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Csk-binding Protein Mediates Sequential Enzymatic Down-regulation and Degradation of Lyn in Erythropoietin-stimulated Cells*

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Evan Ingley[‡§1], Jessica R. Schneider[‡§], Christine J. Payne[‡§], David J. McCarthy[‡§], Kenneth W. Harder[¶], Margaret L. Hibbs[¶], and S. Peter Klinken[§]

From the [‡]Cell Signalling Group and the [§]Laboratory for Cancer Medicine, Western Australian Institute for Medical Research and Centre for Medical Research, University of Western Australia, Perth, Western Australia 6000, Australia and the [¶]Ludwig Institute for Cancer Research, Melbourne Tumor Biology Branch, Royal Melbourne Hospital, Victoria 3050, Australia

We have shown previously that the Src family kinase Lyn is involved in differentiation signals emanating from activated erythropoietin (Epo) receptors. The importance of Lyn to red cell maturation has been highlighted by $Lyn^{-/-}$ mice developing anemia. Here we show that Lyn interacts with C-terminal Src kinase-binding protein (Cbp), an adaptor protein that recruits negative regulators C-terminal Src kinase (Csk)/Csk-like protein-tyrosine kinase (Ctk). Lyn phosphorylated Cbp on several tyrosine residues, including Tyr^{314}, which recruited Csk/Ctk to suppress Lyn kinase activity. Intriguingly, phosphorylated Tyr^{314} also bound suppressor of cytokine signaling 1 (SOCS1), another well characterized negative regulator of cell signaling, resulting in elevated ubiquitination, and degradation of Lyn. In Epo-responsive primary cells and cell lines, Lyn rapidly phosphorylated Cbp, suppressing Lyn kinase activity via Csk/Ctk within minutes of Epo stimulation; hours later, SOCS1 bound to Cbp and was involved in the ubiquitination and turnover of Lyn protein. Thus, a single phosphotyrosine residue on Cbp coordinates a two-phase process involving distinct negative regulatory pathways to inactivate, then degrade, Lyn.

Intracellular signaling cascades activated by erythropoietin (Epo)[2] binding to its cognate receptor, have been the subject of intensive investigation over the past decade (1–4). Activation of signal transduction pathways by Epo maintains viability, stimulates proliferation and induces terminal differentiation of erythroid progenitors and precursors. Upon ligand binding, the receptor dimerizes and Janus kinase 2 (JAK2) becomes activated by transphosphorylation (5, 6). JAK2 also phosphorylates the Epo receptor on tyrosine residues within the cytoplasmic domain, and these serve as docking sites for numerous signaling proteins, *e.g.* signal transducer and activator of transcription 5 (STAT5) associates with the receptor and is phosphorylated by JAK2, leading to STAT5 translocation to the nucleus where it binds DNA and regulates gene transcription (7–9). Other pathways activated by Epo include the Ras/Raf/MAP kinase and PI 3-kinase/Akt cascades (10–13).

Two mechanisms to down-regulate the Epo signaling pathway have been defined. First, the phosphatase SHP-1 docks onto phosphorylated tyrosine residues of the receptor via its SH2 domain, and dephosphorylates the receptor (14). Second, several members of the suppressors of cytokine signaling (SOCS) family of negative regulators are activated upon Epo stimulation, including SOCS1, SOCS3, and cytokine-inducible SH2-domain-containing protein (CIS) (15–17). These proteins inhibit the kinase activity of JAK2 and compete with JAK2 and STAT5 for binding to activated receptors (18). Furthermore, association of SOCS proteins with elongins B/C enables ubiquitination and proteasomal degradation of JAK2 (19).

In addition to the various signaling cascades stimulated by Epo, we demonstrated that the tyrosine kinase Lyn is required for Epo-induced differentiation *in vitro* (20–22). Chin *et al.* (23) confirmed the association of Lyn with the Epo receptor and demonstrated that this tyrosine kinase was also required for STAT5 phosphorylation. Further studies showed that Lyn was involved in CrkL (a Crk-like adaptor protein) activation after Epo receptor stimulation (24). Lyn remains an active kinase in erythrocytes, and is capable of phosphorylating the structural protein Band 3 (25). The effects of Lyn on erythroid cells are not restricted to *in vitro* systems, as $Lyn^{-/-}$ mice display extramedullary stress erythropoiesis and eventually become anemic (26, 27).

The regulation of Src family kinases is complex (28), and the activity of these kinases is influenced significantly by phosphorylation of specific tyrosine residues, *e.g.* Lyn is activated by phosphorylation of Tyr^{397}, and inhibited by phosphorylation of Tyr^{508} (29). Following Src activation, inhibitory kinases Csk/Ctk are recruited by phosphorylated Cbp to down-regulate Src activity (30, 31). In addition, phosphatases play important roles in this interplay as both Shp2 and CD45 dephosphorylate Cbp,

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[1] To whom correspondence should be addressed: Cell Signalling Group, Laboratory for Cancer Medicine Western Australian Institute for Medical Research Level 6, Medical Research Foundation Bldg. Rear 50 Murray St., Perth WA 6000 Australia. Tel.: 61-8-9224-0326; Fax: 61-8-9224-0322; E-mail: eingley@waimr.uwa.edu.au.

[2] The abbreviations used are: Epo, erythropoietin; Csk, C-terminal Src kinase; Cbp, Csk-binding protein; SH2, Src homology 2; SH3, Src homology 3; SOCS1, suppressor of cytokine signaling 1; STAT, signal transducer and activator of transcription; JAK, Janus kinase; BFU-E, burst forming unit-erythroid; CFU-E, colony forming unit-erythroid; GST, glutathione *S*-transferase; HA, hemagglutinin; ANOVA, analysis of variance.

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thereby controlling access of Csk/Ctk to Src kinases (32, 33). It is pertinent to note, that Lyn activity is enhanced by activation of CD45 in hemopoietic stem cells maturing along the erythroid lineage (34).

To biochemically define the Lyn signaling pathway, we have employed the yeast two-hybrid system and demonstrated that hemopoietic-specific protein 1 (HS1) and thyroid hormone receptor-interacting protein 1 (Trip1) are part of the Lyn pathway for red cell maturation (35, 36). Mutation of HS1 and Trip1 in primary erythroid progenitors, or the Epo-responsive J2E cell line (37), impedes differentiation (35, 36). At the time the yeast two-hybrid screen was conducted, one of the Lyn-binding proteins isolated was a novel molecule. However, shortly after the protein was cloned independently by two groups as Cbp/PAG (30, 31).

In this study we examined the role of Cbp in regulating Lyn within erythroid cells, our data demonstrate that suppressing the kinase activity of Lyn via Cbp is the first phase in the down-regulation of this key signaling molecule. We also show that SOCS1 attaches to Cbp, leading to ubiquitination and degradation of Lyn during the second stage of Lyn inactivation. Thus, this study defines sequential steps in Lyn inactivation, which are coordinated by Cbp.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The Myc-tagged Cbp expression constructs pCMV-Cbp and pCMV-CbpY314F (31) were generously provided by Dr. M. Okado (Osaka University, Japan), while the yeast expression vector encoding the Myc-tagged Lyn kinase domain (pMET416-Lyn, Ref. 38) was a kind gift from Dr. J. Bertoglio (INSERM, France), and the ADE2 gene encoding plasmid pASZ11 was graciously provided by Dr. P. Linder (Biozentrum, Switzerland).

The construction of the murine Lyn expression plasmids pCA-Lynwt, pCA-LynY397F, pCA-LynY508F (21), FLAG-tagged murine SOCS1 expression vector pEFBos-SOCS1 (15), murine Lyn full-length and Lyn domain LexA fusion plasmids pBTM116-Lyn, pBTM116-Un, pBTM116-SH3, pBTM116-SH2, pBTM116-kinase (35, 36), murine ARL-6 LexA fusion vector pBTM116-ARL-6 (39), murine Lyn SH2 domain VP16 fusion plasmid pVP16-SH2 (35, 36), and murine GBP3 VP16 fusion plasmid pVP16-GBP3 have been described in detail previously (39).

All plasmid constructs were generated by site-directed mutagenesis using oligonucleotides (sequences available upon request), subcloning into the appropriate vector and confirmation by sequencing. The rat Cbp LexA fusion constructs pBTM116-Cbp, pBTM116-CbpD126, pBTM116-CbpD246, pBTM116-CbpD266, pBTM116-CbpD352, pBTM116-CbpY314F, pBTM116-CbpY381F, pBTM116-CbpY409F, pBTM116-CbpY381/409F, pBTM116-CbpD352Y381F, pBTM116-CbpD352Y409F, pBTM116-CbpD352Y381/409F were generated using pCMV-Cbp or pCMV-CbpY314F as templates and subcloning into pBM116 (40) in-frame with LexA. The Myc-tagged rat Cbp construct pCMV-CbpY381/409F was generated using pCMV-Cbp as a template and subcloned into pCMV-Tag1. The Myc-tagged rat Cbp construct with the proline-rich region mutated to alanine residues pCMV-CbpD-PPY381/409F was generated using pCMV-CbpY381/409F as a template and subcloning into pCMV-Tag1. The murine Cbp glutathione *S*-transferase (GST) fusion construct pGEX-CbpD76 was generated by subcloning Cbp from pVP16-Cbp into pGEX-2T, while pGEX-Cbp, pGEX-CbpY314F, pGEX-CbpY381/409F were generated using pCMV-Cbp, pCMV-CbpY314F, or pCMV-CbpY381/409F as template and subcloning into pGEX-2T. Retroviral vector plasmids pMSCV-Cbp and pMSCV-CbpY314F were made using pCMV-Cbp and pCMV-CbpY314F as template and subcloning onto pMSCV-neo2.2 (41). The His_6-tagged murine Ctk expressing plasmid pcDNA-HIS-Ctk and the HA-tagged murine ubiquitin expressing plasmid pcDNA-HA-Ub, were generated by subcloning murine Ctk or ubiquitin (obtained by RT-PCR from J2E cell mRNA) into pcDNA3-HIS and pcDNA3-HA, respectively. Subcloning the yeast *ADE2* gene from pASZ11 (42) into pMET416-Lyn generated the yeast Lyn kinase domain expression plasmid pRSADE-MET-Lyn, using adenine rather than uracil selection.

Cell Culture, Inductions, Flow Cytometry, and Colony Assays—J2E (37), J397 (21), and R11 (21) cells were maintained in Dulbecco's modified Eagles medium (DMEM) with 5% fetal calf serum (FCS), while COS-7, HEK293, PA317, and Y2 cells were cultured in DMEM/10% fetal calf serum as described elsewhere (36). Cells were stimulated with Epo (5 units/ml) in media containing T_3 depleted FCS (36), and hemoglobin production detected by benzidine staining (21). Splenic erythroblasts were isolated from phenylhydrazine-treated $Lyn^{+/+}$ and $Lyn^{-/-}$ mice as described previously (26).

J2E subclones expressing Lyn[Y397F], Cbp, Cbp[Y314F], or SOCS1, were generated by infection with amphitropic retroviruses produced by PA317 packaging cells transfected with the pMSCV constructs; multiple unique clonal lines were isolated using methylcellulose cultures as previously described (36).

Murine fetal liver progenitor cells were obtained from 12-day-old fetuses of CBA mice as described previously (43). Cell suspensions were harvested and infected with ecotropic retroviruses produced by Y2 packaging cells transfected with pMSCV constructs by co-cultivation for 2 days; 5,000 (CFU-E) or 20,000 (BFU-E; 0.4 mg/ml G418) cells were then plated per dish as previously described (43). Benzidine-positive colonies were counted 2 or 7 days later, to determine CFU-E and BFU-E, respectively.

Standard and Phosphotyrosine-specific Yeast Two-hybrid Systems—The standard yeast two-hybrid system (35, 40) utilized the *Saccharomyces cerevisiae* L40 strain. Wild-type Lyn (Lyn) and a dominant-negative Lyn (Y397F) cDNAs were used to screen a yeast two-hybrid library derived from the lymphohemopoietic progenitor cell line EML C.1 (44) as described previously (35).

The phosphotyrosine-specific yeast two-hybrid system was performed essentially as described for the standard version except that libraries were screened against baits (*i.e.* Cbp) in the presence of the pRSADE-MET-Lyn vector expressing the kinase domain of Lyn. His and β-gal positive clones were tested for activity with, and without, the addition of methionine (200 mg/liter), which suppresses the MET25 promoter driving the

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expression of the Lyn kinase to identify phosphotyrosine-specific interacting clones.

Protein and Antibody Production—Plasmids expressing GST fusion proteins of Cbp (pGEX-CbpD76, pGEX-Cbp, pGEX-CbpY314F, pGEX-CbpY381/409F) were transformed into Rosetta cells (Novagen) and the fusion proteins were induced with isopropyl-1-thio-β-D-galactopyranoside (1 mM), 4h, 25 °C, before the cells were lysed with BugBuster (Novagen) as described by the manufacturer, and purified on glutathione agarose (Sigma) as used previously (35). Antisera directed against GST-CbpD76 were raised in rabbits according to the protocol previously described (43).

Transfection, Immunoprecipitation, and Immunoblotting—COS-7 cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. MG132 (Calbiochem) was used to inhibit proteasomal activity and added to cultures at 40 μM for 12 h before cell lysis.

Immunoprecipitations and immunoblotting were performed essentially as described previously (21, 36) using a lipid raft lysis buffer (150 mm NaCl, 1% IGEPAL CA-630 (Sigma), 0.5% *n*-dodecyl-β-D-maltoside (Sigma), 0.2% octyl-D-glucoside (ICN Biomedicals), 20 mm Tris, pH 8.0, 0.1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 2 mM benzamidine, 2 mM vanadate, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerol phosphate), and protein G-beads (Sigma). In some experiments *N*-ethylmalemide (5 mM) and MG132 (0.4 μM) were added to the lysis buffer. Antibodies used were anti-Lyn, anti-phosphotyrosine (PY99), anti-phospho-ERK2, anti-ERK2, anti-His, anti-Ctk, anti-GST (sc15, sc7020, sc7383, sc154, sc803, sc470, and sc459; Santa Cruz Biotechnology), anti-HA (mouse HA.11; BAbCO), anti-FLAG (M2, Sigma), anti-Myc (clone 9E10 ascites fluid), anti-phosphotyrosine 4G10-HRP, anti-Myc 4A6 (Upstate), anti-phospho-Lyn(Y507) (Cell Signaling Tech).

Kinase Assays—Lyn exokinase and autokinase activity were determined by immunoprecipitation with anti-Lyn antibodies (sc-15, Santa Cruz Biotechnology) and subsequent incubation with [γ-^{32}P]ATP in the presence or absence of the substrates (acid-denatured enolase or purified GST, GST-Cbp, GST-Cbp[Y314F], GST-Cbp[Y381F/Y409F]), according to the protocol described previously (21).

Statistical Analysis—Quantitated data were analyzed for statistical significance by two-way ANOVA using Excel (Microsoft). *p* values less than 0.05 were taken as significant.

RESULTS

Lyn Associates with Cbp—Cbp was identified as a Lyn-binding protein in a yeast two-hybrid screen. Domain mapping revealed that the Lyn SH3 domain bound Cbp (Fig. 1*A*) via a proline-rich region (amino acids 246–266) (data not shown). Cbp contains a number of tyrosine residues, which may become phosphorylated and associate with the Lyn SH2 domain. Using a modified phosphotyrosine-specific yeast two-hybrid assay, tyrosines at the C terminus of Cbp (Tyr^{381} and Tyr^{409}) were shown to be involved in this phosphorylation-specific interaction (Fig. 1*B*). Co-immunoprecipitation of wild-type Cbp and Lyn in transiently transfected COS cells confirmed the yeast two-hybrid interaction (Fig. 1*C*); significantly, the Y381F/Y409F mutant displayed greatly reduced binding to Lyn. Residual binding observed with the Y381F/Y409F mutant was because of the SH3-polyproline interaction, as deletion of the proline-rich region abolished Lyn binding (data not shown).

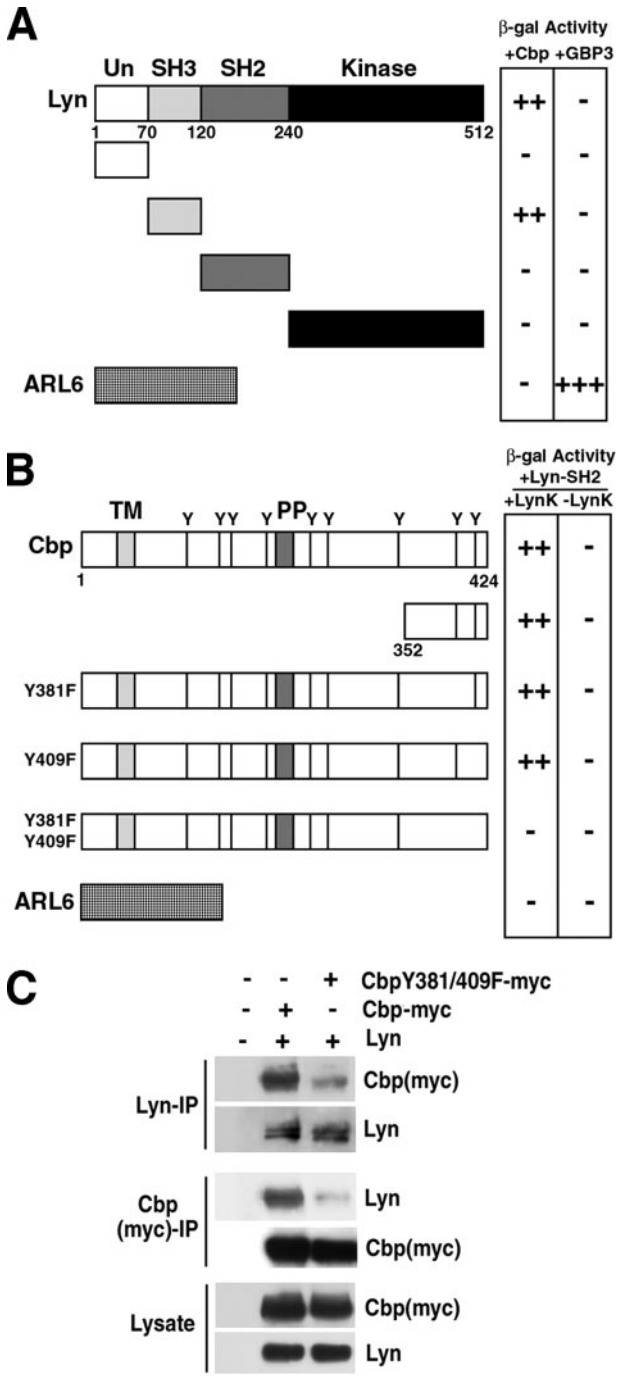

FIGURE 1. **Lyn interacts with Cbp.** *A*, full-length Lyn and its domains (*Un*, unique, SH3, SH2, and kinase) were tested for interaction with Cbp in a yeast two-hybrid assay. ARL-6 and GBP-3 (39) were used as a positive control. Numbers indicate amino acid positions. *B*, using a yeast two-hybrid assay, full-length Cbp together with deletion and point mutants were tested for interaction with the Lyn SH2 domain in the presence or absence of the Lyn kinase domain (*LynK*). *C*, immunoblot analysis of COS cells transfected with constructs expressing Lyn, Cbp, and Cbp[Y381F/Y409F]. After immunoprecipitation (*IP*) blots were probed with anti-Lyn and anti-Myc antibodies. Representative blots of experiments performed in triplicate are shown.

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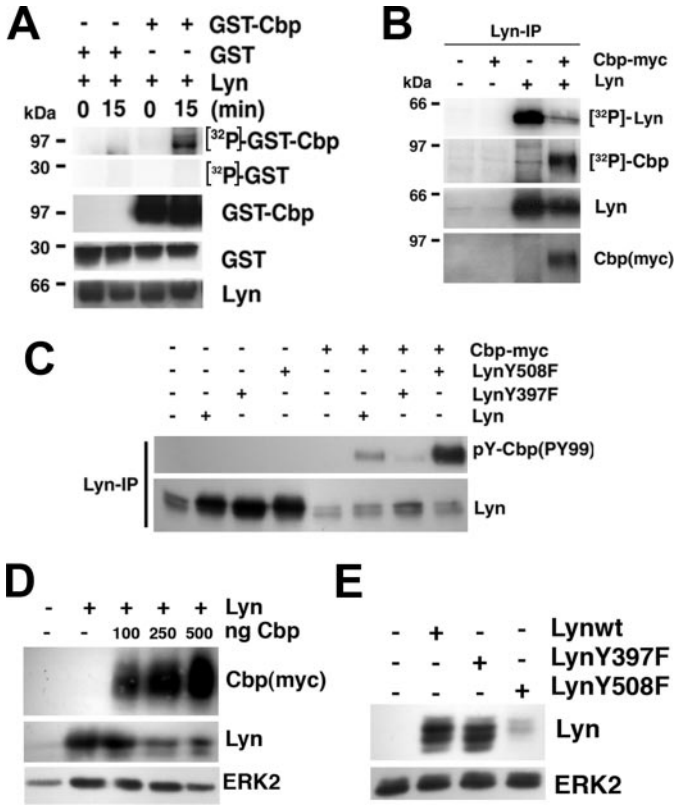

FIGURE 2. **Lyn phosphorylates Cbp.** *A*, GST-Cbp was used as a substrate in kinase assays using [γ-^{32}P]ATP and Lyn[Y508F] immunoprecipitated from transfected COS cells. ^{32}P incorporation was determined by a phosphorimager. Blots were also probed with anti-Lyn and anti-GST antibodies. *B*, COS cells transfected with Lyn and Cbp were immunoprecipitated with anti-Lyn antibodies, then kinase assays performed. ^{32}P incorporation into Cbp and Lyn was determined by a phosphorimager, before probing with anti-Myc and anti-Lyn antibodies. *C*, Lyn and Cbp were immunoprecipitated from COS cells transfected with Cbp and Lyn, Lyn[Y397F] or Lyn[Y508F]. The blots were then probed with anti-phosphotyrosine (*PY99*) and anti-Lyn antibodies. *D*, COS cells were transfected with Lyn and different amounts of Cbp (0–500 ng), then lysates were immunoblotted with anti-Myc, anti-Lyn, and anti-ERK2 (loading control) antibodies. *E*, COS cells were transfected with equal amounts of Lyn, Lyn[Y397F], and Lyn[Y508F], then lysates were prepared 48-h post-transfection. Blots were probed with anti-Lyn and anti-ERK2 antibodies. Representative blots of experiments performed in triplicate are shown.

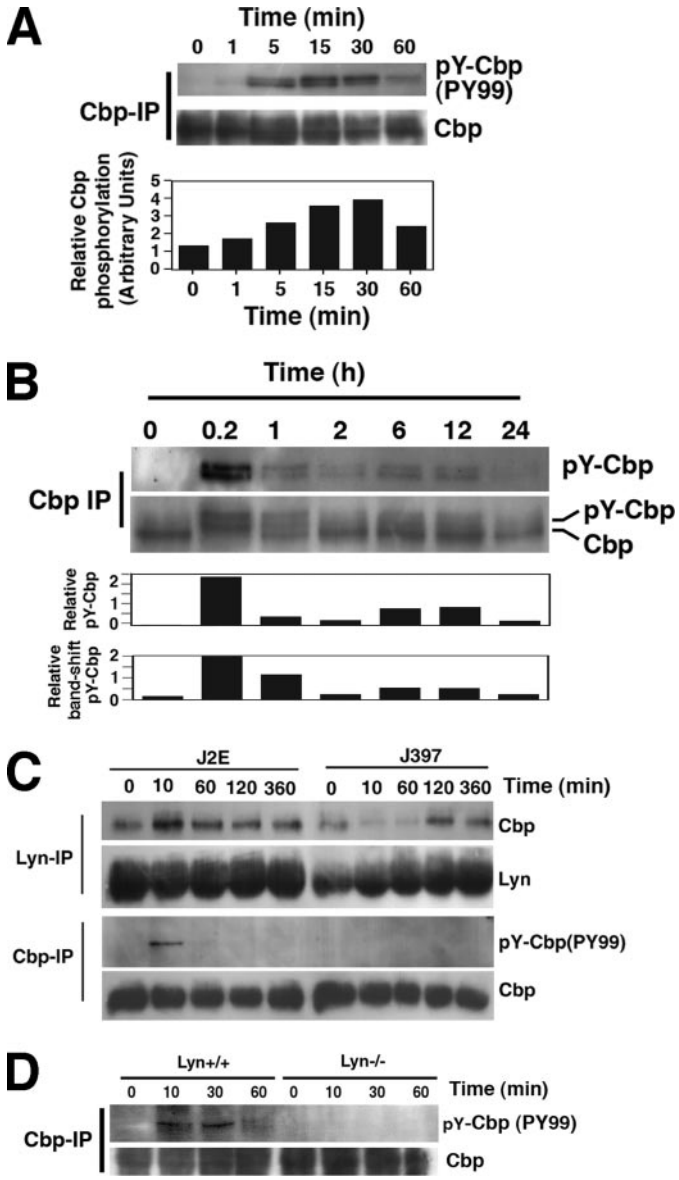

FIGURE 3. **Lyn phosphorylates Cbp in erythroid cells.** *A*, J2E cells were serum-starved for 2 h before Epo induction, then Cbp was immunoprecipitated (*IP*) from lysates prepared at various time points. Blots were probed with anti-phosphotyrosine (*PY99*) and anti-Cbp antibodies. Relative Cbp phosphorylation levels were determined using Quantity One (Bio-Rad). *B*, J2E cell lysates were prepared as in *A* with Epo stimulation for up to 24 h. Cbp was then immunoprecipitated from the lysates. Blots were probed with anti-phosphotyrosine (*PY99*) and anti-Cbp antibodies. Relative Cbp phosphorylation levels were determined using Quantity One. *C*, J2E cells and a clone expressing dominant-negative Lyn (J397) were serum-starved for 2 h before Epo induction, then Lyn and Cbp were immunoprecipitated from lysates prepared at various time points. Blots were probed with anti-phosphotyrosine (*PY99*), anti-Lyn, and anti-Cbp antibodies. *D*, erythroblasts were isolated from spleens of phenylhydrazine-treated *Lyn*$^{-/-}$ and *Lyn*$^{+/+}$ mice, serum starved for 2 h before Epo induction, then Cbp was immunoprecipitated from lysates prepared at various time points. Blots were probed with anti-phosphotyrosine (*PY99*) and anti-Cbp antibodies. Representative blots of experiments performed in triplicate are shown.

These results indicate that Lyn and Cbp are able to associate, and that tyrosine residues 381 and 409 play a significant role in the link between the two molecules. Thus, Lyn becomes another member of the Src family to associate with Cbp.

To demonstrate that Lyn could phosphorylate Cbp directly, wild-type Cbp was fused to GST and incubated with kinase-active Lyn (Lyn[Y508F]) in the presence of [γ-^{32}P]ATP. Fig. 2*A* shows that Cbp was phosphorylated by Lyn *in vitro*. The phosphorylation of Cbp was also confirmed by immunoprecipitation with anti-Lyn antibodies in the presence of [γ-^{32}P]ATP (Fig. 2*B*). Phosphorylation of Cbp was also affected by Lyn activity, the highest level of Cbp phosphorylation was observed with dominant active Lyn (Lyn[Y508F]), followed by wild-type Lyn, then kinase inactive Lyn (Lyn[Y397F]) (Fig. 2*C*). Note that Lyn levels actually decrease in the presence of elevated Cbp (Fig. 2, *C* and *D*). Interestingly, when equal amounts of the various Lyn constructs were transfected into COS cells, much less dominant active Lyn was detected (Fig. 2*E*). Collectively, these experiments show that: (i) Lyn is able to phosphorylate and associate with Cbp, and (ii) Lyn levels vary with Cbp concentration and Lyn activity.

Lyn Phosphorylates Cbp in Erythroid Cells—We then examined the association between Lyn and Cbp in Epo-responsive J2E erythroid cells. Fig. 3*A* shows that phosphorylation of Cbp occurred within minutes after Epo stimulation; significantly,

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the peak in Cbp phosphorylation coincided with maximal Lyn activity (21). Whereas Cbp phosphorylation peaked between 5 and 15 min post-Epo stimulation, lower level phosphorylation could be detected for several hours, especially with the phospho-Cbp band shift (Fig. 3*B*). Lyn preassociated with Cbp prior to Epo stimulation, most probably via the SH3/polyproline interaction (Fig. 3*C*); however, following exposure to Epo, a 2–3-fold increase in Lyn/Cbp association occurred as Cbp became phosphorylated (Fig. 3*C*). These observations support the proposition that the SH2 domain of Lyn associates with phosphorylated Cbp.

In stark contrast, a J2E clone bearing a dominant-negative Lyn (J397) (21) displayed no phosphorylation of Cbp (Fig. 3*C*). As a consequence, markedly reduced Lyn/Cbp binding was observed shortly after Epo stimulation. Similarly, Cbp phosphorylation did not occur when splenic erythroblasts from *Lyn*$^{-/-}$ mice (26, 27) were treated with Epo (Fig. 3*D*). Together, these data demonstrate that Lyn is essential for the phosphorylation of Cbp immediately after Epo stimulation of immature red blood cells.

Cbp Recruits Csk/Ctk—To identify molecules that interact with phosphorylated Cbp and influence Lyn activity, a phosphotyrosine-specific yeast two-hybrid screen was conducted. Two negative regulators of intracellular signaling were identified in this screen, the inhibitory kinase Csk (45) and SOCS1, which suppresses signaling from cytokine receptors (15). Subsequent yeast two-hybrid analyses confirmed that the SH2 domain of Csk bound phosphorylated Cbp; moreover, phosphorylation of Cbp Tyr^{314} by Lyn was crucial for the interaction with Csk (Fig. 4*A*). Previous studies have revealed a key role of Cbp Tyr^{314} in recruiting inhibitory kinases to suppress Src and Fyn (30, 31).

Because our investigations focused primarily on the effects of Lyn/Cbp in erythroid cells, we examined whether Ctk (46), the closely related hemopoietic homologue of Csk, could also associate with Cbp. In the presence of Lyn, Ctk, and Cbp did interact in transiently transfected COS cells; furthermore, mutation of Tyr^{314} resulted in a significant decrease in Ctk/Cbp binding (Fig. 4*B*). An *in vitro* kinase assay was then employed to determine the impact of Cbp and Csk/Ctk on Lyn enzymatic activity. Fig. 4*C* shows that Lyn activity was suppressed substantially by Cbp recruiting endogenous Csk; however, this inhibitory effect was abrogated by the inability of Cbp[Y314F] to associate with Csk. Importantly, exogenous Ctk reduced Lyn kinase activity even further (Fig. 4*C*). Thus, Cbp plays a vital role in modulating the enzymatic activity of Lyn via inhibitory kinases Csk/Ctk.

The recruitment of Ctk was then assessed in erythroid cells. In J2E erythroid cells a 2–3-fold increase in Cbp/Ctk binding was detected immediately after Epo stimulation (Fig. 4*D*); this rise in Cbp/Ctk binding coincided with the peak of Cbp phosphorylation (Fig. 3, *A*–*C*). In contrast, the interaction between Cbp and Ctk was extremely weak in cells containing a dominant-negative Lyn (Fig. 4*D*), because of the failure to phosphorylate Cbp (Fig. 3*C*). Therefore, activated Lyn is required for Cbp to recruit Ctk.

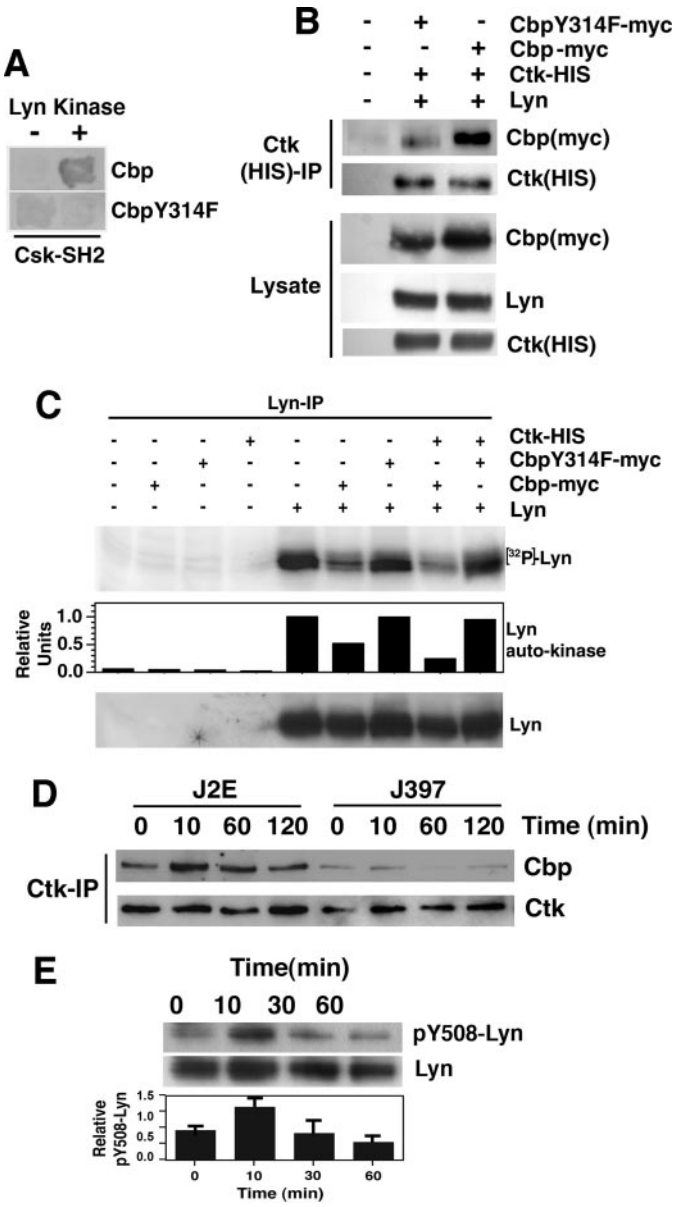

FIGURE 4. **Cbp interacts with Csk/Ctk.** *A*, phosphotyrosine-specific yeast two-hybrid assay was used to demonstrate the interaction of Cbp and Cbp[Y314F] with the SH2 region of Csk in the presence, or absence, of the Lyn kinase domain. *B*, immunoblot analysis of Ctk immunoprecipitates (*IP*) from COS cells transfected with Lyn, Cbp, Cbp[Y314F], and Ctk. Blots were probed with anti-Lyn, anti-His, and anti-Myc antibodies. *C*, Lyn autokinase assays were performed on Lyn immunoprecipitates from COS cells transfected with Lyn, Cbp, Cbp[Y314F], and Ctk. ^{32}P incorporation is expressed relative to Lyn protein levels determined by immunoblotting with anti-Lyn antibodies. *D*, J2E and J397 erythroid cells were serum-starved for 2 h before Epo stimulation, and then lysates were prepared at various time points before immunoprecipitation with anti-Ctk antibodies. Immunoblots were probed with anti-Cbp and anti-Ctk antibodies. *E*, J2E cells were serum-starved for 2 h before Epo stimulation, then lysates were prepared at various time points. Blots were probed with anti-phospho-Lyn(Tyr^{508}) and anti-Lyn antibodies. Relative phospho-Tyr^{508} levels were calculated using Quantity One. The mean and S.D. of triplicate experiments is shown. Representative blots of experiments performed in triplicate are shown.

As the activity of Lyn is down-regulated by phosphorylation of Tyr^{508} (45), immunoblotting was used to monitor the phosphorylation status of Lyn. Increased phosphorylation of Lyn Tyr^{508} was detected shortly after Epo stimulation (Fig. 4*E*). This corresponded with increased recruitment of Ctk to

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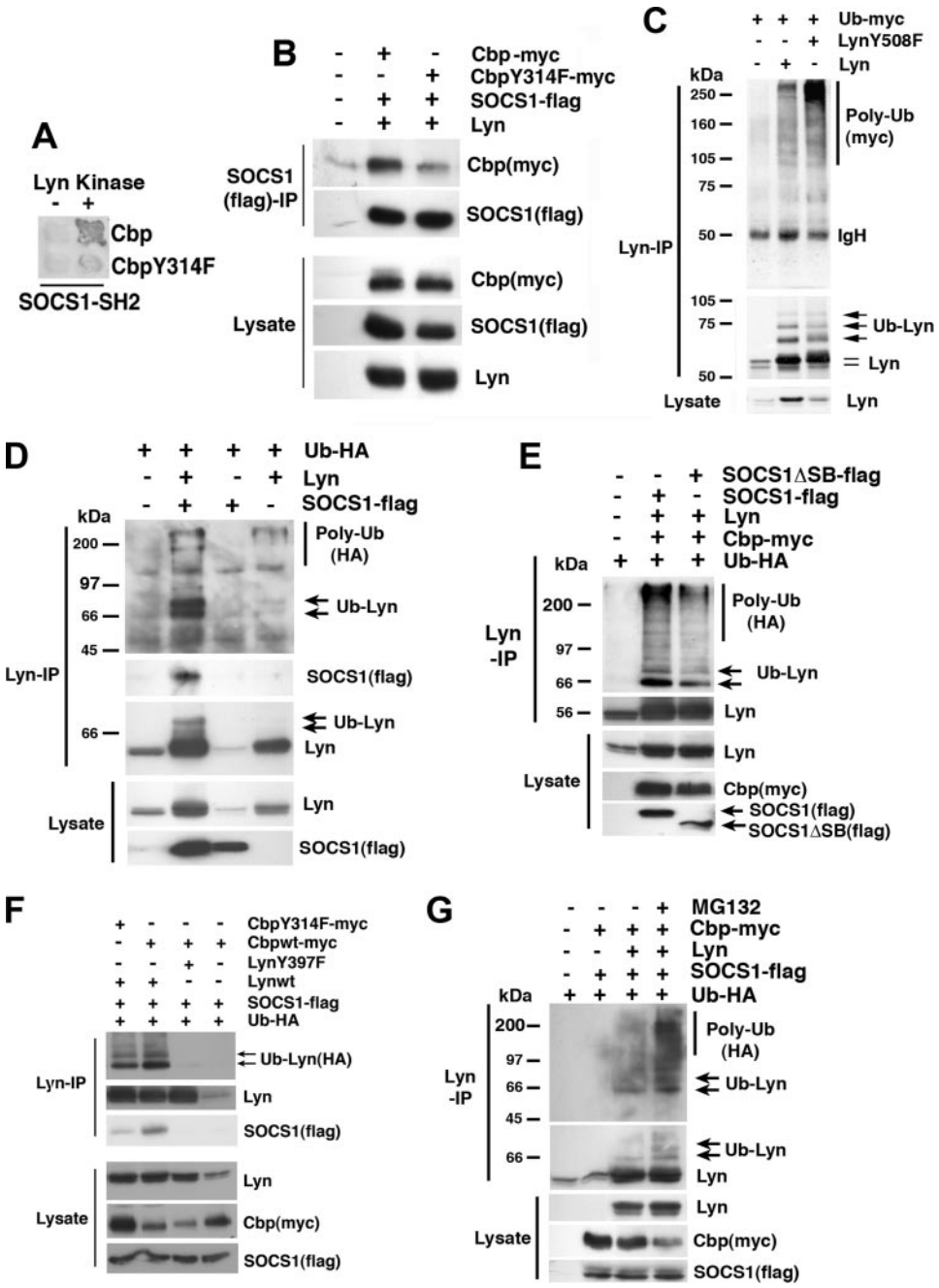

FIGURE 5. **Cbp interacts with SOCS1.** *A*, phosphotyrosine-specific yeast two-hybrid assay was used to demonstrate the interaction of Cbp and Cbp[Y314F] with the SH2 region of SOCS1 in the presence or absence of the Lyn kinase domain. *B*, immunoblot analysis of SOCS1 immunoprecipitates (*IP*) from COS cells transfected with Lyn, Cbp, Cbp[Y314F], and SOCS1. Blots were probed with anti-Lyn, anti-FLAG, and anti-Myc antibodies. *C*, cells were transfected with Lyn, Lyn[Y508F], and ubiquitin (*Ub-myc*) and immunoprecipitated with anti-Lyn antibodies. Blots were probed with anti-Myc and anti-Lyn antibodies. *D*, COS cells were transfected with Lyn, SOCS1, and ubiquitin (*Ub-HA*) and lysates immunoprecipitated with anti-Lyn antibodies. Blots were probed with anti-HA, anti-Lyn, and anti-FLAG antibodies. *E*, COS cells were transfected with Lyn, Cbp, SOCS1, SOCS1ΔSB, and ubiquitin (*Ub-HA*), the lysates were immunoprecipitated with anti-Lyn antibodies. Blots were probed with anti-HA, anti-Lyn, anti-Myc, and anti-FLAG antibodies. *F*, COS cells were transfected with Lyn, Lyn[Y397F], SOCS1, Cbp, Cbp[Y314F], and ubiquitin (*Ub-HA*), the lysates were immunoprecipitated with anti-Lyn antibodies. Blots were probed with anti-HA, anti-Lyn, anti-Myc, and anti-FLAG antibodies. *G*, MG132 (40 μM) was added 12 h prior to lysis of COS cells transfected with Lyn, Cbp, SOCS1, and ubiquitin (*Ub-HA*). Lyn was immunoprecipitated from lysates using anti-Lyn antibodies and blots were probed with anti-HA, anti-Lyn, anti-FLAG, and anti-Myc antibodies. Representative blots of experiments performed in triplicate are shown.

Cbp (Fig. 4*D*) and decreasing Lyn activity (21). The conclusion drawn from these results is that activated Lyn phosphorylates Cbp, which in turn promotes association with Ctk; this leads to phosphorylation of Tyr^{508} and down-regulation of Lyn activity.

Cbp Recruits SOCS1—The SH2 domain of SOCS1 also bound Cbp in a phosphotyrosine-specific manner (Fig. 5*A*). This interaction occurred via Tyr^{314}, indicating that SOCS1 was recruited to Cbp by the same phosphorylated tyrosine residue as Csk/Ctk (Fig. 4*A*). In transiently transfected COS cells, SOCS1 co-immunoprecipitated with wild-type Cbp; however, an appreciable reduction in binding was detected with Cbp mutated on Tyr^{314} (Fig. 5*B*). There was no appreciable co-immunoprecipitation from cells transfected with these components singly. These data demonstrate that Cbp attracts two recognized negative regulators of cell signaling viz Csk/Ctk and SOCS1.

Because SOCS1 is involved in ubiquitination and proteasomal degradation of signaling molecules (19), we speculated it may ubiquinate Lyn. Fig. 5*C* shows that Lyn could indeed be ubiquitinated, and increased polyubiquitination was observed with dominant active Lyn (Lyn[Y508F]). Significantly, ubiquitination of Lyn was elevated in the presence of exogenous SOCS1 (Fig. 5*D*). However, deletion of the SOCS box (ΔSB), which mediates ubiquitination of associated proteins (19), reduced the levels of ubiquitinated Lyn considerably (Fig. 5*E*).

The role of Cbp Tyr^{314} in recruiting SOCS1, and the effect on Lyn ubiquitination, were then evaluated. Data presented in Fig. 5*F* show that the Y314F Cbp mutant recruited SOCS1 poorly and induced less ubiquitination of Lyn than wild-type Cbp. Importantly, dominant-negative Lyn (Lyn[Y397F]), which does not phosphorylate Cbp (Figs. 2*C* and 3*C*), failed in the recruitment of SOCS1 and subsequent ubiquitination of Lyn (Fig. 5*F*). As ubiquitinated proteins are generally destroyed in proteasomes, the large accumulation of polyubiquitinated Lyn in the presence of the inhibitor MG132 indicates that proteasomes are involved in the breakdown of Lyn (Fig.

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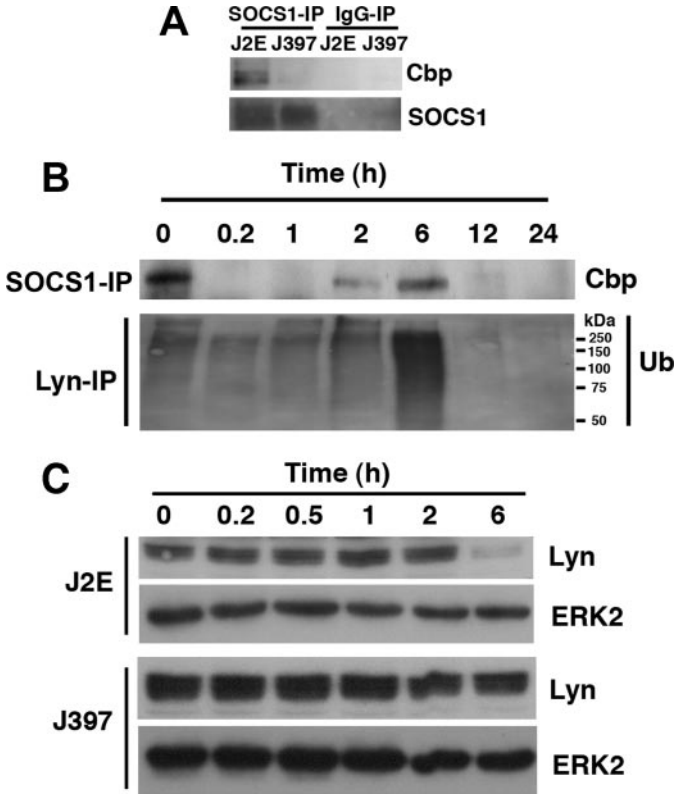

FIGURE 6. **Dynamics of Lyn, Cbp and SOCS1 interaction in erythroid cells.** *A*, J2E and J397 erythroid cells were lysed and immunoprecipitated with anti-SOCS1 or IgG control antibodies; blots were then probed with anti-Cbp and anti-SOCS1 antibodies. *B*, J2E cells were serum-starved for 2 h before Epo stimulation, then lysed and immunoprecipitated (*IP*) with anti-SOCS1 and anti-Cbp antibodies at the time points indicated. Blots were probed with anti-Cbp antibodies. J2E cells were serum-starved for 2 h before Epo stimulation, then lysed and immunoprecipitated with anti-Lyn antibodies at the time points indicated. Blots were probed with anti-ubiquitin (*Ub*) antibodies. *C*, J2E and J397 cells were serum-starved for 2 h, then induced with Epo before lysates were prepared at the indicated time points. Blots were probed with anti-Lyn and anti-ERK2 (loading control) antibodies. Representative blots of experiments performed in triplicate are shown.

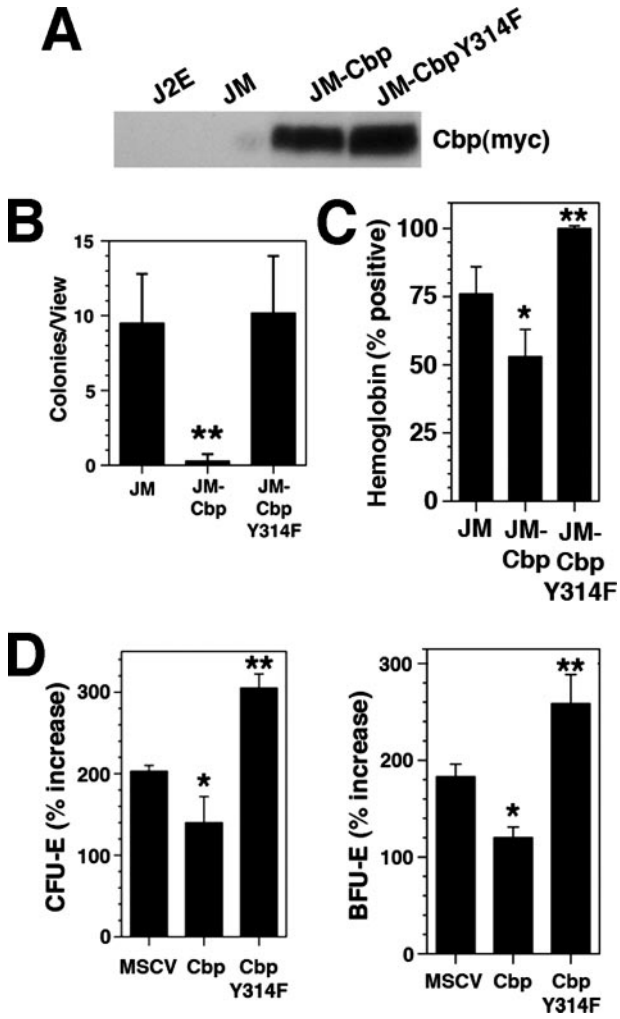

FIGURE 7. **Cbp inhibits Epo-induced differentiation.** *A*, J2E cells were infected with retroviruses expressing Cbp (*JM-Cbp*), Cbp[Y314F] (*JM-CbpY314F*), or vector only (*JM*). Lysates were immunoblotted for expression of exogenous Cbp with anti-Myc antibodies. *B*, cell lines were analyzed for colony formation in methylcellulose. Colony forming efficiency is expressed as colonies per field of view. Representative blots of experiments performed in triplicate are shown. *C*, cell lines were analyzed for Epo-induced hemoglobin production by benzidine staining. *D*, erythroid colony assays using methylcellulose for fetal liver CFU-E and BFU-E infected with retroviruses expressing Cbp, Cbp[Y314F] or vector only (*MSCV*). The percentage increase of Epo-responsive benzidine positive colonies (CFU-E and BFU-E) is shown. The mean ± S.D. is presented ($n = 3$). Statistically significant (two-way ANOVA) values are indicated by *, $p \leq 0.05$ and **, $p \leq 0.01$.

5*G*). It was concluded from these experiments that Cbp phosphorylated on Tyr^{314} by Lyn, recruits SOCS1, which then promotes ubiquitination and degradation of Lyn in proteasomes.

Cbp and SOCS1 in Erythroid Cells—The association between Cbp and SOCS1 was then examined in erythroid cells. Notably, Cbp and SOCS1 form a complex within J2E cells expressing wild-type Lyn, but not cells bearing a dominant-negative Lyn (Fig. 6*A*). The co-immunoprecipitation of SOCS1 and Cbp in uninduced J2E cells is caused by phosphotyrosine-independent mechanisms involving the proline-rich region of SOCS1 (data not shown). However, this association was disrupted immediately after Epo activation; reassociation between Cbp and SOCS1 then occurred 2–6 h later (Fig. 6*B*) when SOCS1 levels were at their highest (Ref. 17, data not shown). Unlike the association between Cbp and Ctk (Fig. 4*D*), binding to SOCS1 occurred when Cbp phosphorylation was less pronounced (Fig. 3*B*).

The impact of Cbp/SOCS1 association on Lyn ubiquitination was then analyzed. Importantly, the maximum interaction between Cbp and SOCS1 at 6 h (Fig. 6*B*) coincided with the peak in Lyn ubiquitination (Fig. 6*B*). The marked decrease in Lyn content detected 6h after Epo treatment (Fig. 6*C*) is, therefore, consistent with the notion that ubiquitination of Lyn leads to degradation (Fig. 5*G*). It is noteworthy that degradation of Lyn was not evident in cells expressing a dominant-negative Lyn (Fig. 6*C*).

Ectopic Cbp Expression in Erythroid Cells—The accumulated data indicated that Cbp played a crucial role in controlling the activity, and levels, of Lyn. To determine the impact of Cbp on erythroid differentiation, J2E cells were stably transfected with wild-type Cbp and Cbp[Y314F] (Fig. 7*A*). Although the morphology of the transfected cells remained pro-erythroblastoid (data not shown), J2E cells expressing wild-type Cbp had significantly reduced colony forming ability (Fig. 7*B*). Enforced expression of Cbp also reduced Epo-induced hemoglobin synthesis; conversely, differentiation was enhanced in cells expressing Cbp[Y314F] (Fig. 7*C*). In addition, Cbp suppressed

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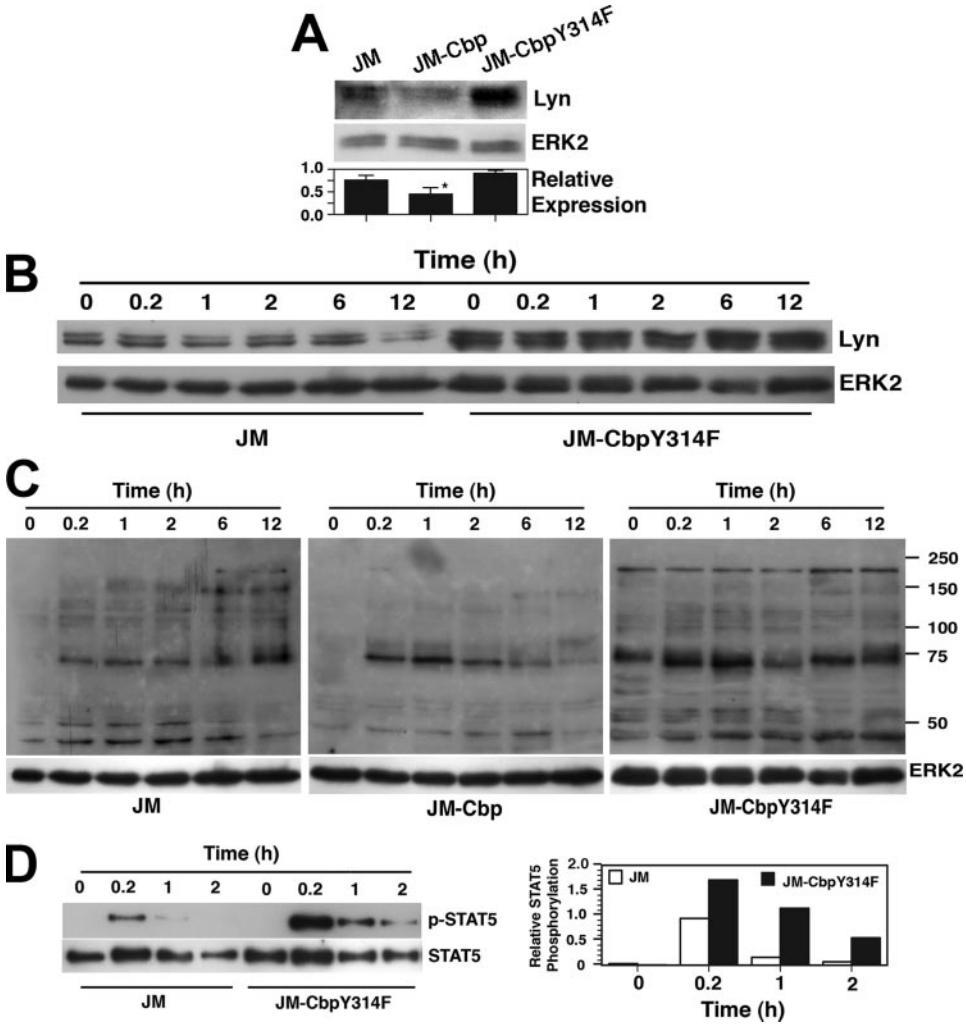

FIGURE 8. **Cbp regulates Epo-induced signaling.** *A*, lysates of cell lines (JM, JM-Cbp, JM-Cbp[Y314F]) were immunoblotted with anti-Lyn and anti-ERK2 (loading control) antibodies. Densitometric analysis of Lyn expression relative to ERK2 levels is displayed below. The mean ± S.D. is presented ($n = 3$). Statistically significant (two-way ANOVA) values are indicated by *, $p \leq 0.05$. *B*, serum-starved JM and JM-Cbp[Y314F] cells were stimulated with Epo, then lysates were prepared at the indicated time points. Blots were probed with anti-Lyn and anti-ERK2 antibodies. *C*, immunoblots of cell lysates generated after Epo stimulation of JM, JM-Cbp, and JM-Cbp[Y314F] cells were probed with anti-phosphotyrosine (*PY99*) and anti-ERK2 antibodies. *D*, serum-starved JM and JM-Cbp[Y314F] cells were stimulated with Epo, then lysates were prepared at the indicated time points and immunoprecipitated with anti-STAT5 antibodies. Immunoblots were probed with anti-phosphotyrosine (*PY99*) and anti-STAT5 antibodies. Relative STAT5 phosphorylation was quantitated using Quantity One (Bio-Rad). Representative blots of experiments performed in triplicate are shown.

Epo-induced expansion of normal erythroid progenitors, *i.e.* burst forming units-erythroid (BFU-E) and colony forming units erythroid (CFU-E); in contrast, Cbp[Y314F] increased the responsiveness of these progenitors to Epo (Fig. 7*D*).

The effects of Cbp and Cbp[Y314F] on Lyn content were quite profound (Fig. 8*A*). Lyn levels decreased in the presence of elevated Cbp, mirroring the inverse relationship between Lyn and Cbp in COS cells (Fig. 2*D*), whereas Cbp[Y314F] raised Lyn content. Significantly, the decline in Lyn protein 6–12-h post Epo-induction did not occur in cells expressing Cbp[Y314F] (Fig. 8*B*). These results emphasize that appropriate Lyn levels are essential for the differentiation of immature red blood cells, and demonstrate the importance of Cbp in maintaining Lyn content.

By altering Lyn levels, Cbp and Cbp[Y314F] also affected intracellular signaling events. The phosphorylation of numerous proteins after Epo stimulation was markedly reduced in cells expressing exogenous Cbp (Fig. 8*C*), just like cells lacking Lyn (22). In contrast, increased phosphorylation was detected in cells containing Cbp[Y314F], even prior to Epo-stimulation. Whereas no alterations to phosphorylation of the Epo receptor or JAK2 were detected in cells overexpressing Cbp or Cbp[Y314F] (data not shown), a significant increase in STAT5 phosphorylation was observed in Cbp[Y314F] cells (Fig. 8*D*). This observation is compatible with the proposed role of Lyn in the phosphorylation of STAT5 in erythroid cells (23).

DISCUSSION

In this article we have demonstrated that the activity and levels of Lyn in erythroid cells are influenced by Cbp. Lyn activity peaks shortly after Epo stimulation (21), and this is followed by a series of exquisitely orchestrated events that initially reduce the kinase activity of Lyn, then remove the protein. Cbp integrates these distinct phases of enzymatic inactivation and subsequent degradation via phosphorylated Tyr^{314}. In unstimulated erythroid cells Lyn and Cbp pre-associate via an SH3/polyproline interaction (Fig. 9*A*). An immediate consequence of Epo stimulation is a rise in Lyn kinase activity (21), which also results in the phosphorylation of Cbp on several tyrosine residues, including Tyr^{314}, Tyr^{381}, and Tyr^{409}. Phosphorylation of Tyr^{381} and Tyr^{409} enables closer association between Lyn and Cbp through the SH2 domain of Lyn. On the other hand, phosphorylated Tyr^{314} enhances recruitment of the negative regulatory kinases Csk/Ctk to the complex, which then phosphorylate Tyr^{508} of Lyn to inactivate the enzyme. These events occur within the first 10–30 min of Epo stimulation, and lead to the rapid reduction in Lyn kinase activity.

In contrast with the acute enzymatic inactivation of Lyn, the next phase of Lyn down-regulation occurs over several hours (Fig. 9*B*). Epo signaling via the JAK/STAT and Lyn pathways induces transcription and synthesis of SOCS proteins hours later. SOCS1 then associates with phosphorylated Cbp, and promotes ubiquitination of Lyn, which ultimately leads to pro-

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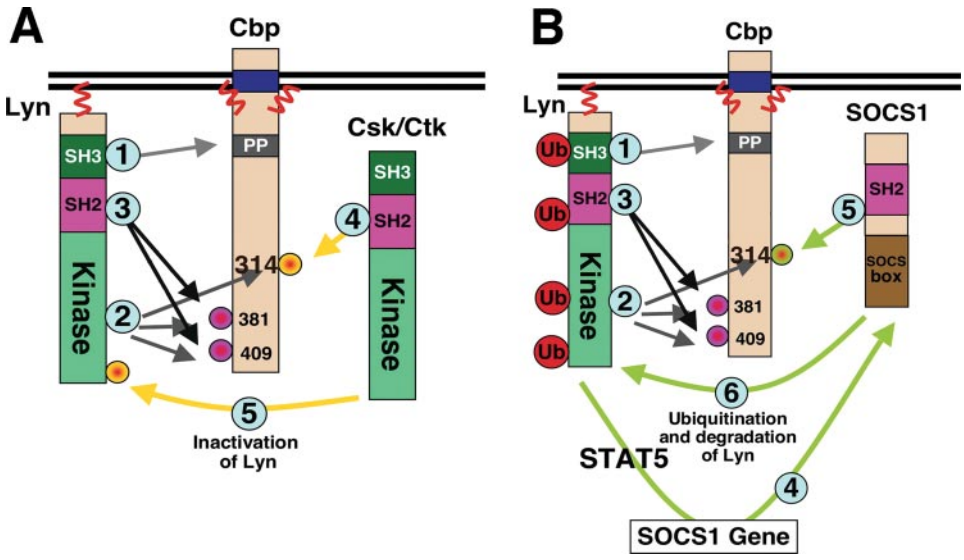

FIGURE 9. **Two-step model for Lyn inactivation and degradation.** *A*, model of Cbp-mediated down-regulation of Lyn via Ctk/Csk, which reduces kinase activity within minutes of exposure to Epo. *1*, the SH3 domain of Lyn preassociates with the polyproline (*PP*) region of Cbp. *2*, after Epo stimulation Lyn phosphorylates Cbp on multiple residues. *3*, the SH2 domain of Lyn specifically binds Tyr^{381}/Tyr^{409} of Cbp. *4*, the SH2 domain of Ctk/Csk is recruited to phosphorylated Tyr^{314} of Cbp. *5*, Csk/Ctk phosphorylate Tyr^{508} of Lyn inducing a decrease in Lyn kinase activity. *B*, model of Cbp-mediated SOCS1 ubiquitination of Lyn, which decreases protein levels hours later. *1–3*, as for *A*). *4*, Lyn kinase activates STAT5 increasing SOCS1 transcription. *5*, the SH2 domain of SOCS1 is recruited to phosphorylated Tyr^{314} of Cbp. *6*, SOCS1 mediates polyubiquitination and proteasomal degradation of Lyn. Representative blots of experiments performed in triplicate are shown.

teasomal degradation of the tyrosine kinase. Thus, Cbp plays a pivotal role in this two-step process that functionally inactivates the enzyme, then degrades the protein.

The data presented here detail how Lyn, like Src and Fyn, is negatively regulated by Cbp (30, 31, 47, 48). The involvement of Cbp in reducing Lyn kinase activity after Epo stimulation of immature erythroid cells, complements studies showing inactivation of Src and Fyn in other cell types (47–49). The crucial role of recruiting Csk/Ctk by Cbp Tyr^{314} was also demonstrated in erythroid cells. Interestingly, Ctk has been previously implicated in the negative regulation of Lyn in platelets (50).

Activation of the FcγRI in mast cells, results in increased phosphorylation of Cbp, an event initiated primarily by Lyn (47, 51). Similarly, we show here that ligand binding to the Epo receptor resulted in a Lyn-mediated phosphorylation of Cbp. It is noteworthy that the cascade of events involving Cbp, Csk/Ctk and SOCS1 did not occur in erythroid cells expressing a dominant-negative Lyn.

Significantly, overexpression of Cbp suppressed erythroid maturation; conversely, expression of a mutated Cbp (Cbp[Y314F]), which is incapable of recruiting negative regulators Csk/Ctk and SOCS1 to Lyn, resulted in increased Epo-induced differentiation. Altering the levels of Cbp, or mutating Cbp, had a marked effect on Lyn levels and intracellular signaling, which correlated closely with the differentiation capacity of the cells. These observations are consistent with an important role for Lyn in erythroid differentiation (21, 22), and the need for precise activation and down-regulation of the tyrosine kinase following Epo stimulation. While neither $Lyn^{-/-}$ nor $Cbp^{-/-}$ (52) mice display overt erythroid defects; it will be interesting to determine whether $Cbp^{-/-}$ mice develop erythropoietic problems when they are aged or stressed.

Ubiquitination of Lyn has been described previously (53, 54). This study defines a novel role for SOCS1 in the ubiquitination of Lyn which may complement the effects of Cbl (54). Our data indicate that activated Lyn induces SOCS1 as part of a feedback loop to regulate Lyn levels. The reduction in total Lyn protein in mice expressing dominant active Lyn (55) is consistent with this model. The observations described in this manuscript demonstrate that the range of kinases targeted by SOCS proteins extend beyond the Janus kinases, to include Lyn as well. It is conceivable that SOCS family members could also be involved in the ubiquitination and degradation of other Src kinase members (53, 56). Another kinase, focal adhesion kinase, has also been shown to be negatively regulated by SOCS proteins (57).

In summary, our model is consistent with a significant role for Lyn in erythroid differentiation, and the need for precise activation and down-regulation of the tyrosine kinase following Epo stimulation (21–23, 26, 27, 34). Importantly, the absence of Lyn, or prolonged activation of Lyn, have deleterious consequences for the hemopoietic compartment *in vivo* (26, 27, 55). The data presented here add to the understanding of the intricate mechanisms involved in the control of Src family kinases.

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Csk-binding Protein Mediates Sequential Enzymatic Down-regulation and Degradation of Lyn in Erythropoietin-stimulated Cells

Evan Ingley, Jessica R. Schneider, Christine J. Payne, David J. McCarthy, Kenneth W. Harder, Margaret L. Hibbs and S. Peter Klinken

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