

Rapid publications

Proteasome-dependent regulation of Syk tyrosine kinase levels in human basophils

Lama A. Youssef, B Pharm, Bridget S. Wilson, PhD, and Janet M. Oliver, PhD

Albuquerque, NM

Background: In human basophils, FcεRI signal initiation, leading to histamine release, relies on activation of Syk protein tyrosine kinase. Basophils from approximately 10% of unselected donors do not degranulate in response to FcεRI cross-linking. Their unresponsiveness has been linked to the absence of Syk protein despite apparently normal levels of Syk mRNA. **Objective:** The aim of this study was to explore pathways of Syk protein degradation as a possible posttranslational mechanism for downregulating Syk protein levels in human basophils and other leukocytes.

Methods: Highly purified basophils, lymphocytes, and monocytes were incubated in the presence or absence of a panel of cell-permeable inhibitors of proteolytic degradation pathway(s). Subsequently, the protein level of Syk tyrosine kinase was determined by means of Western blotting. In vitro assays were conducted through use of immunoprecipitated basophil Syk and a rabbit reticulocyte lysate system.

Results: Three inhibitors of proteasome-mediated degradation—PSI, lactacystin, and ALLN—substantially increased Syk levels in releaser basophils and restored Syk expression in nonreleaser basophils. Caspase inhibitors were less effective, and inhibitors of calpain-mediated proteolysis had no effect. Among other leukocytes tested, only naive CD4⁺ T cells had more Syk after proteasome inhibitor treatment. In vitro ubiquitination assays demonstrated that Syk is readily ubiquitinated in vitro and also that Syk ubiquitination is associated with a substantial decrease in total levels of Syk protein.

Conclusion: These data provide evidence for a ubiquitin/proteasome-dependent mechanism that contributes to Syk regulation in human basophils and might also be relevant to naive T cells. Understanding this regulatory pathway might lead to strategies for suppressing allergic inflammation while preserving essential Syk-mediated functions in other hematopoietic cells. (*J Allergy Clin Immunol* 2002;110:366-73.)

Key words: Basophil, Lyn, Syk, FcεRI, proteasome, ubiquitin

Abbreviations used

ALLN: N-Acetyl-Leu-Leu-Nle-CHO

DMSO: Dimethyl sulfoxide

FcεRI: High-affinity receptor for IgE

NK: Natural killer

PSI: Proteasome inhibitor I [N-benzyloxycarbonyl-Ile-Glu-(O-t-butyl)-Ala-Leu-CHO]

PTK: Protein tyrosine kinase

RBL-2H3: Rat mucosal-type basophilic leukemia cell line

RRL: Rabbit reticulocyte lysate

Ub-Pr: Ubiquitin-proteasome

Basophils are effector cells in the induction and maintenance of an inflammatory allergic response. Clustering of the high-affinity IgE receptor, FcεRI, on the surface of basophils triggers a cascade of biochemical, ionic, and physiologic events that culminates in degranulation and other cellular responses. The initiating event in this cascade is the activation of Lyn, an Src family protein tyrosine kinase (PTK) that catalyzes the tyrosine phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the receptor's β and γ subunit cytoplasmic tails.^{1,2} The subsequent recruitment of Syk tyrosine kinase, via association of its tandem SH2 domains with the FcεRI γ phospho-ITAM, links the enzymatically inert FcεRI to downstream biochemical signaling pathways,²⁻⁴ leading to the secretion of inflammatory mediators such as histamine, the production of cytokines (including IL-4 and IL-13), and other responses.⁵

Syk is broadly distributed throughout hematopoietic lineages and is also found in endothelial, epithelial, and other cell types.⁶ In hematopoietic cells, Syk is recruited not only to the activated FcεRI but also to activated Fcγ receptors, B-cell receptors, T-cell receptors, and several platelet receptors.⁷ Syk^{-/-} mice die before or immediately after birth,⁸ very likely because of both platelet and endothelial cell dysfunction.⁶

Our recent work has focused on a minority (approximately 10%) of normal blood donors whose basophils fail to degranulate in response to FcεRI cross-linking. "Nonreleaser" basophils express normal levels of FcεRI but contain vanishingly low levels of Syk protein.⁹ Because nonreleaser basophils show normal Ca²⁺ mobi-

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Reprint requests: Lama A. Youssef, B Pharm, Department of Pathology, University of New Mexico School of Medicine, CRF 203, 2325 Camino de Salud, Albuquerque, NM 87131.

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lization, secretion, and cytokine synthesis to stimuli that bypass the FcεRI (Ca²⁺ ionophore, phorbol ester, formyl peptide), their defect in FcεRI-mediated signaling very likely results from this absence of Syk protein. Syk mRNA is detected in nonreleaser basophils,¹⁰ suggesting that the absence of Syk protein is determined at a post-translational level.

At present, the cytoplasmic ubiquitin-proteasome (Ub-Pr)-mediated pathway and the vacuolar pathway (including lysosomes and endosomes) are considered the predominant systems for eliminating cellular proteins. Degradation of intracellular proteins via the Ub-Pr proteolytic system is an extremely complex, highly selective, and tightly regulated process that plays crucial roles in a large and diverse multitude of pivotal cellular processes. Ubiquitination-associated proteolysis involves 2 coordinate yet discrete steps: (1) the covalent modification of targeted substrates with chains of a highly evolutionarily conserved 76-amino acid polypeptide (ubiquitin) and (2) degradation of ubiquitin-tagged proteins by the multisubunit 26S complex. The multicatalytic activities of the 26S proteasome allow this proteolysis machinery to degrade a wide array of protein substrates, both short- and long-lived, including protein kinases and phosphatases.^{11,12}

In this investigation, we used a series of cell-permeable inhibitors of proteasome proteolytic activities and an *in vitro* ubiquitination assay to investigate the possible involvement of the Ub-Pr pathway in regulating Syk levels in basophils and other leukocytes. Our results implicate proteasome-dependent mechanisms in the regulation of Syk tyrosine kinase expression levels in human basophils and naive CD4⁺ T cells.

MATERIAL AND METHODS

Reagents

Percoll was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and Optiprep from Invitrogen Life Technologies (Baltimore, Md). Monoclonal anti-actin antibodies and all other chemicals, except as otherwise noted, were purchased from Sigma Chemical Company (St Louis, Mo). Affinity-purified goat antihuman IgE (anti-IgE) was from Biosource (Camarillo, Calif), monoclonal anti-Syk antibodies (IgG2a) were from Zymed (San Francisco, Calif), polyclonal anti-Lyn and monoclonal anti-Cbl antibodies were from Santa Cruz Biotechnology (Santa Cruz, Calif), and polyclonal anti-ubiquitin was from DAKO (Glostrup, Denmark). Hanks (10×) and PBS (10×) solutions were from Gibco-BRL (Grand Island, NY). CD4⁺ T-cell isolation kits, anti-CD45RO antibodies, MidiMacs separation columns, and MidiMacs magnetic columns were from Miltenyi Biotec (Auburn, Calif). All protease inhibitors were from Calbiochem (La Jolla, Calif). FITC-conjugated antihuman CD14 and CD4⁺ and R-PE-conjugated CD45 RA mAbs were from BD Pharmingen (San Diego, Calif). PerCP-conjugated anti-HLA-DR mAb was from Becton Dickinson (San Jose, Calif). Rabbit reticulocyte lysate (RRL) was from Promega (Madison, Wis), and ubiquitin aldehyde was from Boston Biochem (Boston, Mass).

Isolation of peripheral blood basophils and other leukocytes

Basophils were purified to >95% by Percoll gradient centrifugation and negative selection, as described elsewhere.¹³⁻¹⁵ For ultra-purified basophil preparations (>99.9%), negative selection was fol-

lowed by flow sorting based on the forward light scatter/side light scatter characteristics of basophils. The yield from 90 mL of blood ranged from 3.0×10^5 to 2×10^6 basophils per donor.

Other leukocytes were isolated from OptiPrep-gradient fractions. For total lymphocytes, the lymphocyte-enriched fraction after OptiPrep-gradient centrifugation was simply collected and washed with PBS/BSA. For CD4⁺CD45RO⁻ T cells (naive T cells), the lymphocyte-enriched fraction was incubated for 10 minutes with a cocktail of monoclonal hapten-conjugated CD8, CD11b, CD16, CD19, CD36, and CD56 antibodies. MACS anti-hapten MicroBeads (Miltenyi Biotec) and anti-CD45RO antibodies were then added for 15 minutes, and the magnetically labeled cells were depleted through use of MACS columns in a MidiMacs magnetic field. The resulting naive CD4⁺ cells were >80% pure, as determined by flow cytometry after labeling with anti-CD4 and anti-CD45RA mAbs. Monocytes were purified from the OptiPrep monocyte-enriched fraction by incubation for 20 minutes with StemCell Technologies' monocyte enrichment cocktail; this was followed by the addition of goat antimouse IgG MicroBeads and removal of magnetically labeled cells through use of the MACS technology. Monocyte purity, as assessed by flow cytometry after staining with anti-CD14 and anti-HLA-DR mAbs, was routinely >90%.

In vitro culture of leukocytes

Leukocytes (2 to 5×10^5 cells/mL) were suspended in RPMI-1640 medium containing 10% FBS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin and incubated in a 5% CO₂ atmosphere at 37°C for 30 minutes to 4 hours. Cell viability, determined by Trypan blue exclusion, exceeded 99% for the freshly isolated basophils and was still >95% after 4 hours of incubation, even when the medium contained protease inhibitors.

Syk immunoprecipitation and *in vitro* ubiquitination assays

In vitro ubiquitination assays were performed on protein A-Sepharose-bound Syk protein obtained by immunoprecipitation from lysates of unstimulated cells. For immunoprecipitation, cells (approximately 1×10^7 basophils) were pooled from different releaser donors and lysed in 1% Triton, 0.5% NP-40, 70 mmol/L NaCl, 50 mmol/L NaF, 1 mmol/L EDTA, and 1 mmol/L EGTA, 10 mmol/L Tris, pH 7.4, in the presence of 1 mmol/L PMSF and 5 μg/mL of antipain, aprotinin, and leupeptin and 1 mmol/L Na₃VO₄. Protein concentrations were determined through use of a BCA protein assay kit (Pierce, Rockford, Ill). Syk immunoprecipitates were washed extensively with lysis buffer, once with ubiquitination buffer (50 mmol/L Tris, pH 7.4, 0.5 mmol/L MgCl₂, 0.1 mmol/L ATP, 0.1 mmol/L DTT, and 1 mmol/L creatine phosphate). The reactions were conducted as previously described¹⁶ in 35 μL of the same buffer supplemented with 70% (vol/vol) RRL, 10 units of creatine phosphokinase, ±10 μg of ubiquitin, and 2.5 μg of ubiquitin aldehyde for 2 hours at 30°C. The reactions were washed 3 times with lysis buffer and eluted with SDS-sample buffer.

Western blotting and densitometry

For Western blotting, cell pellets or *in vitro* ubiquitination reactions were mixed 1:1 (vol/vol) with Laemmli sample buffer and heated at 95°C for 5 minutes. Solubilized proteins were separated by 8% to 10% SDS-PAGE and transferred to nitrocellulose membranes.^{9,10} Membrane strips were incubated overnight at 4°C with 1 μg/mL of specific antibodies in TBS/BSA and for 1 hour at room temperature with peroxidase-conjugated anti-mouse IgG. Bands were developed through use of the Super Signal Maximum Sensitivity Substrate (Pierce). In some experiments, membranes were stripped with 100 mmol/L 2-mercaptoethanol, 2% SDS (wt/vol), 62.5 mmol/L Tris-HCl (pH 6.7) for 30 minutes at 57°C, and

reprobed with anti-Lyn and anti-actin antibodies and the corresponding peroxidase-conjugated IgG. For densitometry, immunoblots were digitized through use of the Gel-Doc 2000 System (Bio-Rad Laboratories, Hercules, Calif) and analyzed with Bio-Rad Quantity One software.

RESULTS

Proteasome inhibitors increase basophil Syk protein levels

We first investigated whether the steady-state protein levels of basophil Syk are affected by inhibitors of the 26S proteasome, calpain, or caspase proteolytic activities. Releaser and nonreleaser basophils were treated with cell-permeable protease inhibitors or with a corresponding volume of vehicle, dimethyl sulfoxide (DMSO), as a control. Fig 1, A shows the presence of Syk in releaser basophils and its absence from nonreleaser basophils. This illustration also shows that incubating unstimulated basophils for 4 hours with proteasome inhibitor I [PSI; N-benzoyloxycarbonyl-Ile-Glu-(O-t-butyl)-Ala-Leu-CHO; 60 $\mu\text{mol/L}$] remarkably increases Syk protein levels in releaser basophils (3- to 9-fold increase, as determined by densitometry; Fig 1, A) and restores Syk protein expression in nonreleaser basophils (Fig 1, A, *top panel*). Two other inhibitors of proteasome-mediated degradation—lactacystin (40 $\mu\text{mol/L}$), which is considered the most specific proteasome inhibitor,¹⁷ and ALLN (N-Acetyl-Leu-Leu-Nle-CHO; 50 $\mu\text{mol/L}$)—also increased Syk levels in both releaser cells (Fig 1, B) and nonreleaser cells (not shown). In contrast, caspase inhibitor III [Boc-Asp(OMe)-CH₂F; 25 $\mu\text{mol/L}$] caused only a modest increase in Syk levels, and calpain inhibitor V (morpholinoureydyl-Val-homophenylalanyl-CH₂F; 50 $\mu\text{mol/L}$) had no detectable effect on Syk levels (Fig 1, A). Another calpain inhibitor, ALLM (N-Acetyl-Leu-Leu-Met-CHO) also had no effect on Syk levels (not shown).

Levels of the Src-related kinase, Lyn, were similar between releaser and nonreleaser basophils and were not altered by any of the inhibitors (Fig 1, A and B). Thus the increase in Syk levels is not a nonspecific response of signaling proteins to inhibitors of proteasome-mediated protein degradation.

Time-course studies showed that the PSI-induced increase in Syk could be detected by 3 hours in both releaser basophils (data not shown) and nonreleaser basophils (Fig 1, C). Overexposure of the films (Fig 1, D) revealed the presence of extremely low levels of Syk protein in the nonreleaser basophils, which slightly increased after 30 minutes of PSI treatment, and importantly, the accumulation of higher-molecular-weight Syk forms after 3 hours of treatment with PSI. The appearance of upper-shifted Syk species raises the possibility that Syk is ubiquitinated before its degradation. Consistent with this, the relative abilities of the different proteasome inhibitors to increase Syk levels (Fig 1) correlated with their relative abilities to induce high-molecular-weight protein-ubiquitin conjugates above the unmodified Syk protein (as detected by Western blotting with anti-ubiquitin antibodies [not illustrated]).

Recently, the E3 ubiquitin ligase, Cbl, has emerged as a negative regulator of receptor PTKs and nonreceptor PTKs, including the Zap70/Syk tyrosine kinases.¹⁸ Higher levels of c-Cbl in nonreleaser basophils might explain accelerated Syk degradation via the Ub-Pr pathway. However, Western blot analysis using monoclonal anti-Cbl antibodies showed no obvious differences in Cbl protein levels between releaser and nonreleaser basophils (Fig 1, E).

In vitro ubiquitination of Syk is associated with loss of Syk protein that can be rescued by PSI

The ability of basophil Syk to serve as a substrate for ubiquitination was explored through use of immunopurified Syk protein in an in vitro ubiquitination assay. Syk was immunoprecipitated from basophil lysates and incubated in ubiquitination reaction buffer for 2 hours at 30°C in the presence (Fig 2, A, *lanes 2-3*) or absence (Fig 2, A, *lane 1*) of RRL supplemented with ubiquitin and ubiquitin aldehyde (Fig 2, A, *lane 3*). Immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblotting with anti-Syk mAb revealed an accumulation of high-molecular-weight species of Syk, typical of ubiquitinated proteins. The identity of these modified forms was confirmed when the same membranes were subsequently stripped and reprobed with anti-ubiquitin antibodies. The accumulation of ubiquitinated Syk was further enhanced in the presence of exogenous ubiquitin and the deubiquitination enzyme inhibitor, ubiquitin aldehyde (Fig 2, A; compare *lanes 2 and 3*). The most prominent forms appear in a ladder at intervals of approximately 10 kDa, corresponding to what could be mono-ubiquitinated (at ~80 kDa) and bi-ubiquitinated (at ~90 kDa) Syk. In addition, a smear reactive with both anti-Syk and anti-ubiquitin antibodies appeared at a higher-molecular-weight region. These higher-molecular-mass species might represent typical poly-ubiquitinated protein.

The ubiquitination of Syk coincided with a decrease in the Syk protein level (Fig 2, A, *lane 2*), which was more extensive when exogenous ubiquitin was supplied (Fig 2, A, *lane 3*) and was completely blocked by inclusion of 60 $\mu\text{mol/L}$ PSI in the in vitro ubiquitination reaction (Fig 2, B, *lane 7*). Moreover, the decrease in Syk protein was apparent after only 30 minutes of the ubiquitination assay (Fig 2, B, *lane 3*). These in vitro results support our proposal, based on studies in intact cells, that ubiquitinated Syk might be particularly susceptible to degradation by the Ub-Pr pathway. Because rabbit reticulocytes are the source of proteasomes in this assay, these results suggest further that the degradation is not dependent on some special property of the basophil proteasomes.

Proteasome inhibitors do not increase Syk levels in lymphocytes and monocytes

We next examined the effects of the proteasome, calpain, and caspase inhibitors on Syk levels in other

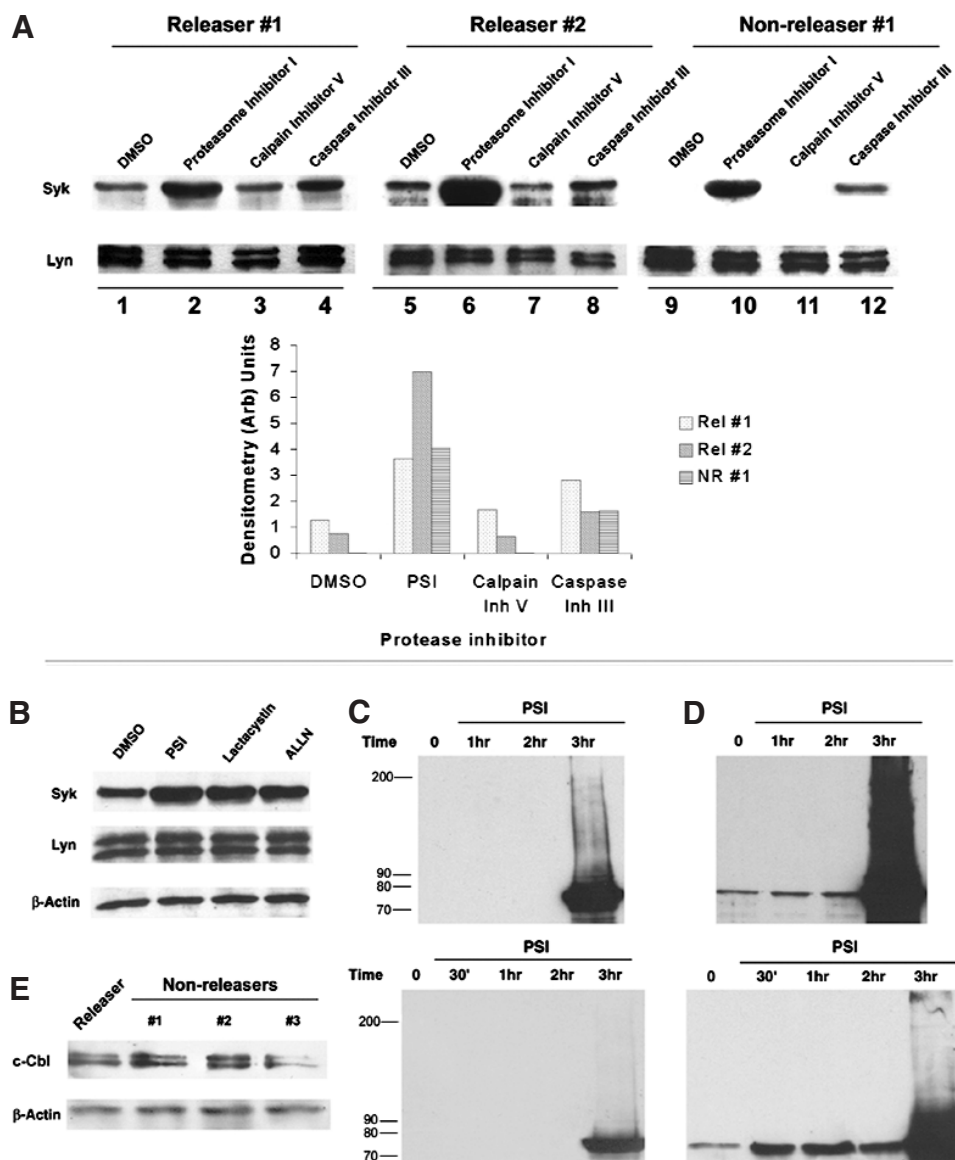


FIG 1. Proteasome and caspase inhibitors increase Syk protein levels in releaser and nonreleaser basophils. **A, Top:** Basophils (>99% pure) were incubated with DMSO or with PSI (60 μ mol/L), calpain inhibitor V (50 μ mol/L), or caspase inhibitor III (25 μ mol/L) for 4 hours. Solubilized proteins (2×10^5 cell equivalents/lane) were resolved by SDS-PAGE and analyzed by Western blotting through use of anti-Syk and anti-Lyn antibodies. **Bottom:** Densitometric analysis of gels shown above. Values are presented as arbitrary (Arb) densitometric units. **B,** Basophils were incubated with DMSO or with PSI (60 μ mol/L), Lactacystin (40 μ mol/L), or ALLN (50 μ mol/L) for 3 hours, then analyzed by Western blotting through use of anti-Syk, anti-Lyn, and anti-actin antibodies. **C,** Nonreleaser basophils were incubated with PSI (60 μ mol/L) for 30 minutes and 1, 2, and 3 hours, then analyzed through use of anti-Syk antibodies. **D,** Longer exposure of the films in Fig 1, C. **E,** Western blot analysis was performed as above through use of anti-Cbl mAbs and then through use of anti-actin mAbs. Each experiment was performed at least 3 times.

leukocytes from the same donors. Syk was readily detected in isolated monocytes and total lymphocytes from both nonreleaser donors (Fig 3) and releaser donors (not shown), and none of the protease inhibitors affected Syk levels in these cells (illustrated for PSI, calpain inhibitor V, and caspase inhibitor III in Fig 3, A). Lyn and actin levels were also unaffected in monocytes and total lymphocytes treated with these inhibitors (Fig 3, A).

Because T cells downregulate Syk expression as they enter the circulation,^{19,20} most of the Syk in total lymphocytes is likely derived from B cells. We therefore fractionated the lymphocyte population to isolate CD4⁺ naive T cells. Fig 3, B shows that incubation of CD4⁺ T cells with PSI for 3 hours increased the Syk protein level (3-fold increase, as determined by densitometry in each of 2 experiments) in comparison with both freshly isolated and vehicle-treated CD4⁺ cells.

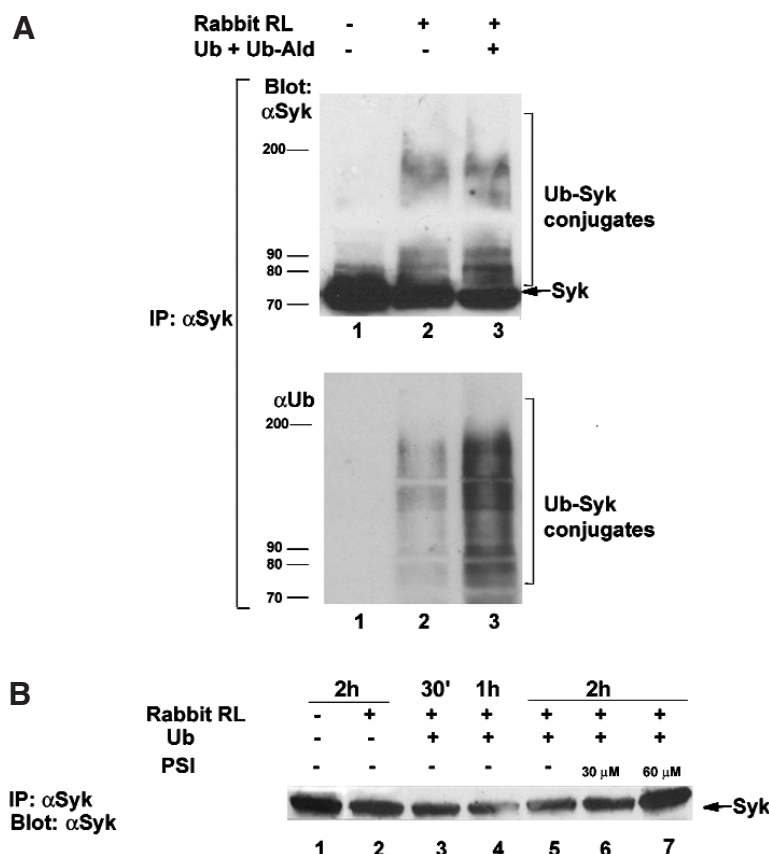


FIG 2. In vitro ubiquitination of Syk. **A**, Protein A-Sepharose-bound Syk protein was immunopurified from 900 μ g aliquots of unstimulated basophil lysates. Immunoprecipitates were subjected to in vitro ubiquitination reactions for 2 hours through use of RRL with added ubiquitin (Ub) and ubiquitin aldehyde (Ub-Ald). Syk immunoprecipitates were eluted with SDS-sample buffer, resolved by 8% SDS-PAGE, and immunoblotted with anti-Syk mAb (top panel) and then with anti-Ub (bottom panel). **B**, Anti-Syk immunoprecipitates from 600- μ g aliquots of basophil lysates were used in the assay. The reactions were carried out for 30 minutes to 2 hours in the presence or absence of PSI. Western blot analysis was performed as above. Molecular weight markers (in kilodaltons) are provided on the left. The same results were obtained in 2 separate experiments.

DISCUSSION

Our previous work linked impaired Ca^{2+} mobilization, histamine secretion, cytokine production, membrane ruffling, and adhesion to extremely low or undetectable levels of Syk in nonreleaser basophils.⁹⁻¹⁰ Two lines of evidence suggested that the lack of Syk in the nonreleaser basophils might be determined at a posttranslational level. First, Syk mRNA was present at comparable levels in nonreleaser and releaser basophils.¹⁰ Second, Syk protein levels were normal in other leukocytes from the nonreleaser donors.⁹ Another group confirmed the absence of basophil Syk from a separate population of nonreleaser donors²¹; unexpectedly, their nonreleaser basophils appeared to lack Lyn²¹ also, whereas we consistently detect Lyn in nonreleaser basophils (Fig 1).^{9,10}

Although Syk/p72 is known to be highly susceptible to proteolysis,^{22,23} little is known about the protease(s) involved in determining its levels in vivo. In recent years,

the pivotal role of the Ub-Pr-mediated proteolysis of critical cellular proteins has become better appreciated. In the present investigation, we found that 3 proteasome inhibitors—PSI, lactacystin, and ALLN—all increased Syk levels in releaser basophils and restored Syk expression in nonreleaser basophils. These results, along with the accumulation of multiple upward-shifted (presumably poly-ubiquitinated) Syk proteins in PSI-treated cells, strongly implicate the Ub-Pr pathway in the control of Syk levels in basophils from both releaser donors and nonreleaser donors.

All known substrates of the proteasome must be ubiquitinated before degradation, the only exception being ornithine decarboxylase, which is degraded by the 26S protease in a ubiquitin-independent process.²⁴ We therefore attempted to provide additional evidence confirming the identity of the Syk high-molecular-weight species accumulated in vivo after proteasome inhibitor treatment. We found that Syk is a substrate for in vitro ubiqui-

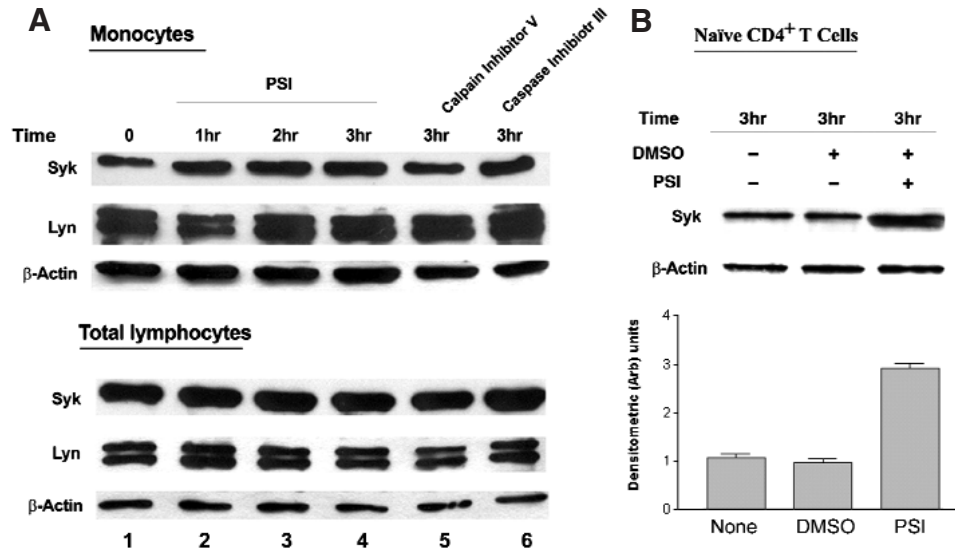


FIG 3. Effects of protease inhibitors on Syk levels in lymphocytes and monocytes. **A**, Monocytes (98% pure) and total lymphocytes (>85% pure) were incubated with DMSO (lane 1) or PSI (60 μ mol/L) for 1, 2, or 3 hours (lanes 2, 3, and 4, respectively) or with calpain inhibitor V (50 μ mol/L; lane 6) or caspase inhibitor III (25 μ mol/L; lane 7) for 3 hours. Proteins from 2×10^5 cells/lane were fractionated by SDS-PAGE, and Western blot analysis was performed as described in the legend to Fig 1. **B**, CD4⁺CD45RO⁻ T cells were incubated for 3 hours in the absence or presence of PSI (60 μ mol/L). Extracts from 3.5×10^5 cells were analyzed by Western blotting through use of anti-Syk and anti-actin antibodies. Results are typical of 3 replicate experiments.

uitination reactions and also that ubiquitination of Syk is associated with a substantial decrease in total levels of Syk protein. Increasing the extent of Syk ubiquitination by adding exogenous ubiquitin to the *in vitro* assay further facilitated the decline in Syk protein levels. Conversely, the addition of proteasome inhibitor protected against the loss of Syk protein. Overall, these results suggest a strong correlation between Syk levels and its targeted destruction by the Ub-Pr pathway.

Very little is known about the signals that target substrate proteins for ubiquitin conjugation and subsequent degradation. We do not at present know the nature of the signal that targets a significant fraction of Syk for proteasome degradation in normal human basophils. Presumably, amplification of this unknown signal results in essentially total Syk degradation in the basophils of nonreleasers. Interestingly, previous studies in RBL-2H3 rat tumor cells demonstrated that Fc ϵ RI cross-linking induces a rapid and reversible multi-ubiquitination of the receptor's β and γ subunits,²⁵ suggesting that the Ub-Pr pathway might also downregulate Fc ϵ RI—but via an activation-dependent mechanism rather than a constitutive mechanism. Importantly, non-phosphorylated forms of Fc ϵ RI γ were found to be ubiquitinated, indicating that phosphorylation might not play a major role in the mechanism by which the ubiquitin multicatalytic system²⁵ recognizes γ .

Two recent reports have investigated Syk ubiquitination and degradation in 2 other different cell types. Rao et al²⁶ have shown that B-cell receptor stimulation in Ramos cells induces the ubiquitination of Syk. Moreover, Paolini et al¹⁶ have provided evidence for ubiquitination and degradation of Syk and Zap-70 PTKs in human natural

killer (NK) cells upon the aggregation of the low-affinity Fc receptor for IgG (CD16). However, in the latter study, a direct link between Syk ubiquitination and proteasome-mediated degradation was less clear. CD16 engagement-induced Syk ubiquitination was evident after only 1 minute and began to decline markedly after 10 minutes, whereas degradation of the kinase was not detected until 8 hours after stimulation. Moreover, the proteasome inhibitor epoxomicin was shown to partially inhibit CD16-induced Syk and ZAP-70 degradation, but no marked increase in Syk level was observed in resting conditions even after 12 hours of incubation of the NK cells in the presence of epoxomicin. These results raise the possibility that the requirements for Ub-Pr-mediated regulation of Syk varies among different cell types.

Neither calpain inhibitor V nor ALLM affected basophil Syk protein levels, indicating that calpain is not importantly involved in the regulation of Syk levels in unstimulated cells. However, the cell-permeable and broad-spectrum caspase inhibitor III caused modest increases in Syk levels in basophils from both releasers and nonreleasers. In NK cells, Syk and ZAP-70 degradation on CD16 engagement was shown to be sensitive to the lysosomal inhibitor ammonium chloride (NH₄CL).¹⁶ Taken together, these results from 2 different laboratories raise the possibility that other proteases—including the caspases, a family of cysteine-dependent, aspartate-directed proteases, and the lysosome—might also contribute to Syk turnover in human basophils and NK cells.

Although Syk levels were either increased (releaser basophils) or restored (nonreleaser basophils) after incubation with proteasome inhibitors, nonreleaser basophils

failed to degranulate, mobilize calcium, or activate MAP kinase after incubation with PSI. Furthermore, all of these responses to FcεRI cross-linking were impaired in releaser basophils treated with PSI (data not shown). The results of further experiments suggested that the explanation very likely lies in an additional effect(s) of proteasome inhibition that occurs upstream of Syk. Specifically, FcεRI β subunit phosphorylation, the precursor to Syk activation, is dramatically impaired in PSI-treated basophils (data not shown). This result reinforces a point made frequently by others—namely, that many aspects of cell behavior and cell signaling are under proteasomal regulation. Previously, we showed that 3 to 4 days of incubation of nonreleaser basophils with IL-3 partially restores both Syk expression and secretion.¹⁰ Further studies are expected to reveal differences between IL-3-treated and PSI-treated nonreleaser basophils that can explain the recovery of Syk protein in both cases and the recovery of secretion only in the IL-3-treated samples.

In hematopoietic cells, Syk expression levels vary widely according to cell type and/or stage of differentiation. In particular, B lymphocytes express 12- to 15-fold higher levels of Syk than peripheral T cells at all stages of differentiation, and thymic T cells express 3- to 4-fold higher levels of Syk than splenic and peripheral T cells.^{19,20} We found that PSI increases Syk levels in CD4⁺ naive T cells but not in monocytes or total lymphocytes (in which Syk would be predominantly from B cells). It thus appears that resting human basophils and T cells might share a proteasome-dependent mechanism of Syk degradation that is not important in unstimulated monocytes and B lymphocytes. The challenge that lies ahead is how to resolve such differential regulation of Syk stability in different leukocyte populations. Some clues might come from the recent discovery of an acridone-related compound, ER-27319, that specifically blocks FcεRI-coupled Syk activation in human mast cells without affecting anti-IgM-induced activation of Syk in human peripheral B cells.²⁷ This observation suggests a cell type-specific Syk protein interaction that might differentially determine the fate of Syk protein.

In summary, the current study provides evidence for the involvement of the Ub-Pr pathway in the regulation of Syk protein levels in human basophils. The Syk-binding E3 ubiquitin ligase, Cbl,²⁸ has been implicated in Syk degradation both in RBL-2H3 mast cells and in a COS-7 reconstitution system.^{29,30} In their recent study, Rao et al²⁶ demonstrated that upon B-cell receptor stimulation, Cbl mediates Syk ubiquitination, marking it for proteasomal degradation. In the present investigation, we found no marked differences in c-Cbl levels between releaser and nonreleaser basophils that might explain the low levels of Syk in nonreleaser basophils. We do not rule out a role for Cbl in downregulating Syk in basophils. It is possible that alterations in Cbl activities and/or partners contribute to Syk instability in the nonreleaser phenotype. Alternatively, regulation of Syk stability might be affected by an as-yet-unknown E3 ligase or chaperone, and a role for deubiquitination enzymes cannot be excluded.

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REFERENCES

- Eiseman E, Bolen JB. Engagement of the high affinity IgE receptor activates src protein-related tyrosine kinases. *Nature* 1992;355:78-80.
- Jouvin MH, Adamczewski M, Numerfo R, Letourneur O, Valle A, Kinet JP. Differential control of the tyrosine kinases Lyn and Syk by the two signalling chains of the high affinity immunoglobulin E receptor. *J Biol Chem* 1994;269:5918-25.
- Costello PK, Turner M, Walters AE, Cunningham CN, Bauer PH, Downward J, et al. Critical role for the tyrosine kinase Syk in signaling through the high affinity IgE receptor of mast cells. *Oncogene* 1997;13:2595-605.
- Hutchcroft JE, Geahlen RL, Deanin GG, Oliver JM. FcεRI-mediated tyrosine phosphorylation and activation of the 72 kDa protein-tyrosine kinase, PTK72, in RBL-2H3 rat tumor mast cells. *Proc Natl Acad Sci U S A* 1992;89:9107-11.
- Wilson BS, Oliver JM. Effector roles of IgE antibodies: targeting allergen to the high affinity IgE receptor for FcεRI-dependent signaling and antigen presentation. In: Zweiman B, Schwartz LB, editors. *Inflammatory mechanisms in allergic diseases*. New York: Marcel-Dekker; p. 197-232.
- Yanagi S, Inatome R, Takano T, Yamamura H. Syk expression and novel function in a wide variety of tissues. *Biochem Biophys Res Commun* 2001;288:495-8.
- Turner M, Schweighoffer E, Colucci F, Di Santo JP, Tybulewicz VL. Tyrosine kinase Syk: essential functions for immunoreceptor signaling. *Immunol Today* 2000;21:148-54.
- Turner M, Mee PJ, Costello PS, Williams O, Price AA, Duddy LP, et al. Prenatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature* 1995;378:298-302.
- Kepley CL, Youssef L, Andrew RP, Wilson BS, Oliver JM. Syk deficiency in non-releaser basophils. *J Allergy Clin Immunol* 1999;104:279-84.
- Kepley CL, Youssef L, Andrew RP, BS Wilson, Oliver JM. Multiple defects in FcεRI signaling in Syk-deficient non-releaser basophils and IL-3-induced recovery of Syk expression and secretion. *J Immunol* 2000;165:5913-20.
- Coux O, Tanaka K, Goldberg AL. Structure and functions of the 20S and 26S proteasomes. *Annu Rev Biochem* 1996;65:801-47.
- Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev* 2002;82:373-428.
- Bjerke T, Nielsen S, Helgestad J, Nielsen BW, Schiøtz PO. Purification of human blood basophils by negative selection using immunomagnetic beads. *J Immunol Methods* 1993;157:49-56.
- Kepley C, Craig S, Schwartz L. Purification of human basophils by density and size alone. *J Immunol Methods* 1994;175:1-9.
- Kepley CL, Wilson BS, Oliver JM. Roles for the protein kinases Lyn, Syk and Zap70 in FcεRI-mediated human basophil degranulation. *J Allergy Clin Immunol* 1998;102:304-15.
- Paolini R, Molfetta R, Piccoli M, Frati L, Santoni A. Ubiquitination and degradation of Syk and Zap-70 protein tyrosine kinase in human NK cells upon CD16 engagement. *Proc Natl Acad Sci U S A* 2001;98:9611-6.
- Fenteany G, Standaert RF, Lane WS, Choi S, Corey EJ, Schreiber SL. Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. *Science* 1995;268:726-31.
- Lupher ML Jr, Andoniou CE, Bonita D, Miyake S, Band H. The c-Cbl oncoprotein. *Int J Biochem Cell Biol* 1998;30:439-44.
- Chan AC, van Ores NSC, Tran A, Turka L, Law CL, Ryan JC, et al. Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TCR signaling. *J Immunol* 1994;152:4758-66.
- Chu DH, van Oers NSC, Malissen M, Harris J, Elder M, Weiss A. Pre-T cell receptor signals are responsible for the down-regulation of Syk protein tyrosine kinase expression. *J Immunol* 1999;163:2610-20.
- Lavens Phillips SE, MacGlashan DW. The tyrosine kinases p53/56lyn and p72syk are differentially expressed at the protein level but not at the messenger RNA level in nonreleasing human basophils. *Am J Resp Cell Mol Biol* 2000;23:566-71.

22. Taniguchi T, Kobayashi T, Kondo J, Takahashi K, Nakamura H, Suzuki J, et al. Molecular cloning of a porcine gene Syk that encodes a 72-kDa protein-tyrosine kinase showing high susceptibility to proteolysis. *J Biol Chem* 1991;266:15790-9.
23. Zionscheck TF, Harrison ML, Isaacson CC, Geahlen RL. Generation of an active protein-tyrosine kinase from lymphocytes by proteolysis. *J Biol Chem* 1988;263:19195-202.
24. Murakami Y, Matsufuji S, Kameji T, Hayashi S, Igarashi K, Tamura T, et al. Ornithin decarboxylase is degraded by the 26S proteasome without ubiquitination. *Nature* 1992;360:597-9.
25. Paolini R, Kinet JP. Cell-surface control of the multiubiquitination and deubiquitination of high-affinity immunoglobulin-E receptors. *EMBO J* 1993;12:779-86.
26. Rao N, Ghosh AK, Ota S, Zhou P, Reddi AL, Hakezi K, et al. The non-receptor tyrosine kinase Syk is a target of Cbl-mediated ubiquitylation upon B-cell receptor stimulation. *EMBO J* 2001;20:7085-95.
27. Moriya K, Rivera J, Odom S, Sakuma Y, Muramoto K, Yoshiuchi T, et al. ER-27319, an acridone-related compound, inhibits release of antigen-induced allergic mediators from mast cells by selective inhibition of Fcε receptor I-mediated activation of Syk. *Proc Natl Acad Sci U S A* 1997;94:12539-44.
28. Joazeiro CA, Wing SS, Huang HK, Leverson JD, Hunter T, Liu YC. The tyrosine kinase negative regulator c-Cbl as a RING-type, E2-dependent ubiquitin-protein ligase. *Science* 1999;286:309-12.
29. Ota S, Hazeki K, Rao N, Lupher ML Jr, Andoniou CE, Druker B, et al. The ring finger domain of Cbl is essential for negative regulation of the Syk tyrosine kinase. *J Biol Chem* 2000;275:414-22.
30. Rao N, Lupher ML Jr, Ota S, Reedquist KA, Druker BJ, Band H. The linker phosphorylation site Tyr292 mediates the negative regulatory effect of Cbl on ZAP-70 in T cells. *J Immunol* 2000;164:4616-26.