Association of p130CAS with Phosphatidylinositol-3-OH Kinase Mediates Adenovirus Cell Entry*

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The Crk-associated substrate, p130CAS, has been implicated in the regulation of the actin cytoskeleton following ligation of cell integrins with the extracellular matrix. Integrin-mediated cell adhesion involves p130CAS association with focal adhesion kinase (p125FAK). Internalization/cell entry of type 2 and type 5 adenoviruses (Ad) is also mediated by αv integrins. However, expression of dominant negative forms of p125FAK does not alter virus entry, and Ad entry occurs normally in p125FAK-deficient fibroblasts. We now provide evidence that Ad internalization, a process which is mediated by αv integrins, also requires p130CAS and phosphatidylinositol-3-OH kinase (PI 3-kinase). Ad induces p130CAS phosphorylation and inhibition of p130CAS phosphorylation by tyrphostin and genistein, or expression of the substrate domain deleted p130CAS blocks Ad internalization. p130CAS was also found to associate with the p85 subunit of PI 3-kinase through its proline-rich domain during virus internalization and expression of p130CAS containing a deleted proline-rich domain (PRD) inhibited adenovirus cell entry. We showed further that the RPLPSPP motif in the proline-rich region of p130CAS interacts with the SH3 domain of p85/PI 3-kinase. These studies reveal the molecular basis by which p130CAS coordinates the signaling pathways involved in integrin-mediated Ad entry.

The identification of cellular receptors that mediate entry of different human pathogens (Salmonella, Listeria, human immunodeficiency virus, herpes simplex virus type-1, adenovirus) has provided an opportunity to investigate the precise biochemical events involved in their pathogenesis (1, 2). Adenovirus has proved to be a useful tool for uncovering the signaling events involved in integrin-mediated viral endocytosis. Adenoviruses are associated with mild/acute infections of the eye, upper respiratory and gastrointestinal tracts (3). Adenovirus types are associated with mild/acute infections of the eye, upper respiratory and gastrointestinal tracts (3). Adenovirus types 2 and 5 adenoviruses (Ad) is also mediated by αv integrins, respectively, PRD, proline-rich domain; SD, substrate domain; SB, substrate binding region; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; aa, amino acid(s).

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† The abbreviations used are: PI 3-kinase, phosphatidylinositol-3-OH kinase; Ad, adenovirus; p125FAK, p125 focal adhesion kinase; p130CAS, p130 Crk-associated substrate; SH2 and SH3, Src homology 2 and 3, respectively; PRD, proline-rich domain; SD, substrate domain; SB, substrate binding region; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; aa, amino acid(s).

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Plasmids and Antibodies—Mammalian expression vectors containing cDNA for the expression of wild-type and substrate domain deleted p130Cas (p130Cas-PRD), p125FAK (p125FAK), and the Y397F point mutant of p125FAK (kindly provided by Dr. Ilic, University of California, San Francisco) were maintained in a 5% CO2 incubator in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 mM L-glutamine, non-essential amino acids, and sodium pyruvate (Life Technologies, Inc., Gaithersburg, MD). Cell monolayers at 30% confluency were either transiently transfected using the LipofectAMINE transfection reagent (Life Technologies, Inc., Gaithersburg, MD), as recommended by the manufacturer or by using standard calcium phosphate precipitation. Under these conditions approximately 60% of SW480 and more than 90% of 293T cells were transfected at 48 h post-transfection as ascertained by the use of a plasmid containing the β-galactosidase reporter gene under control of the same CMV promoter.

Adenovirus, Cell Lines, and Transfection Protocols—Adenovirus type 2 (Ad2, ATCC) was propagated in A549 epithelial cells and purified by banding on CsCl density gradient ultracentrifugation as described previously (6). Purified virions were dialyzed extensively against 10 mM Tris-buffered saline, pH 8.1, containing 10% glycerol and stored at −70 °C. Ad2 was radiolabeled with Na251I using IODO-GEN-coated beads (Pierce, Rockford, IL). The specific activity of labeled virions was approximately 105–106 cpm/μg. The human colon colorectal adenocarcinoma cell line SW480, A549 lung carcinoma cells (obtained from ATCC, Manassas, VA), HEK-293T embryonic kidney cells, and mouse embryonic fibroblasts expression or lacking p125FAK (35), (kindly provided by Dr. Ilic, University of California, San Francisco) were maintained in a 5% CO2 incubator in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 100 mM L-glutamine, non-essential amino acids, and sodium pyruvate (Life Technologies, Inc., Gaithersburg, MD). Cell monolayers at 30% confluency were either transiently transfected using the LipofectAMINE transfection reagent (Life Technologies, Inc., Gaithersburg, MD), as recommended by the manufacturer or by using standard calcium phosphate precipitation. Under these conditions approximately 60% of SW480 and more than 90% of 293T cells were transfected at 48 h post-transfection as ascertained by the use of a plasmid containing the β-galactosidase reporter gene under control of the same CMV promoter.

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Several potential tyrosine phosphorylation sites in FAK are associated with its activity (24, 41). Tyrosine 925 of FAK, which was identified as the binding site for Grb2 protein, as well as three other tyrosines (Tyr1396, Tyr1062, and Tyr1059) in the YXXM motif have been indicated for optimal binding to p85 SH2 domains of PI 3-kinase (42). Therefore, to investigate further the possible role of FAK in virus entry, we quantitated adenovirus internalization and gene delivery in FAK-deficient (FAK+/−) or FAK-expressing (FAK+/+) mouse embryo fibroblasts. Each of these cell types bound equivalent amounts of adenovirus and virus attachment was capable of being blocked by soluble fiber protein (data not shown). FAK-deficient fibroblasts also supported very similar levels of adenovirus internalization as FAK-expressing cells (Fig. 1B). These studies indicated that FAK activity is not essential for αv integrin-mediated adenovirus entry into host cells. To examine further whether FAK might play a role in Ad internalization, we expressed the non-kinase domain of FAK (FRNK), a potent dominant-negative inhibitor of this tyrosine kinase (43) in SW480 cells. Expression of FRNK failed to block adenovirus internalization (Fig. 1C), indicating that FAK is not required for cell entry.

Adenovirus Interaction with Integrin αv/β3 Induces P130CAS Phosphorylation—In our investigation of adenovirus internalization, we noted that there was a significant increase in tyrosine phosphorylation of a 125–130-kDa protein which was also present in p125FAK-deficient fibroblasts (data not shown), consistent with the size of p130CAS. Although p130CAS has been shown to colocalize with integrin-associated focal adhesion complexes, its involvement in Ad cell entry or in endocytosis in general was unknown. To investigate the potential role of p130CAS in virus infection, we first determined whether adenovirus interaction with cells resulted in p130CAS phosphorylation. Incubation of SW480 cells, which express only integrin αvβ5, with Ad, resulted in a 5–7-fold increase in p130CAS phosphorylation compared with untreated cells (Fig. 2A). This response was relatively rapid, occurring 5 min after exposure to virus and persisted for up to 20 min after incubation. To assess whether p130CAS phosphorylation was specifically due to integrin interaction with the adenovirus penton base, we preincubated viral particles with soluble recombinant integrin αvβ5 and then asked whether such viruses were still capable of inducing p130CAS phosphorylation. Preincubation of virus particles with soluble integrin αvβ5 reduced p130CAS phosphorylation by approximately 50% compared with untreated viruses (Fig. 2B). The soluble integrin alone had little effect on p130CAS phosphorylation. These studies indicate that the Ad penton base interaction with αv integrins promotes p130CAS phosphorylation.

p130CAS Phosphorylation/Activation Is Required For Adenovirus Internalization—To determine whether p130CAS phosphorylation is required for Ad endocytosis, we treated cells with tyrosine kinase inhibitors tyrphostin or genistein (5 or 20 μM) for 60 min in serum-free medium, and measured the amount of p130CAS tyrosine phosphorylation and Ad internalization. Treatment of cells with genistein or tyrphostin 25 blocked Ad-induced p130CAS phosphorylation (Fig. 3A, upper panels).
panel) as well as Ad2 internalization (Fig. 3B). In contrast, PD98059 (PD), an inhibitor of the ERK1/ERK2 mitogen-activated protein kinases (45, 46) did not inhibit either Ad endocytosis or p130\textsuperscript{CAS} phosphorylation. These studies indicated that p130\textsuperscript{CAS} phosphorylation is associated with adenovirus cell entry.

p130\textsuperscript{CAS} contains an SH3 domain, a cluster of SH2-binding molecules including p125FAK (20), C3G (47). In contrast, the PRD previously shown to interact with several other signaling molecules such as p130\textsuperscript{CAS} during infection. This study showed that SW480 cells were transfected with wild type (tagged with GST), subdomain wild type (SB), or substrate domain-deleted p130\textsuperscript{CAS} (SD) or a CAS construct truncated at the COOH terminus (\textDelta SB, 5556–806 aa). Cells were incubated with Ad2 at 37 °C for 10 min or untreated. Cell lysates were incubated with glutathione-Sepharose beads. Ad induced tyrosine phosphorylation as described. D, SW480 cells were transfected with wild type (WT) and substrate domain-deleted (SD) p130\textsuperscript{CAS} by immunoblotting for GST tag. The mean ± S.D. of duplicate of triplicate samples.

FIG. 3. Tyrosine phosphorylation of p130\textsuperscript{CAS} is required for adenovirus internalization. A and B, SW480 cells were preincubated with tyrosine phosphorylation inhibitors, tyrphostin 25 (50 μM) or genistein (5 and 20 μM) for 60 min and then assayed for adenovirus-induced p130\textsuperscript{CAS} tyrosine phosphorylation (A) or adenovirus internalization (B) as described under "Experimental Procedures." C, 293T cells were transiently transfected with wild type (tagged with GST), substrate domain-deleted p130\textsuperscript{CAS} (\textDelta SD), or a CAS construct truncated at the COOH terminus (\textDelta SB, 5556–806 aa). Cells were incubated with Ad2 at 37 °C for 10 min or untreated. Cell lysates were incubated with glutathione-Sepharose beads. Ad induced tyrosine phosphorylation as described. D, SW480 cells were transiently transfected with plasmids encoding wild type or substrate domain-deleted p130\textsuperscript{CAS} (SD) and assayed for adenovirus internalization (square, control; triangle, wild type; circle, p130\textsuperscript{CAS} (SD)). The inset shows the expression of both wild type (WT) and substrate domain-deleted (SD) p130\textsuperscript{CAS} by immunoblotting for GST tag. The values represent the mean ± S.D. of duplicate of triplicate samples.

Identification of the Domains in p130\textsuperscript{CAS} and p130\textsuperscript{CAS} That Mediate Their Association—p130\textsuperscript{CAS} contains a SH3 domain at its NH\textsubscript{2} terminus, a substrate domain with multiple tyrosine phosphorylation sites, and a COOH-terminal proline-rich domain (PRD). The SH3 domain of p130\textsuperscript{CAS} was previously shown to interact with several other signaling molecules including p125FAK (20), C3G (47). In contrast, the PRD domain of p130\textsuperscript{CAS} interacts with c-Src (19). The p85 subunit of PI 3-kinase and this correlated with increased PI 3-kinase (45, 46) did not inhibit either Ad endocytosis or p130\textsuperscript{CAS} phosphorylation. These studies indicated that p130\textsuperscript{CAS} phosphorylation is associated with adenovirus cell entry.

p130\textsuperscript{CAS} contains an SH3 domain, a cluster of SH2-binding sites in its substrate domain, and a proline-rich region that is a candidate for SH3 domain binding. The substrate domain of p130\textsuperscript{CAS} contains most of the putative tyrosine phosphorylation sites and accounts for a significant portion of its tyrosine phosphorylation (18, 31). To further examine the role of p130\textsuperscript{CAS} phosphorylation in adenovirus internalization, we transiently expressed wild type CAS, or CAS truncated in either the substrate domain (\textDelta SD, 213–514 aa) or the COOH terminus (\textDelta SB, 5556–806 aa), and assayed tyrosine phosphorylation induced by Ad infection as described. As showed in Fig. 3C, little if any phosphorylation of the overexpressed proteins was detected in the absence of Ad, while in contrast, Ad induced substantial tyrosine phosphorylation of wild type p130\textsuperscript{CAS} as well as p130\textsuperscript{CAS} \textDelta SB. Interestingly, the substrate domain-deleted p130\textsuperscript{CAS} (\textDelta SD) which has the putative tyrosine phosphorylation domain deleted had significantly reduced tyrosine phosphorylation upon Ad infection (Fig. 3C). We next tested whether the expression of p130\textsuperscript{CAS} (\textDelta SD) could alter Ad internalization. Overexpression of p130\textsuperscript{CAS} \textDelta SD inhibited Ad internalization by about 75%, whereas virus uptake was unaffected in cells expressing wild-type p130\textsuperscript{CAS} (Fig. 3D). Together these data suggest that the p130\textsuperscript{CAS} phosphorylation/activity is required for efficient adenovirus internalization.

p130\textsuperscript{CAS} Associates with Activated PI 3-Kinase during Adenovirus Internalization—We previously demonstrated that Ad cell entry requires PI 3-kinase activation, however, it was unknown whether PI 3-kinase associates with other signaling molecules such as p130\textsuperscript{CAS} during infection. To examine this, we incubated SW480 cells with Ad2 particles at 37 °C for 10 min and then immunoprecipitated with either anti-p85 or anti-p130\textsuperscript{CAS}, or a control antibody. PI 3-kinase activity in the immunocomplexes was then assayed with phosphatidylinositol as a substrate and analyzed by TLC. PI 3-kinase activity induced by Ad infection could be detected in both anti-p85 and anti-p130\textsuperscript{CAS} immunoprecipitants, but not in cell samples immunoprecipitated with a control polyclonal antibody (Fig. 4A). To determine if the p85 subunit of PI 3-kinase associates with p130\textsuperscript{CAS} during infection, we then immunoprecipitated SW480 cell lysates with a polyclonal anti-p130\textsuperscript{CAS} antibody, a portion of the immunocomplexes were immunoblotted for associated-p85 after separation on an SDS gel, or used to assay PI 3-kinase activity as described under "Experimental Procedures." Adenovirus infection induced p130\textsuperscript{CAS} association with the p85 subunit of PI 3-kinase and this correlated with increased PI 3-kinase activity in anti-p130\textsuperscript{CAS} complexes (Fig. 4, B and C). The association of p130\textsuperscript{CAS} with p85 was also detected in anti-p85 complexes from A549, 293T, or SW480 cell lysates (data not shown). Together, these data suggest that Ad infection/internalization induces p130\textsuperscript{CAS} and PI 3-kinase/p85 association.

Identification of the Domains in p130\textsuperscript{CAS} Interaction with PI 3-Kinase Promotes Adenovirus Cell Entry

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Adenovirus induces p130<sup>CAS</sup> association with PI 3-kinase. A, serum-starved SW480 cells were incubated with Ad2 at 37 °C for 10 min. Cell lysates were then immunoprecipitated with an anti-p85, an anti-p130<sup>CAS</sup>, or a control antibody. PI 3-kinase activity in the immunocomplexes was assayed using phosphatidylinositol as a substrate. B and C, serum-starved SW480 cells were incubated with Ad2 at 37 °C for varying times, and immunoprecipitated with anti-p130<sup>CAS</sup>. PI 3-kinase activity in these immunocomplexes was examined as described in B, p130<sup>CAS</sup>-associated p85 in these complexes was also examined by blotting with an anti-p85 antibody after separation on an SDS-PAGE gel (C).

Fig. 4. Adenovirus induces p130<sup>CAS</sup> association with PI 3-kinase. A, serum-starved SW480 cells were incubated with Ad2 at 37 °C for 10 min. Cell lysates were then immunoprecipitated with an anti-p85, an anti-p130<sup>CAS</sup>, or a control antibody. PI 3-kinase activity in the immunocomplexes was assayed using phosphatidylinositol as a substrate. B and C, serum-starved SW480 cells were incubated with Ad2 at 37 °C for varying times, and immunoprecipitated with anti-p130<sup>CAS</sup>. PI 3-kinase activity in these immunocomplexes was examined as described in B, p130<sup>CAS</sup>-associated p85 in these complexes was also examined by blotting with an anti-p85 antibody after separation on an SDS-PAGE gel (C).

arated on an SDS-PAGE and detected by immunoblotting using a monoclonal anti-p130<sup>CAS</sup> antibody. The WT-p85, as well as the p85-SH3 domain were capable of binding to p130<sup>CAS</sup>, suggesting that the SH3 domain of p85 is likely responsible for the association with p130<sup>CAS</sup> (Fig. 5A).

p130<sup>CAS</sup> contains a proline-rich domain near its COOH terminus which has been previously demonstrated to interact with the SH3 domain of c-Src (19). We next sought to determine if this region mediates the association with the SH3 domain of p85/PI 3-kinase using GST fusion proteins containing or excluding the PRD of p130<sup>CAS</sup>. A relatively large segment of the p130<sup>CAS</sup> proline-rich domain (aa 600–710) as well as a 16-amino acid peptide (residues 634–649), a consensus sequence that has been proposed to interact with the SH3 domains (50) were both capable of recognizing p85 (Fig. 5B). In contrast, a segment of the p130<sup>CAS</sup>-PRD that lacks this sequence (residues 649–710) did not bind to p85. These findings suggested that the RPLP<sup>SS</sup> motif within the p130<sup>CAS</sup> proline-rich domain is responsible for the interaction with p85/PI 3-kinase. To examine this possibility further in vivo, we first transfected 293T cells with a construct encoding a GST-tagged wild type p130<sup>CAS</sup> or a p130<sup>CAS</sup> mutant in the RPLP<sup>SS</sup> motif, and then performed pull-down experiments for associated p85 (Fig. 5C). WT-p130<sup>CAS</sup> but not a p130<sup>CAS</sup> mutant containing an RPLPSPP motif in the proline-rich domain was capable of associating with endogenous p85. These findings further confirmed that the RPLP<sup>SS</sup> motif in p130<sup>CAS</sup> was responsible for p85 binding.

To investigate whether the p130<sup>CAS</sup>-PRD also regulates adenovirus endocytosis, we next transfected cells with constructs encoding the SH3 domain of p130<sup>CAS</sup> (aa 1–71), p130<sup>CAS</sup> with the deletion of the proline-rich region (p130<sup>CAS</sup>-SH3, Δ556–806 aa) or wild type p130<sup>CAS</sup>, and then examined the effect on adenovirus internalization. As shown in Fig. 6, overexpression of the p130<sup>CAS</sup>-SH3 domain or wild type p130<sup>CAS</sup> did not alter adenovirus cell entry. In contrast, expression of the p130<sup>CAS</sup> ΔSB significantly blocked adenovirus cell entry. These findings are consistent with the notion that the p130<sup>CAS</sup> proline-rich domain plays a key role in interacting with p85 of the PI 3-kinase and that the interaction of p85 with p130<sup>CAS</sup> is important for adenovirus cell entry and Ad infection.

**DISCUSSION**

α<sub>5</sub>β<sub>1</sub>-integrin-mediated adenovirus endocytosis requires signaling events mediated by PI 3-kinase and the Rho family of small GTPases (10). PI 3-kinase and the Rho family GTPases have previously been linked to cell entry of certain pathogenic bacteria (1), as well as to regulate complement and Fc(ig) receptor-mediated phagocytosis (51). Among their many functions, Rho family GTPases play a major role in the reorganization of the actin cytoskeleton. At the cell membrane, the products of PI 3-kinase interact with other cytoskeletal associated molecules such as the small GTPase Rac or the actin regulatory proteins such as gelsolin, profilin, or α-actinin to transmit further signaling reactions (52). Actin filaments, in conjunction with dynamin (53, 54) and other molecules, may be involved in the formation of endosomes at the plasma membrane. Actin filaments have also been proposed to act as a scaffold for signaling complexes at localized regions of the cell plasma membrane thereby prolonging their half-life (55).

In the studies presented here, we provide evidence that p130<sup>CAS</sup> is also a key component in the signaling complex that regulates adenovirus internalization. An unanticipated finding was that PI 3-kinase is capable of interaction with p130<sup>CAS</sup> and that activation of both signaling molecules is required for efficient virus entry.

In earlier studies (9), we discovered that adenovirus interaction with cells induced phosphorylation of focal adhesion kinase (p125<sup>FAK</sup>). Previous studies have shown that p125<sup>FAK</sup> binds to the p85 subunit of PI 3-kinase via a different domain (20, 56) and participates in integrin signaling. Tyrosine phosphorylation of p125<sup>FAK</sup> at residue 397 has been reported to mediate PI 3-kinase activity (40). In chick embryo cells, p130<sup>CAS</sup> localizes to focal adhesion and binds to p125<sup>FAK</sup> (21). However, the biological implication of this association is not known and several investigators have observed that p125<sup>FAK</sup> is not required in tyrosine phosphorylation of various substrates induced by integrin ligation (41, 57, 58). In our initial studies we found that overexpression of p125<sup>FAK</sup> Y397F (Fig. 1), or its COOH-terminal non-kinase construct FRNK did not alter integrin-mediated Ad endocytosis. Moreover, fibroblasts derived from a p125<sup>FAK</sup>-null mouse also supported adenovirus internalization. These findings indicated that p125<sup>FAK</sup> is not involved in Ad entry. This does not rule out the possibility that other tyrosine kinases such as Pyk2 may compensate for p125<sup>FAK</sup> activity. The requirement for p125<sup>FAK</sup> in bacterial uptake (17) but not Ad cell entry may reflect differences in the types of integrins that mediate the entry of these different pathogens.
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...p130CAS substantially inhibited adenovirus internalization (Fig. 3). Overexpression of a substrate domain deletion of p130CAS (aa 634–649), or a control protein lacking the proline-rich domain (aa 649–710), were used to analyze the association of p130CAS with p85. C, GST fusion proteins containing the proline-rich domain of p130CAS (aa 600–710, aa 634–649), or a control protein lacking the proline-rich domain (aa 649–710), were used to analyze the association of p130CAS with p85. C, to further prove the requirement of the proline-rich domain of p130CAS in mediating p130CAS and p85 association, HEK 293T cells were transfected with plasmids encoding wild type p130CAS, or mutant at the proline-rich domain (aa 640 to 642, PLP to LGS) (19) tagged with GST. The cell lysates were incubated with glutathione Sepharose-4B beads and p130CAS-associated p85 was detected by immunoblotting with an anti-p85 monoclonal antibody.

p130CAS is another integration site for various extracellular signals such as those elicited by certain growth factors (27). As a substrate of c-Src, p130CAS has also been reported to regulate tyrosine kinase inhibitors including tyrphostin and genistein (Fig. 3). The interaction between p85 and p130CAS we observed could be through direct or indirect association. It is possible that p85/PI 3-kinase interaction with p130CAS is mediated by c-Src since it has been reported that both the SH2 and SH3 domains of Src bind to p130CAS directly (19) and the same domains could also bind p85 of PI 3-kinase (60, 61). Thus a tripartite complex of p130CAS, Src, and p85/PI 3-kinase may play an essential role in adenovirus uptake. In support of this possibility, we found that overexpression of p130CAS ΔSB or p130CAS ΔSD resulted in reduced virus uptake (Figs. 3D and 6). It is also possible that p85/PI 3-kinase interacts directly with p130CAS and this interaction is potentiated by c-Src.

Since we had previously shown that Ad internalization required actin polymerization mediated by PI 3-kinase and the Rho family GTPases, we wondered if PI 3-kinase might also interact with p130CAS even though an association between these signaling molecules had not been previously demonstrated. Indeed, the vast majority of PI 3-kinase activity generated by Ad interaction with cells was found to be associated with p130CAS (Fig. 4). These findings raise the possibility that localization of PI 3-kinase/p85 with p130CAS may allow subsequent activation via phosphorylation of p85 leading to a conformational change and recruitment of the PI 3-kinase p110 subunit. Pull-down experiments performed with p85 GST fusion proteins indicated that p85 subunit binds to p130CAS via its SH3 domain (Fig. 5). In further mapping studies, we demonstrated that the RXL motif in the p130CAS proline-rich domain is responsible for p130CAS association with p85/PI 3-kinase (Fig. 5). It is interesting to note that the SH3 domain of p130CAS mediates its association with p125FAK (20, 47). While additional studies are needed to determine the precise role of cell signaling molecules in Ad endocytosis, these findings lend further support for the concept that p130CAS plays a role in the reorganization of the actin cytoskeleton.

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REFERENCES
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