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Involvement of p130^{Cas} and p105^{HEF1}, a Novel Cas-like Docking Protein, in a Cytoskeleton-dependent Signaling Pathway Initiated by Ligation of Integrin or Antigen Receptor on Human B Cells*

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The Crk-associated substrate p130^{Cas} (Cas) and the recently described human enhancer of filamentation 1 (HEF1) are two proteins with similar structure (64% amino acid homology), which are thought to act as "docking" molecules in intracellular signaling cascades. Both proteins contain an N-terminal Src homology (SH), three domain and a cluster of SH2 binding motifs. Here we show that ligation of either β1 integrin or B cell antigen receptor (BCR) on human tonsillar B cells and B cell lines promoted tyrosine phosphorylation of HEF1. In contrast, Cas tyrosine phosphorylation was observed in certain B cell lines but not in tonsillar B cells, indicating a more general role for HEF1 in B cell signaling. Interestingly, pretreatment of tonsillar B cells with cytochalasin B dramatically reduced both integrin- and BCR-induced HEF1 phosphorylation, suggesting that some component of the BCR-mediated signaling pathway is closely linked with a cytoskeletal reorganization. Both HEF1 and Cas were found to complex with the related adhesion focal tyrosine kinase (RAFTK), and when tyrosine phosphorylated, with the adapter molecule CrkL. In addition, the two molecules were detected in p53/56^{Lyn} immunoprecipitates, and Lyn kinase was found to specifically bind the C-terminal proline-rich sequence of Cas in an *in vitro* binding assay. These associations implicate HEF1 and Cas as important components in a cytoskeleton-linked signaling pathway initiated by ligation of β1 integrin or BCR on human B cells.

The integrin family of adhesion molecules are involved in transducing biochemical signals into the cell, resulting in diverse biological events. Among these signals are tyrosine phosphorylations of specific proteins such as the focal adhesion kinase p125^{FAK} (Fak)[1] (1). Integrin cytoplasmic domains are associated with actin-containing cytoskeleton components, and one concept of integrin-mediated tyrosine phosphorylations is that oligomerization of integrins reorganizes the cytoskeleton into a framework that supports interactions between components of the intracellular signaling machinery (2). In support of this hypothesis is the observation that inhibitors of cytoskeletal assembly also inhibit integrin-mediated tyrosine phosphorylations (3).

B lymphocytes express several different integrins that are involved in cell localization within specific microenvironments (4, 5). Ligation of integrins on pre-B and mature B cells appears to be involved in regulating cell survival (6–9). The identification of proteins that are tyrosine phosphorylated following integrin ligation is important to understanding how integrin-mediated signaling regulates B cell function. We have previously reported the prominent tyrosine phosphorylation of proteins of 105 to 130 kDa following β1 integrin cross-linking on human B cells (10, 11). Two of these substrates have been identified as Fak (11) and p120$^{c\text{-}CBL}$ (Cbl) (12), the cellular homologue of the oncogene v-CBL.

Following integrin ligation in fibroblasts, another tyrosine phosphorylated protein known as p130^{Cas} (Cas) has been identified (13–15). Integrin-mediated homotypic adhesion in a B cell line also induced tyrosine phosphorylation of Cas (16). Cas (Crk associated substrate) was originally described as a major tyrosine phosphorylated protein in *v-crk*- or *v-src*-transformed cells (17, 18). Cas is an SH3 domain containing molecule with 15 potential Crk-SH2-binding motifs and several potential binding motifs for SH3 domains, suggesting that it may act as a "docking molecule" in intracellular signal transduction. In fact, Cas forms stable complexes with the SH2 domains of *v-crk* family members and *v-src* (16–21) and with Fak through binding to the SH3 domain of Cas (22, 23). Recently, a Cas-related protein known as HEF1 (human enhancer of filamentation 1)[2]

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[1] The abbreviations used are: FAK, p125^{FAK}; Cas, p130^{Cas}; HEF1, human enhancer of filamentation 1; SH, src homology; BCR, B cell antigen receptor; RAFTK, related adhesion focal tyrosine kinase; Cbl, p120$^{c\text{-}CBL}$; RAM, rabbit anti-mouse Ig; CB, cytochalasin B; anti-P-tyr, anti-phosphotyrosine; IVK, *in vitro* kinase assay; PAGE, polyacrylamide gel electrophoresis.

[2] During the time of revision of this manuscript, Minegishi, M., Tachibana, K., Sato, T., Iwata, S., Nojima, Y., and Morimoto, C. (1996) *J. Exp. Med.* **184,** 1365, reported the cloning of Cas-L, which is identical to HEF1, and is tyrosine phosphorylated following β1 integrin ligation in T cells.

has been isolated and characterized (24). Analogous to Cas, HEF1 contains an SH3 domain and multiple Crk-SH2 binding motifs, associates with Fak and v-abl, and localizes to focal contacts. However, in contrast to Cas, HEF1 localizes to the cell nucleus, suggesting that Cas and HEF1 may have distinct functions in cell signaling.

In the present report, we show a significant increase in the tyrosine phosphorylation of Cas and HEF1 induced by β1 integrin ligation on normal or transformed human B cells, with HEF1 being the predominant substrate. Ligation of the B cell antigen receptor (BCR) also induced tyrosine phosphorylation of predominantly HEF1, and similar to integrins, BCR-mediated HEF1 phosphorylation was dependent upon an intact actin network. We further showed that Cas and HEF1 complexed *in vivo* with the related adhesion focal tyrosine kinase RAFTK, the adapter protein CrkL, and Lyn kinase, indicating that both molecules may play an important role in B cell signaling.

EXPERIMENTAL PROCEDURES

Cells, Cell Lines, and Antibodies—Culture of Nalm-6 and ARH-77 cells and preparation and culture of human tonsillar B cells has been described elswhere (10). Antibodies used in this study were directed against: CD29/β1 integrin (K20 mAb provided by Pr. A. Bernard, U146 INSERM, Nice, France); CD18/β2 integrin (10F12 mAb provided by Dr. J. Ritz, Dana-Farber Cancer Institute, Boston, MA); phosphotyrosine (4G10 mAb); $p120^{Cbl}$, $p53/56^{Lyn}$, $p59^{Fyn}$, $p55^{Blk}$, $p130^{Cas}$ (Cas), or CrkL (rabbit polyclonal IgG, Santa Cruz Biotechnology, Santa Cruz, CA); $p130^{Cas}$ (C/H), $p125^{Fak}$, $p56^{Lck}$, or $p59/62^{Hck}$ (mAbs, Transduction Laboratories, Lexington, KY); HEF1 (affinity purified rabbit polyclonal) (24). Anti-Cas antibodies were raised against the last 15 amino acids (949–963) in the C-terminal region of Cas. Anti-HEF1 antibodies were raised against amino acids 426–439 of HEF1 (H) (24). Anti-C/H antibody was raised against amino acids 644–819 of Cas. Affinity purified rabbit anti-Mouse Ig (RAM) and $F(ab)'_2$ goat anti-human IgM/G, IgM, and IgG were obtained from Jackson Laboratories (West Grove, PA). GST fusion proteins of the C-terminal domains of Cas were prepared as described previously (25).

Activation of Cells—Tonsillar B cells and Nalm-6 cell line were resuspended in Iscove's serum-free modified Dulbecco's media for 3 h (Life Technologies, Inc.), and they were stimulated with anti-integrins antibodies plus RAM as described previously (11) or with $F(ab)'_2$ goat anti-Ig for the indicated times at 37 °C. In some experiments, cells were pretreated with 1 μM cytochalasin B (Sigma, St. Louis, MO) for 1 h at 37 °C before stimulation. Cells were then lysed in 1% Nonidet P-40 buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 mM iodoacetamide, 10 mM NaF, 0.4 mM sodium vanadate).

Precipitations and in Vitro Kinase Assay—For immunoprecipitation studies, cell lysates were precleared with protein A-Sepharose beads (Pharmacia Biotech, Inc.) and were then preincubated with specific antibody for 1 h at 4 °C followed by the addition of 25 μl of protein A-Sepharose beads for 1 h at 4 °C. For precipitations with GST fusion proteins, lysates were incubated for 2 h at 4 °C with 25 μg of fusion proteins bound to glutathione beads (Pharmacia). Precipitated proteins were washed with lysis buffer and submitted to kinase assay or eluted by boiling in sample buffer (2% SDS, 10% glycerol, 0.1 M Tris, pH 6.8, 0.02% bromphenol blue). For sequential immunoprecipitation, washed beads were boiled for 5 min in the presence of 2% SDS, and the supernatants were reprecipitated with antibodies in lysis buffer containng 0.1% final SDS concentration. *In vitro* kinase assays were performed by incubating washed GST- or Cas-immunoprecipitates in the absence or presence of 2 units of semi-purified Lyn kinase (Upstate Biotechnology, Inc., Lake Placid, NY) in kinase buffer (10 mM Hepes, pH 7.3, containing 50 mM NaCl, 5 mM $MnCl_2$, 5 mM $MgCl_2$) containing 0.1 mM ATP (Sigma) for 20 min at room temperature. To assay Lyn autophosphorylation activity, kinase buffer containing 10 μCi of [γ-^{32}P]ATP (4500 Ci/mmol) was added to Lyn immunoprecipitates. The reaction was terminated by the addition of an equal volume of sample buffer and boiled at 95 °C for 3 min. Proteins were separated by 7.5% SDS-PAGE under reducing conditions and transferred to Immobilon-P^{TM} membranes (Millipore Corp., Bedford, MA). The Lyn kinase autophosphorylation gel was fixed and incubated in 1 N KOH for 1 h at 55 °C to reduce the background derived from phosphorylated serines and threonines before autoradiography. For Western blots, membranes were blocked using 5% nonfat dried milk in Tris-buffered saline-Tween 20 (20 mM Tris, pH 7.6, 130 mM NaCl, 0.1% Tween-20) and incubated for 1 h with specific antibodies in Tris-buffered saline-Tween 20. Immunoreactive bands were visualized by using secondary horseradish peroxidase-conjugated antibodies (Promega, Madison, WI) and chemiluminescence (ECL, Amersham, UK).

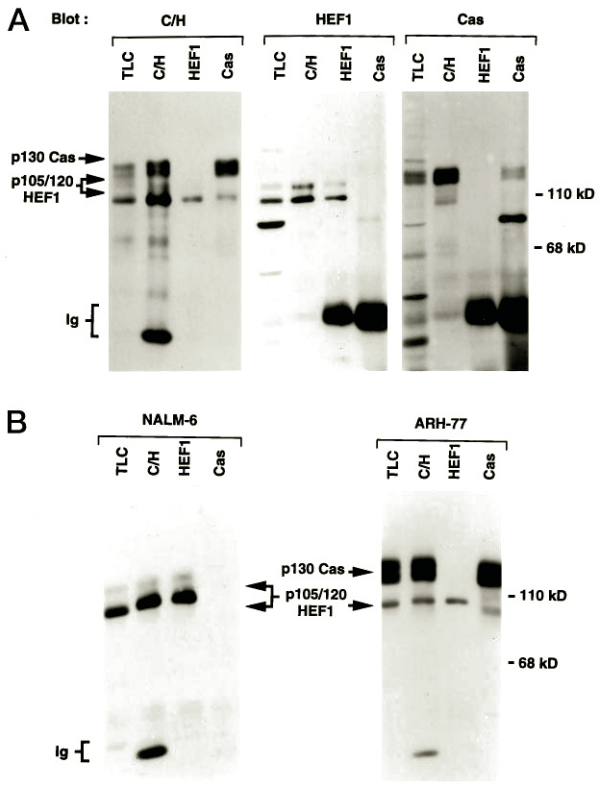

FIG. 1. **Expression of Cas and HEF1 in normal B cells and B cell lines.** *A*, total cell lysates from 5×10^6 unstimulated tonsillar B cells were immunoprecipitated with anti-C/H, anti-HEF1, or anti-Cas antibodies. Total cell lysate (*TLC*) or isolated proteins were separated by SDS-PAGE, transferred to Immobilon P^{TM} membrane, and immunoblotted (*Blot*) with anti-C/H, anti-HEF1, or anti-Cas antibodies as indicated. *B*, total cell lysates from 10×10^6 Nalm-6 or ARH-77 cells were immunoprecipitated with anti-C/H, anti-HEF1, or anti-Cas antibodies and immunoblotted using anti-C/H antibody. The position of Cas and HEF1 proteins (*arrows*) or of immunoglobulin (*bracket*), as well as molecular weight markers (*kDa*) are shown. Blots were imaged by chemoluminescence.

RESULTS

Expression of HEF1 and Cas in B Cell Lines and Normal B Cells—The expression of HEF1 and Cas was determined by using three different antibodies. Total cellular lysates from normal tonsillar B cells were subjected to immunoprecipitation with a monoclonal antibody raised against amino acids 644–819 of Cas (C/H), a polyclonal antibody raised against amino acids 426–439 of HEF1 (24), or a polyclonal antibody raised against the last 15 amino acids (949–963) in the C-terminal region of Cas. Membranes were then immunoblotted with either anti-Cas, anti-HEF1, or anti-C/H antibodies. Fig. 1*A* shows that anti-C/H recognized a complex of 105-, 120-, and a broad 130-kDa band. The 105-kDa and minor 120-kDa bands were immunoprecipitated and immunoblotted by both anti-C/H and anti-HEF1 antibodies but not by anti-Cas antibodies. The broad 130-kDa band, together with some minor bands ranging from 100 to 130 kDa, was immunoprecipitated and immunoblotted by both anti-C/H and anti-Cas antibodies but not by anti-HEF1 antibodies. These results indicated that the main broad 130-kDa band with additional 100–130-kDa minor species represents Cas, the 105- and 120-kDa bands represent

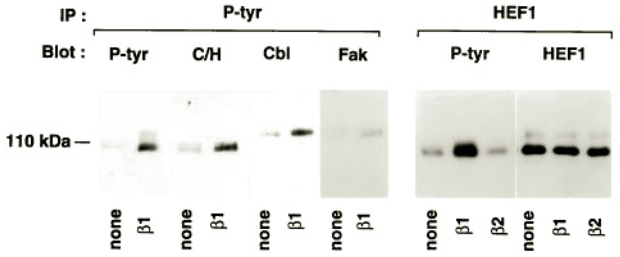

FIG. 2. **HEF1 is a main tyrosine phosphorylated substrate following β1 integrin ligation in Nalm-6 pre-B cells.** 10×10^6 cells/condition were unstimulated (*none*) or stimulated with anti-β1 or anti-β2 antibodies plus RαM for 30 min as indicated. *Right panel*, cell lysates were immunoprecipitated (*IP*) with anti-phosphotyrosine (*P-tyr*) and immunoblotted (*Blot*) with anti-P-Tyr, anti-C/H, anti-Cbl (*Cbl*), or anti-Fak (Fak) antibodies. *Left panel*, cell lysates were immunoprecipitated with anti-HEF1 antibodies and immunoblotted with anti-P-Tyr. The same membrane was stripped and reblotted with anti-HEF1 antibodies to show that equivalent amounts of protein were loaded in each lane.

HEF1, and that the anti-C/H antibody crossreacted with both proteins. The anti-Cas antibody immunoprecipitated an additional 90–95-kDa band (Fig. 1*A*, *right panel*, *Cas lane*) that was not immunoblotted with anti-C/H antibody (Fig. 1*A*, *left panel*, *Cas lane*) and only faintly with anti-HEF1 antibody (Fig. 1*A*, *central panel*, *Cas lane*). The anti-HEF1 antibody immunoblotted on total cellular lysate a similar 90–95-kDa band which was however not immunoprecipitated by this antibody. The nature of the 90–95-kDa bands is presently unknown.

Expression of Cas and HEF1 was then investigated in the pre-B cell line Nalm-6 and the myeloma line ARH-77. Total cell lysates were immunoprecipitated with anti-C/H, anti-HEF1, and anti-Cas antibodies followed by immunoblotting with anti-C/H. As seen in Fig. 1*B*, *left panel*, Nalm-6 cells expressed only $p105/120^{HEF1}$. Northern blot analysis of Nalm-6 for expression of human Cas mRNA confirmed the absence of Cas expression in this cell line (not shown). The anti-Cas antibody did not immunoprecipitate any of the HEF1 species, confirming that this antibody does not cross-react with HEF1. In contrast to Nalm-6, ARH-77 cells (Fig. 1*B*, *right panel*) expressed both $p130^{Cas}$ and the main p105 form of HEF1. Overexposure of the blot revealed the presence of the minor 120-kDa form of HEF1 as well (not shown). Again, the anti-HEF1 did not immunoprecipitate any of the Cas species, further illustrating the specificity of anti-HEF1 and anti-Cas antibodies. This suggests that these homologous proteins may be differentially expressed in B cells.

Tyrosine Phosphorylation of HEF1 and Cas Following β1 Integrin Ligation in Human B Cells—The pre-B cell line Nalm-6 was stimulated with the anti-β1 integrin mAb K20, followed by rabbit anti-mouse Ig for 30 min. Cellular lysates were then immunoprecipitated with anti-phosphotyrosine (P-tyr, 4G10) antibody followed by immunoblotting with anti-P-tyr, anti C/H, anti-Cbl, and anti-Fak. As seen in Fig. 2, *left panel*, the anti-C/H antibody reacted with the major β1 integrin-mediated tyrosine phosphorylated substrate pp105 in Nalm-6 cells, whereas anti-Cbl, and to a lesser extent anti-Fak antibodies, reacted with the pp120–125 bands. Immunoprecipitation with anti-HEF1 antibodies indicated that tyrosine phosphorylation of mainly p105 HEF1 was stimulated by β1 integrin ligation but not by β2 integrin ligation (Fig. 2, *right panel*). Longer exposure revealed some faint but detectable level of tyrosine phosphorylation of the 120-kDa HEF1 species.

We next compared HEF1 and Cas tyrosine phosphorylation in normal tonsillar B cells and ARH-77 cells, which express both proteins. Cellular lysates from these β1 integrin-stimulated cells were immunoprecipitated with either anti-HEF1 or

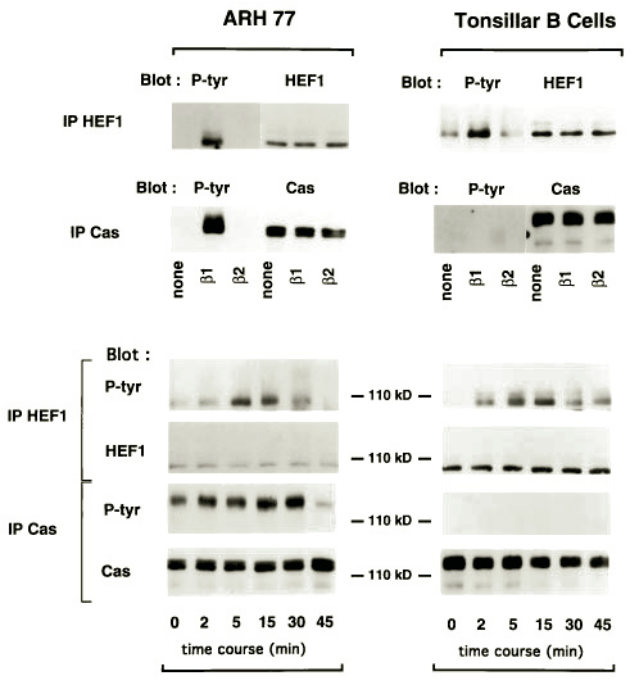

FIG. 3. **Integrin-stimulated tyrosine phosphorylation of Cas and HEF1 can be differentially regulated.** ARH-77 or normal tonsillar B cells were unstimulated (*none*) or stimulated with anti-β1 or anti-β2 antibodies for 30 min as desribed in Fig. 2 (*upper panels*), or they were stimulated with anti-β1 and lysed as a function of time (*lower panels*). Cell lysates from 5×10^6 cells/condition were immunoprecipitated (*IP*) with anti-HEF1 or anti-Cas antibodies and revealed by Western blot (*Blot*) with anti-P-tyr. The same membranes were stripped and reblotted with anti-HEF1 or anti-Cas antibodies to show that equivalent amounts of protein were loaded in each lane.

with anti-Cas, followed by immunoblotting with anti-P-tyr, anti-HEF1, or anti-Cas. As seen in Fig. 3, *upper left panel*, anti-HEF1 and anti-Cas immunoprecipitation demonstrated that β1 integrin ligation stimulated the tyrosine phosphorylation of both HEF1 and Cas in ARH-77 cells but only of HEF1 in tonsillar B cells (*upper right panel*). Similar to that of Nalm-6 cells, a fainter but detectable level of phosphorylation of the 120-kDa form of HEF1 could be detected in both ARH-77 and tonsillar B cells. In contrast to β1 integrin ligation, cross-linking of β2 integrins did not induce tyrosine phosphorylation of either HEF1 or Cas. These results indicate that following β1 integrin-mediated stimulation of B cells, tyrosine phosphorylation of Cas and HEF1 can be differentially regulated, with HEF1 being consistently phosphorylated in normal B cells and seven additional B cell lines examined (RPMI 8866, SB, Ramos, RL, DHL16, DHL6, and RPMI 8226, data not shown).

We investigated the kinetics of β1 integrin-mediated HEF1 and Cas phosphorylation (Fig. 3, *left* and *right lower panels*). An increase in tyrosine phosphorylation of HEF1 in ARH-77 and tonsillar B cells was detectable 2 min after β1 integrin cross-linking and reached maximal levels in 15 min. Similarly, β1 integrin-stimulated tyrosine phosphorylation of Cas was detected 2 min after stimulation in ARH-77 cells, but not in tonsillar B cells, reached maximum at 15 min, and declined thereafter.

HEF1 Is Tyrosine Phosphorylated After B-cell Antigen Receptor Engagement in Normal Tonsillar B Cells—We next examined whether HEF1 was also phosphorylated following BCR stimulation. Tonsillar B cells were stimulated with anti-IgM (b) or anti-IgG (c) $F(ab')_2$ antibodies for 30 min, and cellular lysates were immunoprecipitated with anti-HEF1 or anti-Cas antibodies. As seen in Fig. 4*A*, HEF1 was tyrosine phosphorylated after ligation of either surface IgM or IgG, whereas tyro-

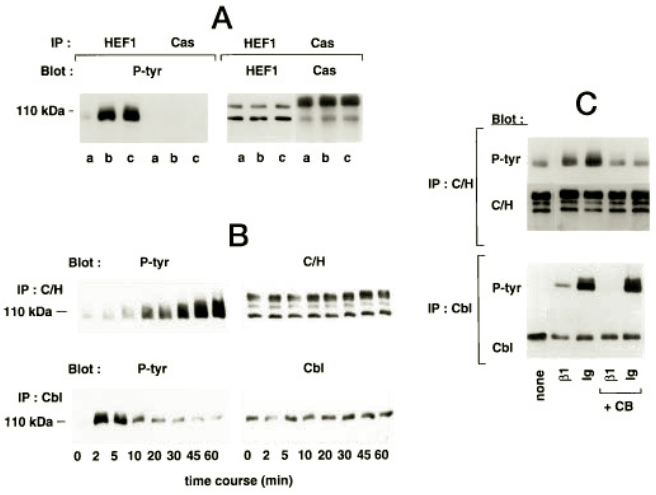

FIG. 4. **Tyrosine phosphorylation of HEF1, but not of Cas, following BCR engagement on tonsillar B cells and comparison to Cbl.** *A*, 10×10^6 tonsillar B cells were unstimulated (*a*) or stimulated with F(ab)′$_2$ goat anti-human IgM (*b*) or IgG (*c*) for 30 min as indicated. Cellular lysates were immunoprecipitated (*IP*) with anti-HEF1 or anti-Cas. Immunoprecipitated proteins were separated by SDS-PAGE and revealed by Western blot (*Blot*) using anti-P-tyr antibodies, or they were reblotted with anti-HEF1 and anti-Cas antibodies to show that equivalent amounts of protein were loaded in each lane. *B*, 10×10^6 tonsillar B cells/condition were stimulated with anti-(Fab′)$_2$ human IgG/M antibody and lysed as a function of time. HEF1/Cas or Cbl proteins were immunoprecipitated using either anti-C/H or anti-Cbl antibodies and immunoblotted with anti-P-tyr antibody. The same membranes were stripped and reblotted with anti-C/H or anti-Cbl antibodies to show that equivalent amounts of protein were loaded in each lane. *C*, 10×10^6 tonsillar B cells were preincubated in media alone or with 1 μM cytochalasin B (+ *CB*) for 1 h, followed by stimulation with anti-β1 antibodies plus RAM or with F(ab)′$_2$ goat anti-human IgM/G. Immunoprecipitated proteins from cellular lysates using anti-C/H or anti-Cbl antibodies were analyzed by Western blot as indicated.

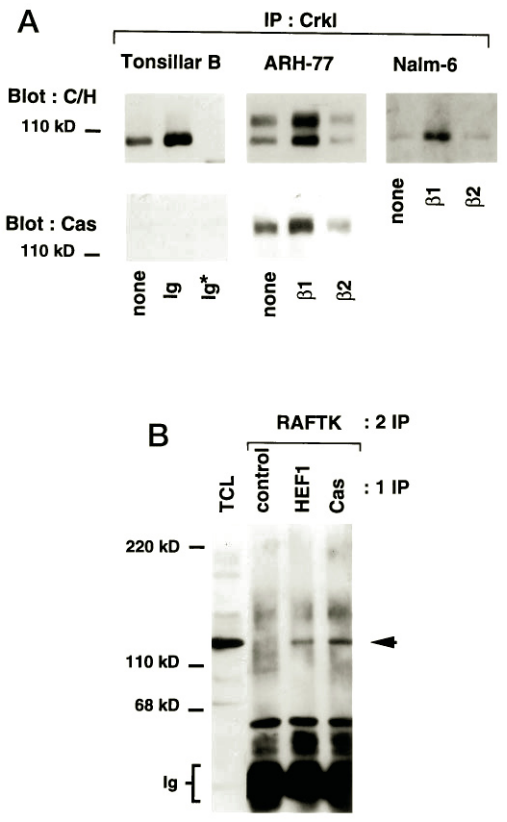

FIG. 5. **Association of the adapter protein CrkL and the related adhesion focal tyrosine kinase RAFTK with HEF1 and Cas.** *A*, Nalm-6 or ARH-77 cells were unstimulated (*none*) or stimulated with anti-β1 or anti-β2 integrin antibodies plus RAM for 30 min as indicated, and normal tonsillar B cells were unstimulated (*none*) or stimulated with F(ab)′$_2$ goat anti-human IgM/G for 30 min. Cellular lysates were immunoprecipitated with anti-CrkL antibodies and immunoblotted with anti-C/H or anti-Cas antibodies. An irrelevant antibody (*Ig**) was used to immunoprecipitate cellular lysate from BCR-stimulated tonsillar B cells to demonstrate the specificity of Crkl antibody. *B*, cell lysates from normal tonsillar B cells were immunoprecipitated with an irrelevant antibody (*control*) or with anti-HEF1 or anti-Cas antibodies (1 *IP*). After elution, the samples were reimmunoprecipitated with anti-RAFTK antibodies (2 *IP*) and immunoblotted with anti-RAFTK antibodies. A total cellular lysate (*TCL*) was run on the same gel. The position of RAFTK is indicated by an *arrow*.

sine phosphorylation of p130^{Cas} was largely undetectable. Again, the 105-kDa form of HEF1 was the main tyrosine phosphorylated species of HEF1.

Cbl also becomes tyrosine phosphorylated following ligation of β1 integrin or BCR (12, 26), therefore we investigated the kinetics of BCR-mediated HEF1 phosphorylation and compared it to Cbl. Tyrosine phosphorylation of HEF1 was detectable 5–10 min after the addition of anti-IgM/G F(ab′)$_2$ antibodies and reached maximal levels in 45–60 min (Fig. 4*B, upper panel*). In contrast, BCR-mediated tyrosine phosphorylation of Cbl was already maximal 2 min after stimulation and then decreased slowly but remained above the basal level after 60 min (Fig. 4*B, lower panel*). These results suggest that the mechanism of phosphorylation of HEF1 and Cbl are different in BCR-mediated signaling.

Both Integrin and BCR-mediated Tyrosine Phosphorylation of HEF1 Require an Intact Actin Network—To further investigate the mechanism of HEF1 phosphorylation, tonsillar B cells were preincubated with cytochalasin B (CB) to inhibit actin reorganization and then stimulated with antibodies directed against β1 integrin or BCR for 20 min. We have previously shown that cytochalasin B markedly decreased β1 integrin-mediated tyrosine phosphorylation of the 105–130-kDa substrates (11). As seen in Fig. 4*C*, the β1 integrin-mediated increase in tyrosine phosphorylation of both HEF1 and Cbl was prevented by cytochalasin B pretreatment (+ *CB*). However, BCR-mediated tyrosine phosphorylation of Cbl was unaffected by cytochalasin B pre-treatment, while phosphorylation of HEF1 was markedly reduced. The absence of an effect on Cbl phosphorylation argues against a toxic effect of cytochalasin B. These results indicate that cytoskeleton organization is required for both integrin or BCR-induced tyrosine phosphorylation of HEF1 in normal mature B cells.

Association of the Adapter Protein CrkL with HEF1 and Cas following B Cell Stimulation—Both Cas and HEF1 contain consensus SH2 binding motifs for the adapter protein Crk. Since CrkL can associate with Cas (27), we examined whether complexes of CrkL with Cas or HEF1 formed following β1 integrin or BCR stimulation. Stimulated Nalm-6, ARH-77, or normal tonsillar B cells were immunoprecipitated with antibody specific for CrkL followed by immunoblotting with anti-C/H or anti-Cas antibodies. An irrelevant antibody was used as an immunoprecipitation control on cellular lysate from BCR-stimulated tonsillar B cells (Fig. 5, *Ig**). As seen in Fig. 5*A*, following β1 or BCR ligation, but not β2 integrin stimulation, an increase in the amount of HEF1 was detected in CrkL immunoprecipitates from all three cell types. In addition, following β1 integrin stimulation of ARH-77 cells, an increase in the amount of Cas was present in CrkL immunoprecipitates. Consistent with the absence of tyrosine phosphorylation of Cas in tonsillar B cells, no detectable formation of Cas·CrkL complex in these cells was noted.

Association of the RAFTK with HEF1 and Cas—Both HEF1 and Cas associate with Fak through their SH3 domains bind-

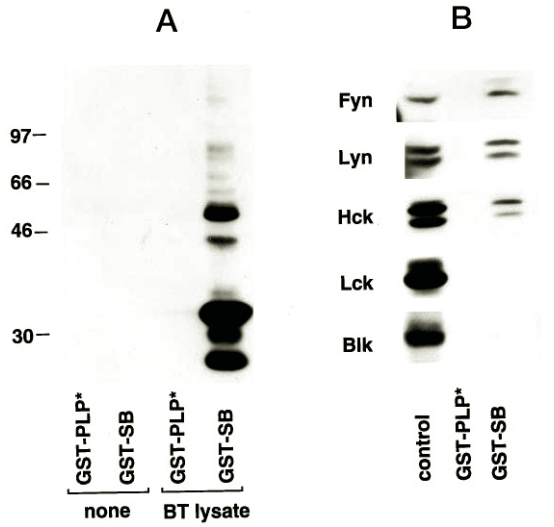

FIG. 6. ***In vitro* association of Src family members from tonsillar B cells with the C-terminal proline-rich region of Cas.** Cell lysates from 10×10^6 unstimulated tonsillar B cells were precipitated with the GST-fusion protein of Cas containing the RPLPSPP sequence (*GST-SB*) or a mutated GST-fusion protein in which the sequence RPLPSPP was converted to RSLGSPP (*GST-PLP**). *A*, precipitates from control lysis buffer (*none*) or from cellular lysates (*BT lysates*) were subject to *in vitro* kinase assay as described under "Experimental Procedures" and immunoblotted using anti-P-tyr antibody. *B*, control total cellular lysate (*control*) or GST-precipitates from tonsillar B cells were subjected to immunoblotting with antibodies indicated on the *left*.

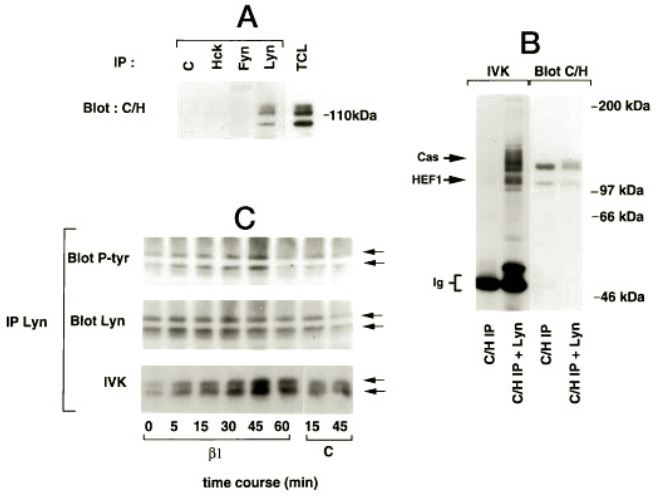

FIG. 7. **Lyn kinase associates *in vivo* with Cas/HEF1 and is stimulated following β1 integrin ligation on tonsillar B cells.** *A*, immunoprecipitations from 10×10^6 unstimulated tonsillar B cells with indicated antibodies (shown on *top*) or with an irrelevant antibody (*C*) were immunoblotted with anti-C/H antibody. A total cellular lysate was run on the same gel as a control (*control*). *B*, Cas/HEF1 immunoprecipitates from 10×10^6 unstimulated tonsillar B cells were subjected to an *in vitro* kinase assay or mixed with 2 units of purified Lyn kinase prior to the assay as indicated on the *bottom*. The phosphotyrosine content was analyzed by Western blot using anti-P-tyr antibody (shown on the *right*), and the membrane was then reblotted with anti-C/H antibodies to show that an equivalent amount of protein had been loaded in each lane. The positions of Cas and HEF1 are indicated. *C*, anti-Lyn immunoprecipitations from 10×10^6 tonsillar B cells stimulated with anti-β1 integrin plus RAM (*β1*) or with an irrelevant antibody plus RAM (*C*) for the indicated times were immunoblotted with anti-P-tyr (*Blot P-tyr*), stripped, and reblotted with anti-Lyn antibody to show that equivalent amounts of protein were loaded in each lane or subjected to *in vitro* kinase assay (*IVK*). *Two arrows* on the *right* indicate the positions of p53 and p56 Lyn, respectively.

ing to the C-terminal polyproline motif of Fak. We have recently shown that RAFTK (28) is tyrosine phosphorylated in B cells following integrin ligation and associates with $p130^{Cas}$ (29). Considering the homology between Fak and RAFTK, we examined whether HEF1 was associated with RAFTK. Cellular lysates of normal tonsillar B cells were immunoprecipitated with an irrelevant antibody (control) or with the anti-HEF1, or the anti-Cas antibody followed by a reimmunoprecipitation using RAFTK antibody and immunoblotted with anti-RAFTK antibody. As seen in Fig. 5*B*, RAFTK was detected in HEF1 and Cas immunoprecipitates but not in the control immunoprecipitate. Stimulation of HEF1 or Cas phosphorylation by integrin ligation did not increase the formation of Cas·RAFTK or HEF1·RAFTK complexes (not shown), suggesting that this association primarily involved the binding of the SH3 domains of HEF1 and Cas.

Association of $p59^{Fyn}$, $p59/62^{Hck}$, and $p53/56^{Lyn}$, but not $p55^{Blk}$ or $p56^{Lck}$, Src Kinases with the C-terminal Proline-rich Region of Cas—In v-Src transformed cells, Src binds via its SH3 domain to the RPLPSPP sequence of Cas (amino acids 733–739) (25). The Src-SH3 mediated binding has been proposed to be important in the tyrosine phosphorylation of Cas (25). To determine if kinases from the Src family could associate with Cas in human B cells, we used a GST-fusion protein of Cas containing the RPLPSPP sequence (Fig. 6, *GST-SB*) or a mutated GST-fusion protein in which the sequence RPLPSPP was converted to RSLGSPP (*GST-PLP**). Incubation of these GST-fusion proteins with a lysate of unstimulated tonsillar B cells followed by an *in vitro* kinase assay indicated that a kinase activity was associated only with GST-SB (Fig. 6*A*). The kinase activity was precipitated from the cellular lysate since control incubation of the GST-fusion proteins with lysis buffer only did not show any activity. Immunoblotting experiments further revealed that Fyn, Lyn, and, to a lesser extent, Hck were precipitated by GST-SB but not by GST-PLP* (Fig. 6*B*). These interactions were specific since Lck and Blk did not bind to GST-SB. Therefore, at least three kinases from the Src-family could potentially interact *in vivo* with the C-terminal proline-rich region of Cas in human B cells.

In Vivo Complex between Cas, HEF1, and $p53/56^{Lyn}$—To demonstrate an *in vivo* association of these kinases with Cas, membranes containing immunoprecipitated Fyn, Lyn, and Hck were reprobed with anti-C/H antibody. As shown in Fig. 7*A*, both HEF1 and Cas were detected in Lyn immunoprecipitates. This result was unexpected since HEF1 does not contain the RPLPSPP SH3 binding motif that is present in Cas (24). Although a longer exposure revealed a low level of these proteins in Fyn immunoprecipitates, they were not detected in immunoprecipitates prepared from control (*C*) or Hck antibodies. Stimulation of cells by β1 integrin or BCR ligation only slightly increased the formation of Cas/Lyn and HEF1/Lyn complexes (not shown), suggesting that the association primarily involved the binding of the SH3 domain of Lyn.

We further investigated whether Cas or HEF1 could be substrates for Lyn kinase. Anti-C/H immunoprecipitates from unstimulated tonsillar B cells were subjected to an *in vitro* kinase assay (*IVK*) in the presence or absence of purified Lyn kinase. As shown in Fig. 7*B*, a significant increase in tyrosine phosphorylation of both Cas and HEF1 was observed when they were added along with Lyn prior to the kinase assay. These results suggest that Cas and HEF1 could serve as substrates for Lyn kinase *in vivo*.

Activation of $p53/56^{Lyn}$ following β1 Integrin Cross-linking on Human Tonsillar B Cells—In addition to BCR ligation, a recent report has shown that Lyn kinase tyrosine phosphorylation is increased following integrin ligation in human B cell lines (30). Therefore, we investigated whether Lyn activation was also increased following β1 integrin ligation in normal

tonsillar B cells. Tonsillar B cells were stimulated with anti-β1 integrin antibody or with irrelevant antibody (*C*) and lysed as a function of time. As shown in Fig. 7*C*, *upper panel*, an *in vivo* increased tyrosine phosphorylation of Lyn was detectable 5 min after β1 stimulation of the cells and reached maximal levels in 45 min. Tyrosine phosphorylation of Lyn *in vivo* correlated with an increased autophosphorylation activity detected in an *in vitro* kinase assay (Fig. 7*C*, *lower panel*). Lyn activation was not induced by the irrelevant antibody.

DISCUSSION

Regulation of B cell survival within specific microenvironments involves integrin engagement (6–9). We previously reported that integrin-mediated signaling pathways in B cells regulates a cascade of tyrosine phosphorylation events (10, 11). In the present study, we have determined that $p130^{Cas}$ and the Cas-like molecule $p105^{HEF1}$ are expressed in B cells and that β1 integrin ligation or BCR engagement on human B cells promoted tyrosine phosphorylation principally of HEF1. Furthermore, HEF1 and Cas phosphorylation following both stimuli appeared to be closely linked with cytoskeletal organization, and we identified several signaling molecules, including $p53/56^{Lyn}$ kinase, RAFTK, and CrkL, associated both with Cas and HEF1.

HEF1 was cloned from a HeLa cDNA library, which when expressed in *Saccharomyces cerevisiae*, strongly enhanced pseudohyphal growth, suggesting a role for HEF1 in regulating cell signaling and morphology (24). Although HEF1 RNA was present in all tissues examined, the highest levels were in placenta, lung, and kidney. HEF1 is 64% similar to Cas at the amino acid level. Both proteins have multiple potential SH2 binding sites and a striking similarity in the SH3 domain and the C terminus. This raises the question as to why B cells express two very similar proteins. In tonsillar B cells as well as in B cell lines, both HEF1 and Cas were present with the exception of the pre-B cell line Nalm-6, which did not express Cas. β1 integrin-mediated tyrosine phosphorylation was mainly detected in HEF1 but not in Cas, except in the more terminally differentiated B cell line ARH-77. Generally, the p105 rather than the p120 form of HEF1 was the predominant species seen and tyrosine phosphorylated. Similarly, HEF1 rather than Cas was phosphorylated following BCR ligation in tonsillar B cells, however Cas could be phosphorylated under BCR ligation in the surface IgG positive cell lines, ARH-77 and SB.[3] Therefore, Cas appears to be phosphorylated only in more terminally differentiated cells, which suggests that Cas and HEF1 may have distinct functions depending on the differentiated state of the cell. Cellular localization studies provide further evidence for distinct functions of HEF1 and Cas and with Cas present at focal contacts, whereas HEF1 localizes to the cell periphery and the nucleus (24).

Similar to Cbl (12, 26), HEF1 is a common substrate in B cells for both integrin and antigen receptors. However, tyrosine phosphorylation of HEF1 and Cbl in fact differ in BCR-mediated signaling pathways. The kinetics of HEF1 phosphorylation was slower than that of Cbl. HEF1 phosphorylation was reduced by prior treatment of cells with cytochalasin B, whereas Cbl phosphorylation was not affected. These findings also suggest that BCR-mediated HEF1 phosphorylation correlated with actin filament reorganization. Interestingly, BCR ligation initiates microfilament assembly (31) and induces a redistribution of signaling molecules such as ras (32) and neurofibromin (33), which is inhibited by cytochalasin. Hence, analogous to integrin-mediated tyrosine phosphorylation (2), some aspects of BCR-mediated signal transduction may require that a functional cytoskeleton serve as a framework that regulates the efficiency of interactions between signaling molecules and allows tyrosine phosphorylation of compartmentalized cellular proteins.

The structure of Cas and HEF1 includes several SH2-binding motifs that are similar to the consensus binding motif for the Crk SH2 domain (34). We showed here that both Cas and HEF1 bind to CrkL. Furthermore, all tyrosine phosphorylated Cas and HEF1 associate with CrkL, and this interaction is mediated by the SH2 domain of CrkL.[4] Since the CrkL SH3 domain has been reported to bind to two guanine nucleotide exchange factors, C3G and mSOS, Cas and HEF1 might provide potential important links of β1 integrin and BCR signaling to the ras and or Rap1 pathways (35, 36). Therefore, by participating in a multimolecular complex formation, Cas and HEF1 may propagate downstream signals.

The focal adhesion kinase Fak can associate with both Cas and HEF1 (22, 24). These interactions are mediated by the highly homologous SH3 domains of Cas and HEF1, associating with the polyproline SH3 binding motif in Fak. We observed an *in vivo* association between the related adhesion focal tyrosine kinase RAFTK with Cas and HEF1. The interaction of Cas and HEF1 with RAFTK is also likely to be mediated through the SH3 domains of Cas and HEF1, binding to the C-terminal polyproline motif of RAFTK that is identical to that present in Fak. Since RAFTK is expressed in certain B cell lines independently of Fak and is phosphorylated under integrin and BCR stimulation (29), the associations of Cas and HEF1 with RAFTK may be important in these signaling pathways.

In contrast to HEF1, Cas contains a C-terminal proline-rich region that is an Src-SH3 binding motif (25). We have shown in an *in vitro* binding assay using a GST-fusion protein containing the C-terminal proline-rich region of Cas that $p53/56^{Lyn}$, $p59^{Fyn}$, and $p59/62^{Hck}$, but not $p55^{Blk}$ or $p56^{Lck}$, could bind to this motif. In addition, we demonstrated the presence of Cas in Lyn immunoprecipitates, whereas Cas was only weakly detected in Fyn immunoprecipitates and not in Hck immunoprecipitates. The anti-Fyn and anti-Lyn antibodies were raised against similar regions of the two molecules, allowing the comparison between them. The anti-Hck antibody was raised against a different region of the kinase, and we can not exclude the possibility that this antibody may have interfered with Cas binding. The interaction of Cas with Lyn *in vivo* likely occurred through the SH3 domain of Lyn because (i) a GST-fusion protein mutated in the C-terminal proline-rich region of Cas was unable to precipitate Lyn, and (ii) Cas phosphorylation following integrin or BCR ligation only minimally increased the presence of Cas in Lyn immunoprecipitates (not shown). HEF1 was also present in Lyn immunoprecipitates. However, HEF1 does not possess the src-SH3 binding motif that is present in Cas. Similar to Cas, HEF1 phosphorylation did not significantly increase its association with Lyn (not shown). Whether HEF1 can associate with Lyn through a non-canonical SH3 binding motif is under investigation. Alternatively, the C-terminal region of Cas may be capable of mediating heterodimerization with HEF1 (24), and therefore, the Lyn immunoprecipitation may involve a ternary Lyn·Cas·HEF1 complex. Lyn kinase activity is stimulated following both BCR ligation (30) and as shown here following β1 integrin ligation, suggesting that Lyn may be a kinase for Cas/HEF1. In support of this is that Lyn kinase could phosphorylate Cas/HEF1 *in vitro* when mixed prior to the kinase assay. Whether Cas/HEF1-associated Lyn is activated and responsible for Cas/HEF1 phosphorylation

[3] S. N. Manie, A. Astier, and A. S. Freedman, unpublished data.

[4] A. Astier, S. N. Manie, S. F. Law, T. Canty, N. Haghayeghi, B. J. Druker, R. Salgia, E. A. Golemis, and A. S. Freedman, submitted for publication.

during the process of integrin or BCR ligation remains to be determined. Cas/HEF1-associated Lyn may also be involved in the phosphorylation of other molecules recruited by Cas/HEF1.

The cytoskeletal dependence for HEF1 phosphorylation following integrin or BCR engagement on normal tonsillar B cells raises the possibility that HEF1 could integrate signals from both receptors. In T cells, signals from integrin and T cell antigen receptor have synergistic effects on proliferation (37–39). Similarly, there is evidence for a functional cross-talk between integrins and BCR from studies of ligation of both receptors, where there appears to be modulation of normal B cell proliferation.[3] Future studies will be directed toward understanding the function of HEF1 in integrin and BCR signaling pathways and gaining insight into the association of adhesion with antigen-induced activation of B cells.

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