Integrin-mediated signal transduction in cells lacking focal adhesion kinase p125^{FAK}

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Abstract We have previously shown that integrin-dependent tyrosine phosphorylation of p130^{Cas} (Cas) could be induced in a mouse fibroblast cell line that does not express focal adhesion kinase p125^{FAK} (FAK). By analyzing FAK-deficient (FAK−/−) cells transiently expressing Cas mutant proteins, we demonstrate here that the Src homology 3 (SH3) domain of Cas is indispensable for adhesion-mediated Cas phosphorylation in this mutant cell line. While the FAK directly binds to Cas-SH3, our findings imply that SH3-binding molecule(s) other than FAK might regulate Cas phosphorylation, at least in FAK−/− cells. In this regard, we observed that FAK−/− cells expressed cell adhesion kinase β (CAKβ), a protein tyrosine kinase of the FAK subfamily. CAKβ expressed by FAK−/− cells was associated in vivo with Cas in a Cas-SH3-dependent manner. Moreover, integrin stimulation induces tyrosine phosphorylation of CAKβ in FAK−/− cells. Thus, our results suggest that CAKβ contributes to integrin-mediated signal transduction in place of FAK in FAK-deficient cells.

Key words: Integrin; Focal adhesion kinase; Cell adhesion kinase β; p130Cas

1. Introduction

The integrins are a major class of receptors used by cells to interact with other cells and with the extracellular matrix. Integrin-mediated adhesive interactions help regulate a variety of fundamental biological processes including cell growth, cell death, differentiation, cell morphology and motility, and tumor growth and metastasis [1,2]. Thus, integrins not only act as simple mediators of cell adhesion but can also transduce biochemical signals across the cell membrane [2,3]. Discovery of a protein tyrosine kinase (PTK) that is specifically localized in focal adhesions has greatly facilitated our understanding of integrin-mediated signal transduction [4,5]. This kinase, termed focal adhesion kinase p125^{FAK} (FAK), physically associates with integrins and is rapidly activated following integrin-mediated cell adhesion [1,3,5]. FAK also acts as an adapter protein for many proteins, including Src family kinases, phosphatidylinositol 3-kinase, C-terminal Src kinase (Csk), the adapter protein Ash/Grb2, tensin, paxillin and p130^{Cas} (Cas) docking protein family members [3,6]. Thus, FAK may play a central role in integrin signaling cascades by mediating the assembly of multiple proteins into cell adhesion sites and thereby propagating signals downstream.

In a previous report [7], we took advantage of mutant fibroblast cell lines lacking expression of various tyrosine kinases to determine their role in adhesion-dependent tyrosine phosphorylation. This approach provided us with the unexpected finding that integrin-mediated tyrosine phosphorylation of FAK-associated proteins, paxillin and Cas, was well preserved in cells derived from FAK-deficient animals. In sharp contrast, tyrosine phosphorylation of Cas but not of paxillin was totally absent in c-Src-deficient cells. This strongly suggested that c-Src primarily regulates Cas phosphorylation [7,8], while FAK is not absolutely required in this process, at least in these mutant cells. However, this does not necessarily discount a regulatory role for FAK in integrin-mediated phosphorylation of Cas and paxillin in wild-type cells. It has been suggested that there is substantial redundancy amongst signaling molecules such as Src family tyrosine kinases, and that when one is eliminated others may substitute [9].

Here we demonstrate an indispensable role of the N-terminal SH3 domain of Cas in inducing integrin-mediated Cas phosphorylation. Importantly, mouse mutant fibroblasts lacking FAK (FAK−/−) expressed CAKβ/Pyk2/CadTK/RAFTK, a non-receptor PTK which belongs to the FAK subfamily [10–13]. Like the prototype FAK, CAKβ was associated with Cas in FAK−/− cells in a Cas-SH3-dependent manner. Thus, CAKβ appears able to functionally substitute for FAK in integrin signal transduction and contribute to Cas phosphorylation in FAK−/− cells.

2. Materials and methods

2.1. Cells and antibodies

FAK−/− fibroblasts were derived from mice whose FAK gene was ablated as described [14]. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Rabbit polyclonal antibodies against Cas (anti-Cas2) were produced as described [15]. Mouse monoclonal antibody against Cas (clone 21) was obtained from Transduction Laboratories (Lexington, KY). Anti-CAKβ rabbit serum, anti-CAKβ(N), was raised against a glutathione *S*-transferase (GST) fusion protein of rat CAKβ residues from −5 to 416 as described [16]. Another antibody, anti-CAKβ(C), was used in some experiments, which was raised against a GST fusion protein of residues 670–716 of rat CAKβ [16]. Anti-phosphotyrosine monoclonal antibody (anti-pTyr), 4G10, was obtained from UBI. HA.11 is a polyclonal rabbit antibody against the hemagglutinin (HA) epitope and was purchased from Babco.

2.2. Transient expression of Cas mutants

Expression plasmids for influenza virus HA-tagged Cas (pSSRa-

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CasHA) and Cas mutant lacking the SH3 domain (pSSRa-ΔSH3HA) were constructed as described previously [17]. Cells were transfected with 10 μg of plasmid DNA per 10 cm dish by the lipofectamine method and were cultured for 48 h. Cells were then detached from the dish by treating with 0.05% trypsin-EDTA, followed by washing three times with serum-free DMEM. Cells were then used in adhesion experiments described below.

2.3. Cell adhesion and preparation of cell lysates

Preparation of culture dishes coated with adhesive ligands, poly-L-lysine (PLL) and fibronectin (FN), was described previously [6]. Cells were plated onto dishes coated with adhesive ligands, and incubated at 37°C for 30 min in serum-free DMEM. Bound cells were then lysed with 1% Nonidet P (NP)-40 lysis buffer as described previously. After removing insoluble materials by centrifugation at 14 000 rpm for 10 min, protein concentrations in the supernatant were determined using the micro BCA protein assay kit (Pierce, Rockford, IL). Cell lysates were stored at −70°C until use.

2.4. Immunoprecipitation and immunoblotting

Immunoprecipitation was performed according to the method described previously [6]. In brief, cell extracts were incubated with various antibodies for 1 h at 4°C, followed by additional incubation with protein-G Sepharose beads for 1 h at 4°C. Beads were then washed 5 times with 1% NP-40 lysis buffer to remove unbound proteins. Immune complexes were treated with sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins on the gel were electrotransferred to nitrocellulose membranes, and then subjected to immunoblotting using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) as a detection system.

3. Results

3.1. The SH3 domain of Cas is essential for adhesion-dependent Cas phosphorylation in FAK−/− cells

In a previous study [7], we showed that Cas could undergo tyrosine phosphorylation upon integrin-dependent adhesion in FAK−/− cells. Moreover, immunohistochemical examination revealed that Cas was capable of localizing in focal adhesions even in the absence of FAK [17]. However, when Cas lacking the SH3 domain (ΔSH3-Cas) was expressed in FAK−/− cells, the mutant protein failed to distribute in focal adhesions [17]. Therefore, we examined whether or not the SH3 domain of Cas was also required for Cas phosphorylation. For this purpose, either wild-type Cas or ΔSH3-Cas, carrying an HA-epitope tag, were transiently expressed in FAK−/− cells, and their tyrosine phosphorylation induced by cell adhesion was examined by immunoprecipitation with anti-HA antibody. As shown in Fig. 1A (lanes 1 and 2), cell adhesion to FN but not to PLL induced significant increase in tyrosine phosphorylation of wild-type Cas. In contrast, ΔSH3-Cas failed to elicit this response (lanes 3 and 4), although expression levels of the mutant protein were not significantly different from those of the wild-type Cas (lanes 1–4, lower panel). This result suggests that the Cas SH3 domain is essential for adhesion-dependent phosphorylation of Cas in FAK−/− cells. In this experiment, we also noted that the migration on SDS-PAGE of Cas obtained from FN-adherent cells was slower than that obtained from PLL-adherent cells. Increased tyrosine phosphorylation does not account for this change in gel mobility, since ΔSH3-Cas also underwent the same degree of shift in its mobility after FN adhesion. These data suggest that Cas undergoes post-translational modification in response to integrin stimulation that is not mediated through the phosphorylation of tyrosine.

ΔSH3-Cas was also expressed in NIH-3T3 cells co-expressing v-Src, and the level of tyrosine phosphorylation was examined by anti-pTyr immunoblotting. As shown in Fig. 1B, lanes 1 and 2, wild-type Cas was intensely phosphorylated in v-Src transformants, and there was no significant difference in the level of phosphorylation between cells adhered to PLL and FN. This is consistent with our previous report [6] which showed that the hyperphosphorylation of endogenous Cas in

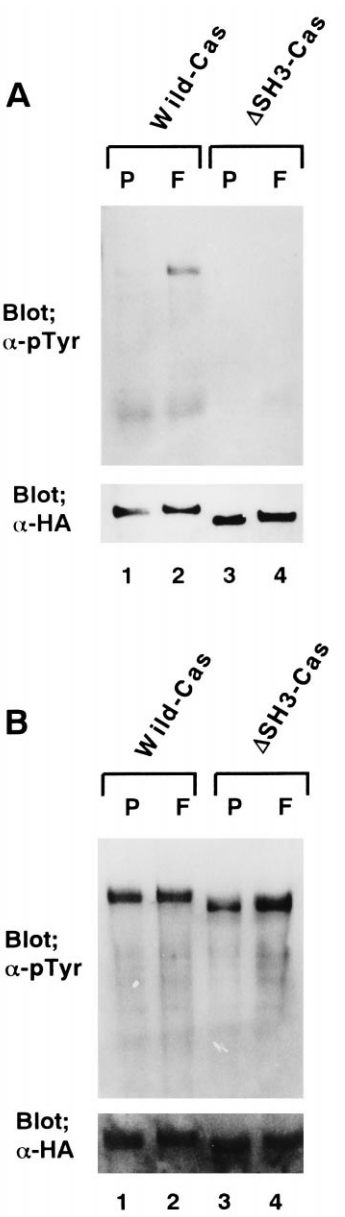


Fig. 1. Adhesion-mediated tyrosine phosphorylation of Cas is dependent on the Cas-SH3 in FAK−/− cells but not in cells transformed by v-Src. A: FAK−/− cells transiently expressing either wild-type Cas (lanes 1 and 2) or ΔSH3-Cas (lanes 3 and 4), carrying an HA epitope tag, were allowed to adhere to plates coated with PLL (P) and FN (F) for 30 min. Cell lysates from each condition were immunoprecipitated with anti-HA and were subjected to anti-pTyr immunoblotting (upper panel). The same membrane was reprobed with anti-HA immunoblotting to examine protein levels (lower panel). B: Epitope-tagged wild-type Cas (lanes 1 and 2) and ΔSH3-Cas (lanes 3 and 4) were immunoprecipitated with anti-HA from lysates of v-Src transformants adhered to PLL (P) or FN (F) for 30 min. The immunoprecipitates were then immunoblotted with anti-pTyr. The same membrane was reprobed with anti-HA immunoblotting to examine protein levels (lower panel).

the transformants was not affected by integrin-mediated cell adhesion. Interestingly, ΔSH3-Cas was also constitutively phosphorylated regardless of cell adhesion (Fig. 1B, lanes 3 and 4). The level of tyrosine phosphorylation of ΔSH3-Cas was almost comparable to that of wild-type Cas. There was no significant difference in the expression between both proteins (Fig. 1B, lower panel). This suggests that hyperphosphorylation of Cas in v-Src-transformed cells does not require the SH3 domain. This also indicates that ΔSH3-Cas used in our experiment can be phosphorylated if appropriate stimuli were provided. Taken together, our results suggest that SH3 domain of Cas is indispensable for adhesion-dependent Cas phosphorylation in FAK−/− cells. FAK, which binds the Cas-SH3 domain, is a strong candidate for PTK regulating adhesion-dependent phosphorylation of Cas [18]. However, this would not explain our previous findings in FAK−/− cells [7]. Thus, at least in FAK−/− cells, other molecule(s) must be responsible for regulating Cas phosphorylation and its intracellular localization.

3.2. *FAK−/− cells express CAKβ, which is associated with Cas in vivo in an SH3-dependent manner*

CAKβ is a recently discovered member of the FAK family [10–13]. Differences in tissue distribution and intracellular localization between FAK and CAKβ suggest that these related kinases have distinct functional properties [10,16,19]. An initial report by Sasaki et al. showed that CAKβ was expressed in rat 3Y1 fibroblasts but not in mouse fibroblast cell lines [10]. Therefore, we examined our FAK−/− cells for expression of CAKβ. NP-40 lysates of 3Y1, FAK−/− cells and NIH-3T3 fibroblast cells were subjected to immunoprecipitation with either control or anti-CAKβ(N) and immunoblotted with anti-CAKβ(N). As described, 3Y1 but not NIH-3T3 expressed CAKβ (Fig. 2, lanes 2 and 6). Interestingly, a significant amount of CAKβ was detectable in FAK−/− cells. Anti-CAKβ(C), another antibody recognizing a different portion of the rat CAKβ, also specifically immunoprecipitated mouse CAKβ from FAK−/− cells (data not shown). These results

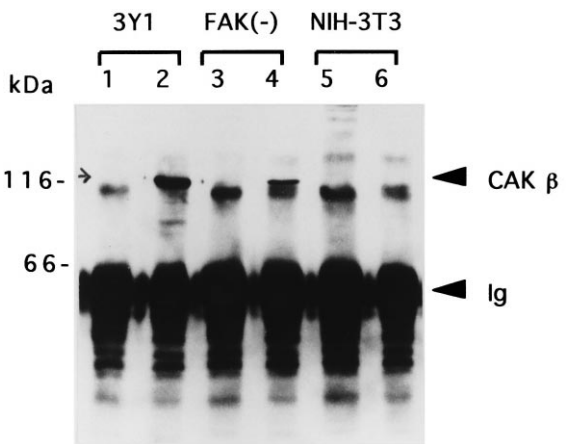
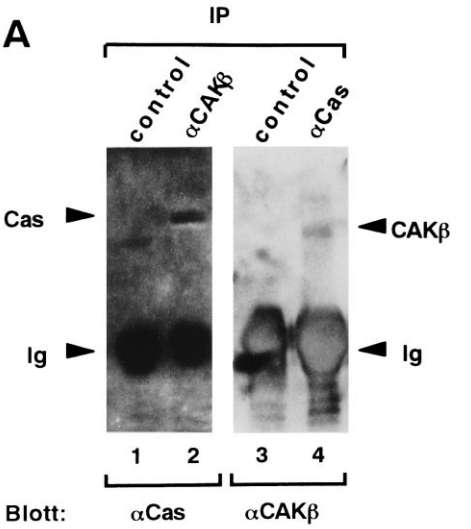


Fig. 2. FAK−/− cells express CAKβ. Cell lysates obtained from 3Y1 rat fibroblasts (lanes 1 and 2), FAK−/− cells (lanes 3 and 4), and NIH-3T3 mouse fibroblasts (lanes 5 and 6) were immunoprecipitated with either control (lanes 1, 3, and 5) or anti-CAKβ(N) (lanes 2, 4, and 6) antibodies. The immunoprecipitates were then immunoblotted with anti-CAKβ(N).



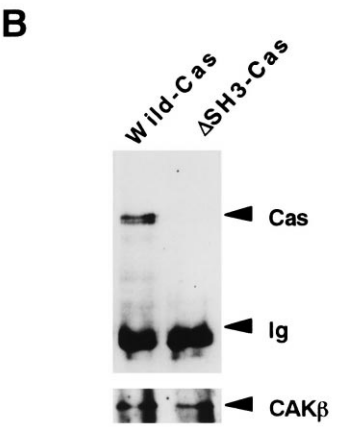


Fig. 3. CAKβ is associated with Cas in FAK−/− cells in a Cas-SH3-dependent manner. A: Lysates obtained from FAK−/− cells were immunoprecipitated with either control or anti-CAKβ(N) antibodies (lanes 1 and 2, respectively) and probed with anti-Cas2. Lysates were also immunoprecipitated with either control or anti-Cas2 (lanes 3 and 4, respectively) and probed with anti-CAKβ(N). B: Lysates from FAK−/− cells transiently expressing either wild-type Cas or ΔSH3-Cas were immunoprecipitated with anti-CAKβ(N), followed by immunoblotting with anti-HA. The same membrane was reprobed with anti-CAKβ(N).

indicate that murine fibroblasts from FAK-deficient mice express FAK-related kinase, CAKβ.

CAKβ contains two highly proline-rich stretches (residues 701–767 and 831–869) in the C-terminal non-kinase region [10]. Proline-rich stretches in the FAK C-terminal domain have been shown to bind to the SH3 domain of Cas [18]. The association between CAKβ and Cas has been recently demonstrated at low stoichiometry [20,21]. Therefore, we next determined whether CAKβ expressed by FAK−/− cells was associated with Cas. NP-40 cell lysates were immunoprecipitated with either control or anti-CAKβ(N) and probed by immunoblotting with clone 21 (monoclonal anti-Cas). It is apparent that Cas was clearly detected only in anti-CAKβ(N) immunoprecipitates (Fig. 3A, lane 2). In parallel experiments, we directly immunoblotted anti-Cas2 immunoprecipitates with anti-Cas (clone 21) to determine the total amount of Cas expressed by FAK−/− cells (data not shown). Densito-

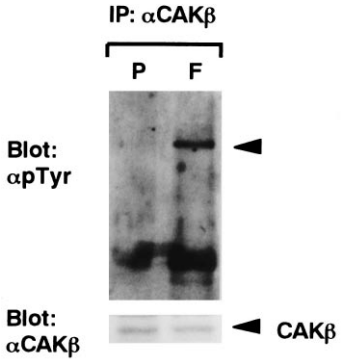


Fig. 4. Integrin-mediated cell adhesion induces tyrosine phosphorylation of CAKβ. Lysates from FAK−/− cells that had been adhered to PLL (P) and FN (F) were immunoprecipitated with anti-CAKβ(N) and then probed with anti-pTyr immunoblotting (upper panel). The same membrane was reprobed with anti-CAKβ(N) to examine protein levels in each lane (lower panel).

metric analysis indicates that approximately 5% of Cas co-precipitates with CAKβ. Conversely, immunoblotting anti-Cas2 immunoprecipitates from FAK−/− cells also reveals the presence of CAKβ (lane 4). This indicates that in FAK−/− cells, an association between Cas and CAKβ does occur in vivo, at low stoichiometry.

We next examined whether or not the association between Cas and CAKβ was mediated by the SH3 domain of Cas. For this purpose, either wild-type Cas or ΔSH3-Cas, carrying an HA epitope tag, was transiently expressed in FAK−/− cells. Cell lysates from each transfectant were subjected to immunoprecipitation with anti-CAKβ(N), followed by immunoblotting with anti-HA. As shown in Fig. 3B, wild-type Cas but not ΔSH3-Cas was clearly detected in anti-CAKβ(N) immunoprecipitates. There was no difference in the amount of CAKβ immunoprecipitated from each transfectant (Fig. 3B, lower panel). Thus, the association between Cas and CAKβ was dependent on the Cas-SH3 domain. Taken together, these data indicate that FAK−/− cells express FAK-related kinase CAKβ, which is associated with Cas via the Cas SH3 domain.

3.3. Integrin-dependent cell adhesion induces tyrosine phosphorylation of CAKβ in FAK-deficient cells

The above findings imply that CAKβ is participating in integrin-mediated signals in FAK-negative cells. We examined, therefore, if integrin-mediated cell adhesion induced tyrosine phosphorylation of CAKβ in FAK−/− cells. As shown in Fig. 4, adhesion to FN-coated plates but not non-specific binding to PLL-coated plates induced a significant increase in tyrosine phosphorylation of CAKβ. Reprobing the same membrane with anti-CAKβ(N) antibody confirmed that the same amounts of CAKβ were loaded into each lane. This result indicates that integrin-dependent cell adhesion induces tyrosine phosphorylation of CAKβ in FAK−/− cells.

4. Discussion

The advent of gene ablation studies in the mouse has provided new insights into the biology of numerous molecules. Mutant mouse embryos generated by targeting the FAK gene displayed a general defect in mesoderm development [14]. The overall phenotype was strikingly similar to the FN-deficient phenotype [22], suggesting that FAK uniquely mediates FN-integrin interactions in cellular processes at this stage of development. However, fibroblast-like cells established from mutant embryos adhered well to FN and were capable of forming focal adhesions in response to cell adhesion [14]. Moreover, adhesion-dependent tyrosine phosphorylation of Cas and paxillin was well preserved even in the absence of FAK [7,8]. These results have suggested that FAK is not essential for organizing focal adhesion and for phosphorylating some of the major phosphotyrosyl proteins localized in focal adhesions. In the present report, we demonstrate that FAK−/− cells expresses CAKβ, a member of FAK-related non-receptor tyrosine kinase subfamily. Like the prototype FAK, CAKβ was found to be associated with Cas in FAK−/− cells in a Cas-SH3 dependent manner. Moreover, we also noted that tyrosine phosphorylation of CAKβ was clearly induced by adhesion of FAK−/− cells to FN. Given an indispensable role of the Cas-SH3 domain in integrin-mediated Cas phosphorylation, CAKβ may be able to substitute the function displayed by FAK in vivo and play a role in mediating integrin-dependent cell function in FAK−/− cells.

Initial studies have shown that intracellular distribution was different between FAK and CAKβ [10]. While FAK is specifically localized in cell-to-matrix contacts at the base of the cells, CAKβ was found to be expressed at cell-to-cell contacts [10]. However, the later immunohistochemical analysis on various cell lines revealed that CAKβ more widely distributed within the cell. In rat fibroblasts, most of CAKβ is localized in the cytoplasm in association with microtubules and microfilaments [16]. In some other cell types, localization of CAKβ into focal adhesions was also clearly demonstrated [19,23]. Moreover, biochemical analysis showed that CAKβ was associated with paxillin and Hic-5, both of which are components of focal adhesions [16,21,24]. Paxillin binding has been shown to be essential for recruiting FAK to focal adhesions [25]. Recent studies by Nakamoto et al. [17] indicated that the SH3 domain and the Src kinase binding site of Cas were both required for targeting Cas to focal adhesions. Cas was clearly distributed in focal adhesions in FAK−/− cells [16]. Thus, CAKβ may play a role in targeting Cas to focal adhesions in FAK−/− cells.

In summary, we showed that CAKβ, a structurally FAK-related PTK, functionally substituted for FAK in the integrin signaling pathway in FAK−/− cells. Nevertheless, it is apparent that CAKβ was not capable of substituting FAK in mouse embryogenesis [14,22]. It is also apparent that tissue and intracellular distribution of FAK and CAKβ was distinct [10,16,19]. Moreover, based on studies on cells which overexpress either FAK or CAKβ [19], these related kinases appeared to differentially mediate substrate phosphorylation. Further studies are necessary to determine differential roles of FAK and CAKβ in cell functions.

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