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Structural Basis for SH2D1A Mutations in X-Linked Lymphoproliferative Disease

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X-linked lymphoproliferative disease (XLP) is a rare and severe immune deficiency, characterized by abnormal immune responses to the Epstein-Barr virus. Recently, the gene responsible for XLP, *SH2D1A*, has been identified and shown to code for a small cytoplasmic protein with an SH2 domain that interacts with SLAM and 2B4, two receptorial molecules involved in signal transduction in T and NK cells, respectively. A variety of *SH2D1A* gene mutations have been reported thus far in XLP males. Here we describe a single-strand conformation polymorphism assay for mutation analysis in XLP. Four novel patients with *SH2D1A* mutations are described. These mutants, and the others previously reported in the literature, have been included in a Registry (SH2D1Abase) that is fully accessible on the World Wide Web. A three-dimensional model of the SH2 domain of the SH2D1A protein has been developed, based on homology with other SH2 domains. The structural consequences of disease-causing *SH2D1A* mutations are discussed. © 2000 Academic Press

X-linked lymphoproliferative disease (XLP; OMIM 308240) is a rare and severe inherited immune deficiency, characterized by an abnormal immune response to the Epstein Barr virus (EBV) (1, 2). Following EBV infection, affected males may develop severe and often fatal infectious mononucleosis, lymphoma, aplastic anemia, and/or dysgammaglobulinemia. The overall prognosis of XLP is very poor, with over 70% of affected patients dying by 10 years of age (3). In most cases, prior to EBV infection the immunological status of genotypically affected males is normal, making the pre-symptomatic diagnosis problematic (4). Recently, a novel gene, *SH2D1A*, previously named also *SAP* or *DSHP*, mutated in XLP families has been identified using positional cloning or a functional cloning approach (5–7). This gene encodes for a short cytoplasmic protein of 128 amino acids, that contains a Src homology 2 (SH2) domain, predominantly expressed in T lymphocytes (5–7). A variety of defects in the *SH2D1A* gene have been reported in XLP males (5–9). On the other hand, a substantial proportion of *bona fide* XLP patients have no apparent defects in the gene (5, 7, 8). These data, together with the recent demonstration that *SH2D1A* defects may occur in patients with atypical presentation (8), including cases of non-Hodkin lymphoma in EBV-seronegative males (9), indicates the need for diagnostic strategies based on mutation screening.

The molecular and cellular mechanisms that account for XLP are still poorly defined. The SH2 domain of the SH2D1A protein appears to play a key functional role. SH2 domains of about 100 residues have been found from a large number of signalling proteins. This module binds tightly to phosphotyrosine (pY)-containing peptides and proteins (10–15). SH2 domains are explicitly involved in protein tyrosine kinase (PTK) signalling pathways, and have been shown to modulate enzyme activity, or to target proteins to certain cellular location (16). The three dimensional structure has been determined for several SH2 domains, in many cases as a complex with high affinity peptides. Their general organization is similar, with a core formed by a continuous β-meander of two connected β-sheets, which are sandwiched between two α-helices. The pY ligand is bound by several conserved residues on the surface of the SH2 domain, and a highly ordered hydrogen bonding network contributes to the binding.

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TABLE 1

Primer Pairs and Conditions for Amplification of the Four Exons and Flanking Splice Sites of the SH2D1A Gene

Exon	Primer 5′	Primer 3′	PCR size (bp)	Ta
1	TTTGCACATCTGGCTGAACT	GCCCATGTCCACCGTATCA	242	64
2	GTGTCCTAGTATATGTGACATT	CAAATACCTCCTTGACACCC	200	60
3	TTTGTATCATTATGAGATAGGTA	CTGAGCTTCCAAACCCTGTC	240	56
4	TTATAAGTTTGAGTTAATCTGT	CATTTGTAGCTCACCGAACTG	210	60

Note. Ta, temperature of annealing.

SH2 domains have several functions. In Src family kinases, in addition to ligand binding, the SH2 domain also regulates the activity of the kinase by binding to a regulatory pY in the C-terminal tail (17–19). The SH2 domain of the SH2D1A protein has been shown to interact with a phosphotyrosine residue of the cytoplasmic tail of SLAM (Signalling Lymphocyte Activating Molecule), a co-receptorial molecule predominantly expressed in T and B lymphocytes, and involved in cell activation (6, 20). More recently, interaction between SH2D1A and 2B4, an activatory receptor of Natural Killer (NK) cells, has been also demonstrated *in vitro* (21, 22). Interaction of SH2D1A with either SLAM or 2B4 would prevent binding of SHP2, an SH2 domain containing phosphatase, to SLAM or 2B4, and thus modulate immune responses in T and NK cells (6, 21). Defective expression and/or function of SH2D1A, as in XLP males, may therefore contribute to inappropriate immune response following EBV infection.

In this paper, we report a DNA-based screening assay for mutation detection in XLP, which has allowed us to identify the molecular defect in four previously unreported patients. All these patients were included in a novel clinical and molecular registry of patients with *SH2D1A* gene mutations, that is fully accessible through the World Wide Web. Finally, we present the structural model of the SH2 domain of the SH2D1A protein, and discuss the effects of naturally occurring mutations, as a first step towards a better understanding of the molecular mechanisms of the disease.

MATERIALS AND METHODS

Patients. Four unrelated males with a clinical history highly suggestive for XLP were studied. In all cases, a positive family history was apparent. DNA samples were available from three males prior to their death, and were collected from peripheral blood from patient D, who is still alive. Patient A died of fulminant hepatitis following EBV infection, and had four male maternal relatives who developed lymphoma or fatal infectious mononucleosis. Patient B was a 7-year-old male who died of non-Hodgkin lymphoma and had one maternal uncle who died with aplastic anemia following EBV infection. Patient C was a 4-year-old male who died of fatal hepatitis following EBV infection; his elder brother had died at 16 months of life of non-Hodgkin lymphoma. Finally, patient D is a 56-year-old male with hypogammaglobulinemia diagnosed after severe EBV infection; he has a nephew who developed EBV-related non-Hodgkin lymphoma at 3 years of age.

SSCP screening assay and mutation analysis at the SH2D1A locus. PCR primers were designed from the intronic region flanking the four exons of the *SH2D1A* gene. The primer sequences, the length of the PCR product and the annealing temperature for the amplification reactions are reported in Table 1.

200 ng of genomic DNA was amplified at the specific annealing temperature in a reaction mix containing 2 mM $MgCl_2$ (Perkin Elmer, Foster City, CA). PCR products were then analyzed by Single Strand Conformation Polymorphism and Heteroduplex formation (SSCP/HD) screening assay. Briefly, the amplification products were diluted 1:1 with loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05 xylene cyanol), denatured for 5 minutes at 95°C, chilled at −20°C for 5 minutes and then run onto a 0.5× MDE gel plus 5% glycerol. Gels were run at 4°C in 0.6× TBE at 70 volts for 20 h and then revealed with silver nitrate. PCR products showing abnormal SSCP/HD pattern were further analyzed by direct sequencing using the Dye Terminator Mix and the automated ABI Prism 310 Sequencer (Perkin Elmer, Foster City, CA).

Sequence analysis and molecular modelling. The SH2D1A SH2 domain structure was modeled based on the crystal structure of the Abl SH2 domain (Protein Data Bank PDB entry 2abl) at 2.5 Å resolution (23). The sequence alignment was built with the GCG (24) and MULTICOMP program packages (25). The model was built by using program Insight II (Molecular Simulations, Inc., San Diego, CA). Insertions and deletions were modeled with a database, which contained either most of the PDB (26) structures or an unbiased selection of PDB (27). The model was refined by energy minimization with the program Discover using the Amber force field in a stepwise manner. First the new loops were minimized while the borders of indels (insertions and deletions) were harmonically restrained and the rest of the molecule was fixed. In the next step the borders of indels and the C_a atoms of the conserved regions, and the finally only the C_a atoms of the conserved regions were harmonically constrained. The model was evaluated with programs WHAT CHECK and PROCHECK (28, 29). The ligand peptide was docked to the SH2 domain based on the Src SH2 domain high-affinity peptide complex (PDB entry 1sps) (30).

SH2D1Abase. SH2D1A gene mutation data as well as information from the affected patients were collected into the SH2D1Abase. A program was developed for the submission of mutation and patient information into the database by using MUTbase program suite (31). The registry was built according to the concepts used in BTKbase (32, 33) and other immunodeficiency mutation databases (IDbases). The database contains four major items: identification of the patient and mutation(s), reference either to published article(s) or a submitting physician, mutation information, and data related to disease and therapy. Data security protects the patients identity, which is not coded into the registry. Data are organized into entries, which can be analyzed with the provided computer tools or with sequence retrieval system (SRS) (34).

TABLE 2

Mutations in *SH2D1A* Gene Identified through the SSCP Screening Assay

Patient	Exon	Mutation	Effect
A	1	ATG302ATT	M1I
B	1	Intron 1, +1 G > A	Splice site
C	2	CGA462TGA	R55X
D	2	CGA462TGA	R55X

RESULTS

Mutation Analysis at the SH2D1A Locus

The coding region and flanking splice sites of the *SH2D1A* gene were analyzed by SSCP/HD. In all four patients with a putative diagnosis of XLP, an abnormal SSCP/HD pattern was observed, and direct sequencing of the amplification product allowed to identify the *SH2D1A* mutation (Table 2).

In particular, in patient A, an abnormality of the SSCP pattern was found in exon 1. DNA sequencing revealed a G to T mutation at the translation initiation codon ATG, thus changing the invariant Met into Ile. A different abnormality of the SSCP pattern in exon 1 was also identified in family B. Direct sequencing of the PCR product revealed a G to A nucleotide change at position +1 of the invariant GT dinucleotide in the donor splice site of intron 1.

Patients C and D, belonging to unrelated families, shared the same abnormal SSCP pattern in exon 2. Sequencing analysis revealed a C to T mutation at position 462, leading to premature termination at residue 55.

Structural Model of the SH2D1A SH2 Domain

Sequence analysis indicated that Abl SH2 domain had the highest sequence similarity among the solved protein structures (Fig. 1). The sequence identity is 31%, which ensures an accurate model as the structural scaffolding of SH2 domains is conserved; in particular, SH2D1A retains the conserved and invariant residues that are present in Abl and in most other SH2 domains (Fig. 2).

The quality of computer modeling relies greatly on the quality of the sequence alignment, which in this case is unambiguous and provides good basis for modeling. The amino acid substitutions were mainly conservative. There are two insertions in the model, and both occur in variable regions between secondary structural elements. A one residue insertion in the loop connecting the first α-helix and the following β-strand leads to a longer helical structure in SH2D1A. The other insertion of five amino acids is in the loop connecting β-strands 5 and 6. This loop has been shown to interact with the ligand residue +3 in several SH2 domain structures (Table 3) (30, 35–38). The stereochemical quality of the SH2D1A model and Abl SH2 structure were analyzed and found to give high scores of globular proteins.

SH2D1Abase

The information of the XLP-causing *SH2D1A* gene mutations (5–9) was collected into a database called SH2D1Abase. In addition to mutation data, the registry also contains clinical and immunological information (immunoglobulin levels, lymphocyte counts, age at diagnosis, symptoms, details of therapy, when available), mRNA and protein data, and illustrates the putative structural consequences of the mutations. Furthermore, the registry also provides information on mutation-caused abnormalities in restriction sites, as well as overall analysis of type and distribution of mutations along the gene. The SH2D1Abase is freely accessible through the World Wide Web at http://www.uta.fi/imt/bioinfo/SH2D1Abase.html. Researchers are encouraged to send their patient and mutation information to the registry.

The current SH2D1Abase contains 48 mutation entries from 39 unrelated families showing 22 different molecular events. There are 6 different missense and three nonsense mutations. Gross deletions cause deletion of the whole gene. The upstream mutation changes the CCAAT-box. The only insertion is caused by a mutation in the stop codon changing it to arginine and insertion of 12 residues at the C-terminus of the protein.

The SH2D1A mutations were compared with other disease-related SH2 domain mutations. The SH2D1A

TABLE 3

Conservation of Ligand Binding Residues in SH2 Domains

	Src	SH2D1A	Abl	GapC	SykN	Btk
pTyr	R155	R13	R134	K356	R22	R288
	R175	R32	R152	R375	R42	R307
	S177	S34	S154	S377	S44	S309
	E178	E35	E155	D378	R45	S310
	T179	S36	S156	N379	N46	K311
	T180	V37	S157	T380	Y47	A312
	K203	R55	R157	K398	T65	V335
+1	K200	Y52	Y172	Q395	H62	R332
	H201	T53	H173	R396	H63	H333
	Y202	Y54	Y174	F397	Y64	Y334
	K203	R55	R175	K398	T65	V335
	R205	S57	N177	C400	E67	C337
	I214	A66	V186	M408	I76	L346
+3	Y202	Y54	Y174	F397	Y64	Y334
	I214	A66	V186	M408	I76	L346
	T215	E67	S187	G409	A77	A347
	Y230	A86	H200	Y423	Y91	Y361
	G237	G93	G208	I428	G98	G368
	L238	I94	L209	V429	L99	L369

gene insertions and deletions reported so far lead to frameshift and premature termination of the protein. Missense mutations are particularly interesting as they may be used to dissect the structural and functional properties of the SH2D1A protein using the three dimensional model.

DISCUSSION

Mutation Analysis in XLP

We used SSCP screening assay for mutation detection in XLP. An aberrant SSCP pattern was identified in all four previously unreported males with a clinical diagnosis of XLP. Direct sequencing of the amplification products that led to aberrant SSCP pattern unequivocally identified *SH2D1A* gene mutations. Of the four mutants, one (M1I in patient A) has not been previously reported, and thus adds to the heterogeneity of *SH2D1A* mutations. In our series, two patients (C and D) carried the same mutation, i.e., the C to T nucleotide substitution at position 462 leading to premature termination at codon 55. The same mutation has been previously reported in four unrelated patients (5, 7, 8). Ethnical differences and haplotype analysis (8) argue against a founder effect to explain the frequency of this mutation in XLP males. Thus, nucleotide 462 is a mutational hot-spot in the *SH2D1A* gene. This mutation involves a CpG dinucleotide, which harbors the majority of disease-causing point mutations in humans, despite of the underrepresentation of the doublet (39). Similarly, the splice site mutation (G to A at position +1 of intron 1) identified in the patient B has been previously reported in two unrelated patients (8, 9), and may also represent a mutational hot-spot. In spite of these common mutations, XLP is characterized by a rather extended heterogeneity of mutations, including missense, nonsense, and splice-site mutations, small insertions and deletions, and gross deletions. Development of the SSCP screening assay should facilitate mutation analysis.

Independently from classical XLP, the possibility that lymphomas may be significantly contributed to by *SH2D1A* gene mutations is controversial. Brandau *et al.* have reported *SH2D1A* gene defects in three EBV-seronegative males with non-Hodgkin lymphoma (9). However, Yin *et al.* (8) have failed to identify such mutations in other patients with lymphoma. Our SSCP assay should now enable screening of a large cohort of patients with various types of lymphoma to address this important issue.

Three Dimensional Model of the SH2D1A Protein Structure

Crystal structures of SH2 domains with high-affinity peptides have shown that several residues are involved in phosphotyrosyl peptide binding (30). The number of residues binding in addition to pY varies from three to at least six of seven. However, also regions outside the actual pY binding region can be essential for affinity and specificity.

SH2D1A participates in the regulation of SLAM and it can recognize the binding site whether it is phosphorylated or not, unlike many other SH2 domains. In order to study its function, the SH2D1A model was superimposed to Src SH2 domain with the high-affinity peptide (30). The peptide was modified to correspond to the SH2D1A ligand, SLAM protein sequence YAQV starting from 281 (Fig. 2). The docked peptide fits well to the SH2 domain structure. The phosphotyrosine ligand binding site of SH2 domains consists of several conserved amino acids as revealed by three dimensional structures of a number of SH2 domains (30, 41, 42). The corresponding residues in the four disease-related SH2 domains are listed in Table 3. Arginines R13 and R32 of SH2D1A are thought to be responsible for the specific recognition of the phosphate group and the tyrosine side chain. In Src SH2 domain structure, other interactions with the phosphate group involve the last amino acid of the b strand 2 and the residues in the adjacent loop (30). In the SH2D1A mode (Fig. 2), S39 is close enough to be involved in ligand binding, but the BC loop has more open conformation than Src. In the uncomplexed structure of Src SH2 domain, the corresponding loop is relatively disordered and it points away from the binding pocket (30). In the Lck SH2 domain crystal structure with (phosphomethyl)-phenylalanine-peptides (42), the loop has turned about 40 degrees compared to Src (30, 43). When binding to peptide, the loop shifts closer to the phosphate group facilitating the hydrogen bond formation. The open conformation of the binding site might explain the fact that SH2D1A SH2 domain can recognize and bind to an unphosphorylated ligand.

Previously, Shc has been shown to bind to Grb2 SH2 domain independent of the phosphorylation state (44). Recognition of the tyrosine is essential for the binding and provides the basis for development of Grb2 inhibitors (45). Further, at least Src family SH2 and SH3 domains can interact (46) presumably by binding unphosphorylated SH3 domain to SH2 domain pY ligand recognition site (47). Thus, the SH2 domain interactions with non-phosphorylated ligands can be more common than anticipated. These interactions can have important regulatory functions, but they can also be replaced by high-affinity ligands.

SH2 domains achieve their binding specificity through recognition of several amino acids in addition to the phosphotyrosine. Several of the amino acids required for recognition are conserved in SH2D1A compared to other SH2 domains (Table 3). The side chain of Y202 forms hydrophobic contacts, and K200 is hydrogen bonded to the glutamate +1 in Src. The corre-

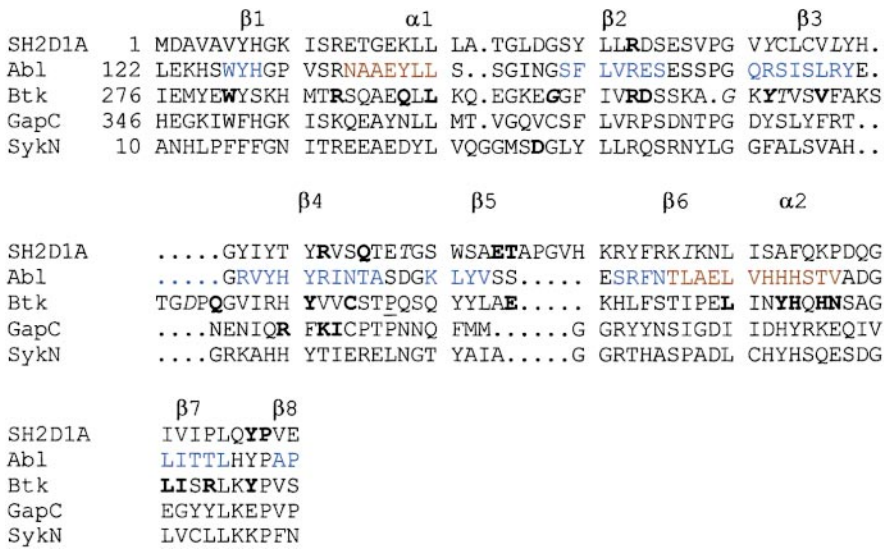

FIG. 1. Sequence alignment of the SH2D1A and Abl SH2 domains. The other SH2 domains involved in diseases are also included, Btk (entry Btk_human), Gap (Gtpa_human), and Syk (Ksyk_human). The secondary structures are indicated by colors, α-helices in red and β-strands in blue. Disease causing mutations are depicted. Substitutions are indicated in bold letters, deletions are indicated with italic letters, and insertions are underlined.

sponding residue Y54 in the SH2D1A model has an identical orientation.

Structural Consequences of XLP-Causing Mutations

Several XLP-causing *SH2D1A* mutations have been identified (5–9, 48) and collected into SH2D1Abase. A large proportion of the mutations lead to truncation of the produced protein either due to nonsense mutation or because of frame shift causing deletions, or due to gross deletions. The truncated proteins do not fold and function correctly even if produced. R55X and Q58X mutations delete half of the SH2 domain. The Y100X mutation is located in the C-terminus of the SH2-domain and the structure of the domain could be preserved; however, the predicted mutant protein would miss the C-terminal extension. The two deletions reported at positions 448 (5) and 481 (7) would lead to unfunctional protein if translated, as would the entire deletion of exon 1 (9) and exon 2 (8).

In addition to the missense mutation M1I reported in this paper, five other missense mutations (R32T, E67D, T68I, G93D, P101L) in the SH2D1A protein have been described (Fig. 2) (5, 8). Mutations of the initiator codon are rather common also in another X-linked immunodeficiency XLA (33). Although not introducing a stop codon, the mutation does not allow transcription initiation.

The R32T mutation introduces a change in the most conserved amino acid among SH2 domains. This arginine is required for phosphotyrosine recognition and binding (30, 43, 49). The corresponding residue causes also XLA when mutated to glysine or threonine (50, 51). *In vitro* mutagenesis of the arginine has led to impaired function of SH2 domains (53, 54).

The E67D and T68I mutations change the conformation of the loop connecting the β5- and β6-strands. The corresponding T68 residue in Btk is also mutated leading to XLA (52). E67 and T68 are located near amino acids involved in residue +3 binding.

P101 at the C-terminus of the SH2 domain is strongly conserved. This residue is located in the short loop connecting the last two β-strands. Substitution of the peptide backbone turning proline by isoleucine will most likely affect folding of the protein, because the kink formed by proline is hardly preserved by other residues.

A point mutation in the stop codon changing it to arginine and insertion of 12 residues at the C-terminus of the SH2D1A protein has also been found (5). The extra residues are likely to change the structure of the C-terminal extension, rather than folding of the SH2 domain.

Mutations in the C-terminal SH2 domain of GAP have been found cause basal carcinomas and mutations in the SH2 domain of Btk lead to XLA (34, 55). Also a single nucleotide insertion in the Syk N-terminal SH2-domain has been found to cause lack of expression (56). A number of mutated residues in these proteins lead to disorders (Fig. 1). Despite of the several mutated sites, only two positions have been identitified in two disorders; R32 and the corresponding residue in Btk and deletion from residue 41. Most likely new missense mutations to be characterized will

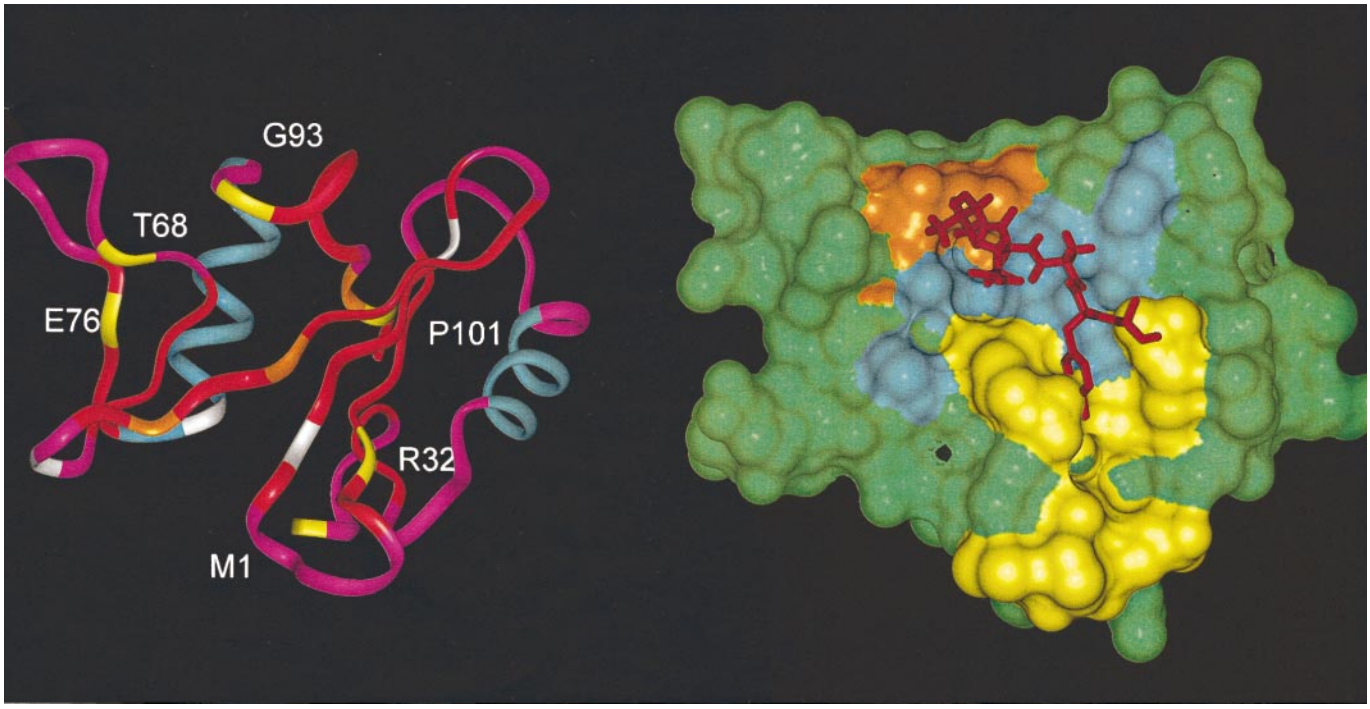

FIG. 2. The model of the SH2D1A SH2 domain three dimensional structure. (Left) The ribbon presentation of the domain. α-Helices in red and β-strands in blue. The positions of the missense mutations are in yellow. Locations of nonsense mutations are in orange and the first residues of deletions in white. (Right) Binding of the SLAM ligand (red) to the SH2D1A SH2 domain (green). The peptide was docked base on the peptide complex of Src (30). The residues forming binding sites for pY and residues +1 and +3 are in yellow, cyan, and orange, respectively. The two figures are in the same orientation.

either affect the ligand binding region or change the fold of the domain.

As a whole, the three-dimensional model of the SH2D1A protein has proven useful for the analysis of the structural consequences of *SH2D1A* mutations identified in XLP patients, and may thus serve as a basis for a better understanding of the molecular pathophysiology of the disease.

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