Biochemical and Biophysical Research Communications **257,** 129–138 (1999)
Article ID bbrc.1999.0427, available online at http://www.idealibrary.com on IDEAL®

The Proto-oncogene p120^{Cbl} Is a Downstream Substrate of the Hck Protein-Tyrosine Kinase

Christopher J. Howlett, Sabine A. Bisson, Mary E. Resek, Allan W. Tigley, and Stephen M. Robbins[1]

Cancer Biology and Immunology Research Groups, Department of Oncology and Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta, Canada

Received February 12, 1999

Hematopoietic cell kinase (Hck) is a member of the Src-family of protein tyrosine kinases. We have found that upon enzymatic activation of Hck by the heavy metal mercuric chloride, there was a rapid increase in the levels of tyrosine phosphorylation of several proteins including the proto-oncogene p120^{Cbl}. Fibroblasts that are transformed with an activated allele of Hck exhibit constitutive Cbl phosphorylation. Upon Fcγ receptor activation, a more physiologically relevant extracellular signal, Cbl is tyrosine phosphorylated and the Src-family selective inhibitor, PP1, can prevent this phosphorylation on Cbl. Hck phosphorylates Cbl *in vitro* and the interaction between Cbl and Hck is direct, requiring Hck's unique, SH3 and SH2 domains for optimal binding. Using a novel estrogen-regulated chimera of Hck we have shown a hormone-dependent association between Hck and Cbl in murine fibroblasts. This work suggests that Cbl serves as a key mediator of Hck induced signalling in hematopoietic cells.

Hck is one member of the Src-family of protein tyrosine kinases (PTKs) that mediate the transduction of a number of different extracellular signals to elicit a wide variety of physiological responses including cellular proliferation and differentiation [1]. The Src gene family encode proteins with a number of characteristic structural features, including a highly conserved catalytic domain occupying the carboxy-terminal half of the proteins; SH3 and SH2 domains which permit the interaction with specific proteins; and an amino-terminal sequence of ca. 70 amino acids that is different in each member of the family [1].

[1] To whom correspondence should be addressed at Department of Oncology and Department of Biochemistry and Molecular Biology, University of Calgary, 3330 Hospital Drive N.W., Calgary, Alberta, Canada, T2N-4N1. Fax: 403-283-8727. E-mail: srobbins@acs.ucalgary.ca.

Hck encodes two isoforms, p59^{Hck} and p61^{Hck} that are derived from alternative initations of translation [2, 3]. Hck is principally expressed in mature myelomonocytic cells and B-lymphocytes [4–7]. Recent evidence suggests that Hck functions in mature leukocytes to communicate signals from various cytokines and Fcγ receptors [8–14]. However, the cellular targets for Hck remain largely unknown.

The c-Cbl proto-oncogene was originally discovered as the transforming gene of the murine Cas NS-1 retrovirus that induces pre-B lymphomas and myelogenous leukemias in mice [15]. The transforming gene, *v-Cbl,* is a truncated version of c-Cbl which encodes the first 355 amino acids of the full length murine Cbl protein [16]. c-Cbl is not transforming when overexpressed [16]. However, when a specific 17 amino acid region is deleted, as discovered in the 70Z/3 pre-B cell lymphoma, Cbl becomes constitutively tyrosine phosphorylated and acquires the ability to transform cells [17].

The c-Cbl gene encodes a 120 kDa protein that is expressed in a number of hematopoietic cells. In addition to its ability to contribute to various hematopoietic malignacies when mutated, a role for Cbl in normal hematopoietic signalling was suggested by the recent findings that it becomes tyrosine phosphorylated in response to a diverse array of extracellular signals (for review see [18]). Although Cbl appears to lack any catalytic activity, it associates with many cytoplasmic molecules and has thus been referred to as a potential adaptor molecule. The proline-rich region of Cbl as well as its phosphorylation on multiple tyrosine residues enables Cbl to interact with a wide variety of signalling proteins through their src-homology 3 (SH3) and src-homology 2 (SH2) domains, respectively (reviewed in [18]). The fact that Cbl becomes tyrosine phosphorylated in response to a wide array of extracellular signals and binds many signalling molecules suggests that it plays a critical function in signal transduction.

There is recent circumstantial evidence to suggest that the Src-family kinases may mediate the tyrosine

0006-291X/99 $30.00

phosphorylation of Cbl. In this paper we provide both *in vitro* and *in vivo* evidence that Cbl is a downstream target of the Src-family PTK Hck. Upon enzymatic activation of Hck either by pharmacological agents or genetic mutation, Cbl becomes tyrosine phosphorylated. We have shown, using glutathione fusion proteins encompassing various domains of Hck, that the interaction with Cbl is direct and requires the unique, SH3 and SH2 domains of Hck for optimal binding to Cbl. In addition, using a novel estrogen-inducible allele of Hck we have observed a regulated physical association between Hck and Cbl in murine fibroblasts. Thus it appears that Cbl is one of the downstream effectors for Hck.

EXPERIMENTAL PROCEDURES

Construction of various Hck cDNAs and virus production. A cDNA encoding the 59 kDa isoform of Hck [3] was used as a template to mutate the TAA stop codon to a TAC by PCR mutagenesis. An EcoR1 site was included in the primer such that it could be ligated to an EcoR1-Cla1 fragment of HE14 cDNA encoding the hormone-binding region of the human estrogen receptor (ER) [19] as described [20], generating p59HckER. To generate a constitutive allele of Hck, the negative regulatory tyrosine residue at amino acid 501 was mutated to Phe (p59Hck^{Y501F}). The p59Hck^{Y501F} and p59HckER alleles were subcloned into LNCX (kind gift of Dr. A.D. Miller) [21] as described previously using the unique Xho1 and Cla1 enzyme sites to excise the various cDNAs [3]. Replication-defective helper-free virus stocks of each cDNA construct were made and used to infect C7-3T3 cells (a subclone of NIH-3T3) to establish stably expressing cell lines as described previously [3].

Antibodies. Rabbit polyclonal antibodies to GST and the hormone binding region of the human estrogen receptor were made using purified GST or a glutathione fusion protein encompassing the hormone-binding region of the human estrogen receptor (amino acids 282-595), respectively. A Cbl monoclonal antibody (7G10) was made using a glutathione fusion protein encompassing amino acids 878-906 of human Cbl (7G10 available through Upstate Biotechnology). This region of the protein is identical between human and mouse and by Western blotting and immunoprecipitation has been shown to recognize Cbl specifically from both species. A Cbl polyclonal antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and the phosphotyrosine antibody 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY).

Cell culture. U937 cells, THP-1 cells and JCaM1 cells (kindly provided by Dr. A. Weiss, U.C.S.F.) were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL, Canadian Life Technologies, Burlington, Ont), 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol. To induce differentiation of U937 cells to the more mature macrophage-like phenotype, cells were suspended in fresh medium containing 12-*O*-tetradecanoylphorbol-13-acetate (TPA) at a concentration of 20 ng/ml, and incubated for 48 to 72 h.

C7-3T3 fibroblast cells expressing the 59 kDa isoform of Hck (p59Hck) [3] or the activated allele p59Hck^{Y501F} were maintained in Dulbecco's modified Eagle minimal medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO-BRL, Canadian Life Technologies, Burlington, Ont) and 600 μg/ml G418 (GIBCO-BRL, Canadian Life Technologies, Burlington, Ont). Murine fibroblasts (C7-3T3) expressing the p59HckER chimera were maintained in phenol-red free DMEM containing 5% cosmic calf serum (HyClone, Logan, UT) and 600 μg/ml G418.

Cell stimulations and preparations of lysates. Differentiated U937 cells were washed once with PBS (Phosphate-buffered saline, pH 7.4), resuspended in RPMI 1640 supplemented with 3% bovine serum albumin, and then incubated with 5 μg/ml human IgG on ice for 30 min. Cross-linking of Fcγ receptors was achieved using 5 μg/ml goat anti-human IgG F(ab')$_2$ (Pierce; Rockford, IL) and incubating at 37°C for the indicated times. Cells were then centrifuged and lysed on ice in Nonidet P-40 (NP-40) lysis buffer, composed of 50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM ethylene glycol-bis(b-aminoethyl ether)-*N'*,*N'*,*N'*,*N'*-tetraacetic acid (EGTA), 100 mM NaF, 10% glycerol, 1% NP-40 (protein grade; Calbiochem, La Jolla, CA), 1 mM PMSF (Boehringer Mannheim, Laval, PQ), 1 mM sodium orthovanadate, and 10 μg/ml each of aprotinin and leupeptin. Insoluble material was removed by centrifugation at 12,000 $\times$ *g* for 15 min at 4°C.

Stimulation of THP-1 cells with $HgCl_2$ was performed as previously described (Robbins et al., manuscript in preparation). Briefly, cells were incubated in PBS containing 0.5 mM $HgCl_2$ for 2 minutes at room temperature, washed once in PBS and then lysed in NP-40 lysis buffer as described above.

For experiments using the tyrosine kinase inhibitor PP1 (Calbiochem, La Jolla, CA), cells were preincubated with 10 μM PP1 for 20 min at 37°C and then stimulated through the Fcγ receptor as described above.

For hormone stimulation of the murine fibroblasts containing either vector alone (LNCX) or HckER, cells were grown to confluency, then serum starved for 16 hours in DMEM containing 0.1% cosmic calf serum before the addition of estradiol (1 μM final concentration) (Sigma, St. Louis, MO) for the indicated times. The cells were lysed in NP-40 lysis buffer and incubated with the Cbl monoclonal antibody (7G10) for immunoprecipitation as described below.

Immunoprecipitations and Western blotting. Cell-free lysates from stimulated or unstimulated cells were incubated with an excess of either polyclonal Hck antiserum as described [3], 5 μg/ml of 4G10, 1 μg/ml of polyclonal Cbl antibody or 3 μg/ml of Cbl monoclonal antibody (7G10) for 1 hr at 4°C. Protein A-Sepharose (or Protein G-sepharose for 7G10) was added, and the mixtures were incubated for an additional 30 min. After the immune complexes were washed several times with NP-40 lysis buffer, the samples were boiled in 2 $\times$ SDS Laemmli's sample buffer. Samples were analyzed on a 10% SDS-polyacrylamide gel. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). Membranes were blocked by incubation in Tris-buffered saline (5 mM Tris, 135 mM NaCl, 5 mM KCl) containing 0.5% NP-40, 0.1% Tween 20 (TBS-TN), and either 5% nonfat dry milk (for non-phosphotyrosine blots) or 5% bovine serum albumin (for phosphotyrosine blots) for 20 min. Membranes were then incubated for 1 hr in either polyclonal Cbl antibody (1 μg/ml), polyclonal hbER antibody (1:2,000) or monoclonal anti-phosphotyrosine antibody 4G10 (1 μg/ml), all diluted in their respective blocking buffers. The membranes were washed extensively in TBS-TN before incubation for 20 min with either donkey anti-rabbit-horseradish peroxidase or sheep anti-mouse-horseradish peroxidase conjugate (Amersham, Arlington Heights, IL), each diluted 1:10,000 in blocking buffer. The membranes were washed as described above and developed using an enhanced chemiluminescence substrate (ECL: Amersham, Arlington Heights, IL).

In-vitro kinase reactions. Cbl immunoprecipitations from JCaM1 cells and Hck immunoprecipitations from 3T3 fibroblasts expressing p59Hck were performed as above except that before boiling in Laemmli's buffer the immune complexes were washed once in kinase assay buffer consisting of 50 mM Tris pH 7.4, 10 mM $MgCl_2$, and 10 mM $MnCl_2$. The washed immune complexes from both immunoprecipitations were either kept separate or mixed together in fresh kinase assay buffer containing 100 μM ATP. After incubation for 15 min at 22°C the samples were boiled in Laemmli's buffer and subjected to electrophoresis and Western blot analysis as described above.

GST fusion proteins, pulldown assays and far Western blotting. The following GST fusion proteins were constructed by cloning the appropriate PCR amplified fragment from the human Hck cDNA into the unique BamH1 and EcoR1 sites of the pGEX-2T vector (Pharmacia): GST-Hck unique (amino acids 1 to 86), GST-Hck SH3 (amino acids 87 to 137), GST-Hck SH2 (amino acids 138 to 249), GST-Hck SH3/SH2 (amino acids 87 to 249) and GST-Hck unique/SH3/SH2 (amino acids 1 to 249). Purification of the GST fusion proteins was performed essentially as described previously [22]. Briefly, bacteria expressing the various GST constructs were harvested 3–4 hours following IPTG induction, washed once with PBS and lysed in PBS containing 1% Triton X-100 and 1 mM PMSF and 10 μg/ml each of aprotinin and leupeptin. After incubation with Glutathione-agarose on a rocker at 4°C for 2 hours, the beads were washed 3 times and either resuspended to a 50% slurry in PBS containing protease inhibitors for 'pulldown assays', or the fusion proteins were eluted in 50 mM Tris-HCl pH 8.0 containing 10 mM reduced glutathione for 'Far-Westerns'.

GST pulldown assays were performed from 2×10^6 THP-1 cells lysed in 0.5 ml of 1% NP-40 lysis buffer. Purified fusion proteins (25 μg) bound to Glutathione S-transferase agarose beads were added to the cell-free extracts and agitated at 4°C for 2 h. The beads were then washed 3 times with NP-40 lysis buffer and the protein complexes were removed from the beads by boiling directly in $2 \times$ SDS sample buffer and further analysed by Western blotting. For the GST pulldown assays from Fcγ receptor stimulated THP-1 cells, the cells were stimulated as described above and incubated with purified GST-Hck unique/SH3/SH2 containing agarose beads as described previously.

Immunoprecipitates and Western blotting for Far Western analysis was performed as described above. The blots were blocked in Tris-buffered saline and 0.05% Tween 20 (TBS-T) containing 3% BSA, then incubated with 5 μg/ml of purified GST or 5 μg/ml of the purified GST-Hck-unique/SH3/SH2 fusion protein diluted in TBS-T containing 1% BSA for 2 h. The membranes were rinsed twice with TBS-T and the fusion protein was detected by incubating the membranes with a mixture of anti-GST polyclonal antibody (1:1,000) and anti rabbit-horseradish peroxidase conjugate (1:10,000) in TBS-T/1% BSA for one h. The membranes were washed in TBS-T and developed with ECL as described above.

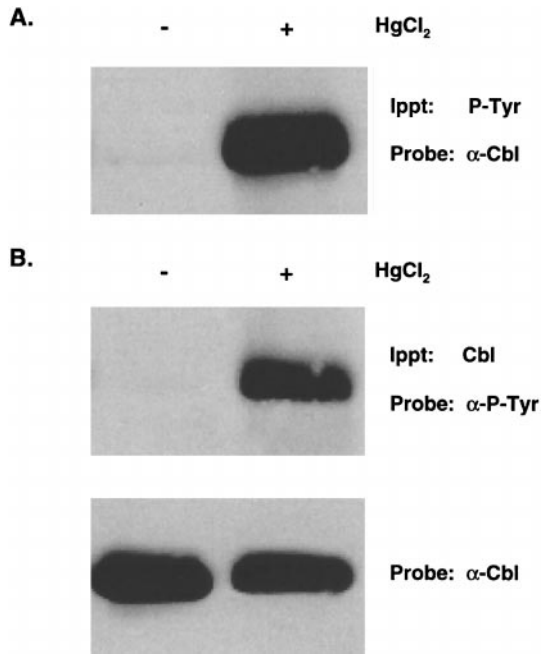

FIG. 1. $HgCl_2$ treatment of human myelomonocytic cells results in the tyrosine phosphorylation of Cbl. THP-1 cells were treated with 0.5 mM $HgCl_2$ for 2 minutes and then lysed in 1% NP-40 lysis buffer. The cell lysates from $HgCl_2$-stimulated (+) or unstimulated (−) cells were either (A) immunoprecipitated with the anti-phosphotyrosine antibody 4G10 and the resulting blot was probed with a Cbl polyclonal antibody or (B) immunoprecipitated with a monoclonal antibody to Cbl (7G10) and analyzed by Western blotting, first probing with an anti-phosphotyrosine monoclonal antibody (4G10) (upper panel) and then reprobing the blot with a Cbl polyclonal antibody (lower panel).

RESULTS

Mercuric chloride treatment of cells results in the tyrosine phosphorylation of Cbl. We and others have previously shown that exposure of various hematopoietic cell lines to the thiol-reactive heavy metal, mercuric chloride ($HgCl_2$) results in the rapid and robust tyrosine phosphorylation of many cellular proteins (Robbins et al., manuscript in preparation) [23–25]. Although the mechanism by which $HgCl_2$ induces intracellular signalling is unknown, exposure of the cells to $HgCl_2$ results in the activation of the Src-family kinases Hck (Robbins et al., manuscript in preparation) and Lck [23]. These results suggested that mercuric chloride treatment of hematopoietic cells could provide a biochemical tool for identifying downstream substrates of the Src-family kinases. After $HgCl_2$ treatment of the human promyelmonocytic cell lines U937 and THP-1, a major tyrosine phosphorylated protein was detected, migrating at a molecular weight of ca. 120 kilodaltons (kDa) (data not shown). The proto-oncogene $p120^{Cbl}$, herein referred to as Cbl, has been shown to become tyrosine phosphorylated upon stimulation by various agonists in hematopoietic cells. Based on these observations we decided to investigate whether the 120 kDa phosphoprotein detected from $HgCl_2$-treated cells was indeed Cbl. Antibodies to Cbl were used to probe a Western blot (Figure 1A) of mercuric chloride treated U937 cell lysates that had been immunobead purified with phosphotyrosine-specific antibodies. Immunoreactive Cbl protein was only detected in the phosphotyrosine purification from mercuric chloride treated lysates (Figure 1A, +), but not from untreated cells (Figure 1A, −), indicating that Cbl becomes tyrosine phosphorylated upon mercuric chloride stimulation. To eliminate the possibility that Cbl was detected by virtue of its co-immunoprecipitation with a tyrosine phosphorylated protein and was not itself phosphorylated, Cbl was specifically immunoprecipitated from the above described lysates and the immunoprecipitated protein was probed with antibodies specific for phosphotyrosine. As can be seen in Figure 1B (lower panel), Cbl was equally immunoprecipitated from both untreated and $HgCl_2$-treated lysates, but only in the treated lysates was Cbl tyrosine phosphorylated (Figure 1B, upper panel). Since $HgCl_2$ activates Src-family kinases, the above described results suggest that a Src-family kinase such as Hck, activated during

the $HgCl_2$ treatment, would be a likely candidate to phosphorylate Cbl *in vivo.*

An activated allele of Hck induces the tyrosine phosphorylation of Cbl in murine fibroblasts. To investigate if Cbl plays a role downstream of Hck, NIH 3T3 cells were infected with viruses that expressed either vector alone or an activated allele of Hck containing a tyrosine to phenylalanine change at amino acid 501, p59Hck^{Y501F}. As can be seen in the photographs in Figure 2 the cells containing the control vector exhibited a flat, non-transformed morphology, whereas cells that expressed the activated allele, p59Hck^{Y501F}, were refractile and exhibited many aspects of transformation including altered morphology, an ability to form foci, and anchorage-independent growth as assessed by the ability to grow in soft agar (data not shown). There was a concomitant increase in the number of intensely tyrosine phosphorylated proteins in the murine fibroblasts that expressed the activated Hck protein (Figure 2, top panel). Cbl was equally expressed in both the wild-type and p59Hck^{Y501F} fibroblasts (Figure 2, bottom panel) but only in cells that expressed activated Hck (p59Hck^{Y501F}) was Cbl recovered as an intensely tyrosine phosphorylated protein (Figure 2, middle panel). This result clearly indicates that Cbl is constitutively phosphorylated in fibroblasts containing activated Hck, and that the presence of constitutively tyrosine phosphorylated proteins, including Cbl, correlates with a transformed phenotype.

The Src-family selective inhibitor PP1 inhibits tyrosine phosphorylation of Cbl induced by Fcγ receptor cross-linking. We felt that a more physiologically relevant system was crucial to investigate the *in vivo* interactions between Hck and Cbl. Therefore, we utilized human IgG as a natural ligand to cross-link Fcγ receptors. Although few upstream activators of Hck have been identified, Fcγ antibody receptors have been shown to bind to, and activate Hck [9, 12, 14]. Additionally, the use of Fcγ receptor (FcγR) cross-linking antibodies has been shown to result in rapid induction of tyrosine phosphorylation on Cbl [26–28]. Experiments in our laboratory with the human promyelomonocytic cell lines, U937 and THP-1, using cross-linked human IgG for FcγR stimulation have also demonstrated that Cbl becomes rapidly tyrosine phosphorylated (Figure 3, lanes 1–5). In order to determine whether the high affinity IgG receptor (FcγRI), low affinity IgG receptor (FcγRII), or both were responsible for this induction we used specific antibodies to cross-link and activate both FcγRI and FcγRII independently. The activation of both of these receptors with specific antibodies resulted in the tyrosine phosphorylation of Cbl (data not shown), suggesting that the use of cross-linked human IgG was a suitable receptor-mediated *in vivo* cellular system for our studies on Cbl phosphorylation. In order to investigate the role of Hck in the process of Fcγ receptor-mediated tyrosine phosphorylation of Cbl, the Src-selective kinase inhibitor PP1 was utilized. The selectivity and potency of the

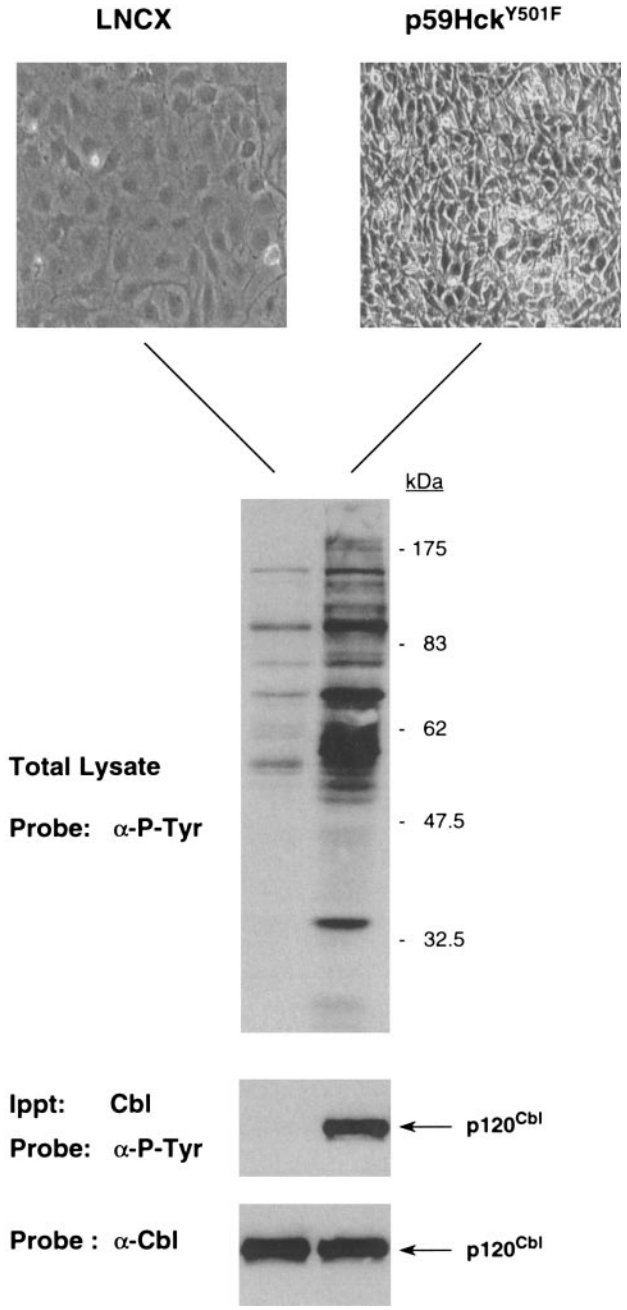

FIG. 2. A transforming allele of Hck induces the constitutive phosphorylation of Cbl. Pools of NIH-3T3 cells containing either empty vector (LNCX) or an activated allele of Hck, p59Hck^{Y501F} were made and photographs taken as indicated. Cell free lysates from the LNCX and p59Hck^{Y501F} containing cells as indicated were analyzed by Western blotting using the phosphotyrosine specific antibody 4G10 (upper blot). Immunoprecipitation of the total lysates was performed using a monoclonal antibody against Cbl (7G10). The corresponding Western blot was probed with either anti-phosphotyrosine (4G10) or anti-Cbl monoclonal (7G10) antibodies as indicated. The molecular weight standards are shown on the right.

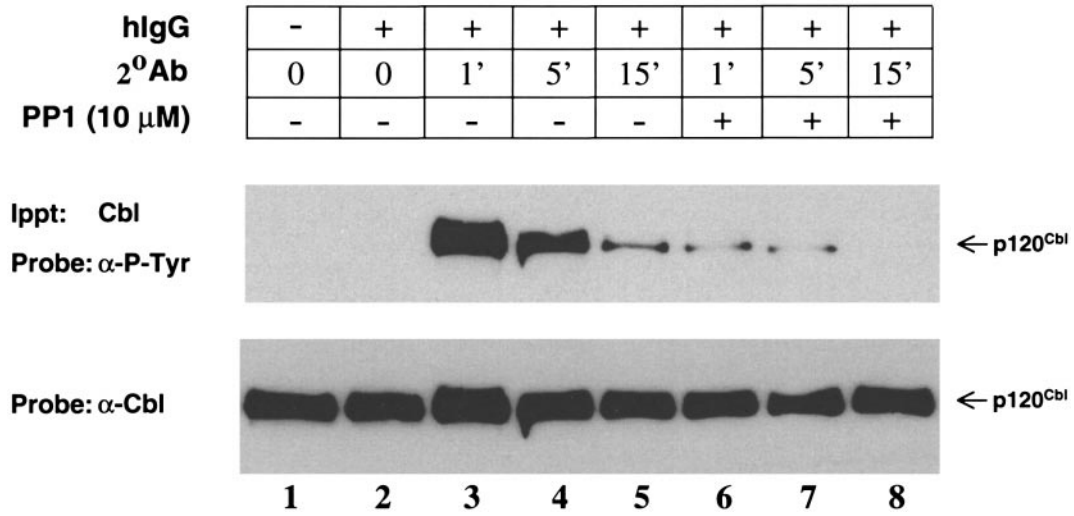

FIG. 3. PP1 inhibits tyrosine phosphorylation of Cbl induced by Fcγ receptor crosslinking. Differentiated U937 cells were washed in PBS and either incubated with (lanes 6–8) or without (lanes 1–5) 10 μM PP1 for 20 minutes. The cells (1×10^7/time point) were then incubated with hIgG for 30 minutes (+). Cross-linking of Fcγ receptors was performed for the indicated amount of time (min) using a goat anti-human $F(ab')_2$ antibody. Cells were lysed in 1% NP-40 lysis buffer and immunoprecipitations were performed using a monoclonal antibody to Cbl. Samples were separated by SDS–PAGE and the resulting Western blot was probed with anti-phosphotyrosine antibody (4G10) (upper panel) and then was reprobed with anti-Cbl polyclonal antibody (lower panel). The position of $p120^{Cbl}$ and the lane numbers are marked.

pyrazolopyrimidine for the Src-family of PTKs has been previously demonstrated [29]. When differentiated U937 cells were pre-incubated with PP1, the induction of tyrosine phosphorylation on Cbl induced by Fcγ receptor cross-linking was severely inhibited (Figure 3, lanes 6–8). The lower panel in Figure 3 indicates that approximately equivalent amounts of Cbl were immunoprecipitated from each sample. Although we have not been able to assess the specificity of PP1 for various tyrosine kinases, we have obtained effects on Cbl phosphorylation with the inhibitor well within the range (1–10 μM) where it has been shown to be selective for the Src-family kinases *in vivo.*

Hck can directly phosphorylate Cbl in vitro. In order to determine if Hck can phosphorylate Cbl *in vitro,* immunoprecipitation experiments using antibodies to Hck, Cbl, or both Hck and Cbl were performed for use in kinase assays. Cbl was immunoprecipitated from JCaM1 cells, and Hck was immunoprecipitated from 3T3 fibroblasts expressing wild-type Hck (p59Hck). JCaM1 cells were chosen because they lack a functional Lck protein and hence have a very low basal level of Cbl tyrosine phosphorylation (Howlett and Robbins, unpublished), which facilitated our analysis of these assays. In the presence of ATP Cbl became tyrosine phosphorylated only when the Cbl and Hck immunoprecipitations were mi×ed (Figure 4, lane 3). When Hck was not present Cbl remained unphosphorylated (Figure 4, lane 2). The Hck protein is also intensely phosphorylated (Figure 4, lanes 1 and 3) indicative of its autophosphorylation activity. The ability to phosphorylate Cbl was not unique to Hck since another Src-family kinase, Lyn, was also able to phosphorylate Cbl when co-immunoprecipitated in the presence of ATP (data not shown).

The interaction between Hck and Cbl is direct and exhibits phosphotyrosine-dependent binding characteristics. In order to define which domains of Hck were required for its interaction with Cbl we used Glutathi-

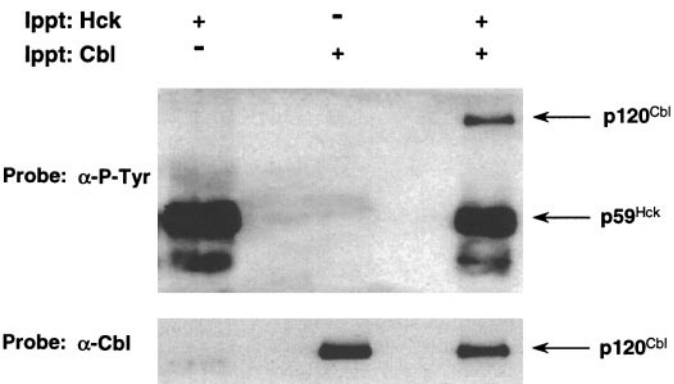

FIG. 4. Hck can phosphorylate Cbl *in vitro.* Lysate from 3T3 fibroblasts expressing p59Hck was immunoprecipitated with Hck-specific antisera (lane 1). Lysate from JCaM1 cells was immunoprecipitated with anti-Cbl antibody (lane 2). Lysates from both cell types were mixed and immunoprecipitated with both Hck and Cbl specific antisera (lane 3). Immunoprecipitated proteins were mixed with kinase assay buffer containing 100 μM ATP, incubated at 22°C for 15 min, followed by direct boiling in Laemmlis' buffer. The samples were analyzed by SDS–PAGE and the corresponding blot was probed with anti-phosphotyrosine (4G10) (top) and then reprobed with Cbl specific antibody (bottom). The positions of $p120^{Cbl}$ and p59Hck are labelled as such.

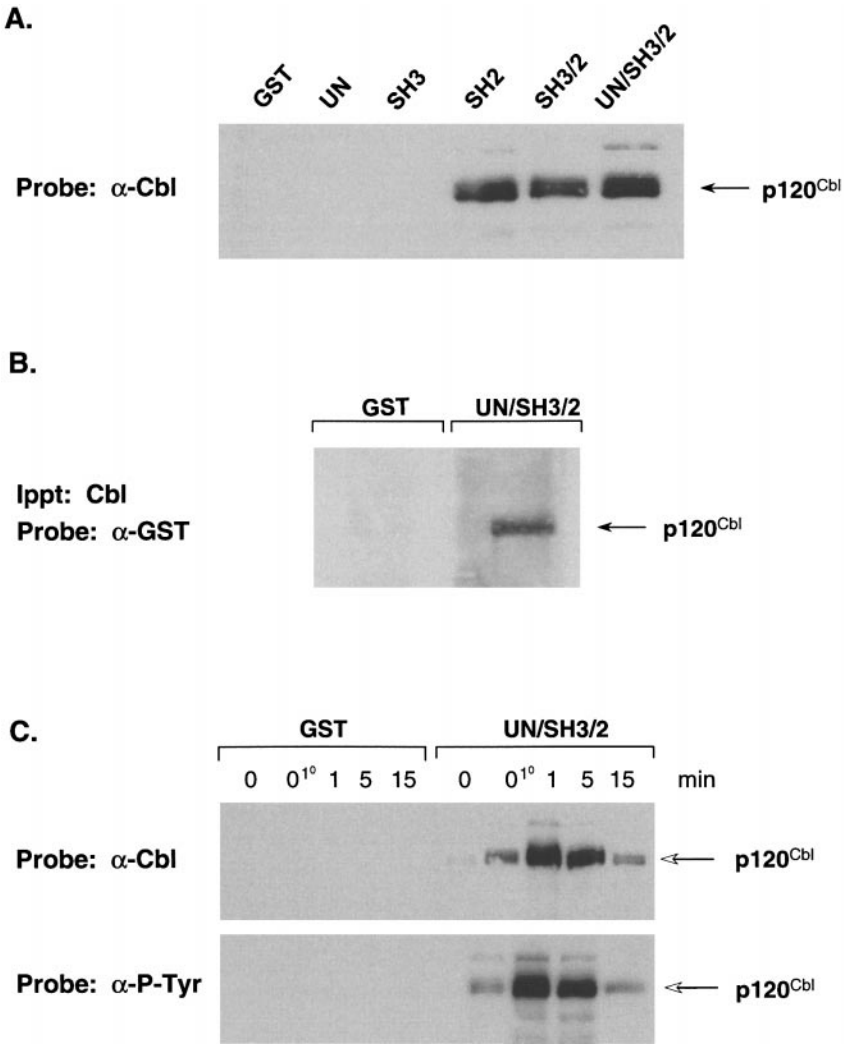

FIG. 5. Direct binding of Hck fusion proteins to Cbl is regulated in a phosphotyrosine-dependent fashion. For the 'Pulldown' experiment (A.) cell lysates were prepared from THP-1 cells (1×10^7 cells/assay) and incubated with either GST or various GST-Hck fusion proteins encompassing the unique (UN), SH3, SH2, both SH3 and SH2 (SH3/2) or the unique, SH3 and SH2 domains (UN/SH3/2) together coupled to Glutathione-agarose beads. Protein complexes were separated by SDS–PAGE and the resulting Western blot was probed with the Cbl monoclonal antibody (7G10). In the 'Far Western' analysis (B.) THP-1 extracts were immunoprecipitated with the Cbl monoclonal antibody and the resulting Western blot was probed directly with either purified GST or the GST fusion protein encompassing the unique, SH3 and SH2 domains of Hck. The bound proteins were detected by ECL after incubating the blot with a specific polyclonal antibody to GST coupled to a horseradish peroxidase conjugated sheep anti-rabbit secondary antibody. (C.) THP-1 cell lysates were prepared from 2×10^6 cells that were untreated (0), or cells that had been incubated with hIgG (O^{10}), and then cross-linked by the addition of goat anti-human $F(ab')_2$ antibody for the indicated times (min). The respective cell lysates were then incubated with 25 μg of Glutathione-agarose beads bound to GST or GST-Hck unique, SH3 and SH2 (UN/SH3/SH2). The protein complexes were equally divided and analyzed by Western blotting with either Cbl or anti-phosphotyrosine (P-Tyr) antibodies as indicated.

one S-transferase (GST) fusion proteins encompassing different domains of Hck in 'pulldown' assays and Far Western blotting. In pulldown assays using the various GST-fusion constructs of Hck, the Hck-SH2 domain was sufficient to pull down Cbl. The efficiency of the interaction did not change appreciably when the construct encompassing both the SH3 and SH2 domains was used, however, binding to Cbl was augmented when the unique domain was added to the two Src homology domains (Figure 5A). There has been no evidence so far whether the interaction between Hck and the proto-oncogene product Cbl is direct or requires an additional 'bridging' protein. Therefore, we used a Far Western approach to address this question. Since the fusion protein encompassing the unique, SH3 and SH2 domains of Hck repeatedly displayed the most robust association with Cbl, we decided to use this construct for Far Western blotting. As shown in Figure 5B, the

GST-Hck fusion protein associated with the immunoprecipitated Cbl, whereas the GST protein alone failed to bind.

As mentioned earlier, Cbl becomes tyrosine phosphorylated in response to many physiological signals, one of which being the stimulation of cells through their Fcγ receptors. We wanted to investigate the effect of FcγR stimulation—and thus phosphorylation of $p120^{Cbl}$—on the binding affinity of the Hck-unique/SH3/SH2 domain to Cbl. As shown in Figure 3, tyrosine phosphorylation of Cbl is apparent as early as 1 minute after Fcγ stimulation and is dramatically reduced by 15 minutes. Therefore, we performed binding assays with the Hck-unique/SH3/SH2 construct on lysates from THP-1 cells that had been Fcγ-stimulated within this timeframe. As shown in Figure 5C, association of the GST-Hck-unique/SH3/SH2 fusion protein with Cbl mirrors the tyrosine phosphorylation status of Cbl, and thus it appears that the association between Hck and Cbl is affected by the phosphorylation status of Cbl.

Physical association of Cbl with an estrogen-regulated allele of Hck. From the above described results we anticipated that there would be a physical association between Cbl and the Src-family kinases such as Hck *in vivo*. In order to better assess the ability for Hck and Cbl to associate within cells we utilized a conditional chimera of Hck. Fusion of the hormone binding region of the estrogen receptor (hbER) to many transcription factors renders at least some of the biological activities of these proteins dependent on the addition of hormone in the growth media [30–32]. This strategy has successfully been used with protein kinases such as Raf [20] and Abl [33]. We therefore fused the hbER to the carboxy-terminal end of a cDNA that encodes p59Hck giving rise to p59HckER. After 24 and 48 h treatments with estradiol there was a dramatic change in cellular morphology (data not shown) with a concomitant increase in the total cellular tyrosine phosphorylated proteins in murine fibroblasts harboring the estrogen-regulated chimera, indicative of the enzymatic activation of p59HckER (Figure 6A). An increase in total tyrosine phosphorylation including $p120^{Cbl}$ was observed as early as 2 h post-estradiol addition (data not shown). The levels of p59HckER protein remained constant upon addition of estradiol (Figure 6A, lower panel). However, the specific activity of the kinase was elevated, presumably due to estradiol-induced conformational changes. Upon treatment of the cells with estradiol for 24 or 48 h, we observed that a substantial amount of the p59HckER chimera co-immunoprecipitated with Cbl (Figure 6B). There was only minimal co-immunoprecipitation of the chimera in the absence of ligand (Figure 6B) despite a high level of p59HckER expression (Figure 6A). The level of Cbl remained fairly constant during the time course of the experiment with estradiol (Figure 6B, lower panel). The ability of Cbl and p59HckER to associate was observed as early as 2 h after the addition of estradiol. However, we have not evaluated earlier time points.

DISCUSSION

The proto-oncogene product $p120^{Cbl}$ becomes a prominent tyrosine phosphorylated substrate upon the receptor-mediated activation of both hematopoietic and non-hematopoietic cells. Several PTKs have been implicated in the phosphorylation of Cbl including the Syk and ZAP-70 family [34, 35]. However, it is clear that Syk is not always essential. In Syk-negative chicken B cells, Cbl is not dependent on Syk for its tyrosine phosphorylation in response to stimulation of the B cell antigen receptor [36]. There have been several recent reports suggesting that Cbl is a downstream target of the Src-family tyrosine kinases [26, 36–43]. Genetic evidence has been provided showing a role for Cbl downstream of c-Src in the function of osteoclast-like cells in mice [38], or downstream of Lyn in chicken and mouse B cells [36, 44].

In this paper we provide several important pieces of evidence both *in vitro* and *in vivo* which substantiate that Cbl is a downstream target of the Src-family protein tyrosine kinase Hck. We have demonstrated that upon activation of Hck by either the heavy metal $HgCl_2$ or by an activating point mutation, Cbl becomes tyrosine phosphorylated. In addition, the Src-family selective kinase inhibitor, PP1, could abrogate the ability of Cbl to become tyrosine phosphorylated in response to extracellular signals such as Fcγ-receptor stimulation. We have also demonstrated that Cbl is a substrate for Hck and that the binding of Hck to Cbl is direct, requiring the unique, SH3 and SH2 domains for optimal binding.

Various biochemical studies have been undertaken which point to an intermolecular association between Cbl and Src PTKs in hematopoietic cells. Using a novel estrogen-regulated allele of Hck we have been able to show a physical association between Hck and Cbl in a hormone-dependent fashion *in vivo*. These findings are consistent with data showing that Cbl co-immunoprecipitates with Lyn and Fyn in B cells and with Lyn in promyelomonocytic cells [26, 36, 39]. The physical association of Cbl with Fyn in T cells has also been observed [37, 41]. The interaction of Src-family kinases with Cbl is thought to be mediated through the SH3 and/or SH2 domains of the Src PTKs binding to Cbl's proline-rich region and phosphorylated tyrosine residues [26, 28, 37, 38, 40, 42]. In agreement with a recent report [42] we have found that the amino-terminal half of Hck encompassing the unique, SH3 and SH2 domains is required for optimal binding to Cbl. However, in contrast to this study we have found that the Hck-SH2 domain alone is capable of interacting with Cbl.

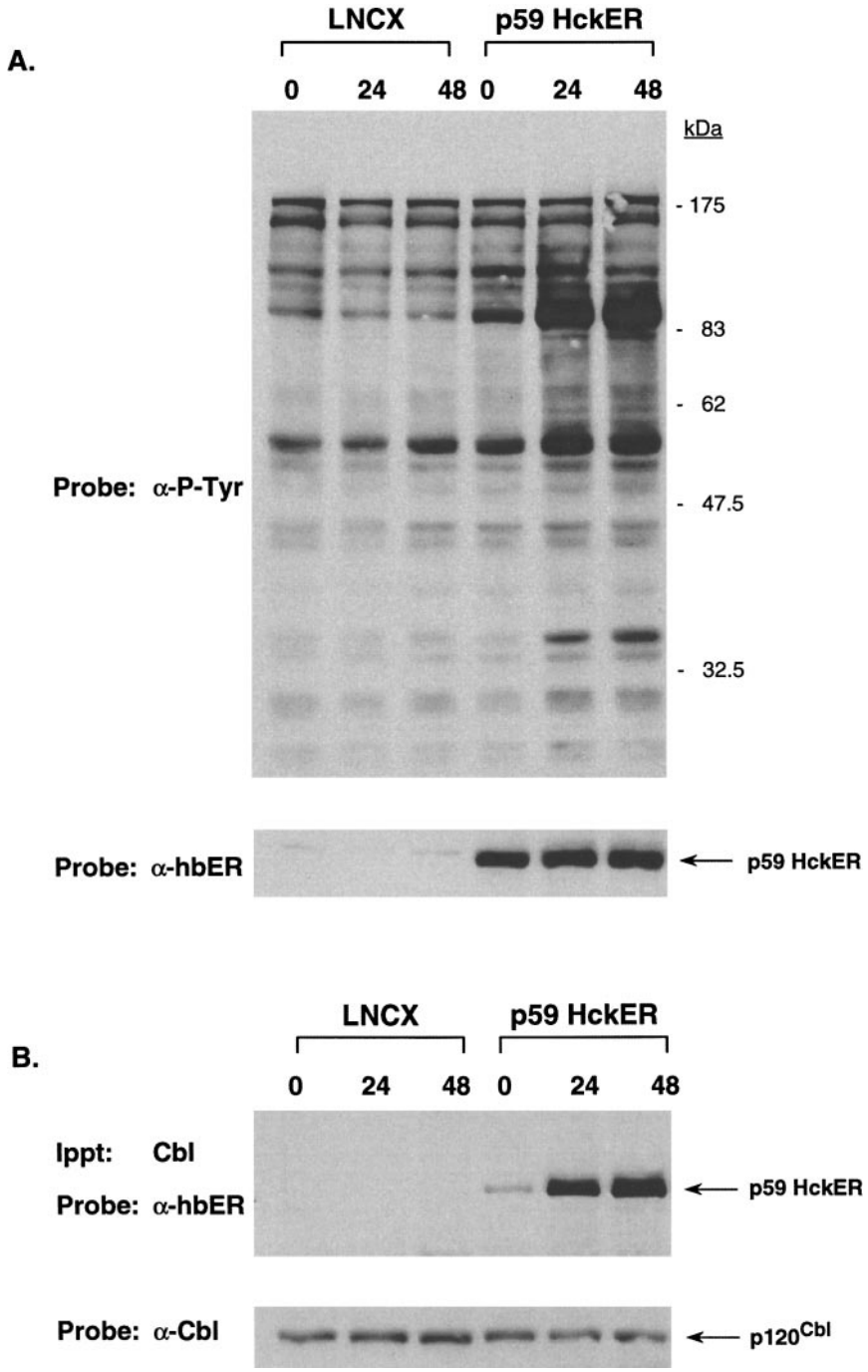

FIG. 6. Physical association of Cbl with an estrogen-regulated allele of Hck, HckER. NIH-3T3 cells transfected with either vector alone (lanes 1–3) or p59HckER (lanes 4–6) were grown to confluency and then treated with estradiol (1 mM final concentration) for the indicated times in h. The cells were then lysed in a 1% NP-40 containing lysis buffer. A) A Western blot of total cell lysates was probed with anti-phosphotyrosine (top panel), and reprobed with anti-hbER (bottom panel). B) Cell lysates were immunoprecipitated with a monoclonal antibody to Cbl (7G10) and the resulting Western blot was probed with either anti-hbER (top panel) or Cbl polyclonal antiserum (bottom panel). The positions of p59HckER and $p120^{Cbl}$ are marked as such.

We have not been able to detect binding of either the unique or SH3 domains of Hck to Cbl which differs from previous reports with the SH3 domains from Fyn [45] and Lyn [26]. This may indicate that different Src-family kinases bind to Cbl by distinct mechanisms, have varying affinities for Cbl or, for technical reasons the SH3 domain of Hck is not in a binding competent configuration. We believe that the latter reason is unlikely since the Hck-SH3 fusion protein is capable of binding another interacting protein, Vav (Bisson and

Robbins, unpublished observations). We have also been able to establish that the molecular association between Hck and Cbl is direct and does not require a 'bridging' protein. This is in agreement with a recent report showing that Cbl interacts with Lyn in a yeast two-hybrid system [43].

Our data would suggest that the interaction between Cbl and Hck is phosphotyrosine-mediated involving the SH2 domain of Hck and the interaction is enhanced by the presence of the unique and SH3 domains. It remains to be determined whether the unique and SH3 domains of Hck have a distinct binding site(s) on Cbl (such as the proline-rich region of Cbl) or whether they merely modulate the affinity of the Hck-SH2 domain for its binding site on Cbl. The ability of Hck to interact with Cbl that contains very little, if any, detectable tyrosine phosphorylation from *in vitro* translated mRNA (data not shown) suggests that phosphotyrosine-independent interactions may also exist. This is not inconceivable since the Lck-SH2 domain has recently been shown to bind a novel 62-kDa protein in a phosphotyrosine-independent fashion [46, 47].

Thus, Hck is one member of the Src-family PTKs that is able to phosphorylate Cbl. The genetic redundacy within this family of kinases would imply that several of its members can fulfill the role of Cbl phosphorylation in response to various extracellular signals [48, 49]. The specificity of the interactions would thus lie in the cellular context and particular extracellular signal received.

ACKNOWLEDGMENTS

We thank Dr. Arthur Weiss (University of California, San Francisco) for the JCaM1 cells. We thank Drs. Morley Hollenberg and Liz Murray for the critical review of the manuscript and helpful discussions during the course of the work. This work is supported by grants from the National Cancer Institute of Canada, from funds from the Terry Fox Run, and an Alberta Heritage for Medical Research (AHFMR) Establishment Award. S.M.R. is an AHFMR scholar, C.J.H. is a recipient of an AHFMR graduate fellowship, and S.A.B. holds an Alberta Cancer Board graduate fellowship.

REFERENCES

1. Brown, M. T., and Cooper, J. A. (1996) *Biochem. Biophys. Acta.* **1287,** 121–149.
2. Lock, P., Ralph, S., Stanley, E., Boulet, I., Ramsay, R., and Dunn, A. R. (1991) *Mol. Cell. Biol.* **11,** 4363–4370.
3. Robbins, S. M., Quintrell, N. A., and Bishop, J. M. (1995) *Mol. Cell. Biol.* **15,** 3507–3515.
4. Holtzman, D. A., Cook, W. D., and Dunn, A. R. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8325–8329.
5. Law, D. A., Gold, M. R., and DeFranco, A. L. (1992) *Mol. Immunol.* **29,** 917–926.
6. Quintrell, N., Lebo, R., Varmus, H., Bishop, J. M., Pettenati, M. J., Le, B. M., Diaz, M. O., and Rowley, J. D. (1987) *Mol. Cell. Biol.* **7,** 2267–2275.
7. Ziegler, S. F., Marth, J. D., Lewis, D. B., and Perlmutter, R. M. (1987) *Mol. Cell. Biol.* **7,** 2276–2285.
8. Anderson, S. M., and Jorgensen, B. (1995) *J. Immuno.* **155,** 1660–1670.
9. Durden, D. L., Kim, H. M., Calore, B., and Liu, Y. (1995) *J. Immunol.* **154,** 4039–4047.
10. English, B. K. (1996) *J. Leukocyte Biol.* **60,** 667–673.
11. Ernst, M., Gearing, D. P., and Dunn, A. R. (1994) *EMBO J.* **13,** 1574–1584.
12. Ghazizadeh, S., Bolen, J. B., and Fleit, H. B. (1994) *J. Biol. Chem.* **269,**, 8878–8884.
13. Linnekin, D., Howard, O. M., Park, L., Farrar, W., Ferris, D., and Longo, D. L. (1994) *Blood* **84,** 94–103.
14. Wang, A. V. T., Scholl, P. R., and Geha, R. S. (1994) *J. Exp. Med.* **180,** 1165–1170.
15. Langdon, W. Y., Hartley, J. W., Klinken, S. P., Ruscetti, S. K., and Morse, H. C. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1168–1172.
16. Blake, T. J., Shapiro, M., Morse, H. C., III, and Langdon, W. Y. (1991) *Oncogene* **6,** 653–657.
17. Andoniou, C. E., Thien, C. B. F., and Langdon, W. Y. (1994) *EMBO J.* **13,** 4515–4523.
18. Liu, Y. C., and Altman, A. (1998) *Cell. Signal.* **10,** 377–385.
19. Kumar, V., Green, S., Staub, A., and Chambon, P. (1986) *EMBO J.* **5,** 2231–2236.
20. Samuels, M. L., Weber, M. J., Bishop, J. M., and McMahon, M. (1993) *Mol. Cell. Biol.* **13,** 6241–6252.
21. Miller, A. D., and Rosman, G. J. (1989) *BioTechniques* **7,** 980–982.
22. Smith, D. B., and Johnson, K. S. (1988) *Gene* **67,** 31–40.
23. Nakashima, I., Pu, M., Nishizaki, A., Rosila, I., Ma, L., Katano, Y., Ohkusu, K., Rahman, S. M. J., Isobe, K., Hamaguchi, M., and Saga, K. (1994) *J. Immunol* **152,** 1064–1071.
24. Pu, M., Akhand, A. A., Kato, M., Koike, T., Hamaguchi, M., Suzuki, H., and Nakashima, I. (1996) *J. Cell. Biochem.* **63,** 104–114.
25. Rahman, S. M. J., Pu, M., Hamaguchi, M., Iwamoto, T., Isobe, K., and Nakashima, I. (1993) *FEBS Lett.* **317,** 35–38.
26. Marcilla, A., Rivero-Lezcano, O. M., Agarwal, A., and Robbins, K. C. (1995) *J. Biol. Chem.* **270,** 9115–9120.
27. Matsuo, T., Hazeki, K., Hazeki, O., Katada, T., and Ui, M. (1996) *FEBS Lett.* **382,** 11–14.
28. Tanaka, S., Neff, L., Baron, R., and Levy, J. B. (1995) *J. Biol. Chem.* **270,** 14347–14351.
29. Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pollok, B. A., and Connelly, P. A. (1996) *J. Biol. Chem.* **271,** 695–701.
30. Boehmelt, G., Walker, A., Kabrun, N., Mellitzer, G., Beug, H., Zenke, M., and Enrietto, P. J. (1992) *EMBO J.* **11,** 4641–4652.
31. Burk, O., and Klempnauer, K. H. (1991) *EMBO J.* **10,** 3713–3719.
32. Eilers, M., Picard, D., Yamamoto, K. R., and Bishop, J. M. (1989) *Nature* **340,** 66–68.
33. Jackson, P., Baltimore, D., and Picard, D. (1993) *EMBO J.* **12,** 2809–2819.
34. Ota, Y., and Samelson, L. E. (1997) *Science* **276,** 418–420.
35. Fournel, M., Davidson, D., Weil, R., and Veillette, A. (1996) *J. Exp. Med.* **183,** 301–306.
36. Tezuka, T., Umemori, H., Fusaki, N., Yagi, T., Takata, M., Kurosaki, T., and Yamamoto, T. (1996) *J. Exp. Med.* **183,** 675–680.
37. Fukazawa, T., Reedquist, K. A., Trub, T., Soltoff, S., Panchamoorthy, G., Druker, B., Cantley, L., Shoelson, S. E., and Band, H. (1995) *J. Biol. Chem.* **270,** 19141–19150.

38. Tanaka, S., Amling, M., Neff, L., Peyman, A., Uhlmann, E., Levy, J. B., and Baron, R. (1996) *Nature* **383,** 528–531.
39. Panchamoorthy, G., Fukazawa, T., Miyake, S., Soltoff, S., Reedquist, K., Druker, B., Shoelson, S., Cantley, L., and Band, H. (1996) *J. Biol. Chem.* **271,** 3187–3194.
40. Ojaniemi, M., Martin, S. S., Dolfi, F., Olefsky, J. M., and Vuori, K. (1997) *J. Biol. Chem.* **272,** 3780–3787.
41. Tsygankov, A. Y., Mahajan, S., Fincke, J. E., and Bolen, J. B. (1996) *J. Biol. Chem.* **271,** 27130–27137.
42. Anderson, S. M., Burton, E. A., and Koch, B. A. (1997) *J. Biol. Chem.* **272,** 739–745.
43. Dombrosky-Ferlan, P., and Corey, S. J. (1997) *Oncogene* **14,** 2019–2024.
44. Nishizumi, H., Taniuchi, I., Yamanashi, Y., Kitamura, D., Ilic, D., Mori, S., Watanabe, T., and Yamamoto, T. (1995) *Immunity* **3,** 549–560.
45. Reedquist, K. A., Fukazawa, T., Druker, B., Panchamoorthy, G., Shoelson, S. E., and Band, H. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4135–4139.
46. Park, I., Chung, J., Walsh, C. T., Yun, Y., Strominger, J. L., and Shin, J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 12338–12342.
47. Joung, I., Strominger, J. L., and Shin, J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5991–5995.
48. Lowell, C. A., Fumagalli, L., and Berton, G. (1996) *J. Cell Biol.* **133,** 895–910.
49. Meng, F., and Lowell, C. A. (1998) *EMBO J.* **17,** 4391–4403.