THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 22, Issue of May 30, pp. 14320–14326, 1997
Printed in U.S.A.

Differential Signaling after β1 Integrin Ligation Is Mediated Through Binding of CRKL to p120^{CBL} and p110^{HEF1}*

(Received for publication, September 9, 1996, and in revised form, February 14, 1997)

Martin Sattler‡, Ravi Salgia‡, Gautam Shrikhande‡, Shalini Verma‡, Naoki Uemura‡, Susan F. Law§, Erica A. Golemis§, and James D. Griffin‡¶

From the ‡Division of Hematologic Malignancies, Dana-Farber Cancer Institute, Boston, Massachusetts 02115 and the §Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

CRKL is an SH2-SH3-SH3 adapter protein that is a major substrate of the BCR/ABL oncogene. The function of CRKL in normal cells is unknown. In cells transformed by BCR/ABL we have previously shown that CRKL is associated with two focal adhesion proteins, tensin and paxillin, suggesting that CRKL could be involved in integrin signaling. In two hematopoietic cell lines, MO7e and H9, we found that CRKL rapidly associates with tyrosine-phosphorylated proteins after cross-linking of β1 integrins with fibronectin or anti-β1 integrin monoclonal antibodies. The major tyrosine-phosphorylated CRKL-binding protein in the megakaryocytic MO7e cells was identified as p120^{CBL}, the cellular homolog of the v-Cbl oncoprotein. However, in the lymphoid H9 cell line, the major tyrosine-phosphorylated CRKL-binding protein was p110^{HEF1}. In both cases, this binding was mediated by the CRKL SH2 domain. Interestingly, although both MO7e and H9 cells express p120^{CBL} and p110^{HEF1}, β1 integrin cross-linking induces tyrosine phosphorylation of p120^{CBL} (but not p110^{HEF1}) in MO7e cells and of p110^{HEF1} (but not p120^{CBL}) in H9 cells. In both cell types, CRKL is constitutively complexed to C3G, SOS, and c-ABL through its SH3 domains, and the stoichiometry of these complexes does not change upon integrin ligation. Thus, in different cell types CRKL and its SH3-associated proteins may form different multimeric complexes depending on whether p120^{CBL} or p110^{HEF1} is tyrosine-phosphorylated after integrin ligation. The shift in association of CRKL and its SH3-associated proteins from p120^{CBL} to p110^{HEF1} could contribute to different functional outcomes of "outside-in" integrin signaling in different cells.

Integrins play a role in cell movement and apoptosis and also act as costimulatory molecules. The integrin receptors are α/β heterodimeric transmembrane proteins that mediate cell-cell or cell-extracellular matrix interactions. Activation of integrin receptors leads to the formation of focal adhesions where integrin cytoplasmic domains are connected with actin-containing cytoskeleton components, thereby providing a link between the extracellular environment and intracellular elements. Tyrosine phosphorylation of cellular proteins is an early event after integrin receptor stimulation and is believed to initiate a series of signaling events involving protein-protein interactions leading to changes in viability, proliferation, or other functions in various cells (1, 2). One tyrosine kinase that is localized to the focal adhesion and is activated after integrin ligation has been identified as p125^{FAK} (3). This kinase may have a negative regulatory role in the formation of focal adhesions (4). Also, another non-receptor tyrosine kinase (related adhesion focal tyrosine kinase) has been found to be partially associated with the actin cytoskeleton and is activated by integrins (5, 6). Recently, investigators have begun to identify the major cellular proteins that are tyrosine-phosphorylated after cross-linking of integrins by ligands. For example, p120^{CBL} is tyrosine-phosphorylated after β1 integrin ligation in the human B cell line Nalm-6 and after β1 and β2 integrin ligation in the megakaryoblastic cell line MO7e (7, 8).

In many signal transduction pathways activated by tyrosine kinases, adapter molecules have been shown to play a key role in mediating transient protein-protein interactions. We have previously shown that the adapter protein CRKL is associated with the focal adhesion protein paxillin in cells transformed by the oncogenic tyrosine kinase BCR/ABL (9). CRKL is a 39-kDa protein with one SH2 and two SH3 domains (10). CRKL has a high homology to c-CRK-II and belongs to the CRK family of adapter proteins, which includes v-CRK, c-CRK-II, and c-CRK-I (11–13). The CRK and CRKL SH3 domains have been shown to specifically bind to c-ABL, SOS, or C3G (14–19). The SH2 domain of CRKL has been shown to bind to p120^{CBL} in cells transformed by oncogenic tyrosine kinases (19, 20), and CRKL binds p120^{CBL} inducibly after epidermal growth factor receptor stimulation (21) or after T cell receptor stimulation (22).

In this study, we examined the involvement of CRKL in signal transduction pathways activated after cross-linking of β1 integrins in two hematopoietic cell lines, the megakaryoblastic cell line MO7e and a T cell line, H9. In both cell lines, β1 integrin stimulation resulted in the rapid association of CRKL with a single major tyrosine-phosphorylated cellular protein. Surprisingly, however, this protein was of a different apparent molecular mass in the two cell lines. We found that p120^{CBL} was the major tyrosine-phosphorylated CRKL-binding protein in MO7e cells, and p110^{HEF1} was the major tyrosine-phosphorylated CRKL-binding protein in H9 cells. In both cases the binding was mediated through the CRKL SH2 domain, while proteins constitutively associated with the CRKL SH3 domain, including C3G, SOS, and c-ABL, did not appear to be affected by β1 integrin stimulation. These results indicate that CRKL and its associated signaling proteins can interact with more than one signaling pathway activated by β1 integrin ligation.

* This work was supported by José Carreras International Leukemia Foundation Fellowship FIJC-95/INT (to M. S.), National Institutes of Health Grants CA60821 (to R. S.), R29-CA63366 (to E. A. G.), and CA36167 (to J. D. G.), American Cancer Society Fellowship PF4383 (to S. F. L.), and American Cancer Society Grant CB-74749 (to E. A. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Div. of Hematologic Malignancies, Dana-Farber Cancer Inst., 44 Binney St., Boston, MA 02115. Tel.: 617-632-3360; Fax: 617-632-4388.

MATERIALS AND METHODS

Cells—The human megakaryoblastic cell line MO7e (obtained from Dr. Steve Clark, Genetics Institute, Cambridge, MA) was maintained in Dulbecco's modified Eagle's medium (Mediatech, Washington, D. C.), 10 ng/ml granulocyte-macrophage colony-stimulating factor (Genetics Institute), and 20% (v/v) fetal calf serum (PAA Laboratories Inc., Newport Beach, CA) at 37 °C with 10% CO_2. The BCR/ABL-expressing MO7e cell line MO7/p210 was generated by transfection with the pGD vector containing the sequence for the p210$^{BCR/ABL}$ cDNA (obtained from Dr. George Daley, MIT, Cambridge, MA). For stimulation studies, MO7e cells were washed with Dulbecco's phosphate-buffered saline (DPBS)[1] and deprived of growth factors for 20 h at 37 °C in serum-free medium with 1% (w/v) bovine serum albumin (Sigma). The human T cell line H9 (obtained from Dr. Jerome Ritz, Dana-Farber Cancer Institute) was maintained in RPMI 1640 (Mediatech) and 10% (v/v) fetal calf serum (PAA Laboratories Inc.) at 37 °C with 5% CO_2. Starved H9 cells were prepared by washing with DPBS and were deprived of serum for 2 h at 37 °C in serum-free medium.

Stimulation of Cells and Preparation of Cellular Lysates—Starved MO7e or H9 cells were first incubated for 15 min on ice with antibodies against CD29/β1 integrin (4B4, obtained from Dr. C. Morimoto, Dana-Farber Cancer Institute), CD3 (OKT3, Coulter Corp., Miami, FL), or an irrelevant antibody (3C11C8, an anti-interferon-γ murine monoclonal antibody) and then stimulated by cross-linking using affinity-purified rabbit anti-mouse Ig (Dako Corp., Carpinteria, CA) at 37 °C for 10 min. For α integrin subunit cross-linking, starved MO7e and H9 cells were incubated for 30 min on ice with antibodies against α4 integrins (8F2 from Dr. C. Morimoto and B5G10 from Dr. M. Hemler, Dana-Farber Cancer Institute) or against α5 integrins (2H6 from Dr. C. Morimoto and A5-PUJ2 from Dr. M. Hemler) and then stimulated by cross-linking for 20 min as described above. Either starved H9 cells or MO7e cells (washed three times in DPBS after starvation and resuspended in Dulbecco's modified Eagle's medium) were used for stimulation with fibronectin (Life Technologies, Inc.) in the same fashion. Cell lysates were prepared as described (23).

Immunoprecipitation and Western Blotting—Western blotting using a chemiluminescence technique was performed as described (23). Immunochemical detection of tyrosine-phosphorylated proteins in Western blots utilized monoclonal antibody 4G10 (kindly provided by Dr. B. Druker, Oregon Health Science University, Portland, OR). Polyclonal rabbit antisera against p120^{CBL} (Santa Cruz Biotechnology, Santa Cruz, CA), CRKL (Santa Cruz Biotechnology), p110^{HEF1} (HEF1 SB) (24), and mouse monoclonal antibodies against c-ABL (AB-3 from Oncogene Science, Manhasset, NY) and CRKL (the mouse monoclonal was generated as described elsewhere (25) and only used for Western blotting) were used for this study. The pGEX vector containing the SH2 and SH3-SH3 domains of CRKL was obtained from Dr. J. Groffen, Children's Hospital, UCLA, Los Angeles, CA. The GST-fusion proteins were expressed in *Escherichia coli* (DH-5α) by isopropyl-1-thio-β-D-galactopyranoside induction and isolated from sonicated bacterial lysates using glutathione-Sepharose beads (Pharmacia Biotech Inc.) according to the manufacturer's directions.

Flow Cytometry Analysis—MO7e and H9 cells (0.5×10^6 cells/sample) were incubated with murine monoclonal antibodies against integrin receptors including α1 (TS2/7), α2 (A2–2E10), α3 (A3–2F5), α4 (B5G10), α5 (A5-PUJ2), α6 (A6-ELE) (all anti-α integrin receptor antibodies were obtained from Dr. M. Hemler, Dana-Farber Cancer Institute), β1 (4B4), or an irrelevant monoclonal antibody (3C11C8) for 20 min on ice and then washed once with DPBS. Cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse serum (Southern Biotechnology Assoc., Birmingham, AL) for an additional 20 min and subsequently washed twice in DPBS before analysis using a Coulter Epics XL flow cytometer (Coulter Corp.) for analysis.

Far-Western Blotting—Using previously established techniques (26), far-Western blotting was performed as described previously (19). In brief, immunoprecipitated proteins were transferred after SDS-PAGE to Immobilon-P (polyvinylidene difluoride) membrane (Millipore) and blocked with 5% nonfat dry milk in 0.1% Tween 20 in phosphate-buffered saline, pH 7.4. The specific direct *in vitro* binding was evaluated by probing the membrane with GST-fusion proteins and visualized with a combination of anti-GST monoclonal antibody (Santa Cruz Biotechnology) and horseradish peroxidase-coupled anti-mouse IgG antibody by chemiluminescence.

[1] The abbreviations used are: DPBS, Dulbecco's phosphate-buffered saline; GST, glutathione *S*-transferase.

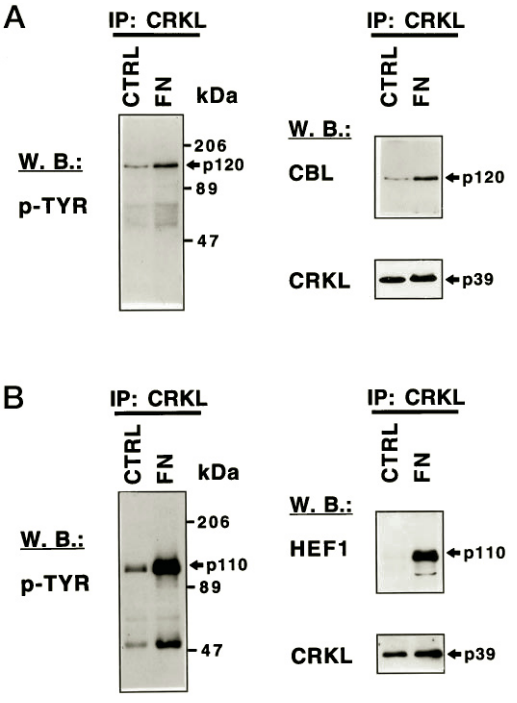

FIG. 1. **CRKL forms a stable complex with p120^{CBL} in MO7e cells and p110^{HEF1} in H9 cells after fibronectin stimulation.** Unstimulated (*CTRL*) or fibronectin (*FN*)-stimulated MO7e cells or H9 cells (20×10^6 cells) were used for immunoprecipitation. *A*, growth factor-deprived MO7e cells were stimulated for 15 min with fibronectin (*FN*). Cell lysates were immunoprecipitated with antisera to CRKL and immunoblotted with either anti-phosphotyrosine antibody (*p-TYR*), antisera to p120^{CBL} (*CBL*), or CRKL as indicated. *B*, serum-starved H9 cells were stimulated for 15 min with fibronectin (*FN*). Cell lysates were immunoprecipitated with antisera to CRKL and immunoblotted with either anti-phosphotyrosine antibodies (*p-TYR*), antisera to p110^{HEF1} (*HEF1*), or CRKL as indicated.

RESULTS

CRKL Binds to p120^{CBL} after Fibronectin Stimulation in MO7e Cells and to p110^{HEF1} in H9 Cells—To investigate the potential role of CRKL in integrin signaling we looked for tyrosine-phosphorylated proteins that coprecipitate with CRKL, since tyrosine phosphorylation of cellular proteins is an early event following integrin receptor ligation. To determine if CRKL associates with tyrosine-phosphorylated proteins, we investigated two different hematopoietic cell lines with known differences in tyrosine phosphorylation of cellular proteins after β1 integrin stimulation.

For initial experiments we stimulated MO7e and H9 cells with fibronectin, a natural ligand for some integrin receptors including VLA-4 (α4β1) and VLA-5 (α5β1), which are the major β1 integrin receptors in MO7e cells as well as in H9 cells. In MO7e cells, fibronectin induced association of CRKL with a prominent 120-kDa tyrosine phosphoprotein (Fig. 1*A*, *left panel*). We have previously shown that β1 integrin ligation induces tyrosine phosphorylation of p120^{CBL} in MO7e cells. We therefore asked if the 120-kDa protein coprecipitating with CRKL in MO7e cells was p120^{CBL}. This blot was stripped, and the phosphoprotein was identified as p120^{CBL} by immunoblotting (Fig. 1*A*, *upper right panel*). The same results were obtained when the immunoprecipitation and blotting antibodies were reversed (data not shown). The *lower right panel* in Fig. 1*A* demonstrates that equal amounts of CRKL were loaded in each *lane*.

In H9 cells, CRKL was also found to associate with tyrosine-phosphorylated proteins. However, a 110-kDa tyrosine-phosphorylated protein coprecipitated with CRKL after fibronectin stimulation (Fig. 1*B*, *left panel*). This protein did not react with

a p120^{CBL} antibody (data not shown). Based on its molecular mass and the presence of multiple potential CRKL SH2 binding motifs (Tyr-*X*-*X*-Pro), we examined p110^{HEF1} for possible coprecipitation with CRKL. The blot was stripped, and the phosphoprotein was identified as p110^{HEF1} by immunoblotting (Fig. 1*B*, *upper right panel*). The *lower right panel* in Fig. 1*B* demonstrates that comparable amounts of CRKL were loaded. These results demonstrate that integrin receptor activation with fibronectin can induce the formation of a CRKL-p120^{CBL} complex in MO7e cells and a CRKL-p110^{HEF1} complex in H9 cells. However, we did not detect significant association of p120^{CBL} with CRKL in H9 cells or with p110^{HEF1} in MO7e cells at any time points tested between 0 and 60 min (data not shown).

p120^{CBL} and p110^{HEF1} Are Differentially Tyrosine-phosphorylated after β1 Integrin Ligation in MO7e Cells and H9 Cells—Since we observed differential association of tyrosine-phosphorylated p120^{CBL} and p110^{HEF1} with CRKL in MO7e cells or H9 cells, respectively, we asked if these proteins were differentially tyrosine-phosphorylated after β1 integrin ligation in these cells. Stimulation of the megakaryocytic MO7e cells or the T cell line H9 with a monoclonal antibody to cross-link β1 integrins induced rapid tyrosine phosphorylation of cellular proteins compared with unstimulated cells (Fig. 2*A*, *left panel*). Mock stimulation with an irrelevant antibody (3C11C8, an anti-interferon-γ murine monoclonal antibody) did not induce tyrosine phosphorylation (data not shown). The major tyrosine-phosphorylated proteins in MO7e cells include proteins with apparent molecular masses of 145, 120, 95, 70, and 40 kDa, whereas in H9 cells two prominent proteins of 110 and 95 kDa were tyrosine-phosphorylated. H9 cells treated with an irrelevant antibody also did not induce tyrosine phosphorylation of cellular proteins. The tyrosine phosphorylation pattern induced by fibronectin was virtually identical to the β1 integrin-induced pattern.

We identified the 120-kDa protein as p120^{CBL} in phosphotyrosine immunoprecipitations of stimulated MO7e cells but not H9 cells (Fig. 2*A*, *middle panel*). In contrast, the 110-kDa protein in the phosphotyrosine immunoprecipitation of H9 cells was found to be the recently cloned p130^{CAS}-related protein p110^{HEF1} (Fig. 2*A*, *right panel*). In addition, p120^{CBL} and p110^{HEF1} were also inducibly (but again selectively) tyrosine-phosphorylated with fibronectin stimulation in MO7e and H9 cells, respectively (data not shown). Interestingly, p110^{HEF1} is not tyrosine-phosphorylated in MO7e cells. The increased tyrosine phosphorylation of p120^{CBL} and p110^{HEF1} is likely to mediate the specific interaction with CRKL after integrin cross-linking (Fig. 1). The differences in phosphorylation of p120^{CBL} or p110^{HEF1} could not be attributed to differential expression of p120^{CBL} and p110^{HEF1} as expression of these proteins in MO7e and H9 cells by Western blotting was comparable (Fig. 2*B*). In addition to p110^{HEF1}, the antiserum to p110^{HEF1} recognized a 95-kDa protein in Western blot experiments (Fig. 2*B*, *right panel*). The identity of the 95-kDa protein is not known at this time. However, our preliminary data suggest that it may be the SH3 domain-containing p130^{CAS}-related protein p95$^{EFS/SIN}$ (27, 28) (data not shown).

The failure to tyrosine-phosphorylate p110^{HEF1} in MO7e cells and p120^{CBL} in H9 cells could be due to defects in signaling pathways leading to tyrosine phosphorylation of these proteins. To address this issue, other pathways known to induce phosphorylation of p120^{CBL} and p110^{HEF1} were examined. We found p110^{HEF1} in phosphotyrosine immunoprecipitates of MO7e cells expressing the oncogenic tyrosine kinase BCR/ABL but not in untransfected cells, demonstrating apparent phosphorylation of p110^{HEF1} in response to BCR/ABL (Fig. 2*C*). Also, p120^{CBL} was found to be inducibly tyrosine-phosphorylated after CD3 cross-linking in H9 (Fig. 2*D*). These data suggest that p120^{CBL} and p110^{HEF1} can be tyrosine-phosphorylated in both cell lines by stimuli other than β1 integrin receptor cross-linking. Tyrosine-phosphorylated p120^{CBL} and p110^{HEF1} were also found to be inducibly and selectively associated with CRKL after cross-linking with α4 integrin in MO7e and H9 cells, respectively (Fig. 2*E*). Cross-linking of α5 integrin in MO7e cells produced similar results; however, the increased association of CRKL with p110^{HEF1} was very small in H9 cells (data not shown). We further tested if different signaling was due to differences in α integrin or β1 integrin receptor expression. We found that both cell lines had comparable expression of the β1 integrin as well as α4, α5, and α6 integrins. Expression of α1, α2, and α3 integrins was lower or negligible (Fig. 2*F*). Overall these results demonstrate that similar integrin receptors can activate distinct signaling proteins in different cell lines.

In Vitro Association of CRKL GST-fusion Proteins with p120^{CBL}and p110^{HEF1}—The above results suggest the potential induction of one or more multimeric protein complexes containing CRKL, p120^{CBL}, or p110^{HEF1}. The binding of CRKL to p120^{CBL} and p110^{HEF1} appears to require tyrosine phosphorylation of these proteins. Since CRKL has one SH2 and two adjacent SH3 domains, we sought to determine the mechanism of CRKL binding to p120^{CBL} and p110^{HEF1} using GST-fusion proteins containing various segments of each protein. The SH2 domain of CRKL precipitated p120^{CBL} from lysates of stimulated (but not unstimulated) MO7e cells (Fig. 3*A*). The blots were stripped and reprobed with antibodies against c-ABL demonstrating that GST-CRKL-SH3 but not GST-CRKL-SH2 constitutively precipitated c-ABL (Fig. 3*A*). We also found constitutive coprecipitation of C3G and SOS with the ABL-SH3 domain (data not shown). Using lysates from H9 cells, the SH2 domain of CRKL precipitated p110^{HEF1} after fibronectin stimulation, while the GST-CRKL SH3 domain did not (Fig. 3*B*).

The *in vitro* GST-fusion protein precipitations with p120^{CBL}, c-ABL, and CRKL do not indicate if binding of the SH2 or SH3 domains is direct or indirect. We therefore used a far-Western technique to examine possible direct *in vitro* interactions. Cellular lysates from unstimulated and β integrin-stimulated MO7e cells were used for immunoprecipitations with anti-p120^{CBL} or anti-CRKL antibody. Fig. 3*C* shows that GST protein alone does not bind to proteins in p120^{CBL} or CRKL immunoprecipitations. Direct binding of a single 120-kDa protein band in CRKL immunoprecipitations using the GST-CRKL SH2 protein as a probe was found only after β integrin ligation. This protein was identified as p120^{CBL} in the p120^{CBL} immunoprecipitation and the CRKL SH2 far-Western blot. We also observed binding of the CRKL SH3 domain to a set of proteins between 140 and 160 kDa; however, this interaction was not changed upon β integrin ligation (data not shown). The binding of SH2 domains to p120^{CBL} is likely to require phosphotyrosine, since no binding was observed to p120^{CBL} in lysates from unstimulated cells where tyrosine phosphorylation of p120^{CBL} is not induced. These results indicate that in MO7e cells, CRKL is linked through its SH2 domain to a pathway involving p120^{CBL}, whereas in H9 cells CRKL is linked to a pathway involving p110^{HEF1}.

β Integrin Ligation Does Not Alter the Binding of CRKL to SOS, C3G, or c-ABL—We also asked if integrin ligation changes any complexes of CRKL with SH3-binding proteins. Fig. 4 demonstrates that β1 integrin ligation did not alter the coprecipitation of CRKL with c-ABL, C3G, and SOS. The same

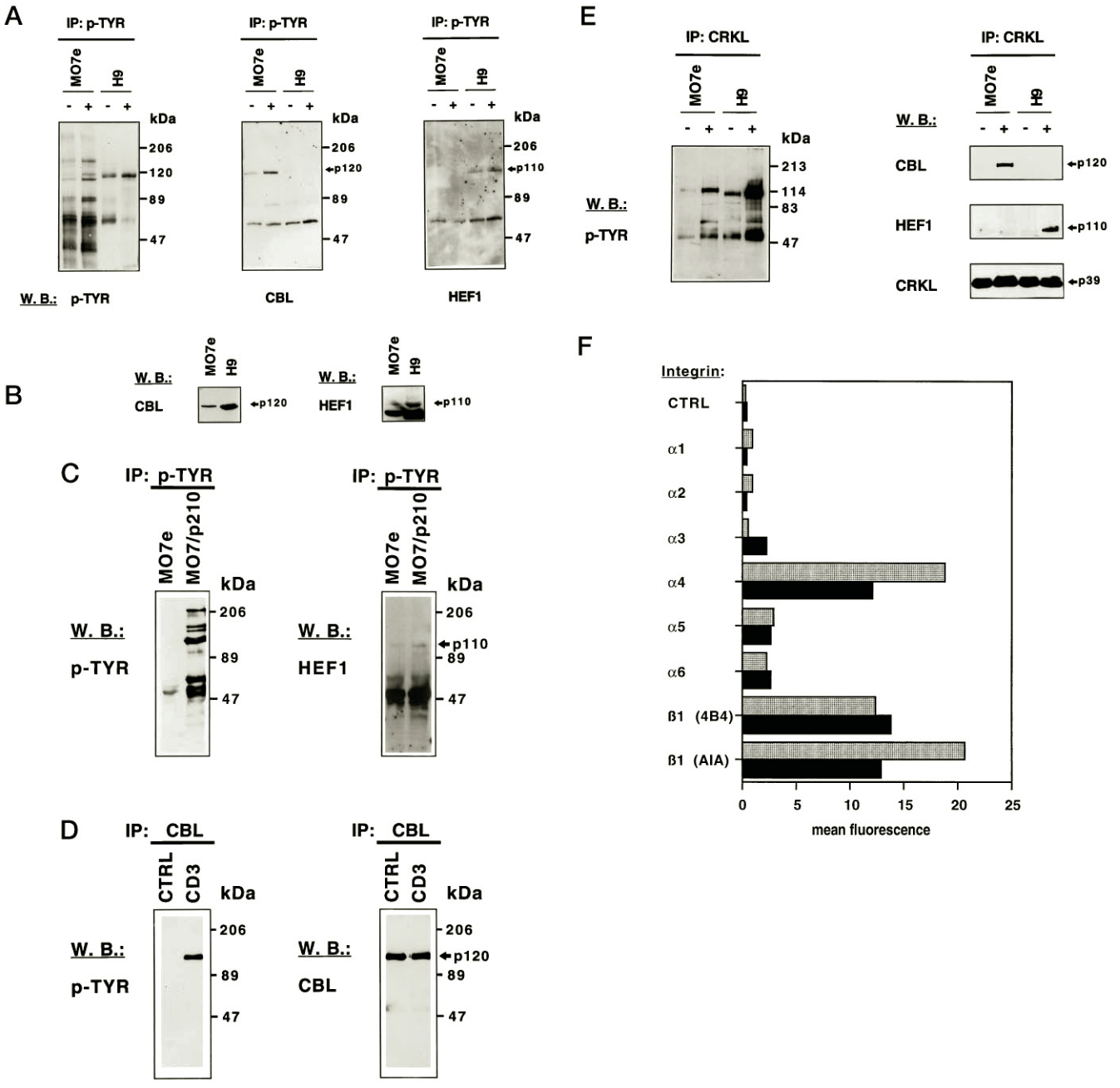

FIG. 2. **Differential activation of p120^{CBL} in MO7e cells and p110^{HEF1} in H9 cells.** *A*, unstimulated (−) or β1 integrin-stimulated (+) MO7e cells or H9 cells (20×10^6 cells) were used for immunoprecipitation. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies (*p-TYR*, PY20) and immunoblotted with either anti-phosphotyrosine antibodies (*p-TYR*, 4G10), antisera to p120^{CBL} (*CBL*), or antisera to p110^{HEF1} as indicated. *B*, total cell lysate of MO7e cells or H9 cells was separated by SDS-PAGE, and protein expression of p120^{CBL} and p110^{HEF1} was detected by Western blotting. *C*, unstimulated MO7e cells or MO7/p210 cells (15×10^6 cells) were used for immunoprecipitation. Cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies (*p-TYR*, PY20) and immunoblotted with either anti-phosphotyrosine antibodies (*p-TYR*, 4G10) or antisera to p110^{HEF1} as indicated. *D*, H9 cells (15×10^6 cells) incubated with an irrelevant antibody (*CTRL*) or CD3 receptor-stimulated were used for immunoprecipitation. Cell lysates were immunoprecipitated with antisera to p120^{CBL} (*CBL*) and immunoblotted with either anti-phosphotyrosine antibodies (*p-TYR*) or antisera to p120^{CBL} (*CBL*) as indicated. *E*, lysates of MO7e cells or H9 cells (30×10^6 cells) incubated with an irrelevant antibody (−) or α4 integrin-stimulated (+) were used for immunoprecipitation. Cell lysates were immunoprecipitated with antisera to CRKL and immunoblotted with either anti-phosphotyrosine antibodies (*p-TYR*), antisera to p120^{CBL} (*CBL*), antisera to p110^{HEF1} (*HEF1*), or an antibody to CRKL. *F*, expression of α integrins and β1 integrins on MO7e cells (*gray bars*) and H9 cells (*black bars*) was analyzed by flow cytometry using specific monoclonal antibodies or an irrelevant monoclonal antibody (*CTRL*) as a negative control. The relative fluorescence intensities are indicated as mean values.

results were obtained when the immunoprecipitation and blotting antibodies were reversed (data not shown). We did not observe detectable induction of tyrosine phosphorylation of c-ABL after integrin ligation (data not shown). These data demonstrate that integrin cross-linking does not alter the constitutive complexes of CRKL with c-ABL, C3G, and SOS.

DISCUSSION

The biological effects of cross-linking integrins may vary widely from cell to cell, ranging from stimulation of proliferation to induction of apoptosis. When integrins are cross-linked through binding with a natural ligand such as fibronectin, a series of signaling events are initiated. This signaling is asso-

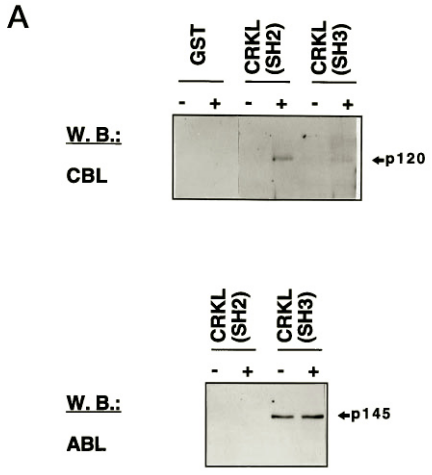

FIG. 3. **Precipitation of p120^{CBL} and p110^{HEF1} with CRKL GST-fusion proteins.** Unstimulated (−) or β1 integrin (+)-stimulated MO7e cells or H9 cells were used for precipitations. *A*, lysates of 7.5 × 10^6 MO7e cells before (−) and after (+) β integrin stimulation (7.5 min, 37 °C) were incubated with 10 μg of GST-fusion protein and GST immobilized on glutathione beads. GST-fusion proteins of the SH2 domain (*SH2*) and both SH3 domains (*SH3-SH3*) of CRKL were used for precipitations. Coprecipitation of p120^{CBL} (*CBL*) or c-ABL was detected by Western blotting. *B*, lysates of 15 × 10^6 H9 cells before (−) and after (+) β1 integrin stimulation (30 min, 37 °C) were incubated with 10 μg of GST-fusion protein and GST immobilized on glutathione beads. GST and GST-fusion proteins of the SH2 domain (*SH2*) and both SH3 domains (*SH3-SH3*) of CRKL were used for precipitations. Coprecipitation of p110^{HEF1} (*HEF1*) was detected by Western blotting. *C*, lysates of 10 × 10^6 unstimulated (−) or β integrin-stimulated cells (+) were incubated with antibodies against CRKL or p120^{CBL} (*CBL*) as indicated. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane, and specific direct binding of GST-fusion proteins to p120^{CBL} was detected by far-Western blotting. GST and GST-fusion proteins of the CRKL SH2 domain (*CRKL-SH2*) were used.

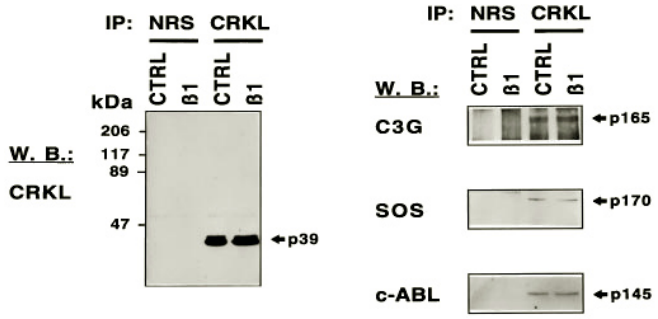

FIG. 4. **β1 integrin ligation does not alter the constitutive association of CRKL with c-ABL, C3G, and SOS.** Lysates of unstimulated (*CTRL*) or β1 integrin (*β1*)-stimulated MO7e cells (20 × 10^6 cells) were used for immunoprecipitation. Cell lysates were immunoprecipitated with preimmune serum (*NRS*) or antisera to CRKL and immunoblotted with antisera to CRKL, c-ABL, SOS, or C3G as indicated.

ciated with the following changes in the actin cytoskeleton: formation of a cytoskeletal complex of proteins that includes actin, vinculin, talin, p125^{FAK}, paxillin, and tensin, activation of tyrosine phosphorylation, and activation of other signal transduction pathways such as the p21^{RAS} pathway. Overall, this outside-in signaling of integrins is likely to be an important part of the signals sent by the microenvironment to influence cell behavior (1, 2).

However, the mechanisms of outside-in signaling are not well understood. This is due in part to the complexity of studying a system with many related receptors (the integrin family) that are expressed heterogeneously on different cell types, coupled with the fact that different integrins may share the same ligand. Since the biological effects of outside-in signaling may vary widely in different cells, it is of interest to determine how integrin cross-linking in one cell may augment proliferation but induce apoptosis in another cell type. It would be anticipated that different integrins may activate different signaling pathways in the same cell and also that the same integrin could potentially activate different pathways in different cells. Despite this prediction, there are few examples of differential signaling by integrins and even fewer examples where specific integrin-activated signaling pathways have been directly linked to a biological event.

In this study we have investigated the specific role of CRKL (an adapter protein that has one SH2 domain and two SH3 domains) in integrin signaling as part of a larger effort to understand the cellular functions of CRKL. During preliminary studies in the human megakaryoblastic cell line MO7e, we had noted that after cross-linking of β1 integrins by monoclonal antibody, CRKL was induced to bind through its SH2 domain to a 120-kDa protein identified as p120^{CBL}. p120^{CBL} was shown to be one of the most prominently tyrosine-phosphophorylated proteins induced after integrin activation in these cells (8) and virtually the only tyrosine phosphoprotein coprecipitating with CRKL. However, in another hematopoietic cell line, the T cell line H9, we noted that CRKL did not coprecipitate with p120^{CBL} after integrin cross-linking, despite the fact that the H9 cell line was found to have the same pattern of β1 integrin expression as MO7e as well as abundant expression of p120^{CBL} (29, 30). This unexpected result was made more interesting by the finding that CRKL was induced to coprecipitate with another tyrosine phosphoprotein in H9 cells, p110^{HEF1}, which is a signaling protein related to p130^{CAS}. Again, p110^{HEF1} was virtually the only tyrosine phosphoprotein coprecipitating with CRKL after integrin stimulation, and the interaction was mediated by the CRKL SH2 domain. Like p120^{CBL}, p110^{HEF1} has multiple copies of potential CRKL SH2 binding motifs (phospho Tyr-*X*-*X*-Pro) (7, 24). These combined observations suggest that CRKL is not only involved in integrin-mediated outside-in signaling, it can also participate in different pathways depending on which upstream molecule (p120^{CBL} or p110^{HEF1}) is phosphorylated (probably at the phospho Tyr-*X*-*X*-Pro motifs previously shown to represent binding sites for CRK and CRKL SH2 domains). This provides for the possibility of an intracellular

signaling "switch" that could couple integrin signaling to different biological effects.

In contrast to the effects of integrin-induced tyrosine phosphorylation on the binding of the CRKL SH2 domain to signaling molecules, the proteins that were bound to the CRKL SH3 domains were not affected by integrin cross-linking. The known CRKL SH3-binding proteins include c-ABL, C3G, and SOS. These proteins were first described as binding to the CRKII SH3 domain; however, we and others have shown that they also bind to the CRKL SH3 domain (14–19). SOS has known guanine-exchange factor activity for p21^{RAS}; in contrast, C3G appears to have specific guanine exchange activity for p21^{RAP1}. C3G does not have substrate specificity for p21^{RAS} (31), but its substrate p21^{RAP1} appears to regulate, at least in part, the signal from p21^{RAS} to the RAF kinase. C3G also shows sequence similarity to CDC25 and SOS family proteins (17) and preferentially binds to the N-terminal SH3 domain (16). The exact function of the tyrosine kinase c-ABL is unknown, although c-ABL has been shown to be involved in transcriptional activation (32) and possibly is activated in response to certain types of DNA damage (33). Interestingly, c-ABL can interact with the actin cytoskeleton through an actin binding site in its C terminus (34). During integrin signaling, c-ABL, C3G, or SOS could be linked to either p120^{CBL} or p110^{HEF1} by CRKL, although no direct evidence of such multimeric proteins was demonstrated in this study.

The protooncoprotein p120^{CBL} (for Casitas B-lineage lymphoma) is a widely expressed 120-kDa protein. It is the cellular homolog of v-Cbl, the oncoprotein in the CAS NS-1 retrovirus (35, 36) that induces pre-B cell lymphomas and myelogenous leukemias in mice (37). The p120^{CBL} homolog Sli-1 in *Caenorhabditis elegans* is a negative regulator of the epidermal growth factor receptor tyrosine kinase homolog Let-23 (38). p120^{CBL} is also known to be a substrate of tyrosine kinases in response to T cell (39) and B cell (40) activation, FC-γ receptor cross-linking (41, 42), and growth factors (23, 43–45). In mammalian cells, the function of p120^{CBL} is not known, although several interactions with other signaling proteins have been reported. For example, p120^{CBL} has been shown to associate with active phosphatidylinositol 3-kinase in antigen receptor-stimulated cells or BCR/ABL transformed cells, and it interacts with the SH3 domains of GRB2, NCK, or SRC kinases including LYN and FYN (39, 42, 46). The H9 T cell line, which was derived from the HuT 78 cell line, expresses both a full-size c-CBL protein and a protein containing a C-terminal truncation of c-CBL (47). In H9 cells, we observed tyrosine phosphorylation of full-length p120^{CBL} after CD3 stimulation, indicating that this p120^{CBL} pathway is intact.

p110^{HEF1} (for human enhancer of filamentation 1) is a tissue-specific protein first identified during cloning of human genes that induce morphological changes in *Saccharomyces cerevisiae*. Expression of the p110^{HEF1} C terminus induces pseudohyphae in *S. cerevisiae*. p110^{HEF1} shares 64% homology with p130^{CAS} and similarly has an N-terminal SH3 domain. p110^{HEF1} is also a prominent substrate of oncogenic tyrosine kinases including v-ABL and may function as a docking protein. This protein is structurally related to p130^{CAS}, it appears to be localized to the nucleus and the cell periphery (24). It is not known if p110^{HEF1} in mammalian cells is also involved in organization of the cytoskeleton. Interestingly, p110^{HEF1} has several Tyr-*X*-*X*-Pro motifs (24) that have been shown to be recognized by the CRKL SH2 domain (9, 48). This is consistent with our findings demonstrating coprecipitation of CRKL with p110^{HEF1}.

Our data demonstrate that β1 integrin receptors in MO7e or H9 cells are activating distinct signaling pathways. These differences are probably not mediated through different expression of β1 integrins since we demonstrate that the major β1 integrin receptors (the fibronectin receptors α4β1 (VLA4) and α5β1 (VLA5)) are expressed both in MO7e and H9 cells. Further, cross-linking α4 integrin chains with specific monoclonal antibodies in MO7e and H9 cells also resulted in selective phosphorylation of p120^{CBL} and P110^{HEF1}, respectively. However, the β1 integrin family consists of four known isoforms (A, B, C, and D) that have the same extracellular domains but differ in their cytoplasmic domains. The major isoform β1A is ubiquitously expressed but is substituted in muscle cells by the β1D isoform and thus not expressed in hematopoietic cells (49). β1B integrin is a minor isoform and is coexpressed with β1A in some tissues and cells (50). The β1B isoform might negatively regulate adhesion and mobility (51). The β1C isoform is expressed in hematopoietic cells but does not appear to colocalize to focal adhesions and has been shown to cause growth arrest and inhibit DNA synthesis when transfected and expressed in fibroblasts (52). It is possible that the cell is using these distinct signaling pathways involving p120^{CBL} or p110^{HEF1} depending on the differential expression of another regulatory signaling protein. Interestingly, there is at least one additional pattern of CRKL-related signaling in hematopoietic cells. We recently examined several additional cell lines and found that after β1 integrin ligation in the B cell line Nalm-6, CRKL binds to both tyrosine-phosphorylated p120^{CBL} as well as p110^{HEF1}.[2] This suggests that the mechanisms that activate either pathway are not mutually exclusive. The differential activation of p120^{CBL} and p110^{HEF1} could be directly mediated through a process that leads to activation of different tyrosine kinases that are specific for p120^{CBL} or p110^{HEF1}, and we are currently investigating this possibility.

REFERENCES

1. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) *Annu. Rev. Cell Dev. Biol.* **11,** 549–599
2. Clark, E. A., and Brugge, J. S. (1995) *Science* **268,** 233–239
3. Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., and Brugge, J. S. (1992) *J. Cell Biol.* **119,** 905–912
4. Ilic, D., Furuta, Y., Kanazawa, S., Takeda, N., Sobue, K., Nakatsuji, N., Nomura, S., Fujimoto, J., Okada, M., Yamamoto, T., and Aizawa, S. (1995) *Nature* **377,** 539–544
5. Avraham, S., London, R., Fu, Y., Ota, S., Hiregowdara, D., Li, J., Jiang, S., Pasztor, L. M., White, R. A., Groopman, J. E., and Avraham, H. (1995) *J. Biol. Chem.* **270,** 27742–27751
6. Li, J. Z., Avraham, H., Rogers, R. A., Raja, S., and Avraham, S. (1996) *Blood* **88,** 417–428
7. Blake, T. J., Shapiro, M., Morse, H. D., and Langdon, W. Y. (1991) *Oncogene* **6,** 653–657
8. Manié, S. N., Sattler, M., Astier, A., Phifer, J. S., Canty, T., Morimoto, C., Druker, B. J., Salgia, R., Griffin, J. D., and Freedman, A. S. (1997) *Exp. Hematol.* **25,** 45–50
9. Salgia, R., Uemura, N., Okuda, K., Li, J.-L., Pisick, E., Sattler, M., de Jong, R., Druker, B., Heisterkamp, N., Chen, L. B., Groffen, J., and Griffin, J. D. (1995) *J. Biol. Chem.* **270,** 29145–29150
10. ten Hoeve, J., Morris, C., Heisterkamp, N., and Groffen, J. (1993) *Oncogene* **8,** 2469–2474
11. Mayer, B. J., Hamaguchi, M., and Hanafusa, H. (1988) *Nature* **332,** 272–275
12. Reichman, C. T., Mayer, B. J., Keshav, S., and Hanafusa, H. (1992) *Cell Growth & Differ.* **3,** 451–460
13. Matsuda, M., Tanaka, S., Nagata, S., Kojima, A., Kurata, T., and Shibuya, M. (1992) *Mol. Cell. Biol.* **12,** 3482–3489
14. Feller, S. M., Knudsen, B., and Hanafusa, H. (1994) *EMBO J.* **13,** 2341–2351
15. Matsuda, M., Hashimoto, Y., Muroya, K., Hasegawa, H., Kurata, T., Tanaka, S., Nakamura, S., and Hattori, S. (1994) *Mol. Cell. Biol.* **14,** 5495–5500
16. Knudsen, B. S., Feller, S. M., and Hanafusa, H. (1994) *J. Biol. Chem.* **269,** 32781–32787
17. Tanaka, S., Morishita, T., Hashimoto, Y., Hattori, S., Nakamura, S., Shibuya, M., Matuoka, K., Takenawa, T., Kurata, T., Nagashima, K., and Matsuda, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 3443–3447
18. Feller, S. M., Knudsen, B., and Hanafusa, H. (1995) *Oncogene* **10,** 1465–1473
19. Sattler, M., Salgia, R., Okuda, K., Uemura, N., Durstin, M. A., Pisick, E., Xu, G., Li, J. L., Prasad, K. V., and Griffin, J. D. (1996) *Oncogene* **12,** 839–846
20. de Jong, R., ten Hoeve, J., Heisterkamp, N., and Groffen, J. (1995) *J. Biol. Chem.* **270,** 21468–21471
21. Fukazawa, T., Miyake, S., Band, V., and Band, H. (1996) *J. Biol. Chem.* **271,** 14554–14559

[2] M. Sattler, unpublished data.

22. Reedquist, K. A., Fukazawa, T., Panchamoorthy, G., Langdon, W. Y., Shoelson, S. E., Druker, B. J., and Band, H. (1996) *J. Biol. Chem.* **271,** 8435–8442
23. Sattler, M., Durstin, M. A., Frank, D. A., Okuda, K., Kaushansky, K., Salgia, R., and Griffin, J. D. (1995) *Exp. Hematol.* **23,** 1040–1048
24. Law, S. F., Estojak, J., Wang, B. L., Mysliwiec, T., Kruh, G., and Golemis, E. A. (1996) *Mol. Cell. Biol.* **16,** 3327–3337
25. Uemura, N., Salgia, R., Li, J-L., Pisick, E., Sattler, M., and Griffin, J. D. (1997) *Leukemia* **11,** 376–385
26. ten Hoeve, J., Kaartinen, V., Fioretos, T., Haataja, L., Voncken, J. W., Heisterkamp, N., and Groffen, J. (1994) *Cancer Res.* **54,** 2563–2567
27. Ishino, M., Ohba, T., Sasaki, H., and Sasaki, T. (1995) *Oncogene* **11,** 2331–2338
28. Alexandropoulos, K., and Baltimore, D. (1996) *Genes Dev.* **10,** 1341–1355
29. Bazzoni, G., Carlesso, N., Griffin, J. D., and Hemler, M. E. (1996) *J. Clin. Invest.* **98,** 521–528
30. Nojima, Y., Tachibana, K., Sato, T., Schlossman, S. F., and Morimoto, C. (1995) *Cell. Immunol.* **161,** 8–13
31. Gotoh, T., Hattori, S., Nakamura, S., Kitayama, H., Noda, M., Takai, Y., Kaibuchi, K., Matsui, H., Hatase, O., Takahashi, H., Kurata, T., and Matsuda, M. (1995) *Mol. Cell. Biol.* **15,** 6746–6753
32. Welch, P. J., and Wang, J. Y. (1993) *Cell* **75,** 779–790
33. Yuan, Z. M., Huang, Y. Y., Whang, Y., Sawyers, C., Weichselbaum, R., Kharbanda, S., and Kufe, D. (1996) *Nature* **382,** 272–274
34. Van Etten, R. A., Jackson, P. K., Baltimore, D., Sanders, M. C., Matsudaira, P. T., and Janmey, P. A. (1994) *J. Cell Biol.* **124,** 325–340
35. Langdon, W. Y., Hyland, C. D., Grumont, R. J., and Morse, H. D. (1989) *J. Virol.* **63,** 5420–5424
36. Langdon, W. Y., Hartley, J. W., Klinken, S. P., Ruscetti, S. K., and Morse, H. D. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 1168–1172
37. Fredrickson, T. N., Langdon, W. Y., Hoffman, P. M., Hartley, J. W., and Morse, H. D. (1984) *J. Natl. Cancer Inst.* **72,** 447–454
38. Yoon, C. H., Lee, J. H., Jongeward, G. D., and Sternberg, P. W. (1995) *Science* **269,** 1102–1105
39. Donovan, J. A., Wange, R. L., Langdon, W. Y., and Samelson, L. E. (1994) *J. Biol. Chem.* **269,** 22921–22924
40. Cory, G., Lovering, R. C., Hinshelwood, S., Maccarthymorrogh, L., Levinsky, R. J., and Kinnon, C. (1995) *J. Exp. Med.* **182,** 611–615
41. Marcilla, A., Rivero-Lezcano, O. M., Agarwal, A., and Robbins, K. C. (1995) *J. Biol. Chem.* **270,** 9115–9120
42. Tanaka, S., Neff, L., Baron, R., and Levy, J. B. (1995) *J. Biol. Chem.* **270,** 14347–14351
43. Galisteo, M. L., Dikic, I., Batzer, A. G., Langdon, W. Y., and Schlessinger, J. (1995) *J. Biol. Chem.* **270,** 20242–20245
44. Meisner, H., Conway, B. R., Hartley, D., and Czech, M. P. (1995) *Mol. Cell. Biol.* **15,** 3571–3578
45. Odai, H., Sasaki, K., Iwamatsu, A., Hanazono, Y., Tanaka, T., Mitani, K., Yazaki, Y., and Hirai, H. (1995) *J. Biol. Chem.* **270,** 10800–10805
46. Rivero-Lezcano, O. M., Sameshima, J. H., Marcilla, A., and Robbins, K. C. (1994) *J. Biol. Chem.* **269,** 17363–17366
47. Blake, T. J., and Langdon, W. Y. (1992) *Oncogene* **7,** 757–762
48. Andoniou, C. E., Thien, C., and Langdon, W. Y. (1996) *Oncogene* **12,** 1981–1989
49. Belkin, A. M., Zhidkova, N. I., Balzac, F., Altruda, F., Tomatis, D., Maier, A., Tarone, G., Koteliansky, V. E., and Burridge, K. (1996) *J. Cell Biol.* **132,** 211–226
50. Balzac, F., Belkin, A. M., Koteliansky, V. E., Balabanov, Y. V., Altruda, F., Silengo, L., and Tarone, G. (1993) *J. Cell Biol.* **121,** 171–178
51. Balzac, F., Retta, S. F., Albini, A., Melchiorri, A., Koteliansky, V. E., Geuna, M., Silengo, L., and Tarone, G. (1994) *J. Cell Biol.* **127,** 557–565
52. Meredith, J., Jr., Takada, Y., Fornaro, M., Languino, L. R., and Schwartz, M. A. (1995) *Science* **269,** 1570–1572