A Lymphocyte-specific Ltk Tyrosine Kinase Isoform Is Retained in the Endoplasmic Reticulum in Association with Calnexin

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A lymphocyte-specific murine Ltk tyrosine kinase isoform was previously found to reside in the endoplasmic reticulum and to be potently activated upon treatment of cells with alkylating or thiol-oxidizing agents. Based on these observations, a unique role for Ltk was proposed as an endoplasmic reticulum-resident transmembrane kinase regulated by redox changes (Bauskin, A. R., Alkalay, I., and Ben-Neriah, Y. (1991) Cell 66, 685–696). To analyze why this Ltk isoform is retained in the endoplasmic reticulum, we investigated its behavior in over-expressing cells. Our results indicate that lymphoid Ltk exhibits a dual Nexo/Ccty and Ncyt/Cexo transmembrane topology in transfected cells. This unusual behavior may be responsible for retention in the endoplasmic reticulum since mutants with an increased number of positive amino acids downstream of the transmembrane segment exhibit a conventional Nexo/Ccty orientation and proceed to the cell surface. Endoplasmic reticulum-retained Ltk forms a prominent complex with the chaperone calnexin, suggesting that Ltk may be retained by the mechanism that prevents surface expression of inappropriately folded proteins or incompletely assembled protein complexes.

Ltk is a membrane-spanning tyrosine kinase that is most closely related to the product of the human non-Hodgkin’s lymphoma ALK proto-oncogene (1, 2). The restricted expression pattern of murine Ltk in subsets of lymphoid and neuronal cells initially suggested a role as a receptor for an unknown lymphoid or neuronal growth or differentiation factor (3). However, this hypothesis became less likely when one of the most widely expressed Ltk isoforms was found to reside in the endoplasmic reticulum (ER) where the protein showed extensive disulfide-linked oligomerization concomitant with potent activation of its kinase activity upon treatment of cells with alkylating or thiol-oxidizing agents. Based on this observation, a unique function for Ltk was proposed, as a ligand-independent ER-resident kinase regulated by redox changes (4).

Several observations complicated this hypothesis, however. First, its low expression level makes endogenous Ltk protein hard to detect, even by such sensitive methods as immune complex kinase labeling (3). The most convincing results arguing for ER residence were thus obtained with Ltk over-expressing transfected cells (4). In these cells, Ltk undergoes extensive oligomerization, which may have contributed to its ER retention. Second, ER retention and redox regulation was demonstrated for only one of at least four murine Ltk isoforms. The analyzed protein is unusual for harboring only 109 amino acids upstream of its transmembrane (TM) segment and is encoded by a lymphocyte-specific mRNA that includes an unconventional CUG translational start codon (3). A subsequently identified human LTK cDNA, extended further upstream, included a regular AUG start codon and predicted a receptor kinase with a considerably larger putative ligand-binding segment (5). Although the identification of the larger human LTK gene product shed some doubt on the significance of the ER-retained murine isoform, we subsequently found that mRNAs predicting analogous larger murine Ltk proteins also existed but had very restricted neuronal expression patterns (6).

Because transport to the cell surface is believed to occur by default once mammalian proteins enter the ER (7, 8), Ltk must be retained either because it contains an ER retention/retrieval signal (9) or because it is recognized by chaperones that prevent transport of incorrectly folded proteins or partially assembled protein complexes (10). To distinguish between these possibilities, we analyzed the intracellular trafficking of the lymphoid Ltk isoform in over-expressing transfected cells. Our results argue that ER retention may not be the normal fate of Ltk and provide support for the hypothesis that positively charged amino acids adjacent to membrane spanning segments play important roles in determining the TM topology of some integral membrane proteins (11, 12).

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—To generate mutants, we transferred the insert of the pBluescript II cDNA (3) to the pALTER vector and used the Altered Sites mutagenesis system (Promega) to change the asparagines of five N-X-S/T potential glycosylation acceptor sites into serines. The same strategy was used to change uncharged juxtamembrane residues into positively charged ones. Oligonucleotides used to mutate glycosylation sites were: NS1, AAG CAT CCC AGC TGC AGT CAC TG; NS2, AGC TGT GGA TAG TGT CAC TCT CAT; NS3, TCA CCA GCC AGT GTC ACT CTA C; NS4, CAG GCT GGG AGG CAG TGC ACT G; and NS5, GAA CAG TGC AGT GGC TCC TCC TCA. The juxtamembrane mutants were made using primers Ltk-JM-Pos1, CTA GTG AAC CAG AAG CCT AAC AGG CTT GGG CCC ACC, and Ltk-JM-Pos2, AAC CAG AAG TGT CAG CGC GTG CCG GGG ACC AGG CTG CCA. All mutations were confirmed by sequence analysis. Wild-type and mutant cDNAs were expressed using the SV40 promoter containing expression vector pBho (13).

Generation of Hybrid cDNAs—Ltk-human epidermal growth factor receptor (EGFR) hybrids were generated by PCR amplifying human EGFR cDNA segments with hybrid Ltk-EGFR oligonucleotides. The Ltk-TM-EGFR hybrid was made using oligonucleotides (5’) AAA GAG CTA GCT GGG CCA GGT CTG AA GAA TG and (3’) AAA CGT AAG GGC CTG TGG GGG ACC, and Ltk-JM-Pos2, AAC CAG AAG TGT CAG CGC GTG CCG GGG ACC AGG CTG CCA. All mutations were confirmed by sequence analysis. Wild-type and mutant cDNAs were expressed using the SV40 promoter containing expression vector pBho (13).
forms C and D have only been detected in a single murine neuronal cell line, but similar proteins may be more widely expressed in human cells.

TTA AGC TCC CTC TCC TGC AGC AGC. Unique Nhel and HindIII sites (underlined) were used to subclone the amplified fragment into the Ltk expression vector. The Ltk-JM-EGFR construct was made using 5'-primer ATG TGT GCA GTC CTG ATT CTA GTG AAG CGC CAC ATC and 3'-primer Ltk-TM-EGFR primer. Subcloning was by means of BsgI and HindIII sites.

Transfection and Immunoprecipitation—Subconfluent NIH 3T3 cells were transfected as described (14) using 10 μg of Ltk expression vector and 1 μg of pSV2neo as a selectable marker. Ltk expression in G418 (0.5 mg/ml) resistant clones was analyzed by RNA blots and immunoprecipitations.

Transient expression of Ltk in COS cells was achieved by DEAE-dextran transfection as described (6). Briefly, approximately 10 μg of plasmid was transfected per subconfluent 9-cm culture dish, and cells were labeled for 4 h with 0.3 mCi of 35S-labeled amino acids (Translabel; DuPont NEN) 2 days after transfection. In pulse-chase experiments, cells were labeled for 30 min before adding an excess (5 mM) of non-radioactive methionine to the culture medium. Cells were lysed in Triton buffer (1% Triton X-100, 100 mM NaCl, 2 mM MgCl2, and 10 mM Tris HCl, pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of antipain, aprotinin, and chymostatin. Cell lysates were preclared for 1 h with 10 μg of rabbit IgG conjugated to protein A-Sepharose and subsequently immunoprecipitated with 2 μl of rabbit anti-Ltk serum (3) for 16 h at 4°C. Immune complexes collected with protein A-Sepharose were washed six times with radioimmuno precipitation buffer (15). Samples were analyzed by electrophoresis on 12.5% SDS-polyacrylamide gels or digested first with endoglycosidase H (endo H) or N-glycanase using conditions recommended by the manufacturer (Genzyme).

Immunofluorescent Staining—To detect Ltk on the cell surface, live transfected COS cells grown on glass coverslips were placed on ice and incubated with either polyclonal Ltk antisem or preimmune serum for 1 h. After five washes in ice-cold PBS, the cells were incubated on ice with a fluorescein-conjugated F(ab)2 goat anti-rabbit IgG (Caltag Laboratories, San Francisco, CA) for 30 min and washed as before. Dilutions of the antisera in PBS containing 0.1% sodium azide were chosen so that untransfected cells exhibited minimal background fluorescence. The cells were then fixed in 2% paraformaldehyde in PBS and mounted in glycerol containing 2.3% (w/v) DABCO (1,4-diazobicyclo-(2,2,2)-octane, Sigma), 10% (v/v) 0.2 M Tris, pH 8.0, and 0.02% NaNO3.

Staining of permeabilized cells was performed at room temperature. Cells were first fixed in 2% paraformaldehyde in PBS for 30 min, then permeabilized with 0.05% saponin in PBS for 5 min, and subsequently stained and mounted as described above. The antisera were diluted in PBS with 0.05% saponin. Stained cells were viewed with an Olympus BH2-RFL fluorescence microscope at ×600 magnification using oil immersion and photographed using Kodak T-MAX 400 film and the Olympus PM-10ADS photometric system.

RESULTS

ER-retained Ltk Isoform A Exhibits a Dual TM Topology—Four Ltk proteins that differ upstream of their TM segment are encoded by tissue-specific murine mRNAs that arise by alternative splicing and alternative promoter usage. A pair of alternatively spliced transcripts that we have only detected in the C1300/Neuro-2A neuronal cell line (6) includes standard AUG start codons and predicts proteins with 360 and 421 amino acids upstream of their TM domains (Fig. 1, Isoforms C and D). Two mRNAs expressed in several lymphoid cell lines and in brain include CUG start codons and predict proteins with 109 and 170 amino acid upstream segments (Fig. 1, isoforms A and B). The lymphocyte-specific isoform A protein was previously found to be retained in the ER (4).

COS cells transfected with isoform A vectors produce two 72- and 78-kDa anti-Ltk immunoreactive glycoproteins (Fig. 2, lane 2). Endoglycosidase H, which specifically removes asparagine-linked sugar groups that have not undergone modification by oligosaccharyl transferase (16), trims both glycoproteins to a single 69-kDa species (Fig. 2, lane 3). Since asparagine-linked glycosylation occurs in the lumen of the ER (18) and since Ltk contains only two N-X-S/T potential glycosylation acceptor sites upstream of its TM segment, we and others previously suggested that differential glycosylation of either one or both of these sites may explain the two observed glycoproteins (3, 4). However, since one of the upstream glycosylation sites (N2 in the diagram in Fig. 2) maps only 13 residues upstream of the TM segment, and since recent studies suggest that glycosylation sites close to TM segments may not be recognized by oligosaccharyl transferase (17), we further analyzed the nature of the 72/78-kDa doublet. To this end, we transfected COS cells with isoform A mutants in which either one or both of the upstream glycosylation acceptor sites had been disrupted by targeted asparagine to serine substitutions. Fig. 2, lanes 4–6, shows proteins precipitated from cells transfected with glycosylation site mutants NS2, NS1, and NS1+2 (NS refers to the sequence change; the numbers identify the sites mutated; see diagram in Fig. 2). As shown in lane 4, disrupting the N-X-S/T motif closest to the TM segment of Ltk did not alter the glycosylation of isoform A. Since a mutant...
with a single glycosylation site upstream of its TM segment thus continues to incorporate more than one endo H-sensitive oligosaccharide, sites elsewhere in Ltk must also be modified. Indeed, although disruption of the most upstream N-X-S/T motif resulted in the conversion of the 72-kDa protein into a 69-kDa form (lane 5), which was not further reduced in size upon endo H digestion (not shown), the 78-kDa glycoprotein was unaffected by this mutation. The 78-kDa glycoprotein also remained when both upstream N-X-S/T motifs were mutated together (lane 6).

The part of Ltk downstream of its TM segment, which we had assumed to reside in the cytoplasm, harbors three additional N-X-S/T motifs (see diagram in Fig. 2). To test whether glycosylation of these sites accounted for the 78-kDa species, we disrupted all three motifs by asparagine to serine substitutions. Fig. 2, lane 7, shows that disrupting the downstream motifs did not affect the 72-kDa glycoprotein, but resulted in the conversion of the 78-kDa species into a 69-kDa protein that comigrates with unglycosylated Ltk. Mutating individual downstream sites resulted in intermediate size reductions, suggesting that all three sites are glycosylated (not shown). Disrupting all five glycosylation sites at either side of the TM segment of the Ltk prevented all glycosylation (lane 8).

Because addition of asparagine-linked core sugars occurs exclusively in the lumen of the ER (18), isoform A must be inserted into the ER membrane of transfected cells in both an Nexo/Ccyt (type I) and an inverted N cyt/Cexo (type II) orientation (Fig. 3). To rule out that this unusual behavior is caused by over-expression, we generated stably transfected NIH 3T3 clones expressing approximately 100-fold less protein. Similar ratios of the 72- and 78-kDa glycoproteins were detected in all analyzed stable transfectants (e.g. Fig. 2, lane 9). Since a similar 72/78-kDa doublet also was previously detected in a murine B lymphocyte cell line (4) and upon in vitro translation of isoform A mRNA in the presence of microsomes (3), we conclude that isoform A Ltk exhibits a mixed TM topology in vitro and in several cell types expressing widely varying protein levels.

**ER-retained Isoform A Binds Calnexin**—ER retention of several proteins, including unassembled components of integral membrane protein complexes, is mediated by the ER resident chaperone calnexin (19). To examine whether calnexin or other chaperones play a role in the ER retention of Ltk, we precipitated potential Ltk-containing complexes after lysing metabolically labeled transfected COS cells in 0.6% CHAPS. A 90-kDa
protein, identical in size to calnexin, co-precipitated with isoform A Ltk under these conditions (Fig. 4, lane 3). The 90-kDa protein was not detected in precipitates from mock transfected cells (lane 1) and was unambiguously identified as calnexin in sequential immunoprecipitation-immunoblotting experiments, using anti-Ltk to precipitate and anti-calnexin to detect the blotted protein or vice versa (not shown). Interestingly, the 78-kDa glycoprotein was the predominant calnexin-associated species in these experiments (Fig. 4, lane 4).

**Ltk Mutants with a Type I TM Topology Proceed to the Cell Surface**—Since calnexin preferentially binds the 78-kDa inverted Ltk protein, we tested whether Ltk mutants with a predominant type I TM topology escaped ER retention. It is important to note in this respect that isoform A lacks an obvious N-terminal signal sequence (3) and shows no obvious bias of positive charges flanking its TM segment. Since the TM topology of integral membrane proteins without cleavable signal sequences is believed to be determined by the ratio of charged amino acids flanking their TM segments, with the most positive side in the cytoplasm (11, 12), we analyzed the trafficking of several Ltk mutants that were engineered to contain extra positive amino acids downstream of the TM segment. Initial mutants were made by replacing membrane-flanking segments of Ltk with the corresponding parts of the EGFR. Because the cytoplasmic juxtamembrane segment of the EGFR is highly positively charged (Fig. 5), Ltk hybrids containing this segment would be expected to exhibit an altered TM topology. Indeed, both Ltk-TM-EGFR and Ltk-JM-EGFR hybrids that harbor the EGFR juxtamembrane segment (see map in Fig. 5, and see “Experimental Procedures” for details), did not show the glycoprotein doublet indicative of a mixed TM topology. Rather, the predominant species observed in cells transfected with these mutants resembled type I oriented isoform A both in its size (~72 kDa) and in the modest size reduction upon endo H digestion (Fig. 5, lanes 1–4). To directly test whether the increased number of positive charges in the juxtamembrane region was responsible for the altered behavior of the Ltk-EGFR hybrids, we made two additional mutants (Ltk-JM-Pos1 and Ltk-JM-Pos2) by site-directed mutagenesis. Each mutant harbored two additional lysine or arginine residues at the positions indicated in Fig. 5. COS cells transfected with both mutants predominantly expressed a 72-kDa endo H-sensitive (shown for the Ltk-JM-Pos1 mutant in Fig. 5, lanes 5 and 6). We infer from these results that in a manner similar to that observed for other proteins (11, 12), the TM topology of isoform A Ltk can be modified by changing the number of charged residues downstream of its TM segment.

All Ltk mutants that exhibit a predominant type I TM topology show a diffuse higher molecular weight species in immunoprecipitates (arrow in Fig. 5). We argued that this slower migrating species, which is not seen upon transfection of wild-type isoform A, might represent protein that has undergone Golgi-specific oligosaccharide modifications. To test this, we performed pulse-chase labeling and endoglycosidase digestion experiments. In pulse-chase experiments, cells transfected with the Ltk-TM-EGFR hybrid did not show the slower migrating species at early time points. At later time points, this species became progressively more prominent, consistent with it representing a processed end product (Fig. 6, left panel). The higher molecular weight species was resistant to endo H, but sensitive to N-glycanase digestion, suggesting the presence of Golgi-specific oligosaccharide modifications (Figs. 5 and 6, right panel). We conclude that Ltk mutants with a predominant type I TM topology proceed beyond the ER to the Golgi.

To analyze whether Ltk juxtamembrane mutants proceed beyond the Golgi to the cell surface, we stained live, non-permeabilized transfected cells by indirect immunofluorescence. In contrast to COS cells transfected with a wild-type isoform A construct, 20–30% of cells transfected with the two Ltk-EGFR hybrids or with both site-directed mutants showed unambiguous surface fluorescence (Fig. 7). A type I TM topology thus correlates with cell surface expression of Ltk mutants. However, staining of saponin-permeabilized transfected cells...
showed that most mutant protein remained localized to the ER, indicating that surface transport of the mutant proteins remains inefficient (Fig. 7).

**DISCUSSION**

A unique role for Ltk as a redox sensing tyrosine kinase was proposed, when a widely expressed murine Ltk isoform was found to reside in the ER, and to undergo extensive disulfide-linked oligomerization concomitant with a 20-fold activation of its kinase activity upon treatment of cells with alkylating or thiol-oxidizing agents (4). Because transport to the cell surface is believed to occur by default when mammalian proteins enter the ER, we analyzed in this study why isoform A Ltk is ER retained. Our results indicate that isoform A Ltk exhibits a highly unusual dual TM topology in transfected cells and forms a prominent complex with the ER resident chaperone calnexin. The association with calnexin suggests that isoform A Ltk may be ER retained by the mechanism that prevents surface expression of incompletely folded proteins or partially assembled protein complexes.

Its unusual dual TM topology may reflect the fact that isoform A lacks a functional N-terminal signal sequence. Indeed, as has also been observed for other integral membrane proteins without cleavable signal peptides, the TM topology of isoform A can be altered by increasing the number of positively charged amino acids downstream of the TM segment. Interestingly, all mutants that exhibit a predominant type I TM topology show evidence of proceeding beyond the ER to the cell surface. However, transport to the cell surface remains inefficient since most mutant protein remains endo H-sensitive and remains localized to the ER in immunofluorescent staining experiments.

Several other points regarding the unusual behavior of Ltk are worth making. First, although calnexin predominantly associates with the 78-kDa inverted isoform A species, both topological forms of Ltk are ER-retained when co-expressed. This may be explained by the fact that isoform A undergoes extensive oligomerization in transfected cells (4). Second, the potent stimulation of the kinase activity of isoform A upon treatment of cells with thiol oxidizing agents may relate to its inverted TM topology and reflect oxidation of amino acids within the catalytic segment of the protein. Finally, although we have not extensively analyzed the trafficking of other Ltk isoforms, we previously noted that isoform C Ltk is also largely ER-retained (6). This ER retention cannot be attributed to an inverted TM topology of this protein since isoform C Ltk harbors a typical N-terminal signal peptide and shows no evidence of a dual TM topology when over-expressed. Different mechanisms may thus contribute to the ER retention of different Ltk isoforms.

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