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Requirements for Localization of p130^{cas} to Focal Adhesions

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p130^{cas} (Cas) is an adapter protein that has an SH3 domain followed by multiple SH2 binding motifs in the substrate domain. It also contains a tyrosine residue and a proline-rich sequence near the C terminus, which are the binding sites for the SH2 and SH3 domains of Src kinase, respectively. Cas was originally identified as a major tyrosine-phosphorylated protein in v-Crk- and v-Src-transformed cells. Subsequently, Cas was shown to be inducibly tyrosine phosphorylated upon integrin stimulation; it is therefore regarded as one of the focal adhesion proteins. Using an immunofluorescence study, we examined the subcellular localization of Cas and determined the regions required for its localization to focal adhesions. In nontransformed cells, Cas was localized predominantly to the cytoplasm and partially to focal adhesions. However, in 527F-c-Src-transformed cells, Cas was localized mainly to podosomes, where the focal adhesion proteins are assembled. The localization of Cas to focal adhesions was also observed in cells expressing the kinase-negative 527F/295M-c-Src. A series of analyses with deletion mutants expressed in various cells revealed that the SH3 domain of Cas is necessary for its localization to focal adhesions in nontransformed cells while both the SH3 domain and the C-terminal Src binding domain of Cas are required in 527F-c-Src-transformed cells and fibronectin-stimulated cells. In addition, the localization of Cas to focal adhesions was abolished in Src-negative cells. These results demonstrate that the SH3 domain of Cas and the association of Cas with Src kinase play a pivotal role in the localization of Cas to focal adhesions.

p130^{cas} (Cas [Crk-associated substrate]) was originally identified as a major tyrosine-phosphorylated protein in cells transformed by p47$^{v\text{-}crk}$ (v-Crk) and was also shown to be tyrosine phosphorylated in p60$^{v\text{-}src}$ (v-Src)-transformed cells (35, 36). The tyrosine phosphorylation levels of Cas correlate well with the transforming phenotypes of cells and are therefore thought to play an essential role in the process of cell transformation, although the exact mechanism through which Cas mediates transformation remains to be elucidated.

Cas contains an SH3 domain followed by the substrate domain, a proline-rich motif and several tyrosine residues near the C terminus (35). The SH3 domain is known to bind to FAK (focal adhesion kinase) (33), FRNK (FAK-related nonkinase) (13), and PTP1B (protein tyrosine phosphatase 1B) (24). The substrate domain, which has 15 potentially phosphorylated tyrosine residues, binds to v-Crk (6, 29) and several other proteins (6). The proline-rich region near the C terminus and Y762 provide the binding sites for the SH3 and SH2 domains of Src kinase, respectively (29). These facts indicate that Cas is an adapter molecule, which can assemble and transmit cellular signals via interaction through the SH2 and SH3 domains of a wide variety of signalling molecules. Two recently cloned molecules, Efs (embryonal Fyn-associated substrate)/Sin (Src-interacting or signal-integrating protein) (1, 16) and HEF1 (human enhancer of filamentation 1)/Cas-L (21, 27), have similar primary structure to Cas and are assumed to comprise a new family of docking proteins.

Cells attach to the extracellular matrix (ECM) proteins such as fibronectin, laminin, and vitronectin through structures known as focal adhesions (reviewed in references 7 and 9), where the stress fibers or actin bundles end and many cytoskeletal proteins and signalling molecules are integrated. Integrins are cell surface receptors, which bind to ECM proteins and transmit signals across the plasma membrane. Stimulation of integrins by ECM proteins elicits tyrosine phosphorylation of several proteins in focal adhesions. Among these proteins are FAK (10), paxillin (8), tensin (4), cortactin (43), and Cas (30, 32, 43).

The tyrosine phosphorylation of Cas by integrin stimulation is supposed to be mediated by Src family kinases, especially c-Src (5, 11, 42). FAK itself might not be necessary for the phosphorylation of Cas (42). However, FAK, which can bind to both Cas and Src family kinases, might recruit Src family kinases to phosphorylate Cas (33, 42). Upon integrin stimulation, the tyrosine-phosphorylated Cas binds to c-Crk, and guanine nucleotide exchange factors C3G and Sos were found in the Cas-Crk complex (42). Thus, Cas is thought to serve as a docking protein in focal adhesions.

The subcellular fractionation study showed that Cas moves from the cytoplasm to particulate fractions following tyrosine phosphorylation in v-Crk-transformed 3Y1 cells (35), suggesting that the subcellular localization of Cas plays an important role in the transforming process. On the other hand, immunofluorescence studies of Cas further support the idea that Cas works as a focal adhesion protein (13, 21, 32). Immunostaining with monoclonal antibody (MAb) 4F4 (18), which recognizes the tyrosine-phosphorylated form of Cas, revealed that the tyrosine-phosphorylated Cas is found in focal adhesions as well as along stress fibers in REF52 cells (32). Subsequently, by using an antibody that recognizes both phosphorylated and unphosphorylated Cas, Cas was shown to be stained in focal

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adhesions in REF cells attached to fibronectin-coated coverslips (13) and in v-Crk transformed 3Y1 cells (21).

In this study, we compared the cellular localization of Cas in NIH 3T3 and that in 3T3 cells transformed by 527F-c-Src (an activated form of c-Src) by immunofluorescence. The 3T3 cells transformed by 527F-c-Src (3T3-aSrc) form structures called podosomes or rosettes, which are the sites of focal contacts with the ECM. Although podosomes are not necessarily the counterparts of focal adhesions in normal cells, they contain many cytoskeletal proteins including actin and many focal adhesion proteins (7). We found that although a small fraction of Cas is localized in focal adhesions in NIH 3T3 cells, Cas moves from the cytoplasm to the podosomes as a result of the introduction of 527F-c-Src. We further demonstrated that both the SH3 domain and the Src binding domain of Cas are required for the efficient localization of Cas to focal adhesions.

MATERIALS AND METHODS

Cell lines and antibodies. 3T3-aSrc is a stable transformant of NIH 3T3 cells transfected with activated *src* (527F-c-*src*) as described previously (14). Abl-negative fibroblasts and Src-negative fibroblasts were kindly provided by B. J. Mayer (Howard Hughes Medical Institute) and by H. E. Varmus (National Institutes of Health), respectively. Fyn-negative and FAK-negative mouse fibroblasts were established as described previously (15, 44). Fibroblast cells except COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% bovine serum, and COS-7 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

Anti-Cas2 is a polyclonal antibody against Cas (35). MAb 327 is a monoclonal antibody against Src (23) and was a kind gift from J. S. Brugge (ARIAD Pharmaceuticals). hVIN-1 is an anti-human vinculin MAb which served as a marker for focal adhesions and was purchased from Sigma. HA.11 is a polyclonal rabbit antibody against the hemagglutinin (HA) epitope and was purchased from BAbCO. Fluorescein-labeled secondary anti-rabbit goat antibody and Texas red-labeled secondary anti-mouse donkey antibody were purchased from Jackson Laboratories, Inc. Cy-5-labeled secondary anti-mouse donkey antibody was purchased from Amersham. Tetramethylrhodamine-5-isothiocyanate (TRITC)-labeled phalloidin was purchased from Sigma.

Construction of plasmids. Most of the mutated forms of Cas were described elsewhere (29). We made three additional mutants for this study; the ΔYQXP and ΔYDXP mutants lack residues 213 to 321 and residues 322 to 514, respectively. The RF mutant is a combination of the RPLP* mutant and the Y762F mutant (29), which means that it has a mutated proline-rich sequence that destroys the capacity for binding to the Src SH3 domain (29) and a Y762F mutation that destroys the capacity for binding to the Src SH2 domain (29).

Expression plasmids for influenza virus HA-tagged Cas were constructed by PCR as follows. First, the HA tag sequence was introduced into the 3′ terminus of the Cas cDNA by PCR which amplifies the 3′ coding sequence of the Cas cDNA containing the *Bgl*II site of Cas cDNA. The primer sequences were 5′-ACC AAC CAG CCA CCC AAG-3′ (5′), and 5′-CGG AAT TCG CTC AGG AAG CGT AGT CTG GGA CGT CGT ATG GGT ACC CCT GCT TTG AGG CGG CAG CCA G-3′ (3′). After digestion with *Eco*RI and *Bgl*II, the PCR product was ligated with the 5′ *Eco*RI-*Bgl*II fragment of the Cas cDNA into the *Eco*RI site of the pSSRα expression vector (41).

The *Eco*RI fragment containing Cas-HA cDNA was excised from this construct and was cloned into pUC18 (pUC18-CasHA). The mutants of Cas cDNA were subcloned into the pSSRα vector as described previously (29). The *Acc*I fragments were excised from these vectors and cloned into pUC18-CasHA digested with *Acc*I. The *Eco*RI fragments were again excised from them and were cloned into the pSSRα vector, resulting in the expression vectors for HA-tagged mutants of Cas.

The expression plasmid for the kinase-negative 527F-c-Src was constructed by replacing the *Pvu*II fragment of the 527F-c-Src in the pSSRα vector (29) with that of the K295M mutant of chicken c-*src* (28).

Immunofluorescence. Cells were grown on uncoated coverslips (Matsunami) for 16 to 24 h or on mouse fibronectin (Gibco)-coated coverslips for 40 min. They were washed twice with phosphate-buffered saline (PBS) and fixed with 3.7% formaldehyde in PBS. The fixed cells were washed twice with PBS and permeabilized with 0.2% Triton X in PBS. The cells were rinsed and then blocked in PBS plus 1% bovine serum albumin (Sigma). Primary antibodies were used at the following dilutions for 3 h at room temperature in a humidified chamber: 1/200 for anti-Cas2, 1/2,000 for anti-HA.11, 1/200 for hVIN-1, and 1/50 for MAb 327 (0.1 mg/ml). The coverslips were washed three times with PBS and treated with secondary antibodies at the recommended dilutions. After being washed three times with PBS, the coverslips were mounted in a 1:1 mixture of 2.5% DABCO (Sigma) in PBS and glycerol. The cells were observed under an MRC1024 confocal microscopic system (Bio-Rad). For the staining of F-actin, TRITC-conjugated phalloidin was incubated for 1 h at room temperature, and the coverslips were washed and mounted similarly.

Transfection of mutated Cas into cells. COS-7 cells were transfected with expression plasmids by the DEAE-dextran method essentially as described previously (29). FAK-negative cells were transfected with 12.5 μg of plasmid DNA per 10-cm dish by the calcium phosphate method and cultured for 48 h. For obtaining the stable transformants, 3T3-aSrc cells were transfected with 2 μg of the cDNA for Cas or Cas mutants together with 0.1 μg of pSV2bsr (Kaken Seiyaku) and Lipofectamine (Gibco) reagents as specified by the manufacturer and was selected with 5 μg of blasticidin hydrochloride (Kaken Seiyaku) per ml. For the transient transfection, 2 μg of the cDNA for Cas or Cas mutants was transfected into 3T3-aSrc cells, NIH 3T3 cells, or Src-negative cells with Lipofectamine reagents, and the cells were cultured for 48 h.

Western blotting. Cells were lysed in 1% Triton buffer (10 mM Tris-HCl [pH 7.4], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate). For protein analysis, each 100 μg of the total-cell lysates was subjected to Western blot analysis as described previously (40). Anti-HA.11 was used at a dilution of 1/1,000, and anti-Cas2 antibody (35) was used at a dilution of 1/2,500. The ProtoBlot Western AP system (Promega) was used for detection.

RESULTS

Localization of Cas in nontransformed cells and in 527F-c-Src-transformed cells. First, we examined the subcellular localization of Cas in NIH 3T3 cells by immunofluorescence microscopy (Fig. 1A to C). NIH 3T3 cells were grown overnight on uncoated coverslips and were costained with anti-Cas ntibody (anti-Cas2) (Fig. 1B), anti-vinculin antibody (hVIN-1) (Fig. 1C), and TRITC-labeled phalloidin to reveal the actin filament formation (Fig. 1A). Immunostaining with anti-Cas2 (Fig. 1B) gave mainly cytoplasmic staining patterns. However, faint but clear staining in focal adhesions was observed (Fig. 1B). This was confirmed by the colocalization with the staining by antivinculin (Fig. 1C). Nuclear staining was not observed. The localization pattern of Cas in COS-7 cells was similar (Fig. 1G to I) to that in NIH 3T3 cells.

Next, 3T3-aSrc (527F-c-Src) cells were immunostained with anti-Cas2 (Fig. 1E), anti-vinculin (Fig. 1F), and phalloidin (Fig. 1D). Some of the 3T3-aSrc cells were morphologically rounded, vinculin was localized solely in the cytoplasm, and no focal adhesion staining was observed. In those cells, Cas was also localized in the cytoplasm (data not shown). The other 3T3-aSrc cells contained podosomes. These structures are visualized by the staining with anti-vinculin and phalloidin (Fig. 1D and F). Cas was localized mainly in the podosomes, and the cytoplasmic staining was weak (Fig. 1E). Nuclear staining was not observed.

These results indicate that Cas is localized mainly in cytoplasm and partially in focal adhesions in nontransformed cells. On the other hand, when the cells are transformed by the activated Src kinase, Cas migrates to the podosomes.

Distribution of Cas mutants in 527F-c-Src-transformed cells. To determine which parts of Cas are responsible for the localization of Cas to focal adhesions, we constructed a series of HA-tagged Cas mutants (Fig. 2) and examined their subcellular localizations. First, we observed the localization of Cas mutants in 3T3-aSrc cells stably expressing these mutants (Fig. 3A) by immunostaining with anti-HA (Fig. 4) and with anti-vinculin (data not shown), because, as described above (Fig. 1E), the localization of Cas to podosomes could be clearly evaluated in these cell lines.

HA-tagged Cas (CasHA) was clearly stained in the podosomes, as shown in Fig. 4A, and the cytoplasmic staining was weak. The nuclear staining in these experiments was considered to be caused by the nonspecific background of anti-HA antibody used here, because it was still observed in the absence of the expression of HA-tagged proteins and because immu-

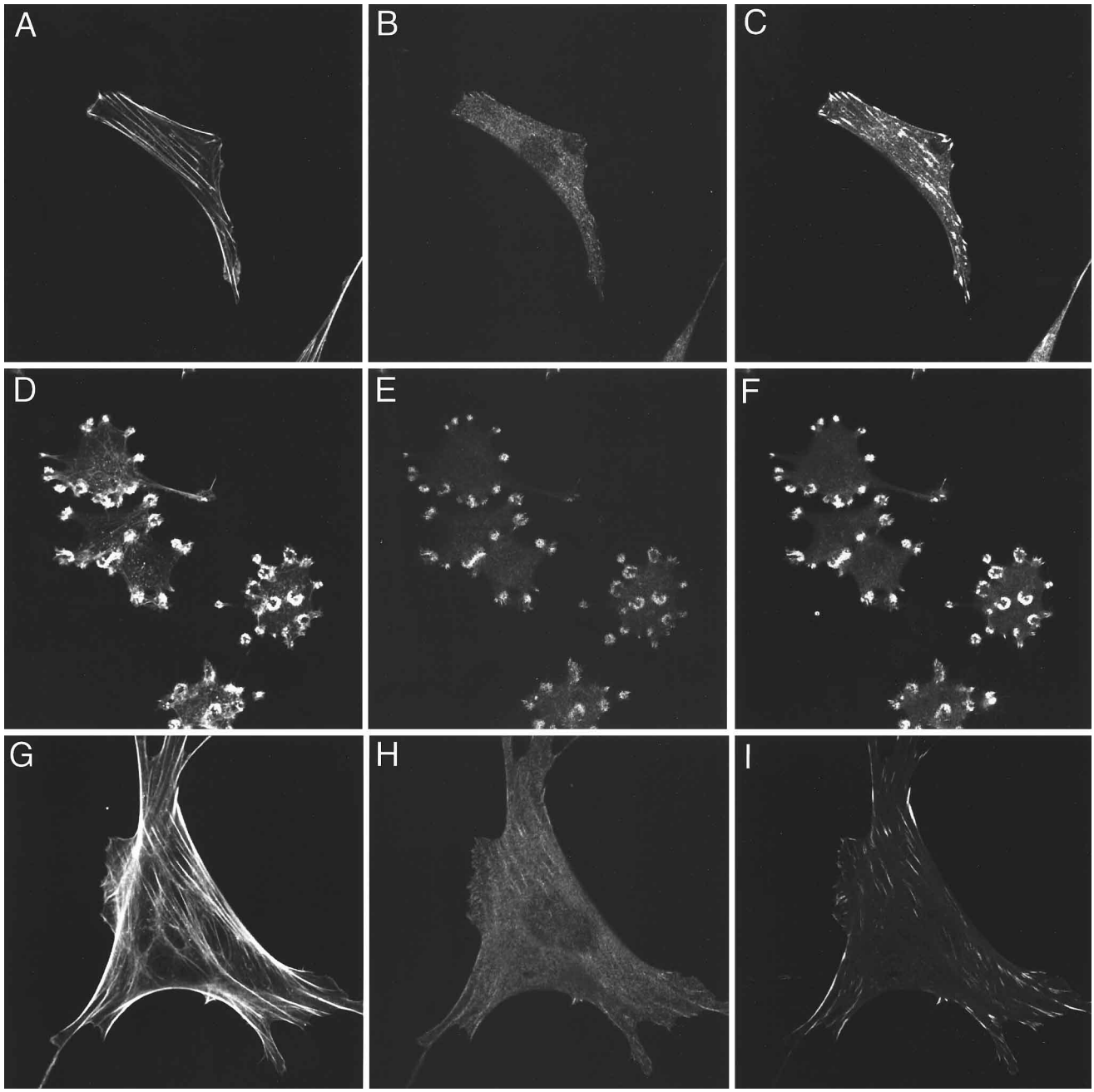

FIG. 1. Localization of Cas in nontransformed NIH 3T3 and COS-7 and aSrc-transformed NIH 3T3 cells. NIH 3T3 (A to C), 3T3-aSrc (D to F), and COS-7 (G to I) cells were grown overnight on the uncoated coverslips and fixed. Immunofluorescence was performed with TRITC-phalloidin (A, D, and G), anti-Cas2 (B, E, and H), or anti-vinculin (C, F, and I) as described in Materials and Methods.

nostaining with anti-Cas did not exhibit nuclear staining (data not shown).

The ΔSB mutant with a deletion of the Src binding domain and the RPLP mutant with a mutation in the Src SH3 binding proline-rich sequence (29) were not localized to podosomes (Fig. 4C and D) in spite of having similar expression levels to the CasHA-expressing cell line (Fig. 3A). In contrast, the ΔYDXP mutant which lacks all the YDXP motifs in the substrate domain, and Y762F mutant, which has the mutation in the Src SH2-binding tyrosine residue (29) were still localized to focal adhesions (Fig. 4B and E).

In spite of repeated attempts, we could not obtain 3T3-aSrc cells stably expressing the ΔSH3, ΔYQXP, or ΔSD mutant of Cas. Therefore, we examined the subcellular localization of these mutant proteins by transient expression in 3T3-aSrc cells and immunostained them with anti-HA and anti-vinculin. The expression level of each mutant is shown in Fig. 3B. CasHA and the ΔSD mutant were located predominantly in podosomes (Fig. 5A and C). However, staining in focal adhesions or in podosomes was not detected in the 3T3-aSrc cells expressing the ΔSH3 mutant (Fig. 5B). In the ΔSB mutant, cytoplasmic staining was predominant, in contrast to wild-type Cas, and the

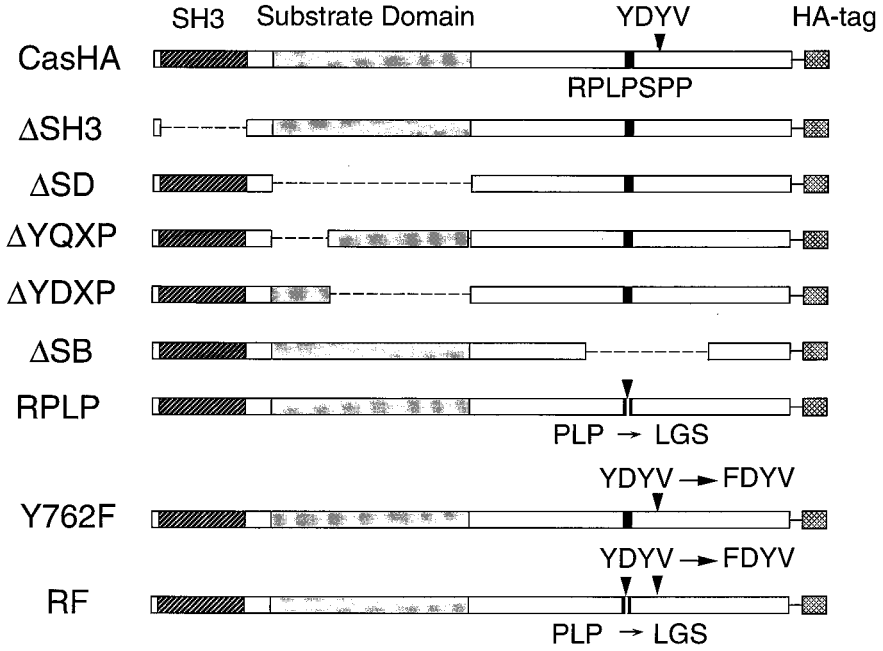

FIG. 2. Schematic diagram representing the various Cas constructs used. HA-tagged Cas constructs were introduced into pSSRα vector. Details for each mutant are given in Materials and Methods.

staining of focal adhesions or podosomes was barely detectable (Fig. 5D), demonstrating that the Src binding domain enhances the localization of Cas to podosomes.

These results suggest that the SH3 domain of Cas is essential and the Src binding domain is also important for the recruitment of Cas to podosomes in 3T3-aSrc cells.

The kinase activity of Src kinase is not required for the localization of Cas to focal adhesions. To determine whether the kinase activity of 527F-c-Src is required for the localization of Cas to focal adhesions, we transiently expressed the kinase-negative mutant (K295M mutant) of 527F-c-Src (527F/295M-c-Src) in NIH 3T3 cells and examined the localization of Cas in these cells. When the 527F/295M-c-Src was expressed, Cas as well as the 527F/295M-c-Src was stained predominantly in focal adhesions (Fig. 6C and D), as was the case with the kinase-positive 527F-c-Src (Fig. 6A and B). In contrast, the localization of Cas to focal adhesions was not as prominent in the mock-transfected cells (Fig. 6E) as in the 527F/295M-c-Src transfected cells. This result suggests that the kinase activity of Src is not required for enhancement of the localization of Cas to focal adhesions. However, since NIH 3T3 cells express endogenous, kinase-active c-Src, we expressed the 527F/295M-c-Src in Src-negative cells.

In contrast to NIH 3T3 cells, the localization of Cas to focal adhesions was not detected in Src-negative cells (Fig. 7B), in spite of the formation of focal adhesions as visualized by the immunostaining with anti-vinculin (Fig. 7A). With the introduction of 527F/295M-c-Src (Fig. 7C), the localization of Cas to focal adhesions became prominent (Fig. 7D). The mock-transfected cells did not show localization of Cas to focal adhesions (Fig. 7E).

Distribution of Cas mutants transiently expressed in COS-7 cells. As shown above, Cas is partially localized to focal adhesions in nontransformed cells. We next searched for the region that is necessary for the localization of Cas to focal adhesions in nontransformed cells. We transiently expressed CasHA and its mutants in COS-7 cells (Fig. 3C), plated them on uncoated coverslips, and performed immunostaining with anti-HA tag (Fig. 8B, D, F, and H) and anti-vinculin (Fig. 8A, C, E, and G). CasHA and the ΔSD and ΔSB mutants were localized mainly to the cytoplasm and partially to focal adhesions (Fig. 8B, F, and H). In contrast, the deletion of the SH3 domain abrogated its localization to focal adhesions (Fig. 8D).

Since Cas was reported to be tyrosine phosphorylated in response to stimulation with fibronectin (30, 32, 43), we investigated the distribution of Cas mutants expressed in COS-7

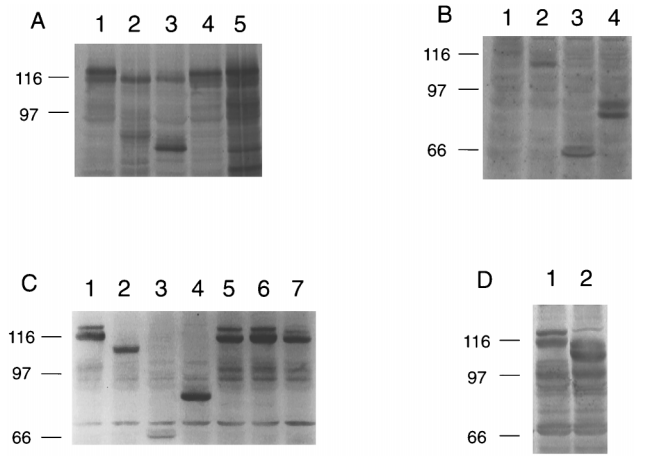

FIG. 3. Expression levels of Cas and Cas mutants. (A) The expression of each mutant of Cas in stably transfected 3T3-aSrc cells was detected by Western blotting with anti-Cas2. Lanes: 1, CasHA; 2, ΔDXP; 3, ΔSB; 4, RPLP; 5, Y762F. (B) The expression of each mutant of Cas in transiently transfected 3T3-aSrc cells was detected by Western blotting with anti-HA.11. Lanes: 1, CasHA; 2, ΔSH3; 3, ΔSD; 4, ΔSB. (C) The expression of each mutant of Cas in COS-7 cells was detected by Western blotting with anti-HA.11. Lanes: 1, CasHA; 2, ΔSH3; 3, ΔSD; 4, ΔSB; 5, RPLP; 6, Y762F; 7, RF. (D) The expression of each mutant of Cas in FAK-negative cells was detected by Western blotting with anti-HA.11. Lanes: 1, CasHA; 2, ΔSH3.

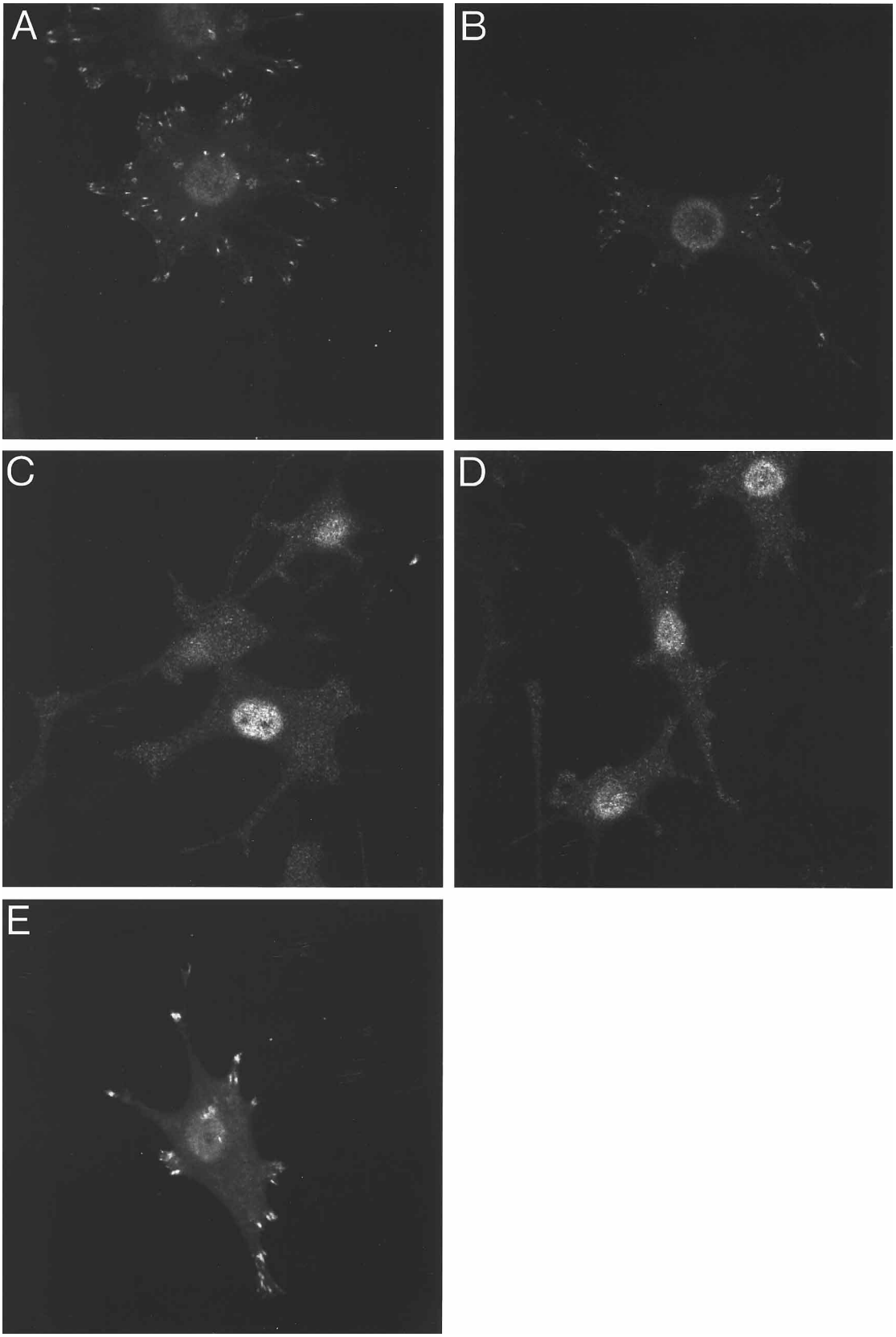

FIG. 4. Localization of stably expressed Cas mutants in 3T3-aSrc cells. 3T3-aSrc cells expressing CasHA (A), ΔYDXP (B), ΔSB (C), RPLP (D), and Y762F (E) were grown overnight on the uncoated coverslips and fixed. The immunofluorescence study was performed with anti-HA.11 as described in Materials and Methods.

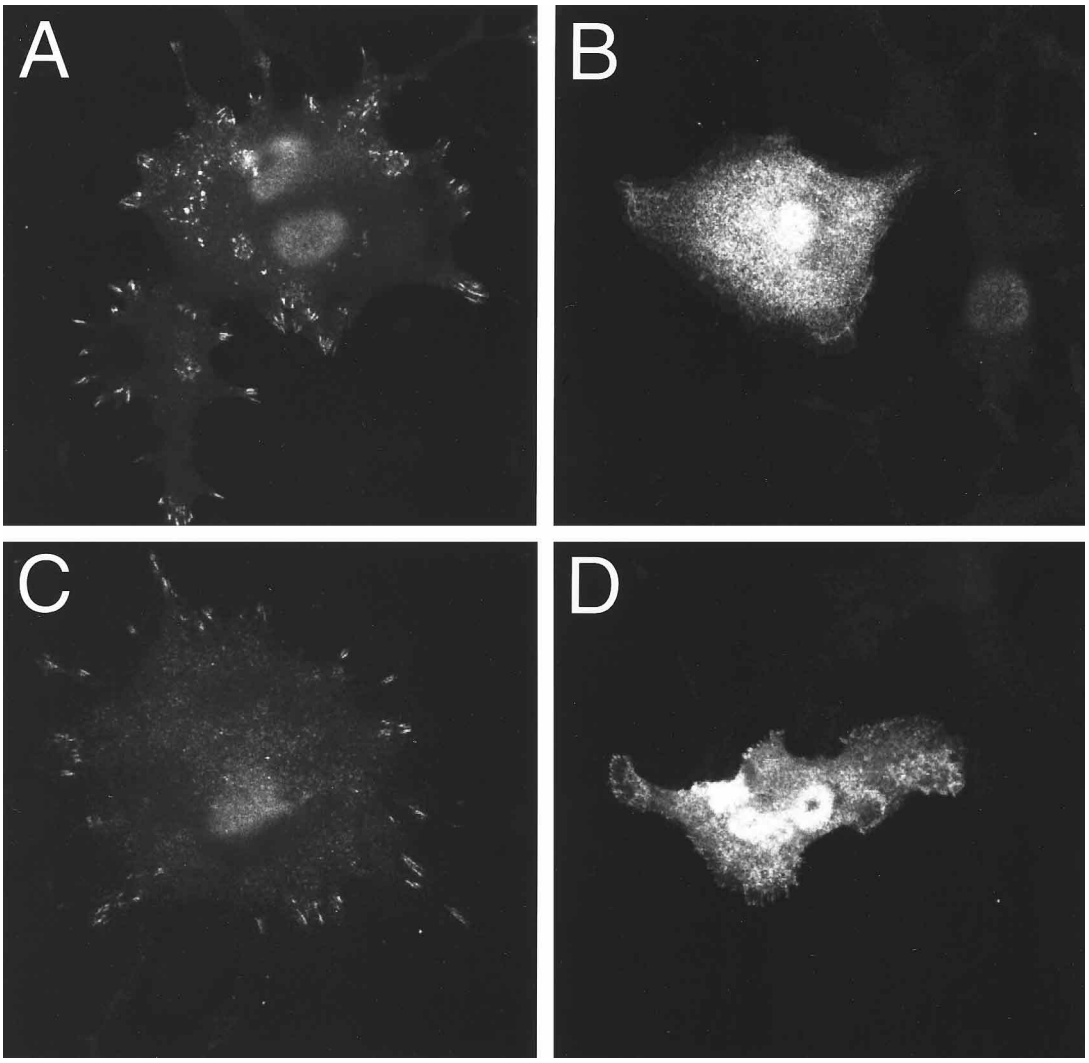

FIG. 5. Localization of transiently expressed Cas mutants in 3T3-aSrc cells. 3T3-aSrc cells were plated on the uncoated coverslips 2 days before transfection of the expression plasmids for CasHA (A), ΔSH3 (B), ΔSD (C), and ΔSB (D) with Lipofectamine reagent. Two days after the transfection, the cells were fixed and immunostained with anti-HA.11 as described in Materials and Methods.

cells (Fig. 3C) stimulated by fibronectin. CasHA and the ΔSD mutant were localized in focal adhesions around the cell periphery as well as in the cytoplasm (Fig. 9A and C), while the ΔSB and ΔSH3 mutants remained solely in cytoplasm (Fig. 9B and D). The RPLP mutant and the Y762F mutant were localized to focal adhesions to some extent (Fig. 9E and F). However, in the cells expressing the RF mutant, which has mutations in both the Src SH3 binding proline-rich motif and the Src SH2 binding tyrosine residue, the distribution pattern of this mutant was exclusively cytosolic (Fig. 9G).

Subcellular localization of Cas in kinase-negative cell lines. Cas is known to be associated with several tyrosine kinases. The SH3 domain is shown to bind to FAK (33). The substrate domain is supposed to be associated with Abl, as shown by in vitro experiments (26). The Src binding domain is reported to bind to 527F-c-Src (29), c-Src (35), and other Src family kinases including Fyn (37). Therefore, to gain insight into the involvement of tyrosine kinases in the localization of Cas, we immunostained the FAK-negative, Abl-negative, Src-negative, and Fyn-negative cell lines with anti-Cas antibody. In FAK-negative cells, Abl-negative cells, and Fyn-negative cells, Cas was localized to focal adhesions as well as to the cytoplasm (Fig. 10A to C). In contrast, in Src-negative cells, the focal adhesion staining was not observed with anti-Cas antibody (Fig. 7B), as described above.

To determine whether the localization of Cas to focal adhesions in the FAK-negative cells requires the SH3 domain of Cas, we expressed the ΔSH3 mutant in FAK-negative cells (Fig. 3D). Although CasHA was localized in focal adhesions (Fig. 11A and B), the ΔSH3 mutant showed a cytoplasmic staining pattern, and no staining was observed in focal adhesions (Fig. 11C and D). This result indicates that some molecule other than FAK mediates the localization of Cas to focal adhesions through the Cas SH3 domain in FAK-negative cells.

DISCUSSION

In this study, we clarified the region of Cas required for its efficient localization to focal adhesions by immunofluorescence as summarized in Table 1. We further identified Src kinase as the protein possessing a specific role in the recruitment of Cas to focal adhesions.

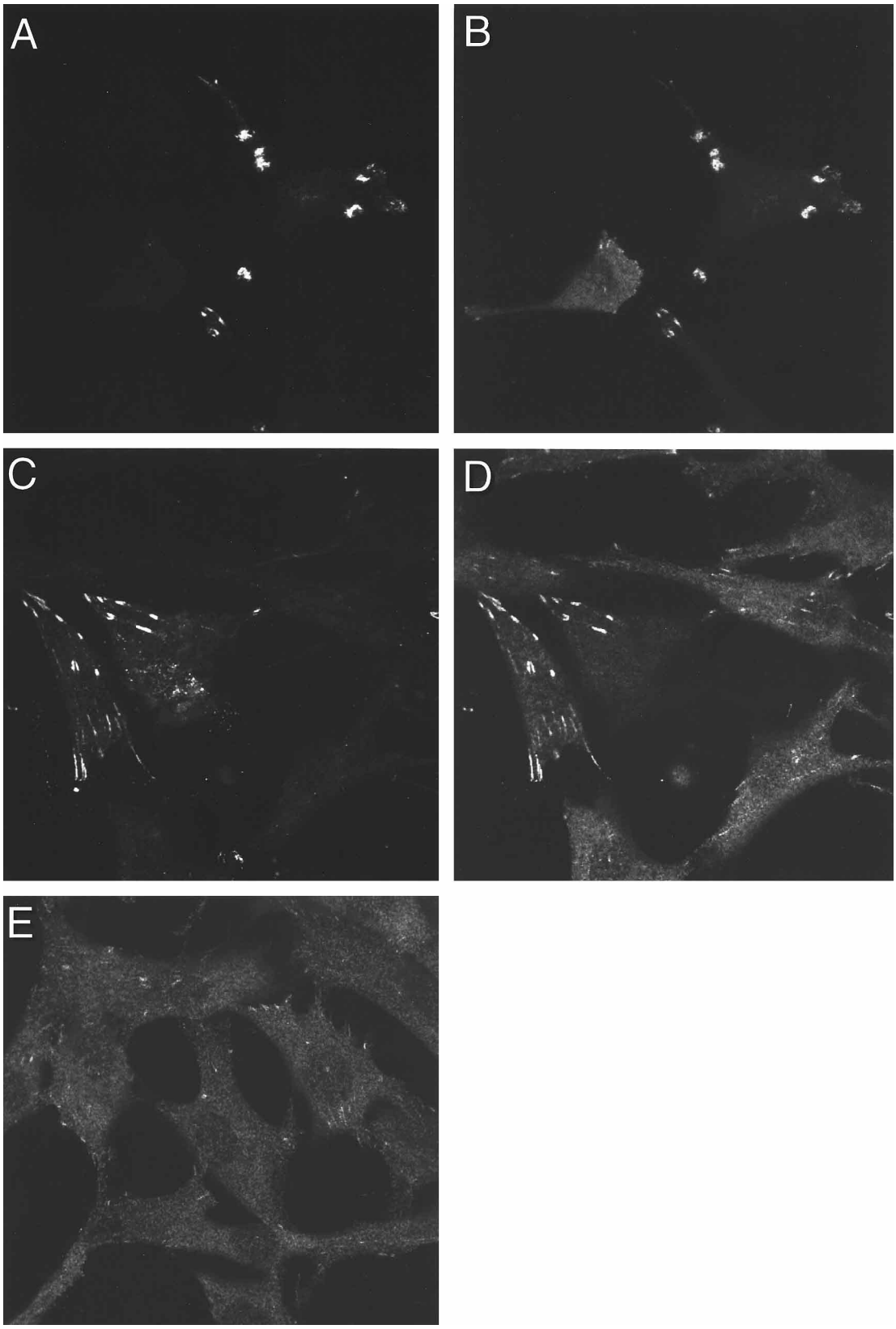

FIG. 6. Cas is localized to focal adhesions in kinase-negative 527F-c-Src-expressing NIH 3T3 cells. NIH 3T3 cells were plated on uncoated coverslips 2 days before transfection of the expression plasmids for 527F-c-Src (A and B), 527F/295M-c-Src (C and D), and the vector only (E) with Lipofectamine reagent. Two days after the transfection, the cells were fixed and immunostained with anti-Cas2 (A, C, and E) and MAb 327 (B and D) as described in Materials and Methods.

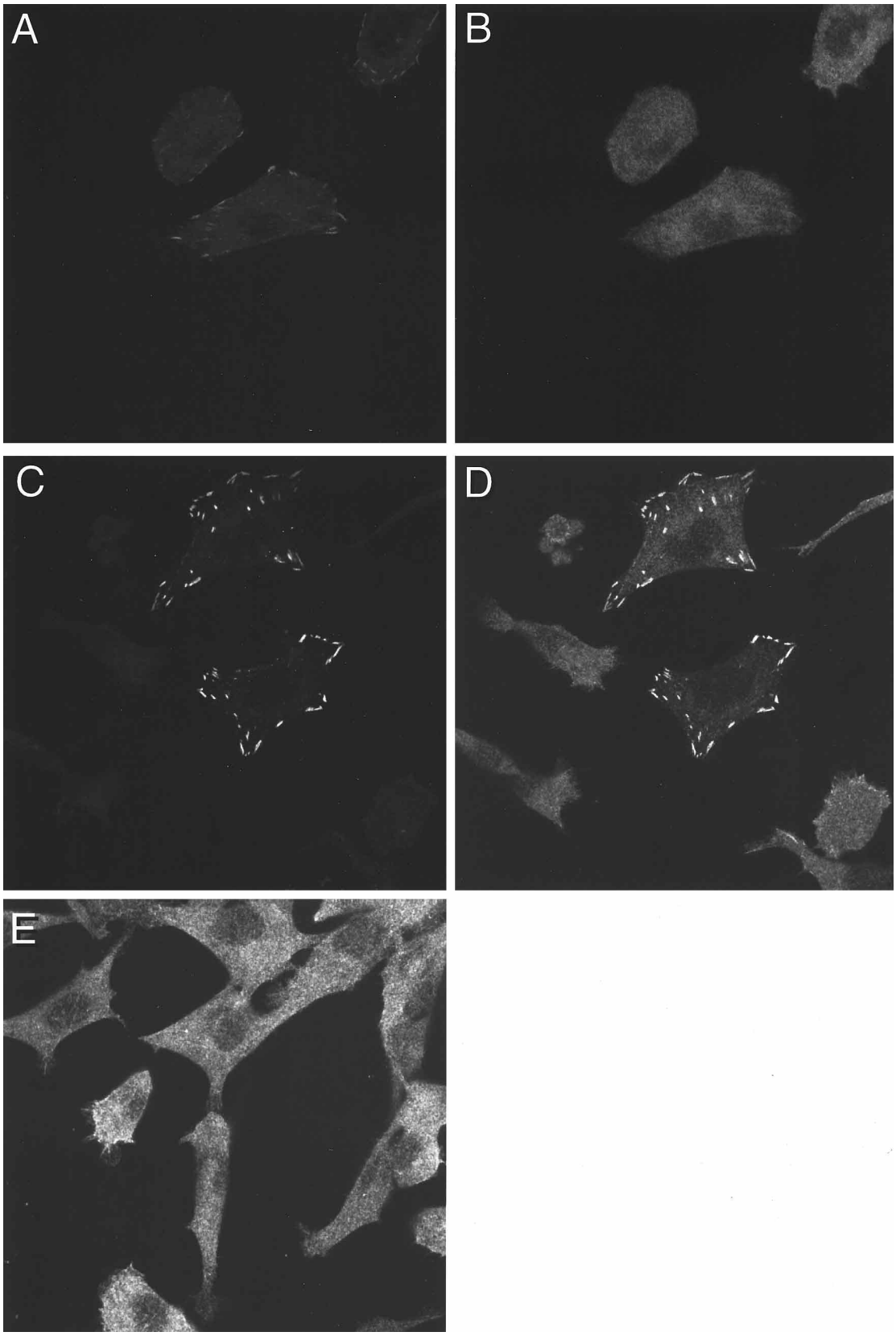

FIG. 7. Cas is localized to focal adhesions in kinase-negative 527F-c-Src-expressing Src-negative cells. Src-negative cells were plated on uncoated coverslips and immunostained with anti-hVIN-1 (A) or anti-Cas2 (B) 2 days later. Src-negative cells were plated on uncoated coverslips 2 days before transfection of the expression plasmids for 527F/295M-c-Src (C and D) and the vector only (E) with Lipofectamine reagent. Two days after the transfection, the cells were fixed and immunostained with MAb 327 (C) and anti-Cas2 (D and E) as described in Materials and Methods.

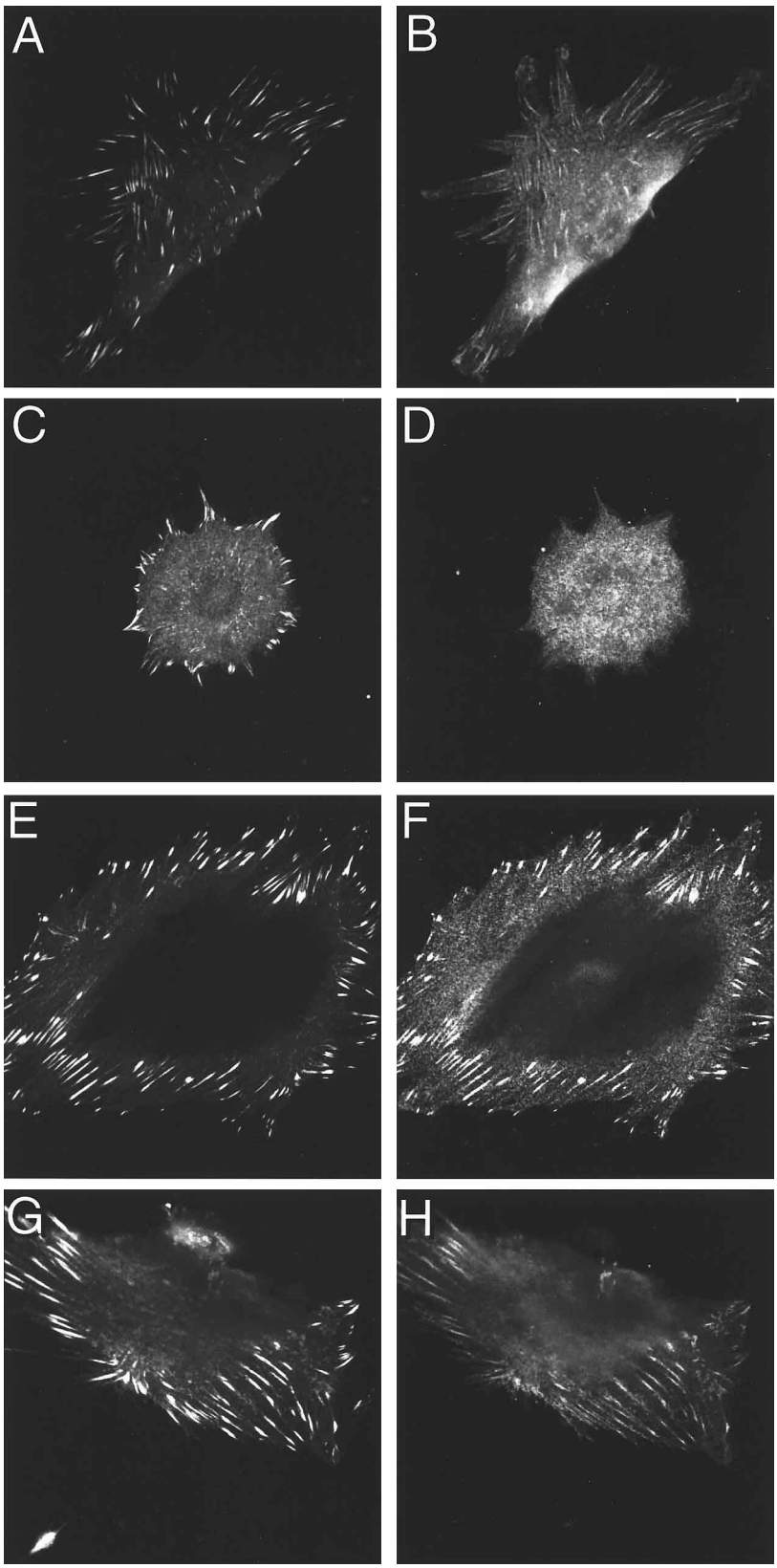

FIG. 8. Localization of Cas mutants expressed in COS-7 cells. COS-7 cells were transfected with the expression plasmids for CasHA (A and B), ΔSH3 (C and D), ΔSD (E and F), and ΔSB (G and H). Two days after the transfection, the cells were plated on uncoated coverslips and grown overnight. The cells were fixed and were immunostained with anti-hVIN-1 (A, C, E, and G) or anti-HA.11 (B, D, F, and H) as described in Materials and Methods.

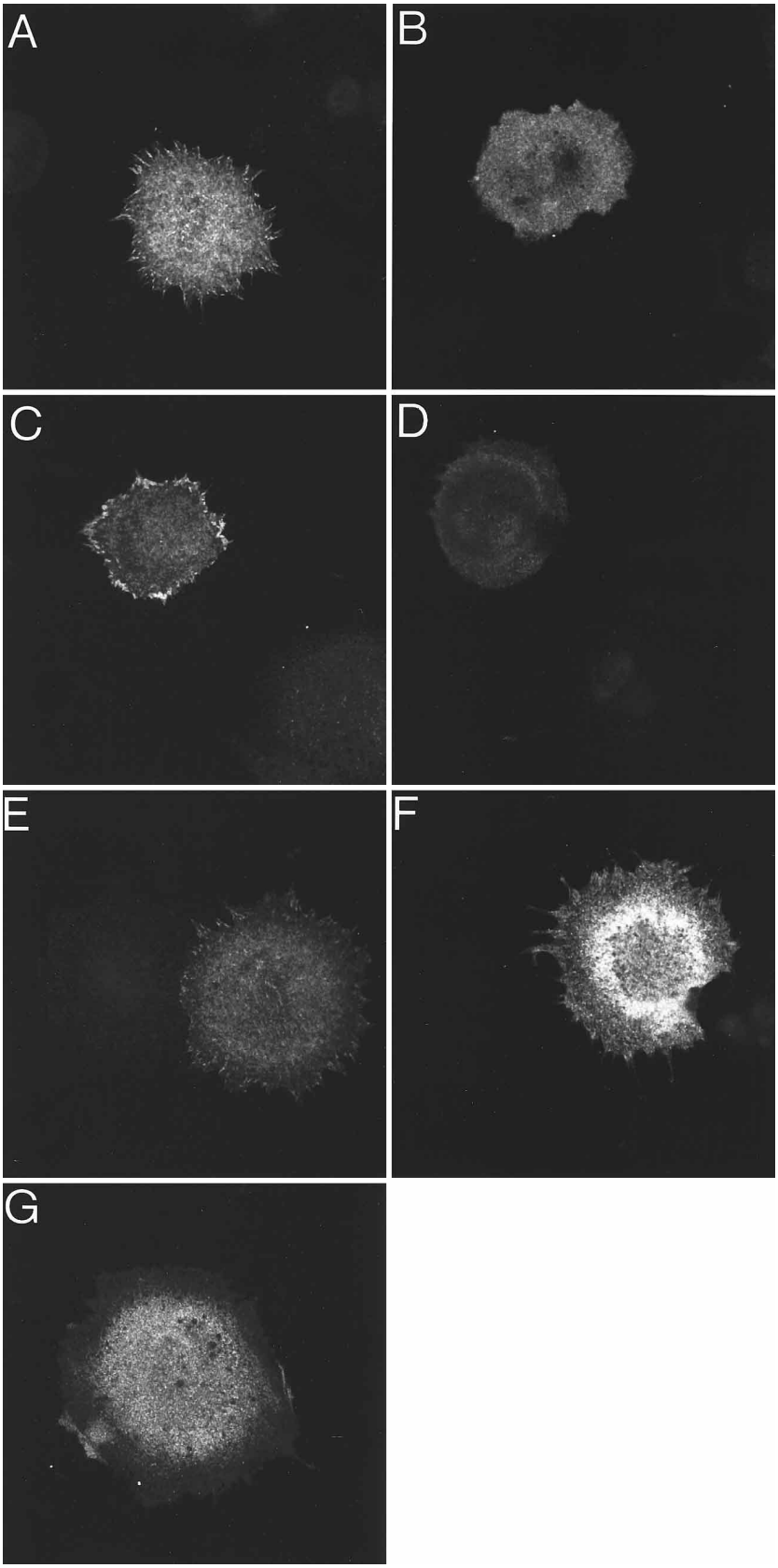

FIG. 9. Localization of Cas mutants upon integrin stimulation. COS-7 cells were transfected with the expression plasmid for CasHA (A), ΔSH3 (B), ΔSD (C), ΔSB (D), RPLP (E), Y762F (F), or RF (G). Two days after the transfection, the cells were plated on fibronectin-coated coverslips and incubated for 40 min. The cells were fixed and were immunostained with anti-HA.11 as described in Materials and Methods.

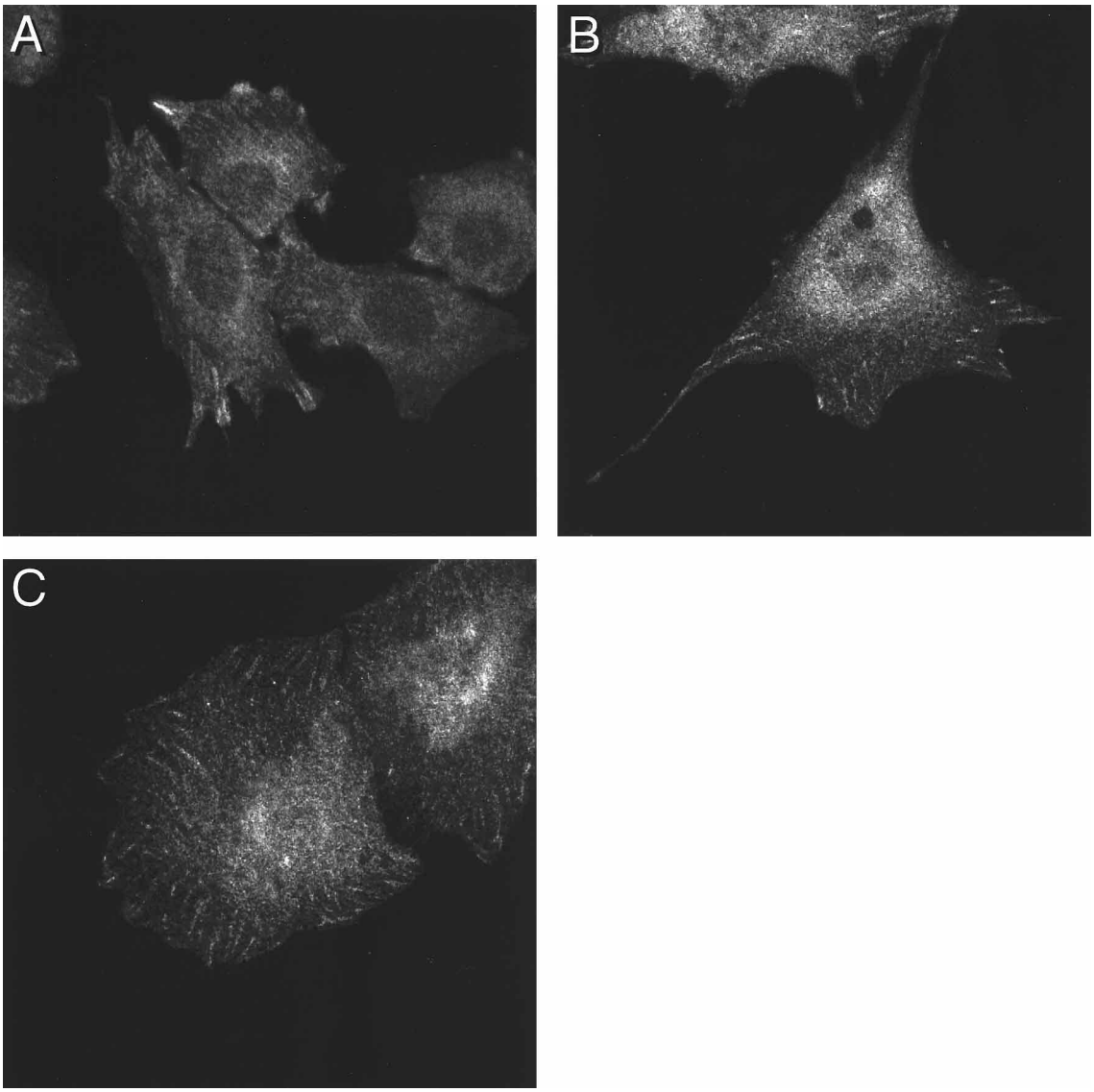

FIG. 10. Localization of Cas in kinase-negative cells. FAK-negative (A), Abl-negative (B), and Fyn-negative (C) cells were grown overnight on uncoated coverslips and were fixed. Immunofluorescence was performed with anti-Cas2 (A to C) and anti-hVIN-1 (data not shown) as described in Materials and Methods.

The SH3 domain of Cas is essential for its localization to focal adhesions. The deletion mutant which lacks the SH3 domain of Cas was located exclusively in cytoplasm in both 3T3-aSrc cells and COS-7 cells. This result indicates that the SH3 domain of Cas is essential for the localization of Cas to focal adhesions. The SH3 domain of Cas was reported to bind to FAK and FRNK (13, 33), and the expression of the constitutively active FAK in REF-52 cells results in the constitutive phosphorylation of Cas (42). Therefore, it might be postulated that the association with FAK directs Cas to the focal adhesions. However, Cas is also localized to focal adhesions in FAK-negative cells (Fig. 10A), and this localization is mediated by the SH3 domain (Fig. 11). These findings indicate that the association of Cas with FAK is not essential for the localization of Cas and that some molecule other than FAK directs Cas to focal adhesions. Otherwise, there might be some redundancy of FAK concerning the association with Cas, namely, indicating that some member(s) (2, 17, 22, 38) of the FAK family kinases might interact with Cas in place of FAK in these FAK-negative cells.

The substrate domain is not necessary for the localization of Cas to focal adhesions. The substrate domain binds to v-Crk upon transformation by v-Crk (6, 29) and is presumed to bind to c-Crk, especially following attachment on fibronectin (11, 31, 42). In both 3T3-aSrc and COS-7 cells, the transiently expressed ΔSD mutants were still localized to focal adhesions, indicating that the binding of the substrate domain to c-Crk or other proteins does not contribute to the localization of Cas to focal adhesions in 527F-c-Src-transformed cells or in the nontransformed cells attached on fibronectin. Rather, the substrate domain of Cas may act as a binding sites for c-Crk or other signaling molecules after Cas has moved to focal adhesions (6, 11, 42).

Roles of Src kinase and the Src binding domain of Cas in the localization of Cas to focal adhesions. In nontransformed NIH 3T3 cells and COS-7 cells, Cas was distributed predomi-

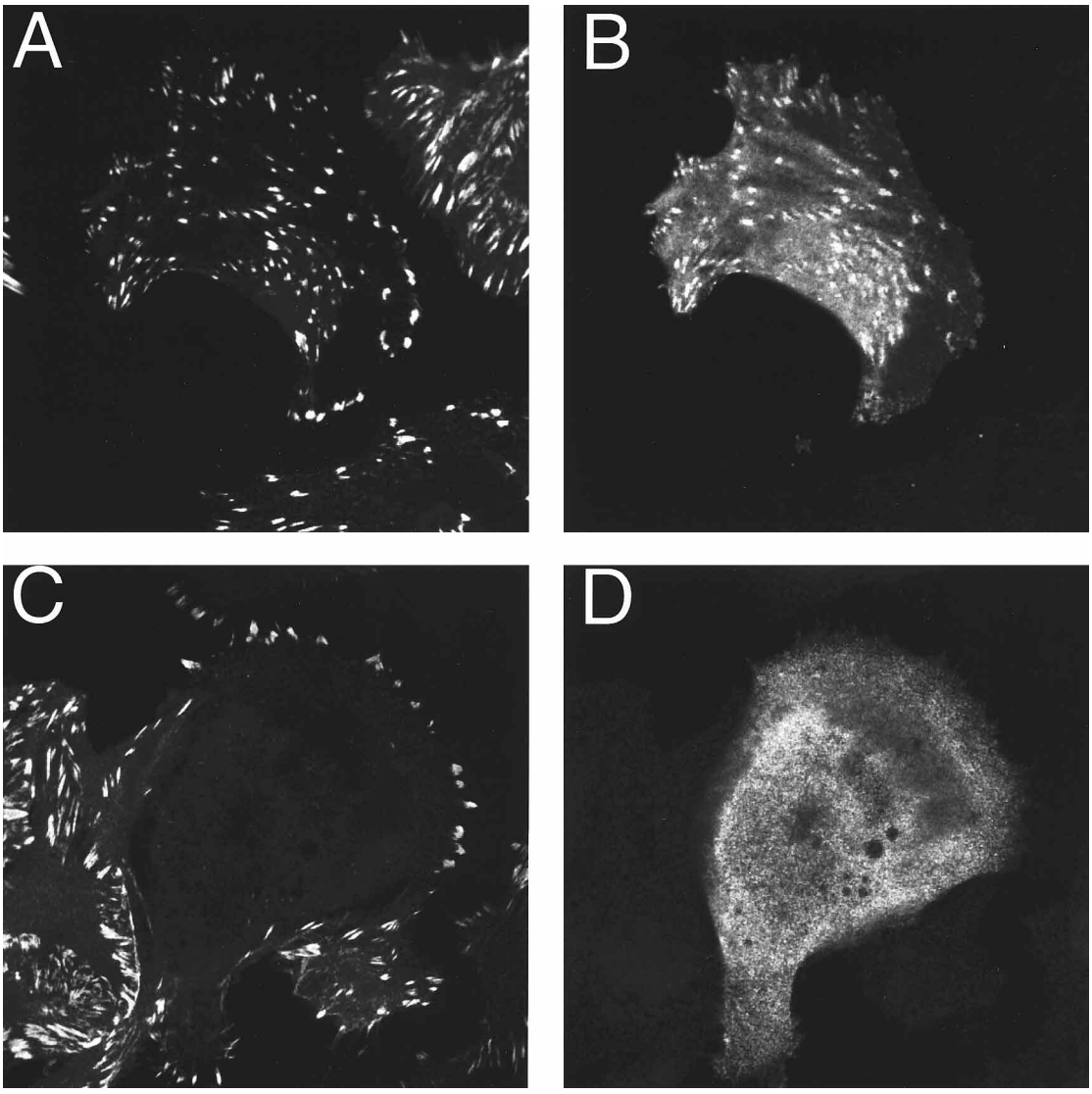

FIG. 11. Deletion of the SH3 domain abolishes the localization of Cas to focal adhesions. FAK-negative cells were plated on uncoated coverslips 2 days before transfection of the expression plasmids for CasHA (A and B) and ΔSH3 (C and D) by the calcium phosphate method. Two days after the transfection, the cells were fixed and immunostained with anti-hVIN-1 (A and C) and anti-HA.11 (B and D) as described in Materials and Methods.

nantly to the cytoplasm but only weakly to focal adhesions. On the other hand, in 527F-c-Src-transformed cells (3T3-aSrc cells), the distribution of Cas was mainly in podosomes and cytosolic staining was attenuated. These data show that Cas moves from the cytoplasm to podosomes by the expression of 527F-c-Src.

The kinase activity of 527F-c-Src was not required for the translocation of Cas. This observation, as well as the dispensability of the substrate domain, suggests that 527F-c-Src-mediated localization of Cas to focal adhesions is caused not by the tyrosine phosphorylation of Cas with activated Src but by the binding of Cas to 527F-c-Src itself (29). Immunostaining of NIH 3T3 and 3T3-aSrc with anti-phosphotyrosine antibody shows the focal adhesion pattern (reference 25 and data not shown), suggesting that Cas and other proteins are tyrosine phosphorylated in focal adhesions. Cas is localized to focal adhesions and becomes tyrosine phosphorylated when the kinase-negative 527F-c-Src is expressed (unpublished results). These findings suggest that the tyrosine phosphorylation of Cas

TABLE 1. Localization of Cas to focal adhesions or podosomes in various cells

Cas mutant	Localization to focal adhesions in[a]:				
	aSrc/stable	aSrc/transient	COS-7	COS-7/FN	FAK−/−
CasHA	++	++	+	+	+
ΔSH3	NT	−	−	−	−
ΔSD	NT	++	+	+	NT
ΔDXP	++	NT	NT	NT	NT
ΔSB	−	−	+	−	+*
RPLP	−	NT	NT	+	NT
Y762F	++	NT	NT	+	NT
RF	NT	NT	NT	−	NT

[a] ++, predominant localization to focal adhesions or podosomes; +, partial localization to focal adhesion and predominant localization in cytoplasm; −, no localization to focal adhesions or podosomes; NT, not tested; COS-7/FN, COS-7 cells attached on fibronectin; *, data not shown.

is promoted by the localization of Cas to focal adhesions, where various kinases such as FAK, Abl, and c-Src are found (3, 12, 19, 20, 34, 39). Many of them are implicated in the tyrosine phosphorylation of Cas (5, 11, 26, 33, 42). Cas is reported not to be tyrosine phosphorylated in response to integrin stimulation in Src-negative cells (5, 11, 42). Since Cas is not localized to focal adhesions in Src-negative cells, it is possible that the lack of Cas tyrosine phosphorylation is because of the absence of Cas in focal adhesions. Therefore, further investigation of the kinases responsible for the tyrosine phosphorylation of Cas in focal adhesions should be required.

In 3T3-aSrc cells and in COS-7 cells stimulated with fibronectin, deletion of the Src binding domain of Cas resulted in total loss or significant reduction in the recruitment of Cas to podosomes or focal adhesions. In contrast, in unstimulated cells, the localization of Cas to focal adhesions was not affected by the deletion of the Src binding domain. As described above, Cas is preferentially localized to podosomes in 3T3-aSrc cells but remains predominantly in the cytoplasm in NIH 3T3 and COS-7 cells. Therefore, we propose the following hypothesis. The Cas SH3 domain is essential for the localization of Cas to focal adhesions, but the affinity to focal adhesions mediated by the SH3 domain is weak. In NIH 3T3 cells and in unstimulated COS-7 cells, the SH3 domain but not the Src binding domain is responsible for the localization of Cas to focal adhesions. As a result, the affinity of Cas to focal adhesions is reduced compared with that to cytoplasm. In 3T3-aSrc cells, since 527F-c-Src is located in podosomes (19), the localization of Cas to podosomes is mediated by both the SH3 domain and the Src binding domain. This would increase the affinity of Cas to podosomes, resulting in its predominant distribution to podosomes. Following the adhesion of fibroblasts to fibronectin, c-Src was reported to be activated and redistributed to newly formed focal adhesions (20). This might increase the affinity of Cas to focal adhesions in a similar fashion to the increase in the affinity of Cas to podosomes in 3T3-aSrc cells. The reason why the ΔSB mutant did not migrate to focal adhesions in stimulated cells and in 527F-c-Src-expressing cells in spite of possessing the SH3 domain may be explained by the idea that the ΔSB mutant would be competed by the endogenous Cas that has both the SH3 domain and the Src binding domain.

In Src-negative cells, Cas is not localized to focal adhesions in spite of the presence of the Cas SH3 domain. Src-negative cells are reported to have a defect in spreading on fibronectin (20), and the lack of Src kinase might change the composition or the tyrosine phosphorylation levels of focal adhesions, abolishing the localization of Cas to focal adhesions.

The kinase-negative Src recruited Cas to focal adhesions even in the Src-negative cells. On the other hand, c-Src rescues the defect in spreading of Src-negative cells in a kinase-independent manner (20). It is possible that c-Src exerts its function in spreading on fibronectin through the interaction with Cas.

Possible models for the localization of Cas to focal adhesions. As shown above, the SH3 domain of Cas is essential for its localization to focal adhesions, whereas Src kinase plays a major role in the localization of Cas and activated Src has been shown to bind to the Src binding region of Cas (29). One possible model that explains this contradiction is that the Cas SH3 binding protein which recruits Cas weakly to focal adhesions can form a ternary complex with activated Src and Cas, enhancing the affinity of Cas for focal adhesions. In this model, activated Src acts as an adapter molecule that links Cas and the putative Cas SH3 binding protein. Therefore, identification of the putative Cas SH3 binding protein would clarify the precise mechanism of the localization of Cas to focal adhesions and the signal transduction involving focal adhesions.

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