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B cell receptor signaling involves physical and functional association of FAK with Lyn and IgM

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Abstract B cell receptor (BCR) stimulation induces phosphorylation of a number of proteins, leading to functional activation of B lymphocytes. Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase, involved in a variety of signaling pathways. In this study, we show that FAK is tyrosine-phosphorylated and activated following BCR stimulation. We also demonstrate constitutive association of FAK with the Src-family kinase Lyn and with components of the BCR. Association of Lyn with FAK which was not correlated with BCR-induced activation of both kinases, appeared to be mediated via the binding of Lyn to the COOH-terminal part of the FAK molecule. Our results indicate that FAK is a component of the BCR complex and that it participates in BCR signaling.

Key words: B lymphocyte; Focal adhesion kinase; Lyn kinase; Signal transduction

1. Introduction

B cell antigen receptor (BCR) is a molecular complex consisting of a transmembrane immunoglobulin (Ig) chain, which recognizes antigen, and Ig-α/Ig-β dimers, which function as signal transducers [1]. The Src-family protein tyrosine kinase Lyn is thought to be a key initiator of BCR signaling [2] by virtue of its physical association with the receptor and its immediate activation upon BCR cross-linking [3,4].

Focal adhesion kinase (FAK) is a 125-kDa non-receptor, proline-rich protein tyrosine kinase [5–7]. It possesses a central catalytic domain and large NH_2- and COOH-terminal non-catalytic regions devoid of Src homology (SH)2 and SH3 sequences [5]. In addition, the COOH-terminal domain contains a focal adhesion targeting sequence (FAT), which is responsible for targeting FAK to focal contacts and is involved in signaling-induced cytoskeletal rearrangement [8,9]. In some cell types, the COOH-terminal domain of $p125^{FAK}$ is expressed autonomously as a 41-kDa protein termed focal adhesion kinase (FAK)-related non-kinase (FRNK), representing a kinase-negative domain that functions as an endogenous regulator of FAK activity [10,11].

Activation of FAK involves autophosphorylation of Tyr-397, which lies within a sequence motif representing a high-affinity binding site for the SH2 domain of Src-family protein tyrosine kinases (PTKs) [10,12]. The formation of Src–FAK complex leads to increased catalytic activity of FAK, and phosphorylation of other tyrosine residues on FAK that results in creation of specific docking sites for multiple proteins [13]. FAK interacts with other signaling molecules through its SH2-binding motifs or proline-rich regions. For instance, Fyn, Src, Csk, the p85 subunit of phosphatidylinositide 3-kinase, and GRB2 are known to associate with FAK through their SH2 domains, whereas $p130^{cas}$ (Crk-associated substrate) binds the proline-rich region of FAK through an SH3 domain. Other potential physiological substrates of FAK, such as cytoskeletal proteins paxillin and tensin, bind via specific sequences [14]. FAK is tyrosine-phosphorylated in response to distinct cellular stimuli including integrin-mediated cell adhesion, mitogenic neuropeptides, bioactive lipids, polypeptide growth factors, and bacterial toxins. FAK-mediated signal transduction is implicated in embryonic development and in the control of cell migration [15] proliferation, and apoptosis [16,17].

Previous studies have shown FAK expression in lymphocytes [18,19], and its activation upon stimulation of β1-integrin and TCR [20–23]. Here we investigate the role of FAK in B lymphocytes and demonstrate that FAK was activated following the surface IgM cross-linking. We also show FAK association to Src-family kinase Lyn and to components of BCR. We postulate the role of FAK to be in linking early BCR signaling events to cytoskeletal reorganization.

2. Materials and methods

2.1. Antibodies

$F(ab')_2$ fragment of goat anti-mouse IgM was from Cappel (Durham, NC, USA). Goat anti-mouse IgM, and anti-Flag M2 antibodies were from Sigma (St. Louis, MO, USA), and goat anti-mouse IgG from Southern Biotech Ass. Inc. (Birmingham, AL, USA). Antibodies specific to N- or C-terminal FAK (A-17, C-20), and anti-p107 were from Santa Cruz Biotech. Inc. (Santa Cruz, CA, USA). Rabbit polyclonal anti-FAK antibodies JF1 were described previously [15]. Biotin-conjugated anti-phosphotyrosine MAb (4G10) and MAb to FAK (2A7) were from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Mouse monoclonal anti-Lyn antibody (Lyn 8) was from Wako Pure Chemicals (Osaka, Japan). HM79, a hamster monoclonal antibody against mouse Ig-β was a kind gift of Dr. Kisaburo Nagata, Tokyo Metropolitan Institute [24].

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Abbreviations: FAK, focal adhesion kinase; FRNK, FAK-related non-kinase; GFP, green fluorescent protein; Pyk2, proline-rich tyrosine kinase-2; PTK, protein tyrosine kinase; SH, Src homology

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2.2. Transient transfections

Fibroblast cell line 293T was cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS at 37°C under 5% CO_2. Cells (1×10^5/10 cm dish) were plated overnight and transfected by the calcium phosphate method with 8 μg of plasmids expressing FAK or FRNK, and then cultured for 48 h before harvesting. Cells were lysed in 1 ml of TNE lysis buffer per 10 cm dish (1% NP-40, 50 mM Tris, pH 8.0, 150 mM NaCl, 20 mM EDTA, 0.1 mM sodium orthovanadate, 10 mM sodium fluoride, 10 μg/ml aprotinin). To recover Lyn immune complexes, 200 μl of cell lysates was immunoprecipitated with 2 μg of anti-Lyn antibody and analyzed on sodium dodecyl sulfate–plyacrylamide gel electrophoresis (SDS–PAGE). The constructs expressing mouse FAK and FRNK were a kind gift of Jiro Fujimoto (IMSUT, University of Tokyo, Japan) and Dusko Ilic (University of California, San Francisco, CA, USA) [25], respectively. In short, the cDNA encoding mouse FAK was cloned into pME18S expression vector, and the sequence of FRNK was tagged with the double hemagglutinin (HA) and cloned in-frame into expression vector pEGFP-C1 (Clontech Laboratories, Palo Alto, CA, USA), to obtain green fluorescent protein (GFP)-FRNK-HA tagged fusion protein.

2.3. Cell activation and immunoprecipitation

WEHI 231 cell line and J2-transformed murine B lymphocytes were cultured in RPMI 1640 containing 10% FCS and 50 μM 2-ME, at 37°C under 5% CO_2. Surface IgM of WEHI 231 cells or immortalized mouse cell lines (2×10^7 cells) was cross-linked with either 10 μg of goat anti-IgM (Sigma) or F(ab′)$_2$ fragment of anti-IgM (Cappel) at 37°C for designated period (1 or 5 min). Activation was stopped by addition of 1 ml of ice-cold TNE lysis buffer. Unstimulated cells were first lysed and thereafter the anti-IgM antibody was added in order to normalize lysate composition. IgM immunoprecipitates were recovered by incubating with protein G-Sepharose (Pharmacia Biotech Inc.). To recover Ig-β, FAK, or Lyn immune complexes, the lysates were first precleared with excess amount of protein G-Sepharose, then incubated with anti-Ig-β, anti-FAK (2A7) or anti-Lyn antibodies, respectively. Immune complexes were collected on protein G-Sepharose beads. When FAK was immunoprecipitated by C-20 or N-17 antibodies the lysates were precleared with protein A-Sepharose, which was used as well for complex capture. Upon intense washing in ice-cold lysis buffer, pellets were resuspended in SDS sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerol, 200 mM DTT, and 0.02% bromophenol blue) and boiled for 5 min.

2.4. Immunoblotting

Proteins in immunoprecipitates or lysates were separated on 7.5% SDS–polyacrylamide gels followed by blotting to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked in 5% BSA (Boehringer, Mannheim, Germany) in TBS buffer (10 mM Tris, pH 7.4, 150 mM sodium chloride). For anti-phosphotyrosine immunoblotting, the membranes were incubated with 1 μg/ml of biotinylated 4G10 antibody (Upstate Biotechnology Inc.). Blots were further incubated with streptavidin-horseradish peroxidase (sa-HRPO). Reactive bands were visualized by the use of enhanced chemiluminescence detection system (Du Pont). For immunodetection of other antibodies, alkaline phosphatase (ALP)-conjugated anti-rabbit or anti-mouse antibodies were used (Promega, Madison, WI, USA) and visualized by the NBT/BCIP colorimetric method. Donkey anti-goat IgG-ALP was detected by the Vista ECF (Amersham Pharmacia Biotech) system. Membranes developed by ECF reagent (Amersham Pharmacia Biotech) were submerged to quantitation and scanning on a Storm phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA).

2.5. Immunoprecipitated FAK kinase assay

FAK was immunoprecipitated from cell lysates (corresponding to 5×10^6 cells/sample) with 2A7 MAb. Upon intense washing, each pellet was resuspended in 20 μl of kinase buffer (40 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 0.1 mM sodium orthovanadate). The reaction was carried out at 37°C for 10 min in the kinase buffer including 0.25 μCi [γ-^{32}P]ATP (NEN, Life Science Products), and terminated by the addition of SDS sample buffer. The proteins were resolved by SDS–PAGE (8%), and ^{32}P-incorporated proteins were analyzed after autoradiography or by densitometer scanning using phosphorimager BAStation-2500 (Fuji Film, Japan).

3. Results

3.1. FAK is tyrosine-phosphorylated and activated upon BCR cross-linking of WEHI 231

Tyrosine kinase FAK has been implicated in the regulation of signaling pathways that are relevant to growth, differentiation, and activation of cells. The function of FAK in B cells was studied in WEHI 231 cells. Surface IgM was ligated with intact anti-IgM antibody for 1 or 5 min and the tyrosine phosphorylation level of proteins from whole cell extracts was determined. We detected stimulation-dependent phosphorylation of numerous cellular proteins with maximal phosphorylation detected at 1 min (Fig. 1A), as reported previously. To examine the tyrosine phosphorylation levels of FAK the kinase was immunoprecipitated from lysates of unstimulated or IgM-stimulated WEHI 231 cells. Anti-FAK (JF1) immune complexes were analyzed by immunoblotting for the presence of phosphoproteins. An increase in the level of tyrosine phosphorylation of FAK was observed following BCR cross-linking, with maximum phosphorylation at 1 min that diminished by 5 min. Reprobing of the blot with anti-

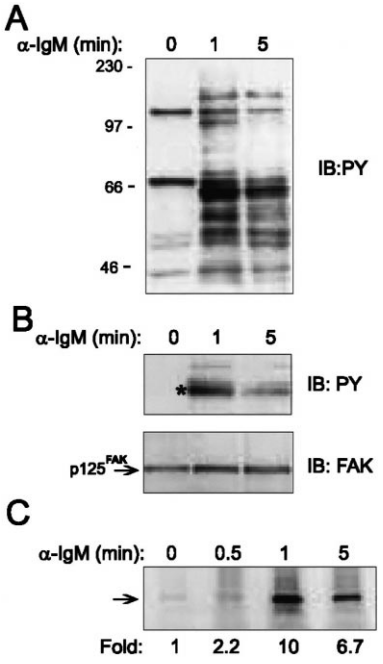

Fig. 1. BCR signaling involves tyrosine phosphorylation and activation of FAK. A: WEHI 231 cells were stimulated with anti-IgM antibody for the indicated time. Protein from whole cell lysates (2×10^5 cells/lane) were fractionated on SDS–PAGE (7.5%), transferred to nitrocellulose filters, and analyzed for the presence of tyrosine-phosphorylated proteins (IB: PY). Molecular size markers are indicated. B: Anti-FAK (JF1) immunoprecipitates (IPs) prepared from 2×10^7 unstimulated (0), and anti-IgM-stimulated (1 and 5 min) WEHI 231 cells. IPs were separated by SDS–PAGE (7.5%). The blotted filters were probed with anti-phosphotyrosine antibody (upper panel, IB: PY). Phosphorylated FAK is indicated by an asterisk. To demonstrate that equal amounts of proteins were immunoprecipitated, the same filters were reprobed with anti-FAK (JF1, IB: FAK). C: In vitro kinase reaction. Precleared lysates were immunoprecipitated with anti-FAK (2A7) antibody. After intense washing, the IPs were subjected to in vitro kinase reaction for 10 min in the kinase buffer including [γ-^{32}P]ATP. Numbers below the lanes indicate the fold increase in band intensity for a particular assay.

FAK antibodies confirmed that equivalent amounts of precipitated proteins were loaded (Fig. 1B). These data suggest that FAK is involved in BCR transduced signaling in WEHI 231 cells. Comparable results were obtained by immunoprecipitating FAK with either polyclonal (JF1) or monoclonal (2A7) anti-FAK antibodies (data not shown). In addition, anti-FAK blots (Fig. 1B, lower panel) reveal two immunoreactive bands having different relative mobility, suggesting that FAK exists in multiple isoforms. This result is consistent with data published for other cell lines; several splice variants of FAK were isolated from neuronal tissues [26].

Since kinase activity and phosphotyrosine levels of FAK are related, proteins immunoprecipitated with anti-FAK (2A7) antibody from IgM-stimulated or non-stimulated WEHI 231 cells were assayed for kinase activity. Levels of FAK autophosphorylation were determined by the levels of incorporation of ^{32}P. Maximal autokinase activity was reached after 1 min of BCR cross-linking, representing a 5- to 10-fold average increase in kinase activity. FAK activation diminished by 5 min of BCR stimulation, suggesting the involvement of FAK in early BCR signaling (Fig. 1C).

3.2. FAK signaling complex involves Lyn

The above data clearly demonstrate the involvement of FAK in early BCR signal transduction, having activation kinetics similar to those of the Src-family PTKs. Since activated FAK interacts with Src-like kinases [8,12,13], we have hypothesized that FAK interacts with Lyn during BCR signaling.

To investigate the relationship of FAK with Lyn, we examined whether these proteins associated with each other. Lysates of untreated or anti-IgM treated WEHI 231 cells were incubated with either anti-Lyn or anti-FAK antibodies, and the immune complexes were subjected to immunoblotting with anti-phosphotyrosine antibody. The analyses of FAK immunoprecipitates revealed activation-dependent induction of several phosphoproteins, among which were the proteins of apparent molecular mass of 125 and 50–60 kDa. When this same blot was probed with anti-Lyn antibody, equimolar amounts of Lyn and FAK proteins were detected regardless of anti-IgM treatment (Fig. 2, left panel). Reciprocal results were obtained by probing Lyn immunoprecipitates with anti-phosphotyrosine antibody. Apart from the tyrosine-phosphorylated p53/56 band the phosphotyrosine of an apparent molecular mass of 125 kDa was detected upon anti-IgM treatment. Reprobing the same membrane with anti-FAK antibodies revealed that this protein has the same mobility as FAK protein. These data showed Lyn kinase to be constitutively associated with FAK and associated FAK to be tyrosine-phosphorylated 1 min after BCR stimulation. The kinetics of FAK tyrosine phosphorylation appears similar to those of Lyn (Fig. 2, right panel). Our findings strongly imply that both molecules are engaged in very early events of BCR signaling.

3.3. The carboxy-terminal part of FAK is sufficient for the association with Lyn

Autophosphorylation of FAK Tyr-397 provides a direct binding site for the SH2 domain of Src-family PTKs. Thus, formation of a FAK–Src signaling complex is thought to be dependent on phosphorylation of Tyr-397 and the activity of both kinases [27]. Association of Lyn and FAK kinases in a

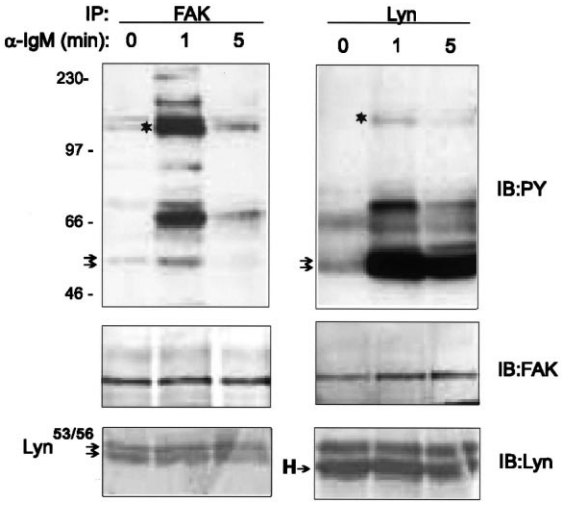

Fig. 2. FAK constitutively associates with Lyn in WEHI 231 cells. FAK (N-17, left panel) and Lyn (right panel) were immunoprecipitated from lysates of unstimulated (0), and anti-IgM-stimulated (1 and 5 min) WEHI 231 cells (1×10^7 cells/assay). The immunoprecipitates were separated by SDS–PAGE (7.5%). To detect tyrosine-phosphorylated proteins, the filters were immunoblotted with anti-phosphotyrosine antibody (IB: PY). Asterisks indicate apparent position of FAK, and the double arrowheads of Lyn migration. Molecular size markers are shown on the left side of the panel. Amounts of immunoprecipitated FAK or Lyn were determined by reprobing the same filters with anti-FAK (IB: FAK) and anti-Lyn (IB: Lyn) antibodies. The mobility of $p53^{lyn}$ appears to overlap with the heavy chain of the precipitating antibody (H).

WEHI 231 cell line (Fig. 2), however, implies that the activity of these kinases is not necessary for their interaction. To identify a sequence on FAK required for the constitutive association with Lyn we checked whether the COOH-terminal part of the FAK molecule is sufficient for interaction with Lyn. FAK, or its COOH-terminal isoform FRNK, was overexpressed by transiently transfecting 293T cells with expression plasmids carrying FAK or FRNK cDNAs. Expression of FAK or FRNK proteins and their effect on overall protein phosphorylation levels were determined by immunoblotting with anti-phosphotyrosine antibodies. Lysates from cells overexpressing FAK, but not FRNK, revealed an overall increase of tyrosine phosphorylation and extensive tyrosine phosphorylation of polypeptide with a relative mobility of 125 kDa (Fig. 3, lane 5). We next examined the possible interaction between Lyn and FAK or Lyn and FRNK. Anti-phosphotyrosine immunoblotting of anti-Lyn immunoprecipitates (Fig. 3, lanes 1–3) had identified the phosphoproteins of an approximate molecular mass of 15 kDa that were, upon anti-FAK blotting, identified as FAK proteins. An anti-FAK reactive double band with relative mobility of 66 kDa was detected in anti-Lyn immunoprecipitates of lysates overexpressing FRNK.

The mobility of these bands corresponds to that of GFP-FRNK-HA fusion protein. The identity of the fusion protein was confirmed in separate experiments with anti-HA antibody (data not shown). These results demonstrate co-immunoprecipitation of endogenous Lyn kinase with transiently expressed FAK and FRNK proteins in 293T cells and indicate that the COOH-terminal part of the FAK molecule is sufficient for association with Lyn. Association of endogenous Lyn and FAK kinases in 293T cells was observed when larger

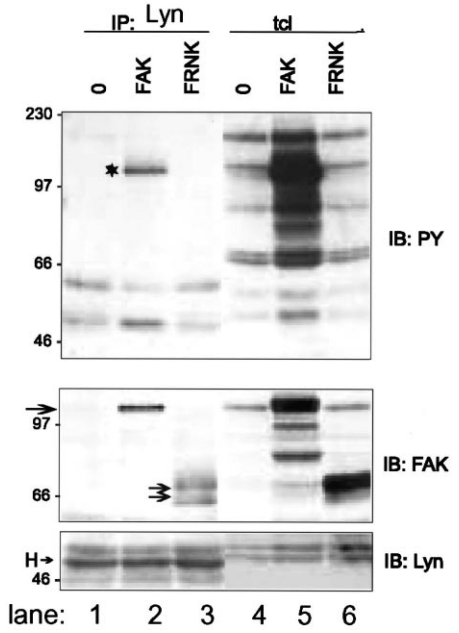

Fig. 3. Lyn associates with FAK and FRNK. 293T cells were transfected with plasmids containing FAK or FRNK cDNAs and lysed in TNE buffer (1×10^7 cells/ml) 48 h after transfection. Lyn was immunoprecipitated from 200 μl of lysates of non-transfected cells (0; lane 1), or cells overexpressing FAK (FAK; lane 2), or FRNK (FRNK; lane 3). The immunoprecipitates and the total cell lysates (tcl, 2×10^5 cells/lane, lanes 4–6) were subjected to SDS–PAGE. Separated proteins were transferred to nitrocellulose filters and immunostained. Upper panel is anti-phosphotyrosine (IB: PY) blot, which was reprobed with anti-FAK (middle panel, IB: FAK) or anti-Lyn (bottom panel, IB: Lyn) antibodies. Position of FAK is indicated by arrow, and of FRNK by double arrow. Endogenous FAK can be detected in tcl of control and FRNK transfected cells (lanes 4 and 6). The mobility of $p53^{lyn}$ overlaps with the heavy chain of the precipitating antibody (H).

amounts of lysates were used (data not shown). These findings support constitutive association of Lyn and FAK in unstimulated WEHI 231 cells, where both kinases are believed to be inactive. However, we are unable to conclude, at this stage, whether this interaction is due to the direct binding of Lyn to FAK or FRNK, or due to binding of both kinases to an intermediate molecule.

3.4. Evidence of FAK localization within proximity of the IgM complex

To further our understanding of the underlying mechanism of FAK–Lyn interaction, we examined FAK localization relative to other components of the BCR. WEHI 231 cells were stimulated with anti-IgM antibody and lysed in TNE buffer. The same amount of anti-IgM antibody used to stimulate cells was added to the extracts of unstimulated cells in order to normalize lysate composition. IgM complexes were recovered by incubating lysates of stimulated or non-stimulated cells with protein G-Sepharose. Western blotting of IgM immunoprecipitates with anti-FAK antibodies revealed the presence of a 125-kDa FAK immunoreactive band, demonstrating constitutive association of FAK with the BCR complex (Fig. 4A, lanes 1, 2). We have subsequently analyzed anti-Ig-β immunoprecipitates. WEHI 231 cells were stimulated with $F(ab')_2$ fragment of anti-IgM antibody. Then, the lysates were precleared and immunoprecipitated with anti-Ig-β antibodies.

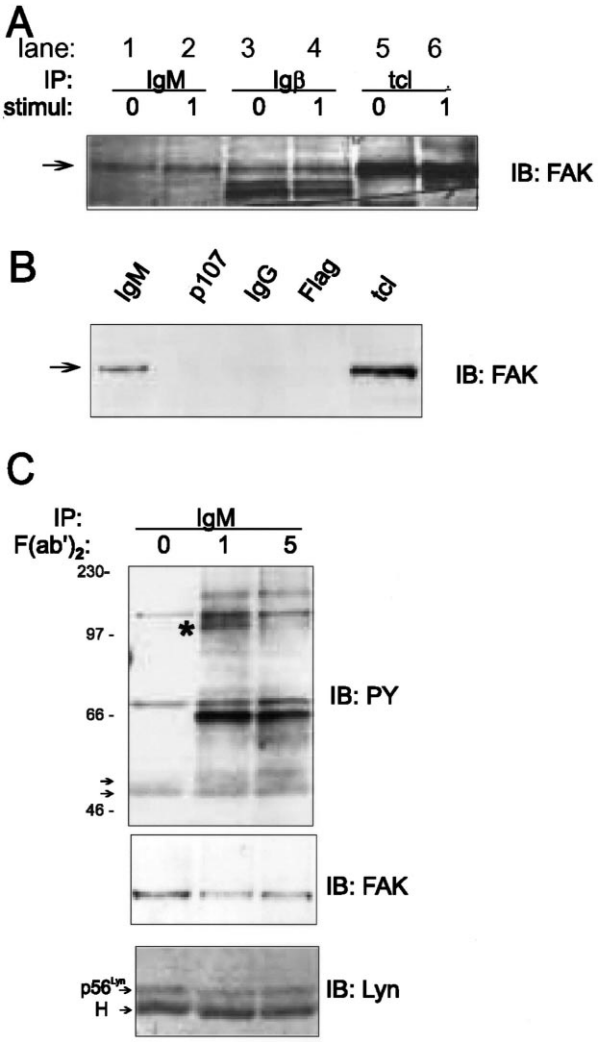

Fig. 4. FAK is constitutively complexed with BCR and is tyrosine-phosphorylated after BCR cross-linking. A: WEHI 231 cells were stimulated by cross-linking of surface IgM by anti-IgM antibody (1, 1 min), unstimulated (0) aliquots were treated with same amount of antibody. The IgM immune complexes were recovered from lysates by protein G-Sepharose (lanes 1, 2). Similarly, cells were stimulated by $F(ab')_2$ fragment of anti-IgM, and Ig-β immune complexes were recovered from lysates of unstimulated (0) or stimulated (1) cells (lanes 3, 4). Proteins in the immunoprecipitates and total cell lysates (tcl) (lanes 5, 6) were separated by SDS–PAGE (7.5%), transferred to nitrocellulose filters, and immunostained with anti-FAK antibody (IB: FAK). Arrow indicates the position of $p125^{FAK}$. B: Lysates of 2×10^7 WEHI 231 cells were precleared with protein G-Sepharose and immunoprecipitated with anti-IgM (IgM), anti-p107 (p107), anti-Flag (Flag), or anti-IgG (IgG) antibodies. Lysate corresponding to 5×10^5 cells was loaded as a control (tcl). C: J2-transformed murine B lymphocyte cells were stimulated by cross-linking with the $F(ab')_2$ fragment of anti-IgM antibody for 1 and 5 min. The cell lysates of unstimulated aliquots (0) were treated with the same amount of antibody. Protein G-Sepharose precleared lysates (10^7 cells/assay) were immunoprecipitated with intact goat anti-IgM antibody. Immunoprecipitates were separated by SDS–PAGE (7.5%), transferred to a nitrocellulose filter, and immunostained with anti-phosphotyrosine antibody (IB: PY; upper panel). The position of apparent FAK migration is marked by asterisks, and of Lyn by double arrows. The amount of FAK co-immunoprecipitated with IgM was determined by reprobing the same membrane with anti-FAK antibody (IB: FAK). The filter was also probed by anti-Lyn antibody (IB: Lyn). Arrow indicates $p56^{lyn}$. $p53^{lyn}$ appears to overlap with the heavy chain of the precipitating antibody (H).

Analyses of anti-Ig-β immunoprecipitates revealed stimulation-independent assembly of FAK with Ig-β of the BCR complex. In addition to the 125-kDa FAK protein, a 110-kDa polypeptide reactive with anti-FAK antibodies was also detected in anti-Ig-β immunoprecipitates, the identity of which was not evaluated (Fig. 4A, lanes 3, 4). In order to rule out the non-specific binding of FAK to IgM, lysates of WEHI 231 cells were subjected to immunoprecipitation with irrelevant mouse monoclonal antibodies such as anti-p107, -Flag, or -mouse IgG. None of these control immunoprecipitates contained FAK protein (Fig. 4B). Our data clearly demonstrate the presence of FAK in anti-IgM or anti-Ig-β immunoprecipitates, suggesting that FAK is localized proximal to the BCR. To confirm the notion of FAK association with Lyn and components of BCR, we have assayed the J2-transformed murine B cells. TNE lysates of unstimulated or $F(ab')_2$-stimulated cells were precleared, immunoprecipitated with anti-IgM or anti-Ig-β antibodies, and assayed for the presence of complexed phosphoproteins and amounts of co-immunoprecipitated FAK and Lyn. Several anti-IgM-associated phosphoproteins were detected in Western blots stained with anti-phosphotyrosine antibodies. By reprobing the same blots with anti-FAK and anti-Lyn antibodies, we revealed a constitutive association of FAK and Lyn with the BCR complex (Fig. 4C). Equivalent results were obtained by anti-Ig-β antibodies (data not shown).

4. Discussion

PTKs play a substantial role in BCR signal transduction. We examined involvement of the focal adhesion kinase in this process. FAK is expressed and tyrosine-phosphorylated in either primary B and T cells as well as transformed cell lines [18,19]. It becomes tyrosine-phosphorylated upon β1-integrin ligation of human B cells [20,21] and TCR cross-linking on Jurkat T cell line and murine CD8+ cytotoxic cells [22,23].

In elaborating the role of FAK in BCR signaling events we provide evidence that FAK becomes tyrosine-phosphorylated and activated as a consequence of surface IgM cross-linking on WEHI 231 cells and murine J2-transformed B lymphocytes. In vitro cross-linking of the BCR induces activation of several proteins. Lyn and FAK become activated after BCR stimulation with apparently similar kinetics. Both FAK and Lyn kinases are associated since FAK was detected in Lyn immunoprecipitates and Lyn was detected in FAK immunoprecipitates. The interaction of FAK and Src kinases is mediated via the phosphorylated Tyr-397 of FAK and the SH2 domain of Src [12]. Phosphorylation of Tyr-397 on FAK facilitates the formation of a FAK–Lyn signaling complex in which both kinases are active. However, in our experiments, interaction of FAK and Lyn kinases was detected in both IgM-stimulated and non-stimulated samples, implying that the activity of these kinases is not necessary for their interaction, and thus indicating activation-independent association of both kinases. Therefore, we proposed a new mode of Lyn–FAK interaction and showed that the COOH-terminal part of the FAK molecule, which corresponds to FRNK, is sufficient for interaction with Lyn. This novel finding can be explained by the presence of several protein–protein interaction sites located in the COOH-terminal part of FAK. There are two proline-rich sequences, P^{712}PKPSRPGYPSP and P^{874}PKKPPRPGAP, that bind SH3 domains of $p130^{cas}$ [28] or Cas-related protein HEF1, Graf, and Cdc42 [29] and, thus, represent a likely candidate for the interaction of FAK and Lyn in an activation-independent manner. Perhaps binding of Lyn and FAK through such an interaction is a prerequisite for the immediate and optimal activation of both kinases after receptor stimulation. Alternatively, interaction of FAK with Lyn may be regulated by FRNK. FRNK is independently expressed in many cell types and is an endogenous regulator of FAK [9,10]. The mechanism by which FRNK regulates FAK is unknown. The physiological relevance of the proposed regulation is suggested by the ability of ectopicly expressed FRNK to block both cell spreading and tyrosine phosphorylation of FAK and paxillin [30]. Src overexpression can override these effects of FRNK [30] by a mechanism that is likely to involve Src-mediated sequestration of FRNK. The mechanism of constitutive association of Lyn and FAK is at this moment unclear, it may be mediated by direct interaction or via interfacing binding proteins.

Lyn is implicated in a variety of biochemical events involved in BCR signaling. First, Lyn constitutively associates with the non-activated receptor through interaction of the N-terminal part of Lyn kinase with the cytoplasmic domain of the Ig-α chain [31]. Based on our findings and consistent with our prediction, FAK was detected in the anti-IgM and anti-Ig-β immunoprecipitable complexes. However, the molecular mode of such interaction is not clear. Our findings demonstrating constitutive association of Lyn with FAK and BCR-mediated FAK activation are complementary with the role of FAK-related proline-rich tyrosine kinase-2 (Pyk2) in T lymphocytes, where Pyk2 is constitutively associated with Fyn [32]. Further analogy can be drawn from the activation of FAK in T cell signaling induced by chemoattractant RANTES and its constitutive association with ZAP-70 tyrosine kinase [33].

BCR-induced tyrosine phosphorylation and activation of FAK as well as its constitutive association with Lyn and antigen receptor components classify FAK kinase as a BCR signaling component. Activation of FAK after ligation of integrin and antigen receptors on B and T cells suggests that the BCR as well as the TCR and integrins share pathways in which FAK may serve as a linker and contribute to functional cross-talk. The formation of signaling complex including FAK and Lyn may contribute to cytoskeletal reorganization in B lymphocytes by linking antigen receptor stimulation to components of the cytoskeleton.

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