p130Cas, a Substrate Associated with v-Src and v-Crk, Localizes to Focal Adhesions and Binds to Focal Adhesion Kinase*

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p130Cas (crk associated substrate) has the structural characteristics of an adapter protein, containing multiple consensus SH2 binding sites, an SH3 domain, and a proline-rich domain. The structure of p130Cas suggests that it may act to provide a framework for protein-protein interactions; however, as yet, its functional role in cells is unknown. In this report we show that p130Cas is localized to focal adhesions. We demonstrate that p130Cas associates both in vitro and in vivo with pp125FAK (focal adhesion kinase), a kinase implicated in signaling by the integrin family of cell adhesion receptors. p130Cas also associates with pp41/43FRNK (pp125FAK-related, non-kinase), an autonomously expressed form of pp125FAK composed of only the C-terminal noncatalytic domain. We show that the association of p130Cas with pp125FAK and pp41/43FRNK is direct, and is mediated by the binding of the SH3 domain of p130Cas to a proline-rich sequence present in both the C terminus of pp125FAK and in pp41/43FRNK. In agreement with recent studies we show that p130Cas is tyrosine-phosphorylated upon integrin mediated cell adhesion. The association of p130Cas with pp125FAK, a kinase which is activated upon cell adhesion, is likely to be functionally important in integrin mediated signal transduction.

Adhesion of cells to the extracellular matrix is mediated primarily by the integrin family of receptors (1). Binding of the integrin receptors to the extracellular matrix results in the clustering of integrins and the recruitment of a wide array of proteins to form polyprotein structures called focal adhesions. Focal adhesions serve to link the extracellular matrix with the actin cytoskeleton of the cell (2). In addition to carrying out a structural role, integrins can also activate a variety of signaling pathways, similar to that of growth factor receptors. These signals include tyrosine phosphorylation, elevation of intracellular pH and Ca2+, and lipid turnover (reviewed in Ref. 3). Consistent with the signaling capability of integrins, cell adhesion can influence a variety of cellular functions including cell growth, differentiation (4), and apoptosis (5).

Integrins are catalytically inactive and based on the paradigm of cytokine receptors it is thought that they signal via the recruitment of signaling molecules (3, 6, 7). A variety of signaling molecules have been identified in focal adhesions. These include the tyrosine kinases pp125FAK (8), pp60Src (9), and C-terminal Src kinase (10), the serinethreonine kinases, protein kinase C (11) and mitogen-activated protein kinase, the small G proteins Ras, Rho, and Rac, protein tyrosine phosphatase 1D, phospholipase Cγ, PI-3K, and adapter proteins such as paxillin and Grb2 (12–14). The recruitment to focal adhesions of all signaling molecules tested to date, except pp125FAK, is inhibited by tyrosine kinase inhibitors or cytochalasin D (13). pp125FAK is tyrosine-phosphorylated upon cell adhesion (15–17), which correlates with an increase in its catalytic activity (18, 19). These observations are consistent with a model in which the early recruitment of pp125FAK enables the tyrosine phosphorylation required by other signaling molecules for focal adhesion recruitment.

Cell adhesion leads to the tyrosine phosphorylation of a limited number of proteins, which in addition to pp125FAK includes paxillin (20), tensin (21), and cortactin (22). It has been suggested that the tyrosine phosphorylation of these proteins may result in the creation of SH2 binding sites for other proteins. This is consistent with the idea that SH2 binding sites are present in the SH2 domain of pp125FAK. The interaction of p130Cas with pp125FAK, a kinase which is activated upon cell adhesion, is likely to be functionally important in integrin mediated signal transduction.

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1 The abbreviations used are: pp125FAK or FAK, focal adhesion kinase; p130Cas or Cas, crk associated substrate; EFS, embryonal Fyn-associated substrate; pp41/43FRNK or FRNK, FAK-related nonkinase; GRAF, GTPase regulator associated with FAK; SH2, Src homology 2; SH3, Src homology 3; GST, glutathione S-transferase; CE, chicken embryo; REF, rat embryo fibroblasts; PAGE, polyacrylamide gel electrophoresis.
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MATERIALS AND METHODS

Cell Culture, Cell Adhesion, and Protein Expression—Primary chicken embryo (CE) cells were prepared and cultured as described previously (28). pp125<sup>FAK</sup> and pp41/42<sup>FAK</sup> were expressed in CE cells using the replicative competent RCAS A retrovirus vector as described previously (15, 29). To study the adhesion-dependent phosphorylation of p130<sup>Cas</sup>, 100-mm bacterial plastic dishes were coated overnight at 4°C, or for 2 h at 37°C, with fibronectin (2.5 µg/cm<sup>2</sup>), or for 1 h at room temperature with poly-l-lysine (25 µg/cm<sup>2</sup>). Cells were removed from culture dishes by trypsinization and washed with phosphate-buffered saline containing soybean trypsin inhibitor. The cells were kept in suspension for approximately 30 min at which time four 10<sup>5</sup> cells/100-mm dish were plated onto coated dishes at 37°C for the indicated times in serum-free medium. Fibronectin, poly-l-lysine, and soybean trypsin inhibitor were all obtained from Sigma. Rat embryo fibroblast (REF) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum, penicillin (100 units/ml), and streptomycin (100 µg/ml) (Life Technologies, Inc.). Cells were lysed as described previously (30) using modified radiolmmune precipitation buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate), containing protease and phosphatase inhibitors (10 µg/ml leupeptin, 100 µg/ml phenylmethylsulfonyl fluoride, 0.045 unit/ml apronitin, 1 mM sodium vanadate, 40 mM sodium fluoride, 40 mM β-nitrophenylphosphate). For the communoprecipitation experiments, modified radiolmmune precipitation buffer without sodium deoxycholate was used. Protein concentrations were determined using the BCA protein assay (Pierce).

Antibodies—Polyclonal antibodies were raised against purified bacterially expressed glutathione S-transferase (GST) fusion proteins of p130<sup>Cas</sup>, composed of amino acids 318–486 (Cas B), or 670–896 (Cas F) (Fig. 1) (31). All amino acid designations are based on the sequence of rat p130<sup>Cas</sup> (25). The monoclonal antibody against p130<sup>Cas</sup> was obtained from Transduction Laboratories.

Mutagenesis of pp125<sup>FAK</sup> and Construction of GST Fusions of p130<sup>Cas</sup>—pp125<sup>FAK</sup> and p130<sup>Cas</sup>—The point mutations P715A, P718A, P715A/P718A, P878A, P881A, and P878A/P881A were made in the context of a truncated form of pp125<sup>FAK</sup> composed of amino acid residues 687-1054 (Fig. 6, Cterm1), using the Altered Sites in vitro mutagenesis system (Promega). The presence of the mutations was confirmed by dideoxy sequencing (U. S. Biochemical Corp.). The mutants were cloned into pGEX2TK (Pharmacia) for expression as bacterial fusion proteins. The quadruple point mutant P715A/P718A/P878A/P881A was generated in pGEX2TK-Cterm1 by splicing together the double point mutants P715A/P718A and P878A/P881A at an internal HindIII site.

For in vitro association experiments regions of p130<sup>Cas</sup> were expressed as GST fusion proteins. GST fusion proteins of p130<sup>Cas</sup> composed of the SH3 domain (amino acids 100–158), the substrate domain (amino acids 211–512) and two different portions of the C terminus, amino acids 513–637 (mid-domain) and amino acids 638–916 (C-Term) (Fig. 1) were generated by cloning the corresponding cDNA fragment into pGEX2TK. The generation of the GRAF (GTPase regulator associated with FAK) SH3 is described elsewhere (32).

Immunofluorescence—To determine the subcellular localization of p130<sup>Cas</sup>, immunofluorescent labeling of REF cells was carried out using two p130<sup>Cas</sup> specific polyclonal antisera, CAS B and CAS F. CAS B was raised against a GST fusion protein composed of residues 318 to 486 within the substrate domain of p130<sup>Cas</sup>. CAS F was raised against a GST fusion protein composed of residues 670 to 896 within the C terminus of p130<sup>Cas</sup> (Fig. 1). Both antibodies gave a staining pattern characteristic of focal adhesions (Fig. 2, panels A and C). To confirm the p130<sup>Cas</sup> staining was localized to focal adhesions, co-staining was carried out using a monoclonal antibody to vinculin, a well characterized component of focal adhesions. The staining of p130<sup>Cas</sup> with both antibodies colocalized exactly with that of vinculin (compare panels A with B, and C with D). Immunostaining with 2A7, a monoclonal antibody specific for pp125<sup>FAK</sup>, another component of focal adhesions, gave the same results (data not shown). To confirm the specificity of the antibodies for p130<sup>Cas</sup> they were incubated prior to cell staining with glutathione-Sepharose beads, complexed with GST or the GST fusion protein to which they were raised. Preclearing with GST fusion protein resulted in a loss of staining, whereas GST alone had no effect on the ability of the antibodies to stain focal adhesions (data not shown). In addition, no staining of focal adhesions was observed when preimmune serum was used (data not shown). Immunostaining with pp125<sup>FAK</sup> gave a similar pattern of focal adhesion staining.

p130<sup>Cas</sup> Associates In Vitro with pp125<sup>FAK</sup>—To determine if p130<sup>Cas</sup> was associated with any known components of focal adhesions, in vitro association experiments were carried out. On the basis of sequence analysis, putative functional domains have been delineated in p130<sup>Cas</sup> (25): these include an SH3 domain, a “substrate domain” rich in potential SH2 interaction sites and a C-terminal domain containing putative Src SH2 and SH3 binding sites (Fig. 1). GST fusion proteins encompassing these putative functional domains of p130<sup>Cas</sup> were made as described under “Materials and Methods.” CE cell lysate was incubated with the GST fusion proteins or GST immobilized on glutathione beads, the beads were washed, and associated proteins were analyzed by Western blotting using a phosphotyrosine specific antibody. As shown in Fig. 3A the SH3 domain of p130<sup>Cas</sup> formed a stable complex with the major tyrosine phosphorylated protein present in CE cell lysates (lanes 1 and 3). This protein was previously identified as pp125<sup>FAK</sup> (8), a tyrosine kinase present in focal adhesions. To confirm the identity of this protein the association assay was repeated and Western blotting was carried out using BC3, a polyclonal antiserum specific for pp125<sup>FAK</sup> (8). As shown in Fig. 3B the SH3 domain of p130<sup>Cas</sup> was capable of forming a stable complex...
with pp125FAK in vitro (top panel, lane 3). The other domains of p130Cas failed to associate with pp125FAK (lanes 4–6) and no nonspecific association with GST was detected (lane 2).

In addition to being immunoreactive with pp125FAK, BC3 also recognizes a pp125FAK-related protein, pp41/43FRNK. pp41/43FRNK is an autonomously expressed form of pp125FAK, composed of only the C-terminal noncatalytic region (15). Blotting of the GST fusion protein associated proteins with BC3 revealed that pp41/43FRNK was also capable of specifically interacting with the SH3 domain of p130Cas in vitro (Fig. 3B, bottom panel, lane 3). This interaction was not detected on the phosphotyrosine blot because pp41/43FRNK is phosphorylated only on serine residues (15). Comparing lane 1 with lane 3 we estimate that approximately 20% of the cellular pp125FAK and approximately 50% of pp41/43FRNK was associated with p130Cas.

To examine the specificity of the association of the p130Cas SH3 with pp125FAK and pp41/43FRNK, equivalent amounts of GST fusion proteins of the SH3 domains of p130Cas, Abl, Src, Fyn, Lyn, cortactin, and GRAF along with full-length Grb2 were incubated with lysate from CE cells and Western blotted for the presence of pp125FAK and pp41/43FRNK using BC3. Among these SH3 domains only p130Cas and GRAF were capable of stably associating with both pp125FAK and pp41/43FRNK (Fig. 4, lanes 3 and 9), with p130Cas exhibiting the most efficient binding. Lyn was capable of associating with pp125FAK (lane 7); however, this binding appeared less efficient.

p130Cas Binding to pp125FAK Is Direct—To determine if the interaction of the SH3 domain of p130Cas with pp125FAK and pp41/43FRNK is direct or is mediated by other proteins, a Far Western blot analysis was carried out. The GSTp130CasSH3 was purified and labeled with 32P on a specific protein kinase A

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Fig. 1. Schematic representation of p130Cas and p130Cas GST fusions. The amino acids are designated by the numbers below the schematic. The black oval represents the "substrate domain" which contains multiple copies of the amino acid motif given in single letter code. YDYV represents a potential interaction site for pp60src in the C terminus of p130Cas. The black lines above the substrate and C-terminal domains delineate the regions against which the antibodies CAS B and CAS F, respectively, were raised. SH3, Src-homology 3 domain; Pro, proline-rich stretch of amino acids.

Fig. 2. p130Cas is localized to focal adhesions. REF cells were grown overnight on fibronectin-coated coverslips (2.5 μg/cm²), fixed with paraformaldehyde, permeabilized, and costained with a mix of Cas B (A) or Cas F (C), and a monoclonal antibody to vinculin (B and D). Representative areas of coincident staining are marked by arrowheads. Cas B and Cas F are polyclonal antibodies raised against GST fusion proteins of the substrate and C-terminal domain respectively, of p130Cas.
The SH3 domain of p130Cas interacts with pp125FAK and pp41/43FRNK. GST-p130Cas domain fusion proteins or GST alone were incubated with 250 μg of CE cell lysate. Associated proteins were eluted by boiling in Laemmli sample buffer and probed by Western blotting using (A) 4G10, a phosphotyrosine specific antibody, or (B) BC3, a pp125FAK and pp41/43FRNK specific polyclonal antibody. The efficiency of association was determined by directly blotting 25 μg of CE lysate (lane 1 in each panel).

The association of the SH3 domain of p130Cas with pp125FAK and pp41/43FRNK with representative SH3 domains. GST fusion proteins composed of the SH3 domains of the proteins indicated, were incubated with 250 μg of CE cell lysate. Associated proteins were eluted and Western blotted for pp125FAK or pp41/43FRNK using the specific polyclonal antisera BC3. The efficiency of association was determined by directly blotting 25 μg of CE lysate (lane 1). GST represents a control for nonspecific binding.

p130Cas interacts with a proline-rich sequence present in the C terminus of pp125FAK. The association of the SH3 domain of p130Cas with pp125FAK and pp41/43FRNK is direct. Far Western blot of 100 μg of CE cell lysate (lane 1), BC3 immune complex (lane 3), or p130Cas SH3-associated proteins (lane 5), from pp125FAK (top panel) or pp41/43FRNK (bottom panel) expressing CE cells. pp125FAK or pp41/43FRNK were expressed in CE cells infected with the retroviral vector RCAS A. 1 mg of the lysate was immunoprecipitated with BC3, a polyclonal antibody specific for both pp125FAK and pp41/43FRNK, or incubated with a GST fusion protein of p130Cas SH3 immobilized on glutathione beads. The complexes were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with 32P-labeled p130Cas SH3. Preimmune and GST represent controls for nonspecific binding.
FIG. 6. Schematic of pp125FAK and the pp125FAK Cterm II fusion protein. The numbers below the schematic represent amino acid numbers P represents proline-rich regions, the sequence of which is given underneath in single-letter code.

FIG. 7. The sequence P712PKPSR present in the C terminus of pp125FAK is the primary binding site for p130Cas. A GST fusion protein of the pp125FAK C terminus (Cterm II) composed of only the C terminus (Fig. 6). GST fusion proteins of these constructs were generated and assayed for their ability to associate with p130Cas in CE cell lysate. Fig. 7 shows a representative Western blot of the association. Among the mutant proteins Cterm II P715A and Cterm II P715A/718A, which contain mutations within the proline-rich region proximal to the catalytic domain of pp125FAK, reproducibly displayed reduced association to p130Cas, localizing the binding site to the type II consensus motif P712PKPSR.

p130Cas Associates with pp125FAK in Cells—To demonstrate that p130Cas and pp125FAK are associated within the cell, coimmunoprecipitation experiments were carried out. pp125FAK was expressed in CE cells infected with a retroviral vector RCAS A and immunoprecipitated from lysates using a monoclonal antibody specific for p130Cas. As shown in Fig. 8A p130Cas was present in these pp125FAK immune complexes as revealed by blotting with a mixture of CAS specific antibodies (lane 3), whereas no p130Cas was present in a control immune complex prepared with rabbit antimouse IgG (lane 2). Similarly immune complexes prepared using the p130Cas-specific monoclonal antibody contained pp125FAK as shown by Western blotting with BC3, a polyclonal antibody specific for pp125FAK (Fig. 8B, lane 3). Rabbit anti-mouse IgG did not associate with pp125FAK (lane 2). These results show that p130Cas and pp125FAK form stable complexes within cells.

p130Cas is Tyrosine-phosphorylated in Response to Integrin-mediated Cell Adhesion—The localization of p130Cas to focal adhesions and its association with pp125FAK prompted us to examine whether p130Cas was tyrosine-phosphorylated upon integrin mediated cell adhesion. CE cells were placed in suspension, and plated onto fibronectin or poly-L-lysine-coated dishes for the indicated times (Fig. 9). p130Cas was tyrosine-phosphorylated in continuously adherent cells (Fig. 9, lane 1). Replating the cells onto fibronectin, on which the cells attach and spread in an integrin dependent fashion, resulted in an increase in tyrosine-phosphorylated p130Cas (lanes 4, 6, 8, and 10). In contrast, replating onto poly-L-lysine, where the cells adhere in a non-specific manner, caused no further increase in p130Cas tyrosine phosphorylation over that observed in cells in suspension (lanes 3, 5, 7, and 9). These results indicate that p130Cas is tyrosine-phosphorylated in an integrin-dependent manner. The kinetics of p130Cas phosphorylation and pp125FAK phosphorylation upon adhesion to fibronectin were similar (data not shown).

DISCUSSION

The structural organization of focal adhesions is dictated by a complex network of protein-protein interactions, involving some well characterized proteins and other more poorly understood components. Here we show that p130Cas, a substrate for...
protein tyrosine kinases in v-src- and v-ckr-transformed cells, is a component of focal adhesions and stably associates with the focal adhesion-associated tyrosine kinase protein, pp125FAK. Previous studies examining the localization of p130Cas, used an antibody against the tyrosine-phosphorylated form of p130Cas and reported variable staining of focal adhesions, nuclei, and stress fibers. Using antibodies directed against purified bacterially produced Cas protein, we confirmed the focal adhesion localization of p130Cas; however, we observed no detectable staining of nuclei or stress fibers in REF or CE cells. An association of p130Cas with pp125FAK was initially suggested based on the results of a yeast two-hybrid screen designed to isolate pp125FAK-interacting proteins. The results reported herein extend these observations and clearly show that p130Cas and pp125FAK can form stable complexes in vivo. Further, the association can be reconstituted in vitro and in this context is mediated predominantly by the binding of the SH3 domain of p130Cas to the sequence P172APKSAR, a typical type II SH3 consensus binding site (38) present in the C terminus of pp125FAK. The physiological relevance of the interaction of p130Cas with pp125FAK is thus supported by three observations; the colocalization of p130Cas and pp125FAK to the same cellular compartment, the isolation of stable complexes of p130Cas and pp125FAK from cells expressing pp125FAK, and the ability to reconstitute the interaction of FAK and Cas in vitro.

The tyrosine phosphorylation of p130Cas during cell adhesion to fibronectin indicates that the association of p130Cas and pp125FAK may be functionally significant and is in accord with recent reports (22, 36, 39). In addition to pp125FAK, other tyrosine kinases, including the Src family kinases and C-terminal Src kinase, have been reported to localized to focal adhesions (9, 10, 13) and are known to be involved in mediating the tyrosine phosphorylation of p130Cas, because it becomes phosphorylated upon adhesion with similar kinetics to that of p130Cas, and the tyrosine phosphorylation of pp125FAK has been shown to coincide with an increase in its kinase activity (18, 19). Using pp125FAK overexpressing cells we observed that p130Cas is phosphorylated in a pp125FAK-dependent fashion in vivo, upon inhibition of cellular tyrosine phosphatases by vanadate. This observation is consistent with pp125FAK playing a role, either directly or indirectly, via the activation of Src (23, 40), in the tyrosine phosphorylation of p130Cas by integrin activation. Further evidence for the functional importance of the binding of FAK to Cas is provided by the conservation of this interaction between Cas family members. The SH3 domain of p130Cas is highly conserved in EFS (embryonal Fyn-associated substrate), a recently isolated p130Cas family member (41). A partial cDNA clone (designated FIPSH3-2, encoding the SH3 domain of EFS, was isolated along with p130Cas during the yeast two hybrid screen designed to identify binding partners of pp125FAK (37).

p130Cas joins a small family of proteins which includes tensin, pavlin and cortactin, that become tyrosine-phosphorylated upon adhesion (20–22). p130Cas is rich in consensus SH2 binding sites, nine of which conform to the consensus for Crk binding. Consensus binding sites also exist for the SH2 domains of Src, tensin, Ab1, Grb2, PI3K, and Nck. It has been shown that the substrate domain of Cas binds to v-Crk, whereas v-Src binds to the C-terminal domain (31). p130Cas is also able to associate with Grb2 in an in vitro association assay. Therefore tyrosine phosphorylation of p130Cas upon adhesion could generate functional binding sites for any of these ligands. These molecules have been implicated in a variety of signaling pathways and their binding to p130Cas could thus mediate the activation of these signaling pathways upon integrin dependent adhesion. Alternatively generation of a tensin binding site on p130Cas could promote the assembly of focal adhesions by facilitating the binding of actin stress fibers (42). p130Cas was identified originally as a protein highly tyrosine-phosphorylated in v-src- and v-ckr-transformed cells (26, 27). A recent study has shown that overexpression of antisense mRNA for p130Cas caused a morphological reversion of the transformed phenotype in cells transformed by onconorphic de-carboxylase, v-src and c-Ha-ras (43), suggesting p130Cas plays a central role in transformation mediated by these oncogenes. The tyrosine phosphorylation of p130Cas upon integrin mediated cell adhesion raises the possibility that one mechanism of the action of these oncogenes is in bypassing normal integrin stimulated signaling. Further elucidation of the role of p130Cas in focal adhesion signaling and structure will be key to understanding its involvement in cell transformation.

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