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Geldanamycin-induced Lyn dissociation from aberrant Hsp90-stabilized cytosolic complex is an early event in apoptotic mechanisms in B-chronic lymphocytic leukemia

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Lyn, a tyrosine kinase belonging to the Src family, plays a key role as a switch molecule that couples the B-cell receptor to downstream signaling. In B-CLL cells, Lyn is overexpressed, anomalously present in the cytosol, and displays a high constitutive activity, compared with normal B lymphocytes. The aim of this work was to gain insights into the molecular mechanisms underlying these aberrant properties of Lyn, which have already been demonstrated to be related to defective apoptosis in B-cell chronic lymphocytic leukemia (B-CLL) cells. Herein, Lyn is described to be in an active conformation as integral component of an aberrant cytosolic 600-kDa multiprotein complex in B-CLL cells, associated with several proteins, such as Hsp90 through its catalytic domain, and HS1 and SHP-1L through its SH3 domain. In particular, Hsp90 appears tightly bound to cytosolic Lyn (CL), thus stabilizing the aberrant complex and converting individual transient interactions into stable ones. We also demonstrate that treatment of B-CLL cells with geldanamycin, an Hsp90 inhibitor already reported to induce cell death, is capable of dissociating the CL complex in the early phases of apoptosis and thus inactivating CL itself. These data identify the CL complex as a potential target for therapy in B-CLL. (Blood. 2008;112:4665-4674)

Introduction

B-cell chronic lymphocytic leukemia (B-CLL) is the most common leukemia in the Western world and is characterized by the accumulation of relatively mature B cells with aberrant coexpression of $CD5^+$ in blood, bone marrow, and peripheral lymphoid organs.[1] The clinical course of B-CLL is heterogeneous, varying from a stable, long-lasting indolent form to rapidly progressive disease and death. Several biologic parameters have been proposed to account for this clinical heterogeneity, including the mutational status of immunoglobulin variable region genes (IgV_H), expression of 70-kDa zeta-associated protein (ZAP-70) and $CD38^+$ antigen, as well as specific cytogenetic alterations.[2,3] On the basis of the mutational status of IgV_H, B-CLL patients can be classified into 2 major groups with different outcomes: one with mutated IgV_H genes (M-CLL) and a relatively stable disease course and one with unmutated IgV_H configuration (U-CLL) and a more aggressive clinical behavior. The latter correlates with the up-regulation of ZAP-70.[4,5] Despite the extensive molecular characterization of B-CLL cells, little is known on the molecular mechanisms involved in its neoplastic transformation and proliferation. In the past, it was assumed that CLL is caused by the accumulation of slowly proliferating cells with defective apoptosis, but more recently B-CLL has been described as a dynamic disorder, in which the accumulation of cells is consequent to increased cell replication associated with decreased cell death, the high replication rate resulting in a rapidly progressive disease.[6,7]

One approach toward the discovery of new therapeutic targets is to explore the nature of the intracellular pathways responsible for modulating the proliferation and/or apoptotic rate of B-CLL cells. In this regard, B-cell receptor (BCR) engagement is known to play an important role by triggering a signaling cascade mediated by Lyn, a tyrosine kinase belonging to the Src family (SFK), which plays a key role in many downstream pathways.[8-10] The activity of SFKs is mainly modulated by the phosphorylation of 2 critical tyrosine residues in the activation loop (Tyr396 of Lyn) and the C-terminus (Tyr508 of Lyn).[11-13] Phosphorylation of C-terminal tyrosine induces an inactive closed conformation of the protein kinase through 2 major intramolecular inhibitory interactions: binding of phosphorylated C-terminal tyrosine to the SH2 domain, and interaction of a polyproline type II helical motif (PPII) in the SH2-kinase linker with the SH3 domain.[14-16] The activation of SFKs involves disruption of these inhibitory interactions through multiple mechanisms, such as dephosphorylation of the tail, displacement of the tail from the SH2 domain, displacement of the PPII motif from the SH3 domain, mutations in the SH3-SH2 connector, and/or mutations in the SH2-kinase linker.[17-23] It is known that, after activation, SFK level is regulated by the balance of 2 opposing mechanisms: degradation by ubiquitinylation or rescue by association with Hsp90, a chaperone interacting with the N-terminal lobe of the SFK catalytic domain.[24-27]

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We have recently demonstrated in B-CLL cells that Lyn is remarkably overexpressed and anomalously localized in the cytosol, displaying remarkable constitutive activity that leads to increased basal tyrosine protein phosphorylation and poor responsiveness to BCR ligation. Restoration of apoptosis by treatment of leukemia cells with specific Lyn inhibitors also points out the importance of high basal Lyn activity in altering the balance between cell survival and apoptosis signals in B-CLL cells.[28] The aim of this work was to gain deeper insight into the molecular mechanisms that give rise to aberrant properties of Lyn in this disease. We herein demonstrate that Lyn is an integral component of an aberrant cytosolic 600-kDa complex, where Lyn is associated both with Hsp90 through its catalytic domain and with HS1 and SHP-1L through its SH3 domain. Moreover, Hsp90 stabilizes the complex by contributing to converting a network of transient interactions into permanent ones, thus maintaining Lyn in an active conformation and preventing its degradation. Geldanamycin (GA), an apoptotic compound that directly binds and inhibits Hsp90, causes disruption of the aberrant cytosolic complex and, in turn, inactivation of Lyn in the early phases of apoptosis in B-CLL cells.

Methods

Materials

GA, 17-(allylamino)-17-demethoxy-geldanamycin (17-AAG), lactacystin, polymer polyGlu$_4$Tyr, lambda protein phosphatase (λ-PPase), and phosphatase inhibitor cocktail 1 and 2 were from Sigma-Aldrich (St Louis, MO). [γ^{32}P]ATP was from GE Healthcare (Little Chalfont, United Kingdom).

Anti-IgM–fluorescein isothiocyanate (FITC) and anti-IgD–FITC antibodies were from Dako Denmark (Glostrup, Denmark). Antiphospho-Lyn (Tyr507) (pY$_T$), antiphospho-SFK (Tyr416) (pY$_A$), and anti-PARP polyclonal antibodies were from Cell Signaling Technology (Danvers, MA). Anti-Lyn, anti-Akt, anti-Cbl, anti–SHP-2, anti–SHP-1, a polyclonal antibody raised against the C-terminal tail of SHP-1 and Hsp90 α/β, were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-HS1 polyclonal antibody was obtained as elsewhere reported.[29] Monoclonal antibodies CD3-FITC, CD19-FITC, CD5-PE, CD16-PE, CD23-PE, CD38-PE, and CD79b-PE, the monoclonal anti–SHP-1/1L, were from BD Biosciences (San Jose, CA). The monoclonal antiphosphotyrosine (clone PY-20) was from MP Biomedicals (Irvine, CA). Anti-STAT3 antibody and monoclonal anti–ZAP-70 antibody were from Upstate Biotechnology (Charlottesville, VA). Anti–β-actin antibody (clone AC-15) was from Sigma-Aldrich.

Patients, cell separation, and culture conditions

After obtaining their informed consent, 40 untreated patients, according to the criteria for diagnosis of B-CLL,[30] were enrolled in the study. The study was approved by the scientific board from the Department of Clinical and Experimental Medicine, Padua University. Informed consent was provided in accordance with the Declaration of Helsinki.

Patient characteristics are listed in Table 1. Peripheral blood mononuclear cells were isolated from the blood of B-CLL patients by density gradient centrifugation through a Ficoll-Hypaque cushion, as previously reported.[32] B cells were isolated as already described.[33] As assessed by flow cytometry, the content of CD19$^+$ B cells was higher than 95% in all samples.

Untouched peripheral blood B cells were isolated from the peripheral blood mononuclear cells of 5 healthy donors by negative selection with a B-cell isolation kit and MACS separation columns (Miltenyi Biotec, Auburn, CA). The purity of isolated peripheral blood B cells was at least 95% (CD19$^+$), as assessed by flow cytometry.

Purified B-CLL cells were either used immediately or cultured (2×10^6 cells/mL) in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% heated inactivated fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37°C in a humidified atmosphere containing 5% CO_2.

Flow cytometric analysis

The following monoclonal antibodies were used for direct immunofluorescence staining: CD3-FITC, CD19-FITC, anti–IgM-FITC, anti–IgD-FITC, CD5-PE, CD16-PE, CD23-PE, CD38-PE, and CD79b-PE. Cells were scored on a FACSCalibur analyzer (BD Biosciences Immunocytometry Systems), and data were processed by CellQuest software (BD Biosciences).

Recombinant proteins

The recombinant GST-Lyn/SH3 domain was expressed and purified according to the protocol described by Takemoto et al.[34] The GST-Lyn/SH2 domain was expressed and purified as previously reported.[35]

Recombinant HS1ΔN-Term (Δ1-207), HS1ΔSH3 (Δ403-486), and HS1-ΔPro-rich (Δ324-393) were expressed and purified as previously described.[36]

Cell lysis and subcellular fractionation

For total lysates, normal and B-CLL cells (5×10^5 for each assay) were rapidly lysed in 62 mM Tris/HCL buffer, pH 6.8, 5% glycerol, and 0.5% β-mercaptoethanol containing 0.5% sodium dodecyl sulfate (SDS).

For subcellular fractionation, B-CLL cells (15×10^6 for each assay) were disrupted on ice by sonication (3 cycles of 5 seconds at 22 Hz intervaled by 15 seconds) or alternatively in a Dounce homogenizer (20 strokes) in 350 μL isotonic buffer (50 mM Tris/HCl, pH 7.5, 0.25 M saccharose, 1 mM sodium orthovanadate, and protease inhibitor cocktail). Homogenates were centrifuged 10 minutes at 10 000*g*, and the supernatant was further centrifuged 1 hour at 105 000*g* to separate cytosol from microsomes. Protein concentration was determined by the Bradford method.

Treatment of cytosol with λPPase

A total of 300 μg of the cytosolic protein was obtained from B-CLL cells as described in "Cell lysis and subcellular fractionation," with isotonic buffer without sodium orthovanadate. The sample was supplemented with 20 mM $MnCl_2$ and λPPase buffer. The mixture was divided into 2 equal aliquots, and 300 units of λPPase was added to one of them. The samples were incubated 45 minutes at 30°C and blocked by phosphatase inhibitor cocktails 1 and 2 and 1 mM sodium orthovanadate.

Fractionation by centrifugation on glycerol gradient

A total of 150 μg of cytosolic protein from B-CLL cells was loaded on a 3.9-mL glycerol (10%-40%) linear gradient in 25 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, pH 7.4, 1 mM ethylenediaminetetraacetic acid. The tubes were centrifuged 18 hours at 100 000*g* in an SW60Ti rotor (Beckman Coulter, Fullerton, CA) at 4°C and fractionated from the top into 18 fractions.

Western blotting

Samples, from different cell fractions or immunoprecipitates, were run in 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After treatment with 3% bovine serum albumin at 4°C overnight, membranes were incubated with the appropriate antibodies for 2 hours and treated as reported by Contri et al.[28] Membranes, when required, were reprobed with other primary antibodies after stripping with 0.1 M glycine (pH 2.5), 0.5 M NaCl, 0.1% Tween 20, 1% β-mercaptoethanol, and 0.1% NaN_3 for 2×10 minutes.

In vitro tyrosine kinase assays

Lyn activity from various samples was assayed on 200 μM cdc2(6-20) peptide or 1 mg/mL polyGlu$_4$Tyr as described.[28]

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Table 1. Biologic and clinical characteristics of the patients

Patient no.	Age, y	Sex	Rai stage*	WBC count/mm^3	Lymphocytes, %	V_H mutational status†	ZAP70 expression‡
1	51	M	0	33 300	87.1	Mutated	−
2	64	F	0	49 600	83.6	Mutated	+
3	46	F	0	25 000	74.4	Mutated	−
4	58	F	0	37 500	84.0	Unmutated	+
5	67	M	0	18 600	82.8	Mutated	ND
6	64	F	I	68 900	85.5	Mutated	−
7	72	F	I	16 600	92.4	Mutated	−
8	68	M	I	29 600	81.2	Mutated	−
9	44	F	I	50 200	89.5	Mutated	−
10	70	F	I	15 200	76 3	Mutated	+
11	74	M	I	17 700	68.6	Unmutated	+
12	75	F	I	14 200	60.0	Unmutated	+
13	63	M	I	28 800	84.0	ND	−
14	56	M	I	77 000	82.5	Mutated	+
15	49	F	I	37 200	83.4	Unmutated	+
16	60	M	I	11 500	77.6	Mutated	+
17	69	M	I	18 500	81.5	Unmutated	+
18	74	M	II	28 000	85.0	Mutated	−
19	59	M	II	156 200	75.9	ND	−
20	65	M	II	27 680	79.7	Mutated	−
21	65	M	II	142 000	80.2	Unmutated	ND
22	63	M	II	33 000	85.1	Unmutated	+
23	66	M	III	29 700	88.6	Mutated	−
24	60	M	III	39 400	74.0	Unmutated	+
25	60	M	III	73 640	88.7	Mutated	−
26	60	M	III	33 500	79.5	Mutated	+
27	68	M	III	128 800	96.4	Unmutated	+
28	84	M	IV	160 500	76.1	Unmutated	+
29	80	F	IV	27 300	84.6	Mutated	+
30	77	F	IV	46 700	88.8	Mutated	−
31	77	M	IV	120 600	79.1	Unmutated	+
32	74	F	IV	31 700	85.6	Unmutated	+
33	85	M	IV	36 800	91.6	Mutated	−
34	63	M	IV	106 100	85.6	Mutated	−
35	80	M	IV	41 300	87.8	Unmutated	+
36	82	M	IV	150 800	76.0	Mutated	−
37	58	M	IV	32 800	89.0	ND	−
38	60	M	IV	87 600	94.4	Unmutated	+
39	33	F	IV	21 700	81.6	Unmutated	+
40	64	M	IV	51 700	82.1	Mutated	ND

ND indicates not determined; −, negative; and +, positive.
*Staging system developed by Rai et al.[31]
†Mutated was defined as having a frequency of mutations greater than 2% from germline V_H sequence.
‡As determined by immunoblot analysis on purified B cells (purity at least 98%).

Results

The remarkable basal activity of Lyn is related to its aberrant phosphorylation state in B-CLL cells

To understand the molecular mechanisms by which Lyn becomes constitutively active in resting B-CLL cells, cell lysates as well as microsomes and cytosol from untreated B-CLL patients in different clinical stages (Table 1), compared with B lymphocytes from healthy donors, were tested for the autophosphorylation site common to all SFKs (Tyr396 of Lyn, and referred to as pY_A) and for phospho-Lyn site recognizing the C-terminal regulatory tyrosine of Lyn (Tyr508 of Lyn, and referred to as pY_T), diagrammed as shown in Figure 1A, by Western blot analysis. The relative purity of the subcellular compartments was assessed using antibodies against specific markers (Figure 1B). Figure 1C shows the representative results obtained on the sample from one normal donor (left panel) in comparison with that from patient 15 (right panel), whereas Figure 1D displays the densitometric analysis of the Western blot bands corresponding to Lyn on B cells from 5 normal donors (left panel) compared with the data obtained in B-CLL cells from all the 40 B-CLL patients (right panel). As expected in normal B cells, Lyn appeared exclusively in the microsomal fraction and immunoreacted only with anti-pY_T antibody, demonstrating that the enzyme was in the inactive conformation (Figure 1C,D). As previously observed,[28] B-CLL samples showed overexpression of Lyn compared with control cells (Figure 1C,D, compare lane 1 with lane 4) and a remarkable fraction of the kinase ($>$ 30% of total protein) abnormally localized in the cytosol (Figure 1C,D, compare lanes 2 and 3 with lanes 5 and 6, respectively). Immunostaining of the subcellular fractions revealed that the anti-pY_T antibody reacted only with microsomal Lyn, whereas anti-pY_A reacted with both

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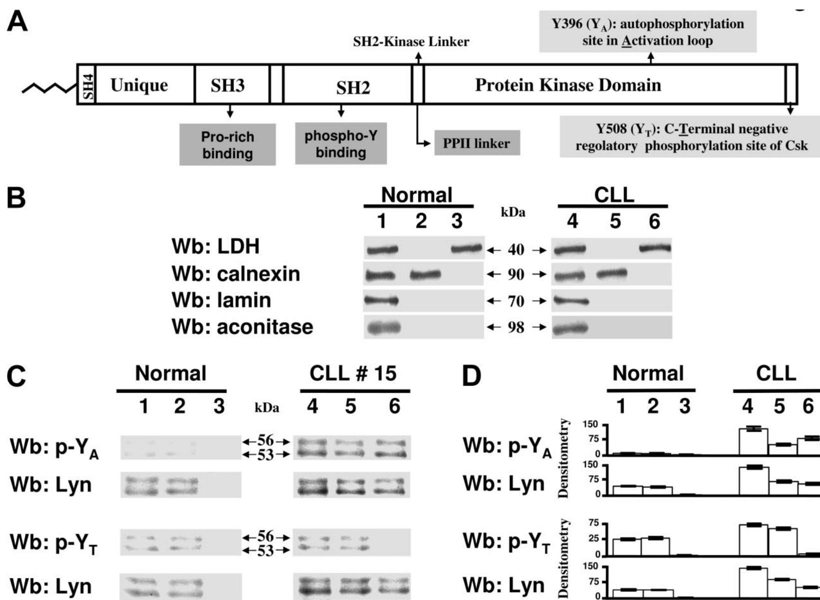

Figure 1. Aberrant phosphorylation state of Lyn in B-CLL. (A) Schematic representation of domain structure and functional properties of Lyn along with the 2 phosphorylation sites recognized by the specific antibodies. (B) Whole B-cell lysates (lanes 1 and 4), microsomes (lanes 2 and 5), and cytosol (lanes 3 and 6) were assayed by Western blot analysis with anti-LDH (cytosolic marker), anticalnexin (microsomal marker), antilamin (nuclear marker), and antiaconitase (mitochondrial marker) antibodies. Western blots are representative of samples from 5 normal donors (left, lanes 1-3), and of those from 10 CLL patients (right, lanes 4-6) are shown. (C) Whole B-cell lysates (lanes 1 and 4), microsomes (lanes 2 and 5), and cytosol (lanes 3 and 6) from one normal donor (lanes 1-3) and from CLL patient 15 (lanes 4-6) were analyzed by immunoblotting with anti-pY_A, anti-pY_T and, after stripping, reprobed with anti-Lyn antibody. Molecular weight (kDa) corresponding to p53 and p56 isoforms of Lyn are indicated in the middle. (D) Densitometric analysis (arbitrary units) of anti-pY_A, anti-pY_T, and anti-Lyn bands of whole cell lysates (lanes 1 and 4), microsomes (lanes 2 and 5), and cytosol (lanes 3 and 6) from 5 normal (lanes 1-3) and 40 B-CLL samples (lanes 4-6) is shown. Data are mean plus or minus SD from 3 separate experiments. Whole cell lysates, microsomes, and cytosol were prepared as detailed in "Methods."

microsomal and cytosolic Lyn, indicating that cytosolic Lyn was in its fully active conformation in unstimulated leukemia cells.

Cytosolic Lyn (CL) participates in a complex stabilized by interactions mediated by its SH3 and catalytic domains

Because Lyn cumulates in an active conformation in the cytosolic fraction of B-CLL cells, we examined the role of its interactions with potential partners, which might lead to an aberrant state of Lyn.

Irrespective of the method of cell disruption, either by sonication ("S" in Figure 2A) or by "Douncing" (Figure 2A,D), Lyn was detected by Western blot analysis after fractionation of the cytosol of leukemia cells from B-CLL patients on a glycerol gradient in the correspondence of a molecular weight of approximately 600 kDa, suggesting its taking part in a multiprotein complex.

To assess whether phosphorylation of the components, including Lyn, of the CL complex, played a role in its stabilization, the cytosol was fractionated, under the conditions already described above, on a glycerol gradient as such or after treatment with the broad-specificity λ-PPase. Figure 2B shows that the molecular weight of the CL complex was not modified by λ-PPase treatment, demonstrating that neither Lyn pY_A nor phosphorylation of other protein partners had a role in the maintenance of the CL complex.

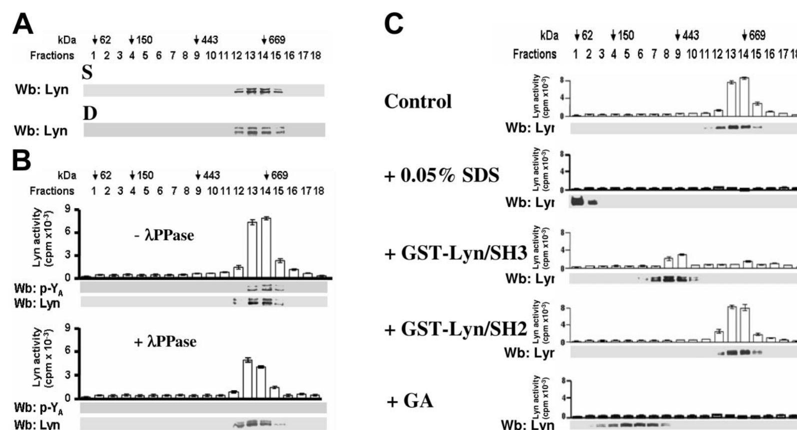

Figure 2. Purification and characterization of Lyn complex from cytosol of B-CLL. (A) Cytosol from 15 × 10^6 freshly isolated B-CLL cells lysed by sonication (top panel, S) or, alternatively, by douncing (bottom panel, D) was loaded on top of a linear glycerol gradient (10%-40%) and centrifuged 18 hours at 100 000*g* in an SW60Ti rotor (Beckman Coulter) at 4°C. Eighteen fractions (200 μL each) were collected from top and analyzed by immunoblotting with anti-Lyn antibody. The figure is representative of experiments performed in triplicate on samples from each of 5 B-CLL patients. (B) Cytosol, from 15 × 10^6 freshly isolated B-CLL cells lysed by sonication, was treated without (upper panel, −λPPase) or with λPPase (bottom panel, + λPPase), and subjected to the separation procedure described in panel A. Eighteen fractions (200 μL each) were collected from top, assayed for Lyn activity tested on Src-specific peptide substrate cdc2(6-20), and analyzed by immunoblotting with anti-pY_A antibody and, after stripping, with anti-Lyn antibody. The figure is representative of experiments performed in triplicate on samples from each of 40 B-CLL patients. (C) Fractions 13 and 14 (CL complex) of the cytosol purified from 75 × 10^6 B-CCL cells and subjected to a linear glycerol gradient under the conditions described in panel A were collected and split into 5 aliquots, which were incubated for 30 minutes at 4°C in the absence (control) or presence of 0.05% SDS, 0.1 μM GST-Lyn/SH3, 0.1 μM GST-Lyn/SH2, and 0.1 μM GA, respectively. The treated samples were then subjected separately to glycerol gradient centrifugation as described in panel A, and aliquots of the resulting fractions were assayed for Lyn activity tested on Src-specific peptide substrate cdc2(6-20), and analyzed by immunoblotting, with anti-Lyn antibody. The figure is representative of experiments performed in triplicate on samples from 10 B-CLL patients. Downward arrows represent position of molecular weight standards on glycerol gradients, glutamate dehydrogenase (62 kDa), alcohol dehydrogenase (150 kDa), apoferritin (443 kDa), and thyroglobulin (669 kDa; Sigma-Aldrich), indicated to estimate the molecular weight of the protein complexes on parallel gradient runs.

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Furthermore, the distribution profile of CL activity of the gradient fractions tested in vitro on the Src-specific peptide cdc2(6-20) showed that, after λ-PPase treatment, the dephosphorylated form of Lyn displayed lower kinase activity, this finding being directly related to the tyrosine dephosphorylation in the activation loop of the kinase (Figure 2B bottom panel compared with top panel). In parallel experiments, PP2, a selective SFK inhibitor,[37] abolished the kinase activity assayed on the nonspecific substrate poly(Glu_4Tyr) random polymer, indicating that the tyrosine kinase activity of the cytosolic complex was exclusively catalyzed by Lyn (data not shown).

To highlight the nature of the interactions in the CL complex, the fractions 13 and 14 of the glycerol gradient containing Lyn (Figure 2A) were collected, differently treated, and fractionated on a further glycerol gradient. The results are shown in Figure 2C. This procedure did not affect protein interactions, as demonstrated by sedimentation of the CL complex at the expected molecular mass, and the kinase activity fully coincided with the CL complex. The addition of SDS to a final concentration of 0.05% to the CL complex under nonreducing conditions showed that disulfide bridges did not mediate Lyn binding to the interacting proteins, as proven by the detergent-induced dissociation of Lyn and its appearance as uncomplexed and inactive protein in fractions 1 or 2.

To analyze the interactions of Lyn with hypothetical binding proteins, we performed competition assays using compounds capable of disrupting their binding to SH3, SH2, and catalytic domains of Lyn itself, thus altering the structure of the CL complex. Western blotting with anti-Lyn antibody showed that addition of the GST-Lyn/SH3 domain induced complex dissociation and the appearance of CL in the fractions 6 to 10, suggesting that the enzyme interacted with other proteins by its SH3 domain. Conversely, the addition of the GST-Lyn/SH2 domain, which is capable of binding phosphotyrosine-containing motifs, did not induce an apparent complex dissociation. The CL complex was also treated with GA, a compound that directly binds to and inhibits Hsp90, thereby causing proteolytic degradation of its client proteins.[38,39] This treatment promoted the dissociation of Lyn from the 600-kDa complex, as revealed by the immunodetection of Lyn in the gradient fractions at lower molecular weight, suggesting that CL may associate with Hsp90. As for the activity of Lyn, it was unaltered when the GST-Lyn/SH2 domain was used in the competition assay, although strongly reduced and totally abolished after treatment with the GST-Lyn/SH3 domain and GA, respectively.

Lyn SH3 domain promotes the interaction of CL with HS1 and SHP-1

To identify the protein partners binding to Lyn through the Lyn SH3 domain, we analyzed the CL complex for the presence of already known proteins interacting with the SH3 domain of SFKs, namely, Cbl, Akt, SHP-1L, SHP-2, HS1, and STAT3.[29,40-44] The CL complex purified from the cytosol of leukemia cells obtained from each of 40 patients was thus subjected to a further glycerol gradient, and aliquots of the resulting fractions were probed with antibodies against Lyn and the aforementioned potential protein ligands, demonstrating that only SHP-1L, HS1, and STAT3 cosedimented with Lyn (Figure 3A).

SHP-1L, the C-terminal alternatively spliced form of SHP-1 with a unique C-terminal tail containing the proline-rich motif PVPGPPVLSP, was identified with an antibody recognizing the protein-tyrosine phosphatase domain shared by SHP-1L and SHP-1.[41,42] The presence of the latter was ruled out because the specific antibody raised against its C-terminal tail showed no immunoreactivity (Figure 3A). SHP-1L physically interacted with Lyn in the CL complex, as demonstrated by immunoprecipitating SHP-1L or Lyn from the glycerol fractions containing the CL complex and analyzing the immunocomplex with anti-Lyn or anti-SHP1/1L antibody, respectively. The addition of either the synthetic Pro-rich peptide (KGGRSRLPLPPLPPPG), known to interact with Lyn SH3 domain,[45] or the GST-Lyn/SH3 domain, during anti-Lyn or anti-SHP-1/1L immunoprecipitation, completely abolished the binding between Lyn and SHP-1L (Figure 3B).

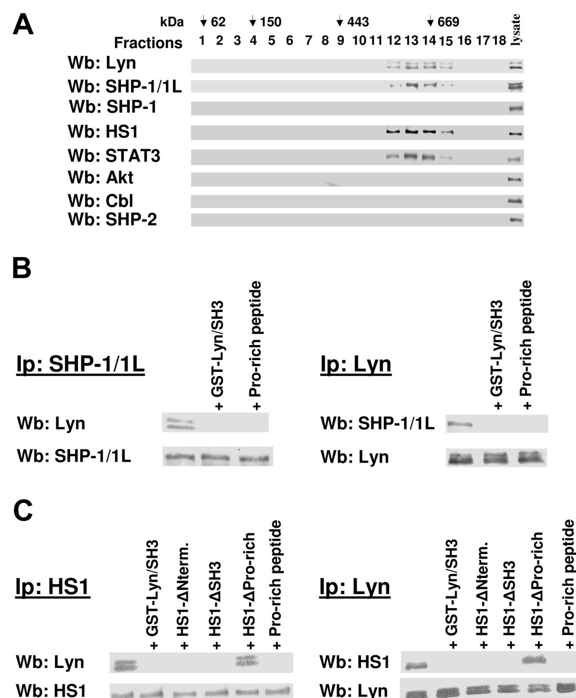

Figure 3. SH3 domain of cytosolic Lyn binds to HS1 and SHP-1L proteins. (A) Cytosol from 15×10^6 freshly isolated B-CLL cells lysed by sonication was subjected to the separation procedure described in Figure 2A. Fractions 13 and 14 (CL complex) were collected and resubmitted to an additional centrifugation step on a glycerol gradient. Aliquots of the gradient fractions were analyzed by immunoblotting with anti-Lyn, anti-SHP-1/1L, anti-SHP-1, anti-HS1, anti-STAT3, anti-Akt, anti-Cbl, and anti-SHP-2 antibodies. Whole cell lysates from 2×10^5 B-CLL cells were probed with the same antibodies as positive controls. Downward arrows represent position of molecular weight standards on glycerol gradients, glutamate dehydrogenase (62 kDa), alcohol dehydrogenase (150 kDa), apoferritin (443 kDa), and thyroglobulin (669 kDa; Sigma-Aldrich), indicated to estimate the molecular weight of the protein complexes on parallel gradient runs. (B) CL complex purified after 2 centrifugation steps on a glycerol gradient, as described in panel A, was collected, and aliquots were treated for 30 minutes at 4°C in the absence or presence of 0.1 μM GST/SH3-Lyn and 0.1 μM Pro-rich peptide, further subjected to immunoprecipitation by anti–SHP-1/1L and anti-Lyn antibodies, and assayed for Lyn and SHP-1/1L, respectively. (C) Same fractions as in panel B were collected, and aliquots were treated for 30 minutes at 4°C in the absence or presence of 0.1 μM GST/SH3-Lyn, 0.1 μM HS1ΔN-term, 0.1 μM HS1ΔSH3, 0.1 μM HS1-ΔPro-rich, 0.1 μM Pro-rich peptide, further subjected to immunoprecipitation by anti-HS1 and anti-Lyn antibodies, and assayed for Lyn and HS1, respectively.

The interaction between HS1 and Lyn in the CL complex was demonstrated by similar immunoprecipitation assays (Figure 3C). Because HS1 contains a variety of structurally significant motifs, including a proline-rich region (amino acids 324-393) and an SH3 domain located at the C terminus,[34] we performed competition assays by adding recombinant truncated forms of HS1 during anti-Lyn or anti-HS1 immunoprecipitation, to identify the HS1 region interacting with Lyn. As shown in Figure 3C, although HS1ΔSH3 completely abolished the interaction between Lyn and HS1, the addition of the HS1 mutant lacking the Pro-rich sequence proved to be ineffective, indicating that the Lyn SH3 domain interacted with the HS1 Pro-rich sequence. This finding was further

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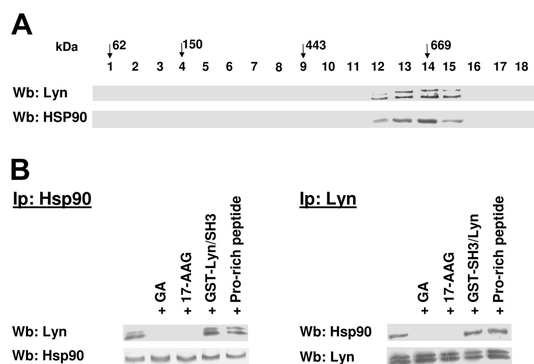

Figure 4. Hsp90 is detectable in Lyn complex and interacts with Lyn. (A) Cytosol from 15 $\times$ 10^6 freshly isolated B-CLL cells lysed by sonication was subjected to the separation procedure described in Figure 2A. Fractions 13 and 14 (CL complex) were collected and resubmitted to an additional centrifugation step on a glycerol gradient. Aliquots of the gradient fractions were analyzed by immunoblotting with anti-Lyn and anti-Hsp90 antibodies. Downward arrows represent position of molecular weight markers on glycerol gradient. (B) CL complex purified after 2 centrifugation steps on a glycerol gradient, as described in panel A, was collected, and aliquots were treated for 30 minutes at 4°C in the absence or presence of 0.1 μM GA, 0.1 μM 17-AAG, 0.1 μM GST/SH3-Lyn, and 0.1 μM Pro-rich peptide, respectively, and further subjected to immunoprecipitation by anti-Hsp90 or anti-Lyn antibodies. Immunoprecipitates were subsequently assayed both for Lyn and Hsp90, respectively. The figure is representative of experiments performed in triplicate on samples from 40 B-CLL patients.

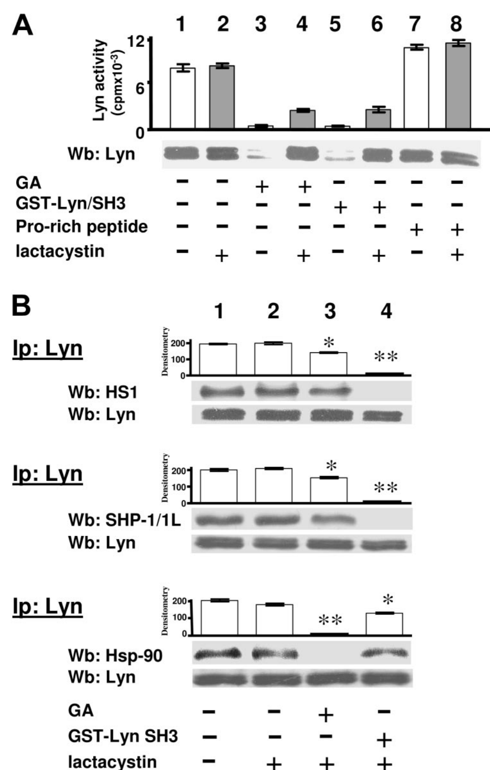

Figure 5. Stabilization of cytosolic Lyn complex by synergic cooperation of SH3 and catalytic domains. Cytosol from freshly isolated B-CLL cells lysed by sonication was subjected to the separation procedure described in Figure 2A and treated without or with 0.1 μM GA, 0.1 μM GST/SH3-Lyn, and 0.1 μM Pro-rich peptide, respectively, in the absence or presence of 10 μM lactacystin for 1 hour at 37°C. (A) Aliquots of each treated sample were analyzed for in vitro Lyn activity on Src-specific peptide substrate cdc2(6-20) and by Western blotting for Lyn. (B) Aliquots of each sample were immunoprecipitated with anti-Lyn antibody and the immunocomplexes probed with anti-HS1, anti–SHP-1/1L, and anti-Hsp90 antibodies, respectively. Blots were then stripped and reprobed with anti-Lyn antibody. The bar graph above the blot panels represents the values of a densitometric analysis (arbitrary units) of anti-HS1, anti–SHP-1/1L, and anti-Hsp90 bands, expressed as mean plus or minus SD. The statistical analyses were performed using a one-way analysis of variance with posttest, and the significance is indicated as a P value. $^*P < .05$, $^{**}P < .001$, compared with control (bar 1). Data are representative of experiments performed in triplicate on samples from 16 B-CLL patients.

confirmed by adding the Pro-rich peptide, which abolished Lyn-HS1 coimmunoprecipitation (Figure 3C) performed on the fractions containing the CL complex, under the same experimental conditions.

On the other hand, no interaction between STAT3 and Lyn was evidenced, as the former was not revealed after immunoprecipitation with anti-Lyn antibody and the latter was not detected after immunoprecipitation with anti-STAT3 antibody, showing that the 2 proteins are not part of the same complex (data not shown).

Lyn catalytic domain interacts with the chaperone Hsp90 in the CL complex

To explore the role played by the catalytic domain of Lyn in the CL complex, and on the basis of data obtained with GA, we verified whether Hsp90 takes part in the cytosolic complex (Figure 2C). The CL complex purified from the cytosol of leukemia cells obtained from each of 40 patients was fractionated on a second glycerol gradient, and aliquots of the resulting fractions were revealed with antibodies to Lyn and Hsp90. Hsp90 and Lyn sedimented in the same glycerol gradient fractions (Figure 4A), and the interaction between the 2 proteins was demonstrated by probing Hsp90 or Lyn immunoprecipitates with anti-Lyn and anti-Hsp90 antibodies, respectively (Figure 4B). Treatment of CL complex with GA or its derivative 17-AAG induced the expected dissociation of Lyn from Hsp90 (Figure 4B), whereas addition of Pro-rich peptide or GST-Lyn/SH3 domain did not disrupt Lyn-Hsp90 interaction. Experiments aimed at investigating the potential binding of Hsp90 with the membrane-anchored Lyn in B-CLL cells showed that the chaperone was not bound to the microsomal tyrosine kinase (data not shown).

Synergistic effect of Lyn domains in maintaining the CL complex

To better define the mechanisms regulating the stabilization of the CL complex, the cytosol of leukemia cells obtained from 16 B-CLL patients (8 U-CLL and 8 M-CLL) was incubated with compounds capable of destabilizing interactions mediated by either the Lyn SH3 domain (GST-Lyn/SH3 domain and the Pro-rich peptide) or the catalytic domain (GA), in the presence or absence of lactacystin, a specific proteasome inhibitor.[46] After treatment with each compound, both Lyn activity and protein level of CL were tested.

In all the samples analyzed, the addition of GST-Lyn/SH3 domain and GA clearly led to degradation of CL (Figure 5A, lanes 3 and 5). In the presence of lactacystin, although Lyn activity was markedly reduced, its protein level was not altered; conversely, treatment with the Pro-rich peptide enhanced the kinase activity of Lyn and protected it from degradation. It was the inhibitory effect of lactacystin, indeed, that enabled us to examine the interactions between CL and its protein ligands on the CL immunoprecipitated from B-CLL cytosol after treatment with GST-Lyn/SH3 domain or GA. GA abolished the interaction between Hsp90 and the CL complex, leaving the association between Lyn with HS1 and SHP-1L nearly intact (Figure 5B). In parallel, treatment with the GST-Lyn/SH3 domain disrupted the interaction of HS1 and SHP-1L with Lyn and led to partial dissociation of Hsp90 from the CL complex.

These results demonstrate not only that Hsp90 contributes to preventing Lyn degradation but also that interactions between Lyn SH3 domain and its protein ligands play a role in the stabilization

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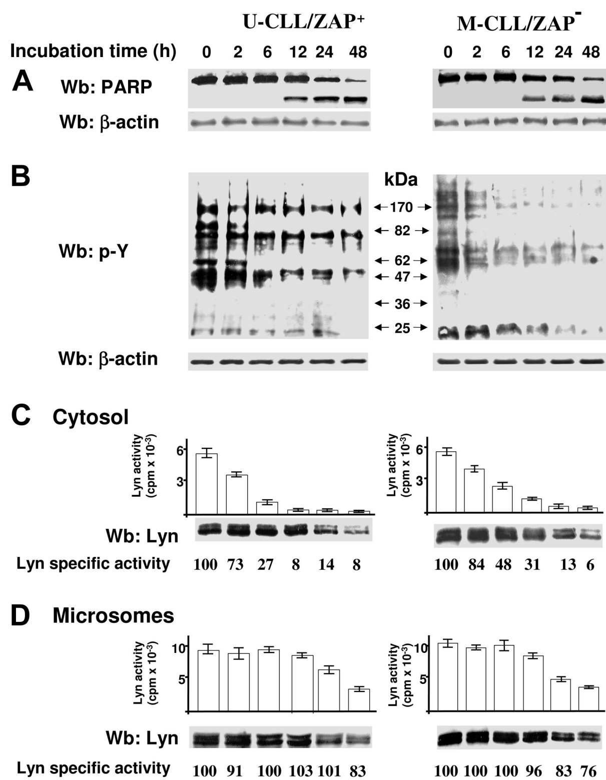

Figure 6. Analysis of Lyn activity and protein level during GA-mediated apoptosis of B-CLL cells. Unmutated CLL/ZAP+ (U-CLL/ZAP+) and mutated CLL/ZAP− (M-CLL/ZAP−) cells were cultured for the indicated times, in the presence of 0.1 μM GA. (A) After GA treatment, U-CLL/ZAP+ and M-CLL/ZAP− cells were lysed and analyzed by immunostaining with antibodies raised against PARP. Blots were stripped and reprobed with anti–β-actin antibody as loading control. (B) After GA treatment, U-CLL/ZAP+ and M-CLL/ZAP− cells were lysed and analyzed by immunostaining with antibody raised against phospho-Tyr (pY). Molecular mass of protein standards are indicated in the middle. Blots were stripped and reprobed with anti–β-actin antibody as loading control. (C,D) After GA treatment, U-CLL/ZAP+ and M-CLL/ZAP− cells were lysed by sonication in an isotonic buffer and subjected to differential centrifugation to separate cytosolic (C) and microsomal (D) fractions. Comparable aliquots were assayed for in vitro Lyn activity on Src-specific peptide substrate cdc2(6-20) and by Western blotting for Lyn. Lyn-specific activity is calculated as ratio of Lyn activity (bar graphs) over densitometric values of Western blot analysis for Lyn (panel below bar graphs) by standardizing the ratios of each control to the value of 100. All calculated SD values are less than 10%. Data are representative of experiments performed in triplicate on samples from each of 16 B-CLL patients.

and preservation of the CL complex, maintaining the Lyn active conformation, regardless of the protein partners containing the SH3-cognate Pro-rich sequence, such as HS1 or SHP-1L.

CL complex becomes destabilized in parallel with GA-induced apoptosis

Because inhibition of Lyn activity, obtained by treating B-CLL cells with specific inhibitors of tyrosine kinases, is sufficient to restore cell apoptosis,[28] we investigated the fate of the CL complex in GA-induced apoptosis.[47]

Freshly isolated leukemic B cells obtained from 16 B-CLL patients (8 U-CLL and 8 M-CLL) were incubated in the presence of GA at different times. The cleavage of poly-ADP-ribose polymerase (PARP-1), which is indicative of apoptosis, was observed after 12 hours of GA treatment, in both U-CLL (Figure 6A left panels) and M-CLL (Figure 6A right panels) samples. On the other hand, CL specific activity started to be decreased 2 hours after GA treatment, reaching 73% and 84% of inhibition in U-CLL and M-CLL, respectively (Figure 6C), whereas microsomal Lyn specific activity decreased only after 24 hours treatment (Figure 6D). It is noteworthy that the aberrant high basal protein Tyr-phosphorylation in B-CLL lysates (Figure 6B) was found to be decreased in parallel with the inhibition of CL activity induced by GA treatment. These data led us to infer that the increased basal tyrosine phosphorylation could be accounted for by the stabilization resulting from synergistic interactions between Lyn and other partners.

To confirm this hypothesis, freshly isolated B-CLL cells from 8 patients (4 U-CLL and 4 M-CLL) were treated with GA at different times, and the cytosol was fractionated on a glycerol gradient. The resulting fractions were tested for Lyn activity and protein level. This analysis showed that Lyn activity, detected only in the correspondence of the 600-kDa complex, decreased over time in parallel with GA treatment (Figure 7A). This treatment changed the distribution profile of CL over a wider range of molecular weights in the glycerol gradient, as revealed by Western blotting, suggesting that CL activity is directly correlated with the stability of the complex (Figure 7A). CL immunoprecipitates from the cytosol of the same samples also displayed decreasing amounts of HS1, SHP-1L, and Hsp90 after GA treatment, supporting the hypothesized involvement of the interaction domains of CL in the stabilization of the CL complex (Figure 7B).

Discussion

In this report, we demonstrate that the Src kinase Lyn, which is abnormally present in the cytosol of B-CLL cells, is an integral component of an aberrant cytosolic complex of 600 kDa (Figure 2). In this complex, CL, which represents 30% of total Lyn, is present in an active conformation and is associated with Hsp90, HS1, and SHP-1L (Figures 3,4). These proteins probably account for some of the anomalous properties of Lyn in B-CLL cells in comparison with normal B cells, such as overexpression with altered turnover, atypical localization in the cytosol, and remarkable constitutive activity.[28] GA, a compound known to bind and inhibit Hsp90, is shown to cause the disruption of the aberrant cytosolic complex and consequently the inactivation of Lyn in the early phases of apoptosis.

Hsp90 targets client protein kinases, including SFKs,[48,49] stabilizing the kinase active conformation and counterbalancing the opposing mechanism of degradation by ubiquitination. Although the association of SFKs with Hsp90 is difficult to detect, probably because of low-affinity binding and repeated cycles of association and release,[50-52] in B-CLL cells CL appears tightly bound to Hsp90, suggesting an abnormal behavior of both proteins or other types of interaction that synergistically contribute to stabilize of the complex. Hsp90 has been recently reported to exhibit an activated conformation in tumor cells, which, in contrast with normal tissues, results in higher affinity to its inhibitors.[53,54] This increased affinity appears to be the result of cochaperone-induced changes in the ATP binding site of Hsp90, which lead to high ATPase activity, whereas Hsp90 from normal tissues is in a latent, apparently unactivated state. Notably, activated Hsp90 is described to bind to and stabilize ZAP-70, which acts as a Hsp90 client protein only in B-CLL cells.[55] Because we detected neither Hsp70 nor cdc37, essential cochaperones of 2 known multichaperone Hsp90 complexes, in association with Hsp90 in the CL complex purified on a glycerol gradient (data not shown), Hsp90 does not appear to be in a traditionally known activation state, suggesting that Hsp90 might be in an altered condition accounting for its tight binding to Lyn. The data presented in this work actually support the view that the

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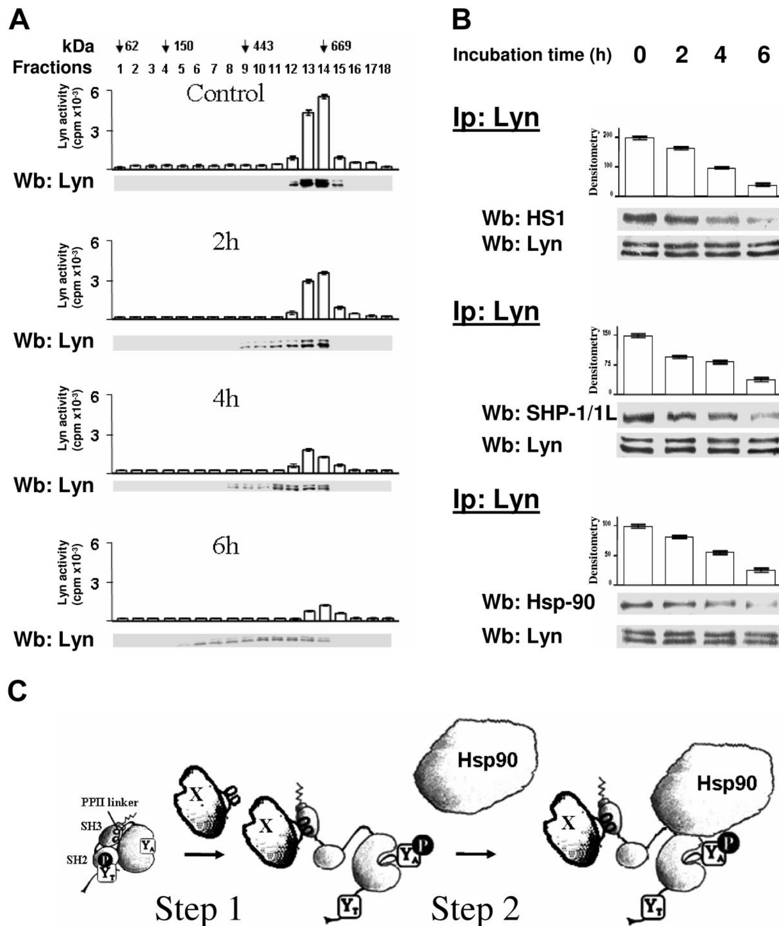

Figure 7. Monitoring of Lyn-complex degradation during GA treatment. B-CLL cells were cultured in the presence of GA for different times, as described in Figure 6. (A) Cells were lysed by sonication in an isotonic buffer and subjected to differential centrifugation to separate microsomal and cytosolic fractions. Cytosol underwent glycerol gradient centrifugation, as described above. Fractions were collected from top and assayed for in vitro Lyn activity on Src-specific peptide substrate cdc2(6-20) and by Western blotting for Lyn. (B) Cytosol, isolated from B-CLL cells as in panel A, was immunoprecipitated with anti-Lyn antibody. Immunocomplexes were then probed with anti-HS1, anti–SHP-1/1L, and anti-Hsp90, respectively. Blots were then stripped and reprobed with anti-Lyn antibody. The bar graph above the blot panels represents the values of a densitometric analysis (arbitrary units) of anti-HS1, anti–SHP-1/1L, and anti-Hsp90 bands, expressed as mean plus or minus SD. Data are representative of 3 experiments performed with 8 B-CLL samples. (C) Proposed model for sequential binding of ligands in the assembly of the CL complex. Step 1 indicates SH3 binding proteins (X) can promote displacement of the PPII motif in the SH2-kinase linker from the SH3 domain, thus inducing an "open" conformation; Step 2, association of Hsp90 with the N-terminal lobe of Lyn catalytic domain stabilizes the complex and maintains the kinase in an active conformation.

high affinity between Hsp90 and Lyn may be the result of the interaction of Lyn with specific ligands through its noncatalytic domains.

It is well established that ligands binding to the SH3 domain of SFKs can efficiently activate them by directly disrupting the intramolecular inhibitory interaction between polyproline linker and the kinase SH3 domain.[11] In this regard, we demonstrate that the interaction between the SH3 domain of Lyn and the Pro-rich regions of discrete proteins, including HS1 and SHP-1L, contributes to stabilize the complex and protect Lyn from degradation (Figures 3,5). In particular, HS1 is a 79-kDa intracellular protein expressed in cells of lymphohematopoietic origin with a pivotal role in the signaling cascade triggered by BCR stimulation, on which Lyn phosphorylates HS1 by "sequential" mechanism[56]; SHP-1L is a 70-kDa cytoplasmic spliced variant of the protein-tyrosine phosphatase SHP-1, predominantly expressed in human hematopoietic tissues and involved in the regulation of hematopoietic signal transduction.[42,43]

HS1 and SHP-1L may be replaced by the Pro-rich peptide KGGRSRLPLPPLPPPG, which contains the optimal motif for binding to the SH3 domain of Lyn without altering the stability of the CL complex (Figure 5B). Furthermore, interaction between the SH3 domain of Lyn and the Pro-rich peptide stimulates the kinase activity of Lyn, leaving the protein level of Lyn unchanged in the cytosol (Figure 5A). We also show that treatment with GA destabilizes the CL complex by disrupting the interaction between Lyn and Hsp90 and by weakening the interactions mediated by the SH3 domain, as observed in the cytosolic fraction of B-CLL cells (Figure 5). However, our results indicate that, in B-CLL cells, Lyn is maintained in an active conformation and preserved from degradation resulting from the interaction not only with Hsp90 but also with several ligands of the Lyn SH3 domain in a synergistic manner with Hsp90, regardless of the partners containing the Pro-rich sequence, such as HS1 and SHP-1L. In this view, we can hypothesize that the CL complex, which is detected in all B-CLL patients, may display a wide variability of the proteins binding to Lyn SH3, without ruling out the possibility that the composition of the CL complex results from the expression level of the interacting proteins.

As emphasized by studies on regulatory mechanisms of SFKs, interaction of the SH3 domain with proteins containing Pro-rich sequences may be modulated by ligands targeting SFKs themselves (eg, C-terminal Src kinase-homologous), which has been shown to destabilize this binding by a noncatalytic inhibitory mechanism.[12,13,57] Conversely, we demonstrate that Hsp90 stabilizes the complex in which the SH3 domain is engaged and maintains the kinase in an active conformation. Hence, interaction of Hsp90 and SH3 ligands with their respective binding domains synergistically converts individual transient interactions into permanent ones, making the complex difficult to degrade (Figure 7C). Further studies are needed to establish a model for sequential binding of ligands in the assembly of the CL complex and why specific proteins are recruited to it. Notably, HS1 has already been shown to play a role related to its phosphorylation state in B-CLL[58]: finding a link between the 2 events may shed light on the pathogenesis of B-CLL.

We observed that a relationship exists between the stability of the CL complex and hence the activated state of Lyn and the

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defective apoptosis of B-CLL cells. We had previously demonstrated that the high basal activity of Lyn, related to the reduced ability of B-CLL cells to enter apoptosis, results from the tyrosine kinase activity of microsomal and cytosolic Lyn.[28] Treatment with GA, which triggers programmed cell death, enabled us to differentiate these 2 subpopulations of Lyn by disrupting the CL complex and thus inactivating CL. It is noteworthy that inactivation of CL by GA occurs earlier than its degradation, indicating that the activity of CL is directly dependent on the stability of the complex. These data, observed in freshly isolated B-CLL cells, are in agreement with those obtained in cytosol extracts treated with compounds capable of destabilizing the interactions of the CL complex mediated by the Lyn SH3 domain (GST-Lyn/SH3) or by the catalytic domain (GA), in the presence of lactacystin, which, as other proteasome inhibitors (eg, MG-132 and PS-341), can induce apoptosis in B-CLL cells, although there is a variation in the sensitivity to treatment of the cells.[59,60] Despite the loss of activity caused by the dissociation of the complex in the presence of GA or GST-Lyn/SH3, Lyn protein level is preserved by the use of lactacystin, confirming that CL activity is related to the stabilization of the CL complex and not to its expression level (Figure 5).

GA-induced inactivation of CL parallels the decrease in the aberrant basal protein Tyr-phosphorylation detectable in B-CLL lysates, indicating that CL, in contrast to microsomal Lyn, is bound to Hsp90, and that its activity relies on the stability of the complex (Figures 6,7). Instead, degradation of Lyn is a late event, involving both the cytosolic and microsomal fractions of Lyn (Figure 6C,D).

These results highlight the prominent role played by CL in aberrant high basal protein Tyr-phosphorylation detected in both ZAP^+ and ZAP^- B-CLL samples (Figure 6B) and seem to exclude a correlation between apoptosis induced by Hsp90 inhibitors and ZAP-70, as is already elsewhere reported.[61] Moreover, the aberrant activation of Lyn through the interaction with specific protein ligands appears to contribute to the pathogenesis of B-CLL and has led us to regard the proteins stabilizing the CL complex as potential targets for a possible therapeutic approach for B-CLL.

Hence, inactivation of the CL complex can be proposed as a further mediator of apoptosis, in addition to depletion of Akt (46) and alteration in the expression of p53,[62] resulting from Hsp90 inhibition in B-CLL, and this is corroborated by the intense work carried out in the last few years focused on Hsp90 inhibitors.[47,55,61-63] In addition, the interactions of the SH3 domain of SFKs could be considered as potential targets in the development of novel drugs capable of disrupting the interaction with protein ligands, also suggesting a new approach in the treatment of pathologic processes in which SFKs are directly involved.

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Authorship

Contribution: L.T. contributed clinical patient samples, performed some of the in vitro research, analyzed the data, and wrote parts of the manuscript; M.F. performed the majority of the in vitro research, analyzed the data, and wrote parts of the manuscript; A.D.-D. provided intellectual input into the phosphorylation studies and reviewed the manuscript; F.F. contributed clinical patient samples and performed some of the in vitro research; M.A.P. performed some of the in vitro research and reviewed the manuscript; E.T. performed some of the in vitro research; C.G. contributed clinical patient samples and performed some of the in vitro research; R.Z. contributed clinical patient samples; G.S. provided intellectual input into the lymphocyte studies and reviewed the manuscript; and A.M.B. designed the research, reviewed all of the data, participated in analysis of data, and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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