. Differences in genotype and allele frequencies in SLE patients and non-SLE controls were compared using chi-square test. The odds ratio and its 95% confidence interval (95% CI) were calculated to provide an estimate of the risk of SLE in a given Ltk genotype compared with the controls. Differences in B1 cell frequencies, serum IgM levels, intensity of LTK autophosphorylation, thymidine incorporation and apoptotic cell frequencies were estimated using Student’s t-test. The P-value <0.05 was considered statistically significant.

Sequencing
For the mouse Ltk sequence, first-stranded cDNA was synthesized from bone marrow-derived total RNA using an oligo(dT) primer and the 2181 bp Ltk gene was amplified using appropriate primer pairs, referred to the database (GenBank X52621). For partial sequence of human Ltk in genomic DNA, PCR products of position 9227–9726 (corresponding to Serine 708–Proline 818) were amplified using appropriate primers, referred to the database (GenBank NM_002344). The PCR products were isolated using agarose gel electrophoresis, then referred to the database (GenBank NM_002344). The PCR fragments were isolated using agarose gel electrophoresis, then digested with restriction enzymes BglII and XbaI, and were ligated into pUC–CAGGS. The chimeric receptor EL3 (hEGFR–hLTK), were subcloned into expression vector pUC-CAGGS and pSR-neo, respectively, and used as templates for site-directed mutagenesis, as described (11). We used the two-step PCR-based method (41) to introduce a single nucleotide substitution in Ltk9465G cDNA. The first PCR was done with primers, 5′-CC TAGGGCCGACCAGGTGTAGCCG-3′ and 5′-GC GGTACACAGGC TAGGGGCTGCCCAG-3′, for the substitution of glutamic acid for glycine at position 750 (NZB-type: 750E), and with primers, 5′-CAGTTGGTGC AGCAACAGCCTGACCCCTAGG-3′ and 5′-GGCGAGCTC AGGTTGTGCTGCCAACACTG-3′, for substitution of lysine for glutamic acid at position 763 (human Ltk9465A-haplotype: 763K). The nucleotides to be substituted are underlined. The second PCR was done with the common flanking primers, 5′-TCTTTTGGGGTGTCTGGCTG-3′ and 5′-TGGGGGTCTTAGGCACTTAA-3′. The PCR fragments carrying substitutions were cut with BglII and ligated into hLtk–pUC-CAGGS or EL3–pSR-neo at the corresponding BglII-digested portion. Introduction of substitutions was verified by sequencing.

In vitro kinase assay and p85 binding assay
COS7 cells were transfected with hLtk–pUC-CAGGS with or without substitutions, using the DEAE-dextran method. For the LTK kinase assay, cell lysates containing total protein of 700 μg in Tris–HCl lysis buffer (0.5%, Triton X-100, 50 mM Tris–HCl, pH 7.4, 2 mM PMSF, 1 mM sodium orthovanadate, 1 mM EDTA, 10 units/ml aprotinin) were incubated with protein A-Sepharose coated by 1D3-1, a mouse mAb recognizing the intracellular domain of hLTK (42), for 1 h at 4°C, followed by three washes with wash buffer (0.1%, Triton X-100, 50 mM Tris–HCl, pH 7.4) and once with kinase buffer (40 mM HEPES, pH 7.4, 10 mM MgCl2, 3 mM MnCl2). The immunoprecipitates were incubated with 10 μCi [γ-P32] ATP for 20 min at room temperature, and the reactions were analyzed using 7.5% SDS–PAGE and autoradiography.

For the P85 binding assay, cell lysates containing a total of 15 μg protein were immunoprecipitated with Z-8, polyclonal rabbit antibody against PI3K p85 subunit, (Santa Cruz Biotech, Santa Cruz, CA, USA), and subjected to 7.5% gel SDS–PAGE. The immunoblotting was done with KM912, a mouse mAb recognizing the extracellular domain of human LTK (42).

For control of LTK or p85 protein levels in the kinase assay and p85-binding assay, cell lysates containing total of 50 μg protein were subjected to 7.5% gel SDS–PAGE. Western blotting was done with KM912 or Z-8, followed by alkaline phosphatase-conjugated goat anti-mouse or -rabbit IgG antibody (CAPPEL, West Chester, PA, USA). The color reaction was done using BCIP/NBT Color Development Substrate (Promega, Madison, WI, USA).

Cell proliferation and cell cycle assays
To examine the effects of amino acid substitutions on cellular function in the LTK kinase domain, Ba/F3 cells were transfected with cDNAs of EL3, EL3-750E or EL3-763K using the retrovirus vector (pSR2MSVtkneo), as described (12). To determine the cell proliferation, cells with the same expression level of transfected genes were selected using flow cytometry with Ab-1, a mouse mAb specific for the extracellular domain of hEGFR (Oncogene Science, Cambridge, MA, USA), and the [H3] thymidine incorporation assay was done. In 96-well plates 105 cells/well were cultured in starvation medium (RPMI1640 containing 0.5% FCS, but not mouse IL-3) for 6 h, then incubated with or without 25 μM of Ly294002 (Sigma, St Louis, MO, USA), a specific PI3K inhibitor, for 30 min. The cells were then stimulated with 50 ng/ml of EGF (WAKUNAGA, Hiroshima, Japan) for 16 h in the presence of 0.5 μCi of [H3] thymidine during the final 3 h and were subjected to liquid scintillation measurements of radioactivity.

For cell cycle analysis, a final density of 1 × 105 cells/ml in 10 cm dish was cultured in starvation medium for 6 h. After incubation with 50 ng/ml of EGF for 16 h, cells were fixed with 70% ice-cold ethanol for 30 min, followed by treatment with
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