The Chediak-Higashi Protein Interacts with SNARE Complex and Signal Transduction Proteins

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Abstract

Background: Chediak–Higashi syndrome (CHS) is an inherited immunodeficiency disease characterized by giant lysosomes and impaired leukocyte degranulation. CHS results from mutations in the lysosomal trafficking regulator (LYST) gene, which encodes a 425-kD cytoplasmic protein of unknown function. The goal of this study was to identify proteins that interact with LYST as a first step in understanding how LYST modulates lysosomal exocytosis.
Materials and Methods: Fourteen cDNA fragments, covering the entire coding domain of LYST, were used as baits to screen five human cDNA libraries by a yeast two-hybrid method, modified to allow screening in the activation and the binding domain, three selectable markers, and more stringent confirmation procedures. Five of the interactions were confirmed by an *in vitro* binding assay.

Results: Twenty-one proteins that interact with LYST were identified in yeast two-hybrid screens. Four interactions, confirmed directly, were with proteins important in vesicular transport and signal transduction (the SNARE-complex protein HRS, 14-3-3, and casein kinase II).
Conclusions: On the basis of protein interactions, LYST appears to function as an adapter protein that may juxtapose proteins that mediate intracellular membrane fusion reactions. The pathologic manifestations observed in CHS patients and in mice with the homologous mutation beige suggest that understanding the role of LYST may be relevant to the treatment of not only CHS but also of diseases such as asthma, urticaria, and lupus, as well as to the molecular dissection of the CHS-associated cancer predisposition.

Introduction

Lysosomes have unique and fascinating abnormalities in Chediak–Higashi syndrome (CHS), a primary immunodeficiency disease. They are greatly enlarged, limited to the perinuclear region, lack critical cargo proteins, and are dilatory in heterotypic membrane fusion (1–3). The clinical features of CHS of recurrent infections, albinism, and bleeding diathesis reflect these defects. Recurrent infections, for example, result from secretory granule protein missorting and impaired leukocyte exocytosis (4–6). Albinism occurs as a result of tyrosinase deficiency in CHS melanocytes due to exocytosis instead of delivery to the melanosome (7). Absence of dense granules in CHS platelets leads to a bleeding tendency (8).

Identification of the causal gene (LYST) failed to explain the lysosomal abnormalities of CHS; LYST encodes a 425-kD cytosolic protein that is ubiquitously expressed but of unknown function (9–12). Structurally, LYST is novel and unlike other proteins implicated in vesicular transport. Although LYST contains C-terminal WD40 repeats, HEAT/ARM domains, a region with weak similarity to stathmin, and multiple potential sites of phosphorylation by casein kinase II (CK2) and protein kinase C (PKC), the significance of these features is unknown.

Understanding the mechanism whereby LYST defects cause CHS is important for treatment both of CHS and diseases associated with inappropriate exocytosis, such as asthma and urticaria. Using a recently described, modified yeast two-hybrid (Y2H) method (13), proteins that interact with LYST were identified. These interactions suggest LYST to act as a scaffold for SNARE complex proteins.

Materials and Methods
Cloning of LYST cDNA Fragments in Y2H Vectors

Fourteen overlapping *LYST* cDNA fragments covering the coding domain were PCR amplified and cloned in the yeast Gal4 activation domain (AD) cloning vector, pAD-GAL4 (modified pGAD-GH, Clontech, Palo Alto, CA, USA), and the Gal4 DNA-binding domain (BD) vector, pGB-GAL4 (modified pGBT9, Clontech). Primers generated in-frame cDNA fragments that preserved LYST protein domains (Table 1). Clones were sequenced to ensure fidelity.

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Table 1. Primers used to amplify *LYST* cDNA fragments

LYST Bait, (bp*)	Primer Sequence, 5′→3′ (forward, upper sequence; reverse, lower sequence)	Restriction Enzyme	Bait Size (bp)
190–1056	GCGGTGGATCCCATGAGCACCGACAGTAACTCAC GCGGAATTCTCATAGTGTGGGCACTACACTGG	*Bam*HI *Eco*RI	866
1051–1950 stathmin-like	GCGGTGGATCCCACACTAACTGAGTTCCTAGCAGGCTTTGGGGACTGC (GAGAGA)₃CTCGAGTCAATCCATACAACAGCATATTCCAATG	*Bam*HI *Xho*l	899
1948–2370	GCGGAACTAGTGGATCCCAAATCTGTAATCATTCC GGAATTCTCACTGAACAACTATATTGCCTTTCTG	*Spe*I *Eco*RI	422
2347–3213	GCGAGTGTGGATCCCCAGAAAGGCAATATAGTTGTTCAG GCGGAATTCTCACTCCTCTTTGTGACTTCTGAAC	*Bam*HI *Eco*RI	866
3190–4032	GCGGTGGATCCCCTGTTCAGAAGTCACAAAGAGG GGAATTCTCAAGCAGAAAGCAAATTTAATTCCAG	*Bam*HI *Eco*RI	842
4009–4821	GCGGTGGATCCCCTGGAATTAAATTTGCTTTCTGC GCGGAATTCTCAGGAGCCCAGTGAAATTATATG	*Bam*HI *Eco*RI	812
4819–5700	GCGGTGGATCCCTCCAAAGCGTTGATGATCCAAG GCGGAATTCTCAAACTCGCAGTGCTAATGCTTG	*Bam*HI *Eco*RI	881
5677–6612	GCGGTGGATCCCACTCAAGCATTAGCACTGCGAG (GAGAGA)₃CTCGAGTCAGGCAACATAAGTATCTGCAATATTTTG	*Bam*HI *Xho*l	935
6586–7449	GCGGTGGATCCCCAAAATATTGCAGATACTTATGTTGCC GCGGAATTCTCATCCCATGTTTCTCACATCTTCCAG	*Bam*HI *Eco*RI	863
7426–8238	GCGGTGGATCCCCTGGAAGATGTGAGAAACATGGG GCGGAATTCTCAGGTCTGGAAAACTGAGGTCTTG	*Bam*HI *Eco*RI	812
8218–9039	GCGGTGGATCCCAAGACCTCAGTTTTCCAGACCG GCGGAATTCTCAATCCAACTGCCATGAGGTTGG	*Bam*HI *Eco*RI	821
9037–9585	GCGGTGGATCCCGATCCAACAGAAGGGCCAAATC GCGGAATTCTCATGTCAGAGCGGTGATGTTACC	*Bam*HI *Eco*RI	548
9502–10590 BEACH dom.	GCGGTGGATCCCAAGGTTCGTGATGATGTATACCAC GCGGAATTCTCACAAGCCTTTTATCCATGACAAAGG	*Bam*HI *Eco*RI	1088
10576–11611 WD-40 dom.	GCGGTGGATCCCTGGATAAAAGGCTTGAAATGGGG GCGGAATTCTCATGAAGTTCATTCGCATTCACCC	*Bam*HI *Eco*RI	1035

*From GenBank # U67615.

Human cDNA Libraries Used in Y2H Screens

cDNA from human fetal brain mRNA (Clontech) was cloned into Gal4 AD of HybriZap 2.1 (Stratagene, La Jolla, CA, USA) to generate a phage cDNA library with 6×10^6 pfu and >90% recombinant clones. Following *in vivo* excision, 300 μg of recombinant plasmid was transformed into yeast strain N106r (*MAT α, ura3, his3, leu2, gal4, gal80, ade2, trp1, cyh*ʳ*, Lys2::GAL1_{UAS}-HIS3_{TATA}-HIS3, ura3::GAL1_{UAS}-GAL_{TATA}-lacZ,*), with selection on synthetic complete medium lacking leucine (SC-Leu). Transformants (7×10^7) were used for forward screens.

A human fetal brain cDNA library (1.5×10^7 pfu, >90% recombinant clones) was constructed in HybriZap modified to contain GAL4 BD. Four hundred micrograms of plasmid was transformed into yeast strain YULH (*MAT a, ura3, his3, lys2, Ade2, trp1, leu2, gal4, gal80, GAL1-URA3, GAL1-lacZ*) and selected on SC-Trp medium, and 1.5×10^8 transformants were used in reverse screens.

Human adult heart, adult keratinocyte, fetal liver, and fetal kidney cDNA libraries in the Gal4 AD vector pACT2 (HL4042AH, HL4030AH, HL4029AH and HL4008AB; Clontech) were amplified and plasmids purified. N106r yeast cells were transformed with 200 μg of plasmid DNA and 10^7 yeast transformants were used in screens.

Forward and Reverse Y2H Screens

In "forward" screens, strain YULH was transformed with LYST baits inserted in the Gal4 BD plasmid, and strain N106r was transformed with the prey cDNA library in the Gal4 AD vector (13). In "reverse" screens, LYST baits in the Gal4 AD plasmid were transformed in N106r, and the prey library fused to the Gal4 BD was transformed in YULH.

LYST bait DNA-binding domain clones were prescreened for intrinsic transcriptional activity. YULH yeast cells were transformed with pBD-GAL4 bait constructs, grown on SC-Trp medium (selection

of pBD), and then on SC-Ura medium overnight at 30°C. Resultant colonies represented self-activating baits and were excluded. Promiscuous LYST baits were also excluded by prescreening against 50,000 prey library clones. Only baits that interacted with fewer than five proteins were used.

Screens entailed mating haploid strains: Following growth to late log phase on selective media, strains YULH and N106r were diluted in YPAD, applied to nitrocellulose membranes, and incubated at 30°C for 8 hr. Products were plated on SC Selective (SCS) medium (SC-Ade-Lys [selection of diploids], -Leu-Trp [selection of pAD and pBD], and -Ura-His [selection of reporter gene-activating protein interactions]). Colonies growing on SCS were replicated on Whatman No. 1 filters and assayed for β-galactosidase activity.

Confirmation of the Specificity of Protein Interactions by Y2H

Interacting proteins were tested for self-activation: YULH yeast cells were transformed with plasmids encoding BD fusion proteins of LYST-interacting proteins, grown at 30°C on SC-Trp medium (selection for BD plasmid) and then overnight on SC-Ura medium. Cells that did not grow lacked self-activating false-positives clones. Interactant self-activation was also tested using YULH and N106r yeast, transformed with LYST-interacting BD or AD fusion protein preys, respectively. These were grown on SC-Trp or SC-Leu media, and a filter-lift β-galactosidase assay was performed. Colonies that did not turn blue contained non–self-activating prey.

The specificity and reproducibility of interactions was evaluated in a matrix-mating test. BD fusions with baits (forward screen) or prey (reverse screen) were retransformed in YULH yeast, and AD fusions of baits (reverse screen) or prey (forward screen) were retransformed in N106r yeast and grown in YPAD medium at 30°C. A matrix mating test was performed by spreading bait-containing haploid yeast over the surface of individual YPAD plates, followed by superimposing prey-containing haploids in a 12 × 8 matrix, and incubating the plates overnight at 30°C. Each plate contained positive (known protein interaction pairs) and negative controls. Resulting diploid colonies were replica-plated both on SCS plates (to select for AD and BD plasmids, diploids, and protein interactions) and SC-Leu-Trp plates (that were used for a filter-lift β-galactosidase assay). Baits and prey from $LacZ^+$ diploids growing on SCS were sequenced.

Expression and Affinity Purification of LYST-Interacting Proteins

LYST-interacting proteins (LIPs) were expressed as His-tag–T7 fusions by ligating PCR fragments of the corresponding genes in frame into PET-28a vector (Novagen, Madison, WI, USA). These constructs were transformed into *Escherichia coli* strain BL21-DE3 (Novagen) and induced with isopropyl-b-D-thiogalactoside (IPTG). Cell pellets from 400 mL cultures were suspended in denaturing lysis buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole and 8 M urea), sonicated on ice, and centrifuged. Supernatants were applied to Nickel-NTA columns (Novagen) pre-equilibrated with lysis buffer and washed with 10 volumes of wash buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 50 mM imidazole and 8 M urea). Successive washes were performed with wash buffers containing urea concentrations of 4 M, 2 M, 1 M, 0.5 M, 0.2 M, 0.1 M, 10 mM, and, finally, no urea. Bound protein was eluted with 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 500 mM imidazole and eluted proteins were desalted by diafiltration (Amicon, Beverly, MA, USA).

Expression and Preparation of LYST Peptide-Solid Support

For expression as glutathione S-transferase (GST) fusions, LYST fragments were cloned in-frame in pGEX-5a (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and transformed into *E. coli* strain BL21 (Amersham Pharmacia Biotech). GST fusion proteins were separated from cellular proteins by glutatione-sepharose column chromatography (Amersham Pharmacia Biotech): Cell pellets from IPTG-induced cultures were suspended in PBS containing 58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 137 mM NaCl (pH 7.5), and protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN, USA). Suspensions were sonicated on ice, centrifuged to remove debris, and supernatants applied to glutathione-sepharose columns. Unbound proteins were removed with washes. Sepharose bound GST-fusion proteins were used in *in vitro* binding assays.

In Vitro Binding Assay

GST-LYST fragment fusion proteins bound to sepharose (200 μL) were mixed with 200 μL of purified, desalted LIP, or control protein, in PBS containing 1% Triton X-100 and incubated at 24°C with rocking for 1 hr. Samples were centrifuged at 13,000 rpm for 1 min, supernatants discarded, and sepharose pellets were washed four times. Bound protein was then eluted with 300 μL of 10-mM reduced glutathione in 50 mM of Tris-HCl (pH 8.0). Eluted proteins were resolved on 8% SDS-PAGE and transferred to nitrocellulose. Membranes were probed with His-tag or T7-tag antibodies (Novagen), or with specific CK2, 14-3-3, or Hrs antisera, and detected with alkaline-phosphatase conjugated anti-mouse secondary antibodies.

Results

Yeast Two-Hybrid Screening

To screen a 425-kD protein effectively, a recently described Y2H modification (13) was used that includes screening in the activation or binding domain, three selectable markers, and stringent confirmation procedures. Cloning into the activation domain enables screening of self-activating baits. The

use of three reporter genes (β-galactosidase, and uracil and histidine auxotrophy) reduces false positives. The modified method also includes quality controls for bait self-activation, prey self-activation, nonspecific binding, and reduplication of interactions by matrix mating with stringent controls, that further reduces false positives. Fourteen LYST cDNA fragments covering the coding domain were cloned into activation- and DNA binding-domain vectors. These baits preserved the proposed structural features of LYST. Human heart, keratinocyte, fetal brain, fetal liver, and fetal kidney cDNA libraries were screened for LIPs. Bait and prey constructs were transformed into complementary haploid yeast and mated on 96-well plates in a high-throughput manner. The interactions shown (Tables 2 and 3) passed all quality controls.

Interactions Identified in Forward Screens

Four known proteins were found to interact with LYST in forward screens (Table 2). The LYST N-terminus interacted with 14-3-3 tau (τ, also called 14-3-3 theta). Seven independent isolates of 14-3-3τ were identified in screens with three libraries (heart, fetal brain, and fetal liver). The 5' end of all of these isolates was within the N-terminal one-third of 14-3-3τ. Because prey libraries had been constructed with oligo-dT primed cDNA, it was assumed that the 3' ends of prey inserts corresponded to the stop codon.

This region of 14-3-3τ was also found to interact with the LYST N-terminus in reverse screens (see below).

Seventy-two colonies, representing nine independent isolates, of CK2β subunit were found to interact with LYST bp3190–4032 (Table 2). LYST–CK2β interactions were identified in all libraries screened. The 5' end of the CK2β isolates was in the vicinity of the start codon.

Screening against a human heart library revealed two independent isolates of an interaction between LYST bp3190–4032 and troponin I.

Hepatocyte growth factor–regulated tyrosine kinase substrate (HRS) was found to interact with LYST fragment 6586–7449. This sequence was isolated once from a screen of a fetal brain cDNA library. The 5' end of the HRS clone corresponded to amino acid 84.

Interactions Identified in the Reverse Screens

Twenty-two LIPs were identified in reverse screens (Table 3). Thirteen were known proteins and nine were novel. All of these interactions were confirmed *in vivo*. The interaction of the LYST N-terminus with 14-3-3τ that had been identified by the forward screen was recapitulated in the reverse screens in four colonies, representing two independent isolates. Another LYST fragment (bp 4009–4821) interacted with the same region of 14-3-3τ. This interaction was found only once.

Table 2. Confirmed LYST interactions identified by forward yeast two-hybrid screens

LYST Bait (bp*)	Prey (GenBank #)	Prey Function	Prey Library	First Nucleotide of Prey Clones	# Yeast Colonies
190–1056	14-3-3τ (X56468)	Vesicular trafficking and signal transduction	Fetal liver	185	1
			Adult heart	221	1
			Fetal brain	290	1
			Fetal liver	299	2
			Fetal brain	308	1
			Fetal liver	322	1
			Fetal brain	342	1
3190–4032	Casein kinase II β-subunit, CK2β (M30448)	Phosphorylation: vesicular trafficking and signal transduction	Fetal brain	1	28
			Adult heart	1	14
			Fetal liver	1	12
			Keratinocyte	1	11
			Fetal kidney	2	1
			Keratinocyte	3	1
			Fetal brain	5	1
			Fetal brain	35	1
			Fetal brain	86	3
	Troponin I (X54163)	Muscle contraction	Adult heart	1	6
			Adult heart	75	3
6586–7449	Hepatocyte growth factor–regulated tyrosine kinase substrate, HRS (D84064)	Vesicular trafficking and signal transduction	Fetal brain	309	1

*From GenBank U67615.

Table 3. Confirmed LYST interactions identified by reverse yeast two-hybrid screens with a fetal brain prey library

LYST Bait (bp*)	Prey (GenBank #)	Prey Function or % AA Identity, Similarity, #AA[†]	First Nucleotide of Prey Clones	# Yeast Colonies
190–1056	14-3-3τ (X56468)	Vesicular trafficking and signal transduction	290	2
			356	2
	14-3-3β (HS1) (X57346)	Signal transduction	465	2
	Calmodulin (D45887)	Ca^{2+}-binding, vesicular trafficking and signal transduction	479	1
	Estrogen receptor-related protein, ERR1 (X51416)	Signal transduction: hormone nuclear receptor and transcription factor	939	3
4009–4821	14-3-3τ (X56468)	Vesicular trafficking and signal transduction	290	1
6586–7449	Importin β-subunit (L38951)	Nuclear protein transport; has ARM and HEAT repeats	1970	1
	Imogen 38 (Z68747)	Pancreas β-cell protein (secretory granule/mitochondrion)	473	1
	DiGeorge syndrome-1, DGS1 (L77566)	Novel gene from DGS critical region (22q11)	454	1
	Norbin, KIAA0607 (AB011179)	98% I, 98% S to rat norbin (AB006461); neurite outgrowth	1462	1
	LIP1, novel (AF141337)	66% I, 85% S, 35 amino acids, human TCP10A protein (U03399)	1	1
			5	3
	LIP2, novel (AF141338)	40% I, 56% S, 110 amino acids, bacterial ribosomal protein L17 (M26414)	1	1
			10	1
			21	1
	LIP4, novel (AF141340)	62% I, 73% S, 89, human hnRNP-E2 (X78136)	496	1
	LIP5, novel (AF141341)	35% I, 61% S, 131, *C. elegans* T23G11.7 (Z81130)	48	1
	LIP6, novel (AF141342)	92% I, 93% S, 139, viral protein NS2-3 (U43603)	1	2
	LIP7, novel (AF141343)	30% I, 59% S, 81, human TCP10A protein (U03399)	1	2
	LIP8, novel (AF141344)	63% I, 63% S, 21, rabbit KAP4L protein (X80035)	1	2
9037–9585	LIP3, novel (AF141339)	72% I, 83% S, 172, rat Zn finger protein Roaz (U92564)	345	1
9502–10590 (BEACH domain)	Atrophin-1 (U23851)	Mutated in dentatorubral pallidoluysian atrophy (DRPLA)	2659	1
	Embryonic-Fyn-substrate 1, EFS1 (AB001466)	Signal transduction	1664	2
	OPA-containing protein (AF071309)	Unknown function	5262	1
	M4 hnRNP (L03532)	RNA-binding, heterogeneous nuclear ribonucleoprotein	782	1
	LIP3, novel (AF141339)	72% I, 83% S, 172, Zinc finger protein Roaz (U92564)	345	1
	LIP9, novel (AF141345)	61% I, 75% S, 180, RNA binding protein etr1 (U16800)	138	1
10576–11611 (WD-40 domain)	Casein kinase II β-subunit (M30448)	Phosphorylation: vesicular trafficking and signal transduction	60	1
	LIP6, novel (AF141342)	92% I, 93% S, 139, viral nonstructural protein NS2-3 (U43603)	1	1
			5	1

*From GenBank # U67615.

[†]#AA designates the number of amino acids in the region of LYST-IP, for which the % identity/similarity is shown.

Abbreviations: I, identity; S, similarity.

In addition to 14-3-3τ, the LYST N-terminus interacted with 14-3-3β (also called HS1 and 14-3-3α), which has 82% amino acid identity to 14-3-3τ. The LYST-interaction region of 14-3-3β was the same as the LYST-interaction region of 14-3-3τ. The 14-3-3β interaction was identified twice in screens of a fetal brain library.

LYST N-terminus also interacted with the C-terminus of calmodulin (CALM), an interaction identified once in a screen of a fetal brain library. The LYST N-terminus also interacted with the C-terminal half of a steroid hormone receptor, estrogen receptor-related protein (ERR1), an interaction detected three times in a screen of a fetal brain library.

LYST fragment 6586–7449 interacted with 11 proteins: the C-terminal halves of the importin β-subunit, imogen 38, and norbin; DGS-I, a gene from the DiGeorge syndrome critical region; and 8 novel genes (LIP1, LIP2, LIP4-LIP8). In addition to LYST 6578–7449, LIP6 interacted with the LYST domain containing WD40 repeats (bp 10576–11611). Two independent isolates of LIP6 were identified among interactions with the WD40 domain. CK2β also interacted with the LYST WD40 repeat domain.

The BEACH domain of LYST (bp 9502–10590) interacted with six proteins: the amino terminal third of atrophin-1 and embryonic Fyn substrate-1 (EFS1), the N-terminus of OPA-containing protein, heterogeneous nuclear riboprotein M4, LIP3, and LIP9. LIP3 also interacted with LYST 9037–9585.

Biochemical Confirmation of LYST Interactions

Four of five interactions of LYST were confirmed by an *in vitro* binding assay (Fig. 1). HRS, 14-3-3τ, and CK2β proteins were purified and incubated with respective LYST peptides. Specifically bound proteins were isolated, resolved electrophoretically, and identified on Western blots. Interactions of LYST with HRS, 14-3-3τ, and CK2β were confirmed: HRS bound to LYST 6586–7449 but not to a negative control. 14-3-3τ bound to the LYST N-terminus and LYST 4009–4821, but not to a negative control. CK2β bound to LYST 3190–4032, but not to a negative control. The interaction of CK2β with the LYST WD40 domain failed confirmation by this method.

Discussion

A modified Y2H method was used to identify potential substrates of LYST action, using 14 LYST baits and 5 prey libraries. The baits were designed to preserve the proposed structural features of LYST. For example, the BEACH domain (10) was included in its entirety in the bait spanning bp 9502–10590, the bait extending over bp 10576–11611 contained the WD-40 domain, and the stathmin-like region was preserved in the bait encompassing bp 1051–1950. Six of the baits (derived from the first ~4700 bp of *CHS*) were common for the large and small isoforms (9–11) of the CHS protein, whereas eight baits were specific only for the large isoform. Prey libraries from tissues that are severely affected in CHS were used in the screens. For example, in CHS patients and animal models, numerous abnormalities have been described in the brain (14,15), kidney (16,17), keratinocytes (18), and hepatocytes (19,20). Authentic interactions were anticipated to involve cytoplasmic proteins because LYST is cytosolic (3,12). Of 24 interacting proteins that passed *in vivo* confirmation tests, 12 were known proteins and 12 were novel. In addition, LYST interactions with HRS, 14-3-3τ, and CK2β were confirmed *in vitro*. Several of the interacting proteins regulate vesicular transport or signal transduction, and provide potential explanations of the mechanism whereby absence of LYST causes CHS.

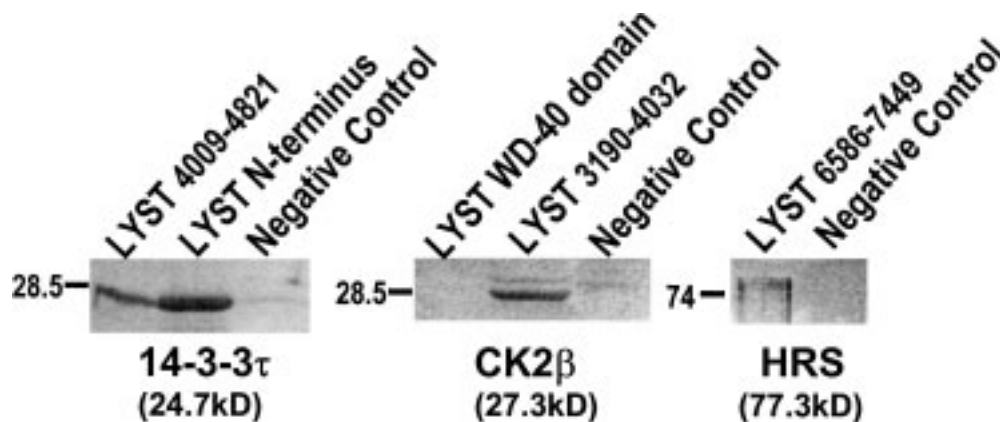Although detected once, the interaction with HRS passed *in vivo* and *in vitro* confirmation tests.

Fig. 1. **Biochemical confirmation of interactions of LYST with 14-3-3τ, CK2β, and HRS by an *in vitro* binding assay.** Following binding, the eluted proteins were resolved by SDS-PAGE, Western blotted, and detected with specific antibodies. LYST N-terminal domain: nucleotides 190-1056. LYST WD-40 (C-terminal) domain: nucleotides 10576–11611. 14-3-3τ negative control: LYST C-terminus. CK2-β negative control LYST 6586–7449. HRS negative control: LYST N-terminus. All interactions were confirmed except that between CK2β and the LYST WD-40 domain. Molecular size standards (in kD) are shown. LYST nucleotide positions are derived from GenBank U67615.

Like LYST, HRS is a cytoplasmic protein that attaches reversibly to the cytoplasmic surface of endosomes (21–23). The region of HRS that interacts with LYST contains a zinc finger domain and an ATPase catalytic site. The HRS–LYST interaction is compelling because HRS inhibits exocytosis by binding to SNAP25, a component of the SNARE protein complex that is important in vesicle docking and fusion (23–25). Ca^{2+} and Zn^{2+} inhibit binding of HRS to SNAP25 and promote exocytosis. HRS is similar in sequence to *S. cerevisiae* Vps27p, which is essential for protein transport to the vacuole (26). In binding to LYST, HRS may become unavailable for interaction with SNAP25 (Fig. 2). Thus, LYST may promote membrane docking by preventing the inhibitory effects of HRS on SNARE assembly. Alternatively, LYST-complexed HRS may have an increased affinity for SNAP25, augmenting the inhibitory effect of HRS on SNARE assembly. In this case, LYST would inhibit membrane docking and fusion.
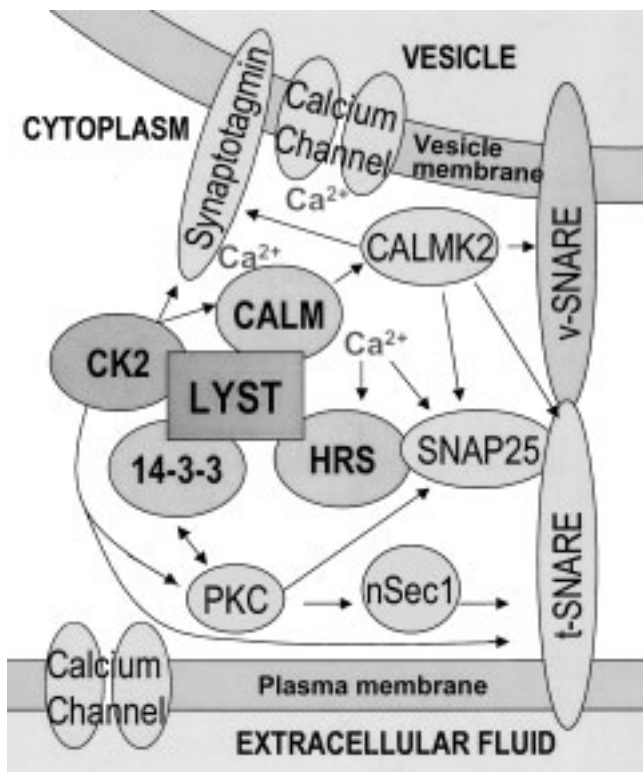

Fig. 2. Interactions of LYST with proteins that regulate vesicle docking and fusion in exocytosis. Not all salient interactions are shown (e.g., synaptotagmin also interacts with syntaxin; syntaxin is phosphorylated by CALMK2). According to this hypothesis, LYST binds to HRS, modifying HRS-mediated modulation of SNAP25, a component of the tSNARE-vSNARE docking complex. LYST also binds to CALM, bringing Ca^{2+} into the proximity of HRS, inhibiting HRS binding to SNAP25. Docking leads to transient opening of Ca^{2+} channels, and Ca^{2+}–CALM complexes activate membrane fusion. LYST interacts with 14-3-3 and CK2, which modify activity of several proteins of the tethering, docking, and fusion machinery.

HRS also interacts with STAM, a relay in intracellular signal transduction (21). Binding of HRS to STAM suppresses signaling, an interesting result; intracellular signal transduction is abnormal in CHS. CHS granulocytes have abnormal concavalin-A–induced capping associated with decreased PKC (27). Normalization of PKC activity rectifies the CHS capping defect and ameliorates the lysosomal abnormalities (28–29). Thus, interaction of LYST with HRS may potentially explain the lysosomal exocytosis and capping abnormalities of CHS.

Another provocative interaction of LYST is with CALM, a cytoplasmic Ca^{2+}-binding protein. LYST interacted with the C-terminus of CALM, which forms the end of a Ca^{2+}-binding site and an α-helix. CALM has previously been shown to bind many proteins in a Ca^{2+}-dependent manner. The LYST-CALM interaction did pass *in vivo* confirmation tests but was not evaluated *in vitro*. CALM has a significant role in membrane fusion: it binds to intracellular organelles, such as secretory granules, and is required for membrane fusion and exocytosis in cell types important in CHS such as platelets and leukocytes (30–32). In particular, SNARE-mediated docking of acidic organelles has been shown to prompt local Ca^{2+} release, causing CALM to bind tightly to the docked vesicle and promote membrane fusion. Furthermore, Ca^{2+} inhibits interaction of HRS and SNAP25. A ternary complex of LYST, HRS, and CALM would be anticipated to juxtapose HRS and Ca^{2+}, inhibiting HRS-SNAP25 binding (Fig. 2). Such LYST/CALM-mediated inhibition of HRS action would be anticipated to enhance SNAP25-regulated membrane docking and fusion (23–25). In CHS, the absence of LYST might prevent juxtaposition of HRS and CALM, potentiating HRS inhibition of SNAP25, and inhibiting membrane docking and fusion.

Other confirmed interactions of LYST were with 14-3-3 proteins 14-3-3τ and 14-3-3β, members of a highly conserved gene family involved in both exocytosis and signal transduction (33). The LYST-interacting domain of 14-3-3τ included an annexin-like region important in promoting Ca^{2+}-regulated exocytosis (34,35). The LYST-interacting domain of 14-3-3β was the same, but also included a PKC-inhibition domain. Neither of the 14-3-3–interacting domains of LYST contained the consensus binding motif (36). An interaction with 14-3-3τ was also observed with LYST2, a novel LYST-related gene that is expressed only in brain (Tchernev et al., unpublished results).

14-3-3 proteins may be either membrane-associated or cytosolic (37,38). They prime Ca^{2+}-dependent exocytosis in a PKC-dependent manner (30,39,40). 14-3-3 proteins are also important in signal transduction: 14-3-3τ binds PKC, is phosphorylated by PKC, and inhibits PKC activity (41). The PKC abnormalities described in CHS are similar to the effects of 14-3-3 on PKC (28,29). 14-3-3 also binds to several kinases, such as Raf-1 and Bcr. 14-3-3 has also been proposed to function as a scaffold protein

that facilitates interactions among components of signaling and exocytic pathways (33).

CK2, a ubiquitous serine/threonine protein kinase, is another LYST interactant associated with signal transduction and vesicular trafficking. Numerous colonies representing interactions between CK2β and LYST fragment 3190-4032 were observed with all prey libraries tested, whereas the interaction of CK2β with the WD-40 domain-containing LYST 10576–11611 was detected only once. The LYST 3190-4032-CK2β interaction was also confirmed *in vivo* and *in vitro*. CK2β is a regulatory subunit that modulates interactions with CK substrates (42). Several of these substrates are important in vesicular transport, including synaptotagmin, syntaxin, synaptobrevin, CALM, PKC, UNC18, and clathrin. A potential function of LYST suggested by interactions with both CK2β and the CK2 substrates CALM, PKC, and HRS, is to provide a scaffold that facilitates juxtaposition of proteins important in vesicular transport and signal transduction.

Other interactants included several proteins involved in signal transduction (BMK1α kinase, embryonic Fyn substrate (Efs1), Fos-transformation effector-1, and KB07, a novel gene similar in sequence to tyrosine kinases) and 12 proteins of completely unknown function. These interactions passed the *in vivo* confirmation tests and may also be significant for LYST function.

The BEACH domain (10) of LYST (bp 9502–10590) interacted with three known proteins (Table 3), some of which participate in signal transduction, as well as with three proteins with unknown function, two of which were novel (LIP3 and LIP9). Although the role of the BEACH domain remains unclear, it is possible that through its multiple interactions, this domain facilitates the association of other proteins involved in vesicular transport.

Interestingly, all known CHS mutations are predicted to result in the production of either truncated or absent proteins (9–11). Compared to the positions of the baits used in this study, in two CHS patients the premature termination occurs in the region of bait190-1056, which we detected to interact with 14-3-3τ in the forward screen, and with 14-3-3τ, 14-3-3β, calmodulin, and ERR1 in the reverse screen. In another patient, the premature stop codon was found at a location corresponding to bait 1051-1950 (containing the stathmin-like domain), and in three other CHS patients the premature termination occurs in the region of bait 3190–4032, which interacts with CK2β in the forward screen (Table 2). Premature stop codons have also been reported at positions corresponding to bait 2347–3213 (43), as well as to bait 1948–2370 and bait 9502–10590 (44), which contains the BEACH domain and interacts with six proteins in the reverse yeast-two hybrid screen (Table 3). Mutations in seven additional patients have recently been described (45), occurring in regions of LYST corresponding to baits 3190–4032,

4819–5700, 6586–7449, 7426–8238, 9037–9585, and 9502–10590, which we found to interact with multiple proteins, including HRS and CK2β. All 16 mutations reported in the literature result in premature termination, indicating that substitutions may lead to a milder phenotype, which may not be recognized as CHS. Mutations producing truncated CHS proteins do not allow evaluation of the effect of individual amino acid changes on the observed protein interactions, because they prevent binding of multiple CHS regions (baits) to their corresponding interacting proteins. However, the interactions that we found using baits from the C-terminal portions of LYST (e.g., BEACH domain and WD-40 motifs) confirm the suggestion (44) that the complete CHS protein is required for its proper function, and show that in all of the described patients the truncated CHS protein will not interact with a number of the detected interacting proteins, leading to defective vesicular transport and clinical manifestations of CHS.

In addition to contributing to an understanding of the role of LYST lysosome biology, identification of LIPs is significant for the treatment of CHS and diseases associated with excessive exocytosis of lysosomal contents. Diseases such as asthma and urticaria largely are the result of inappropriate local degranulation by leukocytes and mast cells. An attractive therapeutic strategy for these disorders would be to recapitulate the lazy lysosome phenotype of CHS in a controlled manner with a small molecule antagonist. The observation that CHS mice are protected from the inflammatory disease lupus nephritis (46) suggests that this approach may also be an effective treatment for autoimmune diseases. Although LYST is not a suitable target for conventional drug development, several LIPs, such as HRS (an ATPase), are potential drug targets. Further validation of the significance of these LIPs in vesicle exocytosis is indicated, for example by colocalization studies in the organelles of interest.

Acknowledgments

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