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Overexpressed Csk Tyrosine Kinase Is Localized in Focal Adhesions, Causes Reorganization of $\alpha_v\beta_5$ Integrin, and Interferes with HeLa Cell Spreading

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The C-terminal Src kinase p50^{csk} phosphorylates Src family tyrosine kinases and down-regulates their activity in vitro. To gain insight into the cellular functions of this potentially antioncogenic enzyme, we have overexpressed the *csk* cDNA by using an inducible promoter in HeLa cells. Despite some differences in basal Src activity in the clones analyzed, Src activity was not significantly suppressed, while the amount of p50^{csk} and Csk activity increased at least 10-fold during 3 days of induction. Immunofluorescence for the induced p50^{csk} was localized in the cytoplasm and distinctly in focal adhesions, in which the amount of phosphotyrosine containing proteins was also increased. Point and deletion mutagenesis experiments showed that localization in focal adhesions was dependent on the SH2 and SH3 domains of Csk but not on its catalytic activity. Csk formed a complex with the focal adhesion protein paxillin in cells, and its SH2 domain was shown to interact with pp125^{FAK} and paxillin in vitro. After Csk induction, the cells became spherical and more loosely attached to the culture substratum, and the $\alpha_v\beta_5$ integrin complex (vitronectin receptor) of focal adhesions was redistributed to a novel type of structure consisting of punctate plaques on the ventral cell surface. These phenotypic changes occurred in several clones analyzed and were totally reversible when Csk was switched off, but they did not occur in cells overexpressing the catalytically inactive Csk R-222 mutant or luciferase. Our results thus show that a fraction of cellular Csk is targeted to focal adhesions via its SH2 and SH3 domains, probably interacting with tyrosyl-phosphorylated focal adhesion proteins. They also suggest that Csk is involved in the regulation of integrins controlling cell attachment and shape.

The human *csk* gene, located in chromosome 15q23-q25, encodes a 50-kDa cytoplasmic protein-tyrosine kinase (TK), p50^{csk}, resembling the Src family TKs but having also some unique features. The p50^{csk} protein contains SH2 and SH3 domains and a characteristic TK domain but has a very short N-terminal tail, lacks the conserved autophosphorylation site (Tyr-416$^{c\text{-}src}$) and the regulatory C-terminal Tyr residue (Tyr-527$^{c\text{-}src}$) and is not myristylated or membrane associated (2, 5, 30, 38, 41). p50^{csk} has been shown to phosphorylate the C-terminal Tyr-527 of c-Src or its equivalent tyrosine residues in other Src family TKs in vitro and thereby strongly suppress their activity (5, 30, 34, 35). Also, p50^{csk} inactivates c-Src in yeasts, which lack endogenous Csk and c-Src (29, 30, 33). Recently, *csk* knockout experiments have established that this kinase is essential for mouse embryo development and that it down-regulates c-Src activity in embryos (24, 31). However, at least two other Csk-related kinases, HYL/Matk (4, 43) and Ctk/Ntk (10, 27), have been described.

In normal cells, pp60$^{c\text{-}src}$ is largely inactive, phosphorylated to a high stoichiometry at Tyr-527, and hypophosphorylated at Tyr-416. When activated, the opposite pattern occurs: the kinase becomes dephosphorylated at Tyr-527 and autophosphorylated at Tyr-416 (11, 22). The current view of this regulatory mechanism is that Tyr-527 has to be phosphorylated in order for the molecule to make a (probably intramolecular) bend so that phosphorylated Tyr-527 (PTyr-527) can interact with the SH2 domain (reviewed in reference 12) or with both the SH2 and SH3 domains of pp60$^{c\text{-}src}$ itself (29, 33, 49). The Tyr-527-phosphorylated, folded molecule would represent the inactive state of the kinase. Apart from their role in Src, the SH2 and SH3 domains have been shown to interact with other proteins, the SH2 domain by binding specifically to amino acid motifs containing a phosphorylated tyrosyl residue and the SH3 domain by binding to proline-rich motifs in a variety of proteins, many of which can be classified as adaptor molecules involved in different signal transduction pathways (20, 39, 47).

Constitutively activated forms of pp60$^{c\text{-}src}$ (e.g., pp60$^{v\text{-}src}$) act as potent transforming oncoproteins. Activation of pp60$^{c\text{-}src}$ can be caused by several mechanisms involving the loss of PTyr-527–SH2/SH3 interactions: (i) constitutive dephosphorylation of Tyr-527, (ii) deletion or mutation of Tyr-527, (iii) mutation or deletion of the SH2 and/or SH3 domains, and (iv) interference with SH2/SH3–Tyr-527 interactions by, for example, the Crk oncoprotein (8, 11, 57).

Cells transformed by v-Src show anchorage- and serum-independent growth, are not contact inhibited, and display typical morphological changes (37). These changes are accompanied or caused by rearrangements of cytoskeletal structures and by alterations in the phosphorylation of a series of proteins, many of which have been shown to be (direct) substrates of pp60$^{c\text{-}src}$ (26, 37). Among such proteins, pp125^{FAK} (focal adhesion kinase) (17, 45), a cytoplasmic TK localized in focal adhesions, and paxillin, a 68- to 70-kDa vinculin-binding protein that is a major structural component of focal adhesions (50), have been identified. Interestingly, cell adhesion to the extracellular matrix through the integrin family of proteins has been shown to induce tyrosine phosphorylation and activation of pp125^{FAK} (7, 25, 56).

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We have here explored the phenotype of cells overexpressing the Csk kinase, which normally controls c-Src activity. For this study, we used a tetracycline-responsive induction system (16). An inducible system was used in order to avoid long-term effects of Csk overexpression and to be able to analyze the time course of the effects of Csk induction.

MATERIALS AND METHODS

Tetracycline-controlled system for regulation of Csk expression. The system is based on transfection of two plasmids into eukaryotic cells (16). One plasmid constitutively expresses a fusion protein, tTA, consisting of the *trans* activation domain of the herpes simplex virus virion protein 16 (VP16) fused to a bacterial tetracycline resistance gene (*tet*) repressor. In *Escherichia coli*, the *tet* repressor binds to a specific recognition sequence in the *tet* operator of Tn*10* in the absence of tetracycline, but in the presence of tetracycline, it binds to the drug and thereby allows transcription of the *tet* genes (19). The other plasmid contains a heptamerized upstream binding site of the *tet* operator, downstream of which a minimal cytomegalovirus promoter was inserted (16). The minimal (enhancerless) promoter is silent in the absence of tTA binding, i.e., in the presence of tetracycline in the culture medium. The cDNA of interest is inserted downstream of this operator/promoter element, thus allowing tetracycline-regulated expression of the desired gene product (16).

Plasmids. The HeLa cell line HtTA-1 contains plasmid pUHD15-1, encoding tTA (16). The constructs containing the tTA binding sequences plus *csk* cDNAs were based on plasmid pUHD10-3 (15). The full-length *csk* cDNA, obtained as a *Sac*II-*Xba*I fragment from either pG*csk* (5) or pG*csk*^{R222} (see below), was inserted downstream of an enhancer element containing the 7 × 19-mer oligonucleotide from the *tet* operator sequence and the minimal cytomegalovirus promoter. These vectors were designated pUHD*csk*^{wt} and pUHD*csk*^{R}, respectively.

The cDNA encoding a kinase-deficient p50^{csk} was made by using a PCR-based strategy to create a point mutation changing the ATP-binding Lys codon (K-222) into an Arg codon (R-222). Two oligonucleotides, flanking the sequence containing the Lys-222 codon, were synthesized. The ends of the oligonucleotides were complementary to *Stu*I sites located on each side of the target sequence. The 3′ antisense primer covered the Lys triplet AAG which was changed to the Arg triplet AGG. A 424-bp fragment was synthesized in the PCR using pG*csk* as the template. This fragment was digested with *Stu*I and used to replace the wild-type (wt) *Stu*I fragment in pG*csk*^{wt}. Since *Stu*I is sensitive to *dam* methylation, the vector was propagated in the *dcm dam E. coli* strain GM1674. The resulting construct was designated pG*csk*^{R}, and the mutation was checked by sequencing of both DNA strands (44).

The ΔSH2 and ΔSH3 mutants were produced by using a loop-out deletion strategy based on the use of the pALTER system (Promega). The human *csk* cDNA was cloned into the pALTER phagemid vector from which single-stranded DNA was produced, using helper phage R408. Sixty-mer oligonucleotides were designed so that their 5′ halves (30 nucleotides) were complementary to the 30 nucleotides preceding the triplets encoding the first amino acids of the SH3 or SH2 domain and their 3′ halves (30 nucleotides) were complementary to the sequences downstream of the last triplets of these domains. Upon annealing of these oligonucleotides to the *csk* single-stranded DNA, in vitro DNA synthesis of the second strand, ligation, and propagation in *E. coli mutS* to allow for replication of the mutated strands, double-stranded *csk* cDNA molecules lacking the sequences encoding the SH3 or SH2 domain were obtained. The SH3 deletion spanned 50 amino acids, and the SH2 deletion spanned 80 amino acids. A *Sac*I-*Sph*I fragment encompassing the entire mutated region was then cloned into pG*csk*^{wt} (5). Finally, a *Sac*II-*Nco*I fragment of the resulting constructs was exchanged for the corresponding fragment of pUHD*csk*^{wt}. The new vectors, pUHD*csk*$^{\Delta SH3}$ and pUHD*csk*$^{\Delta SH2}$, produced proteins approximately 7 and 9 kDa smaller than wt Csk.

The LTR*csk* vector was created by insertion of the full-length *csk* cDNA into the *Sma*I site of the pLTRpoly vector (3). The glutathione *S*-transferase (GST)–SH2^{csk} prokaryotic vector was constructed, and the fusion protein was produced as described elsewhere (32). The GST-SH3^{csk} construct was produced by PCR synthesis of a Csk fragment encoding amino acids 1 to 81, which was cloned into pGEX-1λT (Pharmacia) and produced in *E. coli*. The different Csk constructs and analysis of the tetracycline-regulated expression of their protein products are shown in Fig. 1.

Cell culture, transfections, and induction of Csk expression. The neomycin-resistant HeLa cell line HtTA-1 (16) was grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and 0.5 mg of G418 per ml. For transfection, the calcium phosphate precipitation method of Parker and Stark (36) was used. The Csk constructs were cotransfected with the hygromycin B resistance plasmid pY3 (6), and the cells were grown in the presence of neomycin, hygromycin B (200 μg/ml), and tetracycline (1 μg/ml). Neomycin- and hygromycin B-resistant clones were isolated, split in half, and grown in the presence or absence of tetracycline. After 2 to 4 days of culture, the cells were lysed and the level of Csk expression was determined by Western blotting (immunoblotting). Clones showing tetracycline-dependent induction of Csk expression were chosen for further studies. In standard experiments, the cell lines were kept in medium containing tetracycline; for induction, they were washed three times with phosphate-buffered saline and cultured in the absence of tetracycline for different periods of time. As controls, untransfected HtTA cells and the HeLa cell line HtTA-1/X1 (16) overexpressing luciferase under the control of the tetracycline system were used. As a comparison for the HeLa cells, L6 rat myoblasts were also used in transient transfection experiments using the pLTR *csk* vector.

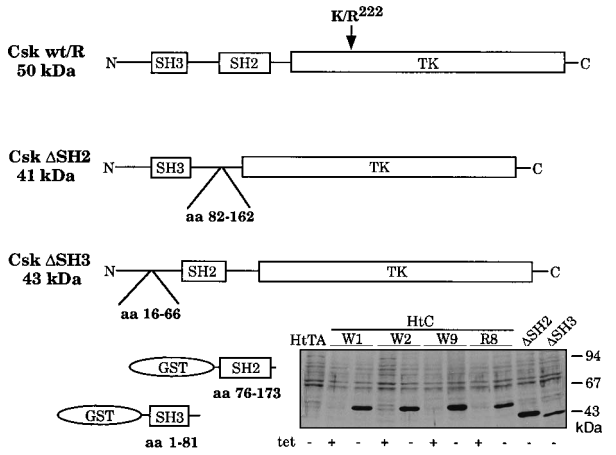

FIG. 1. Recombinant Csk proteins used in this study and their inducible expression. Shown are schematic drawings of the wt Csk, R-222 mutant Csk, ΔSH3, and ΔSH2 proteins expressed in HeLa cells. The amino acid (aa) residues deleted are indicated below. Also shown are the structures of the GST fusion proteins used for isolation of affinity complexes, with the relevant Csk amino acid residues indicated. The anti-Csk Western blot shows lysates from the untransfected HtTA cells and the cells overexpressing the different Csk forms. + and − indicate growth in the presence and absence of tetracycline (tet).

Antibodies, immunoprecipitation, and Western blotting. For immunoprecipitation and Western blotting, we used the rabbit anti-CskC antiserum described previously (5), the anti-Src monoclonal antibody (MAb) 327 (a generous gift from Joan Brugge), the anti-Src family antiserum cst-1 (a generous gift from Sara Courtneidge), anti-FAK and antipaxillin MAbs from Affiniti Research, the anti-PTyr MAbs PY20 (Zymed) and IgG2bk (Upstate Biotechnology, Inc. [UBI]), and the anti-PTyr immunoglobulin (Ig)-horseradish peroxidase fusion protein RC20H (Affiniti Research).

For Western blotting, cells were lysed in 125 mM Tris (pH 6.8)–5 mM EDTA–2.5% sodium dodecyl sulfate (SDS)–1 mM phenylmethylsulfonyl fluoride–aprotinin (20 μg/ml). If the samples were used for PTyr analysis, 1 mM sodium orthovanadate (Na_3VO_4) was included in the lysis buffer. The lysates were sonicated and centrifuged, and the protein concentration of the cleared lysates was determined with the bicinchoninic acid protein assay reagent (Pierce). Then 10 to 15 μg of protein was electrophoresed per lane in 7.5 or 10% SDS-polyacrylamide gels and blotted to nitrocellulose filters by using a semidry blotting apparatus (Ancos). The transfer efficiency and protein amounts were checked by Ponceau staining of the filters, which were then blocked in 4% bovine serum albumin (BSA), probed with the different antibodies, and stained with appropriate horseradish peroxidase-conjugated secondary antibodies. Immunostained proteins were detected by using the enhanced chemiluminescence reagent (Amersham).

For immunoprecipitation, cells were collected in TK lysis buffer (TKB; 20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, aprotinin). For coimmunoprecipitation experiments, cells were incubated in 0.1 mM sodium pervanadate for 20 min prior to lysis. The lysates were kept on ice for 15 to 30 min and centrifuged, and the cleared lysates were either used immediately or frozen at −70°C. Immunoprecipitations were performed on ice. The TKB cell lysates, adjusted to equal protein concentrations, were precleared with protein A-Sepharose (Pharmacia) for the polyclonal sera or protein G-Sepharose for the MAbs, and the antibodies were then added. After 2 to 3 h, protein A- or protein G-Sepharose was added, and the slur was rolled end over end for 30 min. The Sepharose beads were then washed twice with TKB and twice with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.4)–150 mM NaCl.

Indirect immunofluorescence. Cells were grown on either uncoated coverslips or coverslips coated with laminin, collagen I, plasma fibronectin isolated by gelatin-Sepharose chromatography as described previously (14), or vitronectin isolated from plasma by heparin-Sepharose affinity chromatography (54) (20 μg/ml each). The cells were fixed in methanol at −20°C. The following MAbs were used for immunostaining: 102DF5, specific for β_1 integrin (55); 90BB10,

specific for β_3 integrin (55); PIF6, specific for the $\alpha_v\beta_5$ integrin complex (51); J143, specific for α_3 integrin (18); B1E5, specific for α_5 integrin (52); GOH3, specific for α_6 integrin (48); LM142.69, specific for α_v integrin (9); PY20, specific for PTyr; MAb 2A7 (UBI), specific for $pp125^{FAK}$; an antitalin MAb (from Keith Burridge); an antivinculin rabbit antiserum (28); and the polyclonal rabbit anti-CskC antiserum. For the detection of bound antibodies, the specimens were stained with fluorescein isothiocyanate-conjugated sheep anti-mouse IgG, anti-rat IgG (for GOH3 and B1E5), or anti-rabbit IgG (Jackson Laboratories). For the double-stained cells, one primary antibody was detected with fluorescein isothiocyanate-conjugated antibodies, and the other was detected with tetramethyl rhodamine isothiocyanate-conjugated antibodies. After mounting, the specimens were examined in a Leitz Aristoplan microscope.

Enzyme assays. The activity of $p50^{csk}$ was measured in immunoprecipitates from TKB lysates, using two different assays. In one assay, activity was measured as fluorographic signals on films; in the other, the incorporation of [γ-^{32}P]ATP into poly(Glu/Tyr) was measured in a liquid scintillation counter. For the first assay, the last two washes of the immunoprecipitates were done in the assay buffer (10 mM HEPES [pH 7.4], 5 mM $MnCl_2$). As a substrate, 600 ng of baculovirus-derived, purified Src^{K295M} (a generous gift from David Morgan) was used per reaction. The reaction was initiated by the addition of ATP (to 3 μM) and 10 μCi [γ-^{32}P]ATP (3,000 Ci/mmol), incubated for 5 min at 30°C, terminated by heating in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and analyzed by electrophoresis and autoradiography.

The poly(Glu/Tyr) assays were performed with equal amounts of immunoprecipitates from TKB lysates for each time point essentially as described previously (13). After two washes in the assay buffer (10 mM HEPES [pH 7.4], 5 mM $MnCl_2$, 100 μM Na_3VO_4 for Csk; 50 mM HEPES [pH 7.4], 25 mM $MgCl_2$, 4 mM $MnCl_2$, 100 μM Na_3VO_4 for c-Src or Src-family TKs), the reaction was started by addition of poly(Glu/Tyr) (4:1; Sigma) to 1 mg/ml, ATP to final concentrations of 3 μM for Csk and 1 μM for c-Src and Src family TKs, and 3 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol). After careful mixing, the samples were incubated for 10 min at 30°C. The reactions were stopped by bringing the mixture to final concentrations of 3 mM ATP, 30 mM EDTA, and 1.5 mg of BSA per ml; 20-μl samples were spotted in duplicate onto Whatman 3MM filter paper squares. The squares were washed in ice-cold 10% trichloroacetic acid–8% sodium pyrophosphate for 15 min, then three to four times for 15 min each in 5% trichloroacetic acid, and finally in ethanol and dried, and their radioactivity was measured in a liquid scintillation counter. As controls, immunoprecipitates obtained with nonimmune or preimmune sera were used. The radioactivity of the control samples was less than 10% of that of the TK immunoprecipitates. These values were subtracted from the values obtained by using anti-Csk or anti-Src antibody.

RESULTS

Induction of Csk expression. To study the function of $p50^{csk}$, we established cell lines in which overexpression of $p50^{csk}$ could be induced. The parental HeLa cell line, HtTA-1, was transfected with the inducible construct containing wt or mutant R-222, ΔSH2, or ΔSH3 *csk* cDNA. Of nine clones screened for tetracycline-inducible wt Csk expression, four were positive. Three of these, HtCW1, HtCW2, and HtCW9, were analyzed in this study. HtCW2 was chosen as the main subject for the morphological and biochemical analyses. Of the cells transfected with the kinase-deficient R-222 mutant, four of eight screened clones showed good induction and low background. Of these, the clones HtCR1 and HtCR8 were analyzed. For studies of the functions of the SH2 and SH3 domains of Csk, clones HtCΔSH2/9 and HtCΔSH3/19 were used (Fig. 1; Table 1).

Western blotting of lysates from the induced cell lines HtCW2 and HtCR1 is shown in Fig. 2A. Scanning densitometry of luminograms from serially diluted samples showed that the $p50^{csk}$ content of the induced cells was increased 10- to 15-fold at 80 h compared with the cells grown in the presence of tetracycline (data not shown). When the cells were grown in the continuous presence of tetracycline, the level of $p50^{csk}$ remained similar to that of the nontransfected control cells. Clones grown in the continuous absence of tetracycline showed very high $p50^{csk}$ levels (Fig. 2B).

Activity of overexpressed Csk. To analyze if increased amounts of wt $p50^{csk}$ led to an enhanced Csk activity, activity assays were performed with either kinase-deficient $p60^{c\text{-}src}$ or poly(Glu/Tyr) as the substrate. The only signal detected in the former assay is the Csk-mediated phosphorylation of Tyr-527.

TABLE 1. Properties of the tetracycline-regulated Csk cell lines

Clone	Tetracycline	Activity (mean ± SD)[a]		Detection of[b]:		
		Csk[c]	Src[d]	Rounding of cells	Csk in focal adhesions[e]	α_v redistribution
HtCW2	+	1.31 ± 0.02	0.64 ± 0.06	N	N	N
	−	8.43 ± 0.49	0.69 ± 0.06	Y	Y	Y
HtCW1	+	1.47 ± 0.13	2.5	N	N	N
	−	11.40 ± 1.02	1.6	Y	Y	Y
HtCW9	+	0.65 ± 0.08	1.8	N	N	N
	−	8.21 ± 1.18	1.5	Y	Y	Y
HtCR1	+	0.9[f]	ND	N	N	N
	−	0.7	ND	N	Y	N
HtCR8	+	1.05 ± 0.06	1.6	N	N	N
	−	0.94 ± 0.05	3.2	N	Y	N

[a] Measured by using the poly(Glu/Tyr) assay.
[b] Y, yes; N, no.
[c] Values are relative to Csk activity of HtTA, set as 1.
[d] Values are relative to HtTA activity, set as 1. Data for HtCW2 are from four different experiments; data for the other clones are from one experiment. ND, not done.
[e] Determined by anti-Csk and antitalin or anti-FAK double immunofluorescence.
[f] Determined by scanning of autoradiographs of kinase assays, using kinase-deficient c-Src as a substrate.

As shown by the increased phosphorylation of the kinase-deficient $p60^{c\text{-}src}$ by immunoprecipitated $p50^{csk}$ in Fig. 2C, Csk activity increased in parallel with the overexpression of wt $p50^{csk}$ (compare with Fig. 2A), whereas very little change occurred when the kinase-deficient R-222 mutant Csk was induced. Strongly increased Csk activity was also detected in the wt Csk-expressing cells grown continuously in the absence of tetracycline (Fig. 2D).

To study the kinetics and level of the increase of Csk activity, anti-Csk immunoprecipitates from the HtCW2 cells were analyzed in a quantitative assay using poly(Glu/Tyr) as the sub-

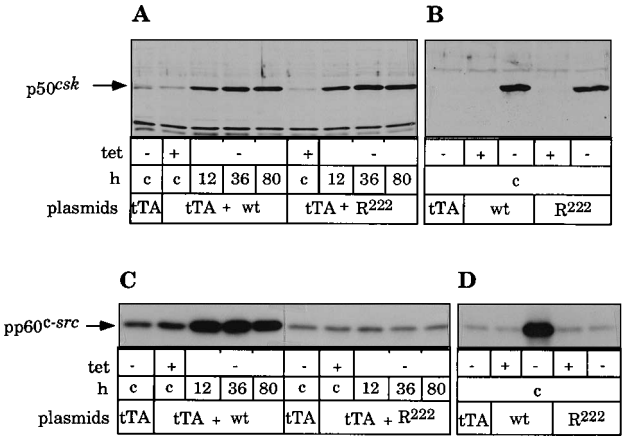

FIG. 2. Kinetics of induction and activity of $p50^{csk}$ in HeLa cells expressing wt Csk or the R-222 kinase-inactive mutant. (A) Immunoblotting of equal amounts of total cell lysates, using anti-CskC antibodies. The presence (+) or absence (−) of tetracycline (tet) and time after removal of tetracycline from the culture medium (h) are indicated below the blot. c denotes the continuous absence or presence of tetracycline. The bottom row indicates the transfected plasmids. (B) Analysis of Csk expression in the cell lines (and controls) grown in the continuous absence of tetracycline. (C) Activity of $p50^{csk}$ in anti-Csk immunoprecipitates from cell lysates. Equal amounts of purified kinase-deficient $pp60^{c\text{-}src}$ were added and kinase assays were carried out in the presence of [γ-^{32}P]ATP. The samples were analyzed by SDS-PAGE followed by autoradiography. Notation is as in panel A. (D) Csk activity in the cell lines grown continuously in the absence of tetracycline.

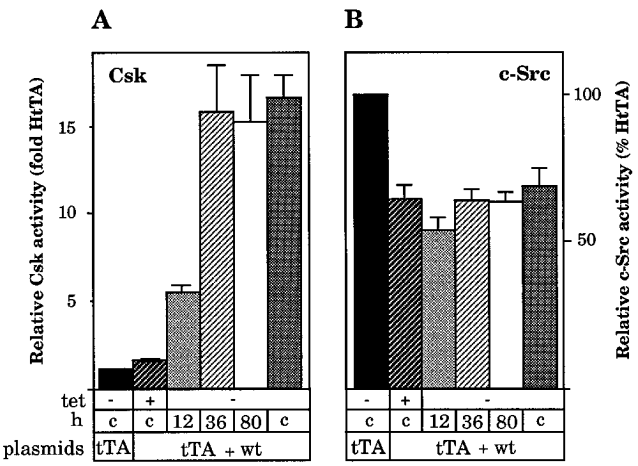

FIG. 3. Csk and c-Src activities in wt Csk-induced cells. The activities of $p50^{csk}$ (A) and $pp60^{c\text{-}src}$ (B) were measured by using the poly(Glu/Tyr) assay. Values for Csk are shown as fold increased activity in comparison with the HtTa control cells; Src values are shown as percentages of the activity immunoprecipitated from HtTA cells. The results were collected from four different measurements, and the values obtained with nonimmune serum were subtracted.

strate (Fig. 3A). This assay confirmed that the increase of Csk activity occurred in concert with the increase of the amount of Csk in the cells, reaching a maximal level 10- to 15-fold higher than the control value. In comparison, in the presence of tetracycline, the cells showed only a small increase of Csk activity, probably reflecting a slight leakage of the promoter under uninduced conditions. The poly(Glu/Tyr) phosphorylation assay of the other clones gave very similar values, and induction of the kinase-deficient form of Csk did not result in any increase of labeling of the substrate (Table 1).

Analysis of $pp60^{c\text{-}src}$ activity in Csk-overproducing cells. As can be seen from Fig. 3B, c-Src activity was approximately 35% down-regulated in HtCW2 cells grown in the presence of tetracycline, apparently as a result of the small increase of Csk activity (Fig. 3A). Upon induction, a slight additional decrease of Src activity (about 10%) occurred at 12 h. However, at later time points, c-Src activity returned to levels seen in the uninduced cells. Similar results were obtained with the cst-1 antiserum, which immunoprecipitates several Src family TKs (data not shown). Analysis of the other wt Csk-expressing clones revealed some differences in the basal c-Src activity and indicated that such activity was slightly suppressed by induction of Csk. In contrast to wt Csk, induction of the R-222 mutant caused a slight increase of c-Src activity (Table 1). Tetracycline did not affect Src activity in the control cells (data not shown). Furthermore, neither wt Csk nor the R-222 mutant caused changes in the phosphorylation of $p59^{fyn}$ or the c-Src substrate p80/85 cortactin (53), paxillin, or $pp125^{FAK}$ in the induced cells (data not shown).

Morphology and properties of Csk-overexpressing cells. The phenotypes of the wt Csk cell lines did not differ significantly from that of the parental cell line HtTA-1 when grown in the presence of tetracycline (Fig. 4A to C; Table 1), but upon induction of Csk expression, morphological changes occurred, as seen in Fig. 4D. After 3 days, a fraction of the cells became more rounded and grew in large focus-like aggregates loosely attached to each other and to the culture dish. Also, many cells grew in tightly interconnected areas or islands. Time-lapse video monitoring of these cultures indicated that most of the cells were capable of undergoing their first mitosis after removal of tetracycline, but after the following cell division, the cells were unable to spread normally again (data not shown). Some cells remained rounded and attached to each other after mitosis through a cytoplasmic bridge. Such a conglomerate of cells is shown in the inset of Fig. 4D. The majority of the cells were less spread than control cells after mitosis and stayed very closely connected. However, cell growth and thymidine incorporation in these cultures were similar to those of the HtTA-1 or uninduced HtCW2 cultures, and the rounded cells did not grow in serum-free conditions or in soft agar (data not shown). The other wt Csk-expressing clones showed very similar properties (Table 1).

When the loosely attached cells were collected by washing them off the culture plates after 3 to 4 days of induction and grown in the absence of tetracycline, they remained spherical and grew in aggregates (Fig. 4E). However, when these cells were shifted to tetracycline-containing medium, they reattached and spread within 2 to 3 days (Fig. 4F). In contrast to the wt Csk-expressing cells, no morphological changes occurred upon induction of the R-222 Csk mutant in the HtCR1 cells (Fig. 4G and H; Table 1) or in the other three clones expressing the mutant protein (not shown). Also, no morphological changes were detected when the control cell line HtTA-1/X1 was induced to overexpress luciferase (data not shown). Thus, the effects seen in the HtCW2 cells were common to all of the analyzed wt clones and specific for the overexpressed catalytically active $p50^{csk}$.

Colocalization of induced $p50^{csk}$ with focal adhesion proteins. To gain more detailed information about the nature of the phenotypic changes observed upon induction of wt Csk expression, HtCW2 cells were stained with antibodies against $p50^{csk}$, focal adhesion components, and integrins, which mediate cell interaction with extracellular matrix adhesion proteins, and compared with uninduced cells. In these studies, the most spherical cells were difficult to examine, both because they were easily lost at fixation and because their shape precluded visualization of certain cellular details. In the tightly growing clusters of cells, focal adhesion structures were difficult to detect. Thus, the more sparsely growing cells, which were better spread on the growth substratum, were chosen for these analyses.

First, cells grown in the continuous absence of tetracycline were examined. Staining of Csk-induced cells with preimmune serum did not produce any significant background, while anti-Csk staining of uninduced cells gave a weak fluorescence (Fig. 5A). Antitalin immunostaining of the same cells showed a typical fluorescence pattern for focal adhesions (Fig. 5B). Upon induction of Csk expression, however, a very strong fluorescence signal was detected with the anti-Csk antiserum (Fig. 5C). In agreement with previous biochemical data, most $p50^{csk}$ appeared to be located in the cytoplasm. However, a portion of $p50^{csk}$ was found in the focal adhesion plaques of the cells (arrows). Furthermore, double staining showed that $p50^{csk}$ colocalized with two known focal adhesion proteins, talin and $pp125^{FAK}$ (Fig. 5C to F). Similarly to wt Csk, a portion of the induced R-222 Csk mutant also resided in focal adhesions (Fig. 5G and H). Identical results were obtained when the other wt- and R-222 Csk-expressing clones were stained (Table 1).

To rule out the possibility that the localization of Csk in the focal adhesions was a peculiarity of the HeLa cells, rat L6 myoblasts were transiently transfected with an LTRcsk^{wt} construct and stained with anti-Csk antiserum. As shown in Fig. 6A, Csk was found in focal adhesions also in these cells.

Localization of Csk in focal adhesions requires both the SH2 and SH3 domains. To study the role of the SH2 and SH3 domains in Csk localization to focal adhesions, their coding

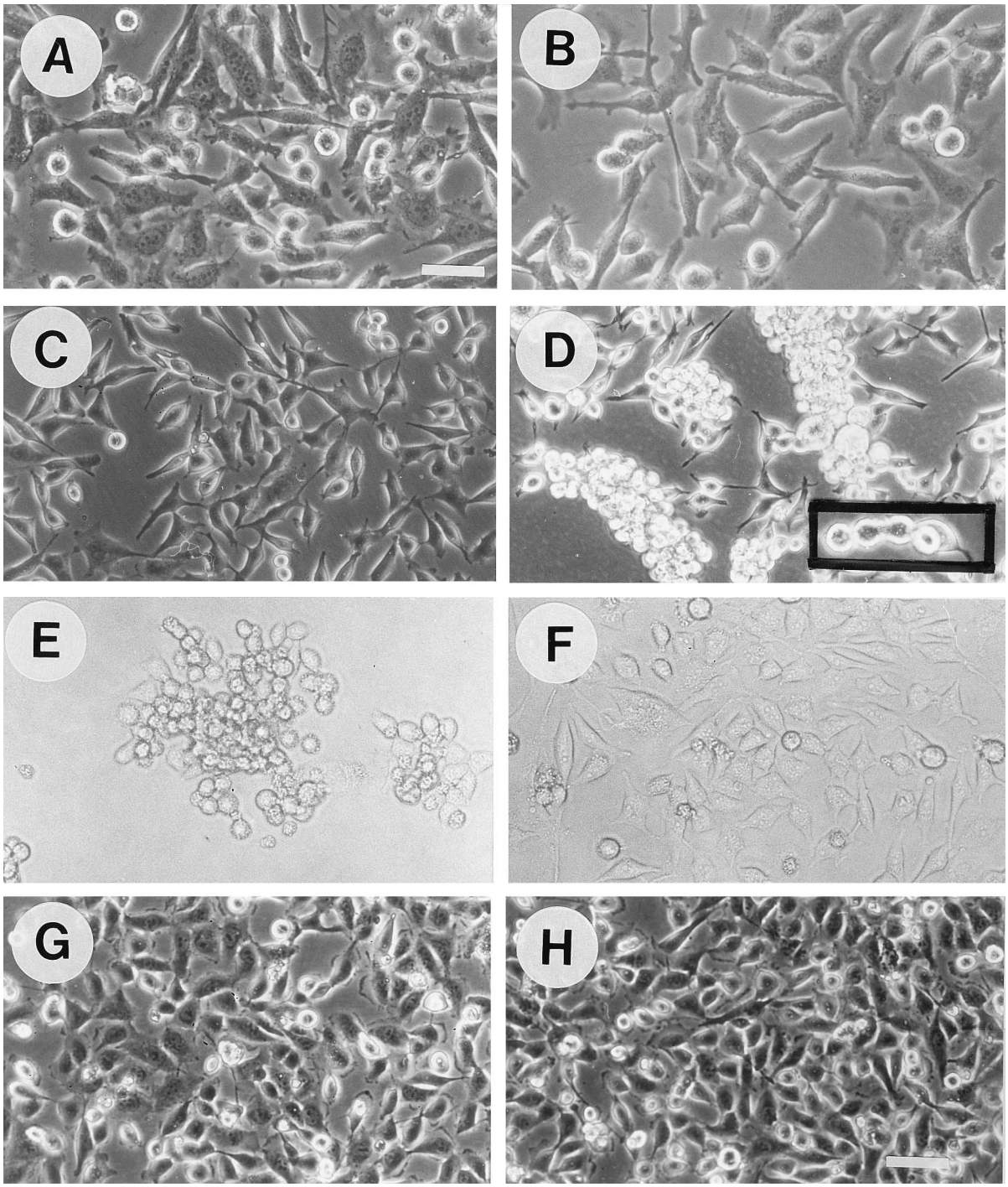

FIG. 4. Phenotypes of the cells induced to overexpress $p50^{csk}$. HtTA parental cells grown in the presence (A) or absence (B) of tetracycline. Scale bar = 55 μm. HtCW2 cells grown continuously in the presence (C) or absence (D) of tetracycline (1 μg/ml). Note the conglomerates and the detachment of spherical cells. The insert in panel D shows a chain of cells that have not separated after mitosis. (E) Spherical, floating, and loosely attached HtCW2 cells collected after 3 days of induction and cultured in the absence of tetracycline. (F) The spherical cells shown in panel E, reseeded and grown for 3 days in medium containing tetracycline. For panels E and F, a transillumination microscope was used. (G and H) HtCR1 cells grown in the presence (G) or absence (H) of tetracycline. No morphological changes are detected upon overexpression of the R-222 mutant form of $p50^{csk}$. Scale bar for panels C to H (shown in panel H) = 70 μm.

sequences were selectively deleted from the *csk* cDNAs (Fig. 1). These constructs were then transfected into HtTA cells and induced in the absence of tetracycline. Anti-Csk immunostaining revealed that neither the CskΔSH2 nor the CskΔSH3 protein was found in focal adhesions (Fig. 6B and C). Thus, both the SH2 and SH3 domains of Csk appeared to be responsible for targeting the protein to focal adhesions. The lack of detection of the deletion mutant proteins in focal adhesions was not due to absence of these structures in the cells, since antitalin staining revealed very prominent focal adhesions (data not

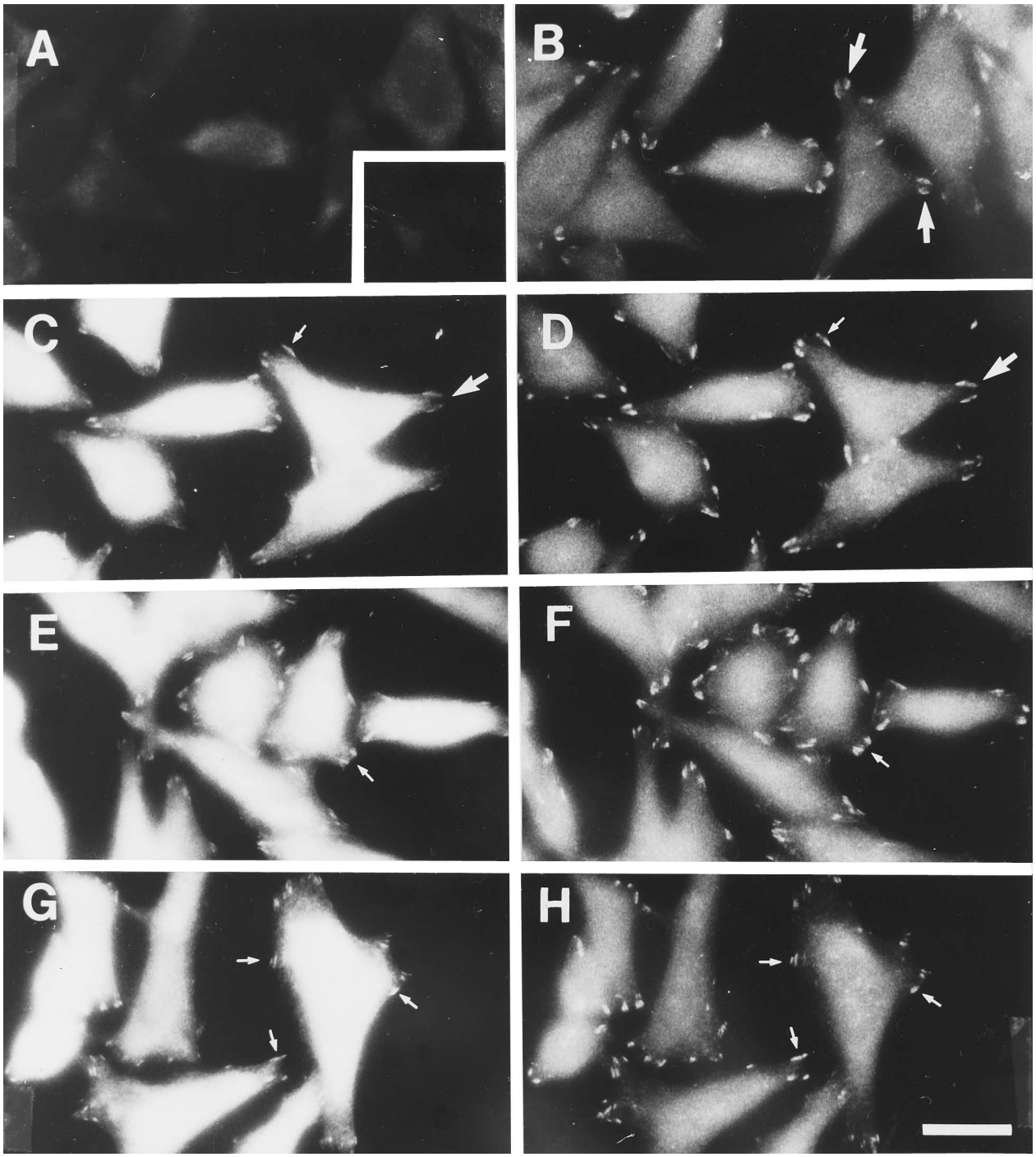

FIG. 5. Colocalization of wt and kinase-deficient p50^{csk} with focal adhesion proteins. (A and B) Double immunostaining of uninduced HtCW2 cells for Csk (A) and talin (B). Note the distinct localization of talin in focal adhesions (arrows). The insert in panel A shows staining of induced HtCW2 cells with preimmune serum (shown at fivefold-lower magnification than the other panels). (C and D) Double staining of induced cells, using anti-Csk antibodies (C) and antitalin antibodies (D). Note the overlapping signals in the focal adhesions (arrows). (E and F) Double staining of induced HtCW2 cells with anti-Csk (E) and anti-FAK (F). Again an overlapping pattern is seen (arrows). (G and H) Double immunostaining of HtCR1 cells overexpressing the kinase-deficient form of Csk with anti-Csk (G) and antitalin (H). Note that the inactive enzyme is also localized in the focal adhesions in a pattern indistinguishable from that of talin (arrows). Scale bar = 10 μm.

shown). The distribution of the mutant proteins differed slightly, however. In general, the cells overexpressing CskΔSH2 appeared more spread on the culture substratum than the wt Csk- or CskΔSH3-overexpressing cells. Further, the CskΔSH3 protein occurred closer to the cell surface than the CskΔSH2 protein (compare Fig. 6B and C).

The SH2 domain of Csk complexes with pp125^{FAK} and paxillin. Since the SH2 and SH3 domains of Csk seemed to mediate the localization of Csk in focal adhesions, we investigated their possible interactions with focal adhesion proteins. To this end, GST-SH2^{Csk} and GST-SH3^{Csk} fusion proteins coupled to glutathione-Sepharose were first used to isolate proteins from

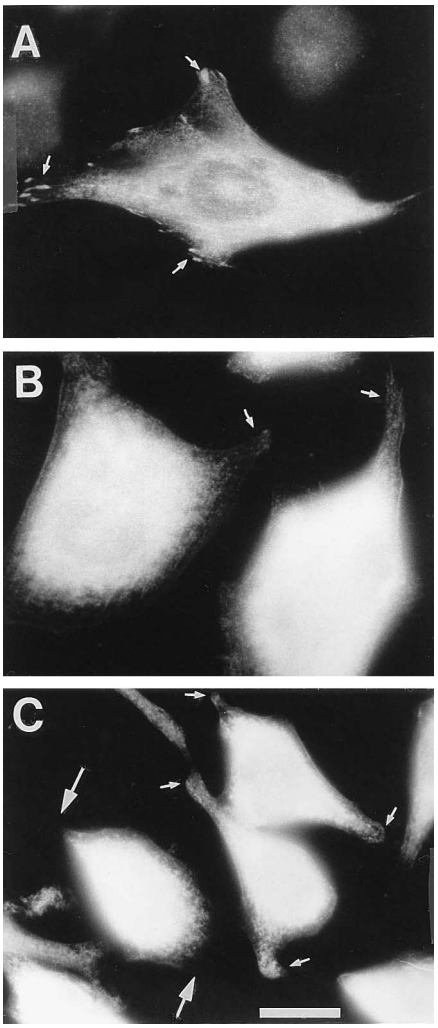

FIG. 6. Analysis of Csk and its deletion mutants in focal adhesions. (A) Anti-Csk immunostaining of focal adhesions (arrows) of L6 rat myoblasts, transiently expressing wt Csk. Anti-Csk immunostaining of HeLa cell lines overexpressing CskΔSH2 (B) or CskΔSH3 (C) shows no staining in focal adhesions (arrows). Note that the CskΔSH2-expressing cells appear larger and flatter than the CskΔSH3-expressing cells and that the CskΔSH3 protein is localized closer to the edges of the cells. Scale bar = 3.5 μm.

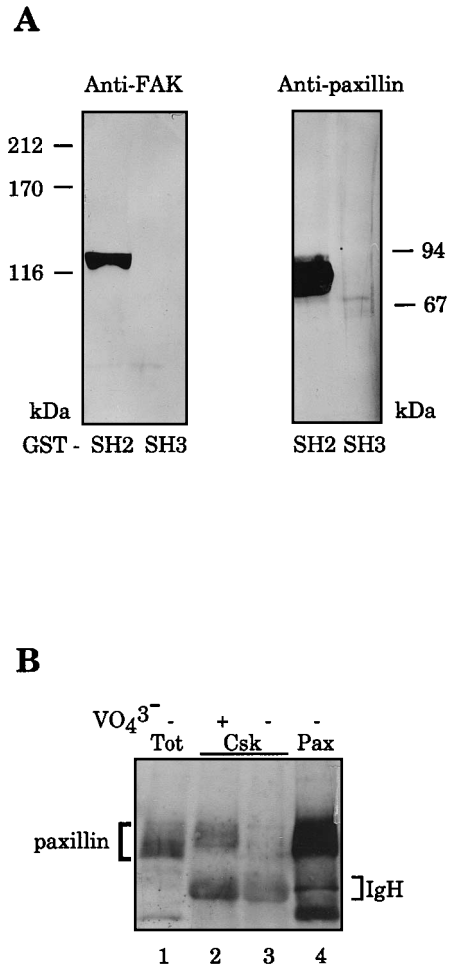

FIG. 7. Interaction of pp125^{FAK} and paxillin with Csk. (A) Interaction of focal adhesion proteins with the SH2 domain of Csk. Sepharose-coupled GST fusion proteins schematically shown in Fig. 1 were used to isolate affinity complexes from lysates of wt Csk-overexpressing cells. The complexes were then analyzed by Western blotting with anti-FAK and antipaxillin antibodies. (B) Interaction of paxillin with intact Csk in cells demonstrated by antipaxillin immunoblotting of anti-Csk immunoprecipitates from wt Csk-overexpressing cells. Lane 1, 15 μg of total HtCW2 cell lysate; lane 2, anti-Csk immunoprecipitate from a lysate of pervanadate-treated HtCW2 cells; lane 3, anti-Csk immunoprecipitate from untreated cells; lane 4, antipaxillin immunoprecipitate. Note that pervanadate treatment of cells increases the binding of paxillin to Csk. Also, the most phosphorylated, high-molecular-weight forms of paxillin preferentially bind to Csk. The position of the Ig heavy chains (IgH) is indicated.

lysates of wt Csk-overexpressing cells. Immunoblotting analysis of such affinity complexes by using anti-FAK and antipaxillin antibodies is shown in Fig. 7A. The results show that the SH2 domain but not the SH3 domain interacts with both pp125^{FAK} and paxillin. A GST fusion protein containing the C-terminal SH2 domain of phospholipase Cγ (kindly provided by Satu Vainikka) did not interact with these proteins in the same assay. Anti-PTyr staining of the blots further indicated that pp125^{FAK} and paxillin were tyrosyl phosphorylated in the cells (data not shown). To determine if these proteins could be seen complexed with intact Csk in cells, coimmunoprecipitation analyses were performed with anti-Csk antiserum and lysates of induced HtCW2 cells. Immunoblotting of such precipitates with antipaxillin MAbs showed that paxillin was coprecipitated with Csk, indicating that these proteins form a complex in the cells (Fig. 7B). Treatment of the cells with sodium pervanadate to inhibit protein-tyrosine phosphatases prior to lysis increased the amount of paxillin complexed with Csk (compare lanes 2 and 3 in Fig. 7B). A similar coprecipitation could not be unambiguously shown for pp125^{FAK}.

Redistribution of the $\alpha_v\beta_5$ integrin (vitronectin receptor) upon Csk induction. To examine if the morphological changes seen upon induction of Csk were related to the expression or localization of integrins, HtCW2 and HtCR1 cells were immunostained with a panel of anti-integrin antibodies. Both uninduced and induced cells had an even cell membrane and cytoplasmic fluorescence for β_1 integrin, a diffuse cell membrane staining for α_3, α_5, and α_6 integrins and lack of staining for β_3 integrin (data not shown). In control HeLa cells (not shown) and uninduced cells, the MAb against the α_v integrin also gave a typical staining of focal adhesion-like structures (Fig. 8A and E). In cells overexpressing wt Csk, however, the α_v subunit was excluded from the focal adhesions. Instead, the α_v integrin immunoreactivity was now mostly confined to distinct, punc-

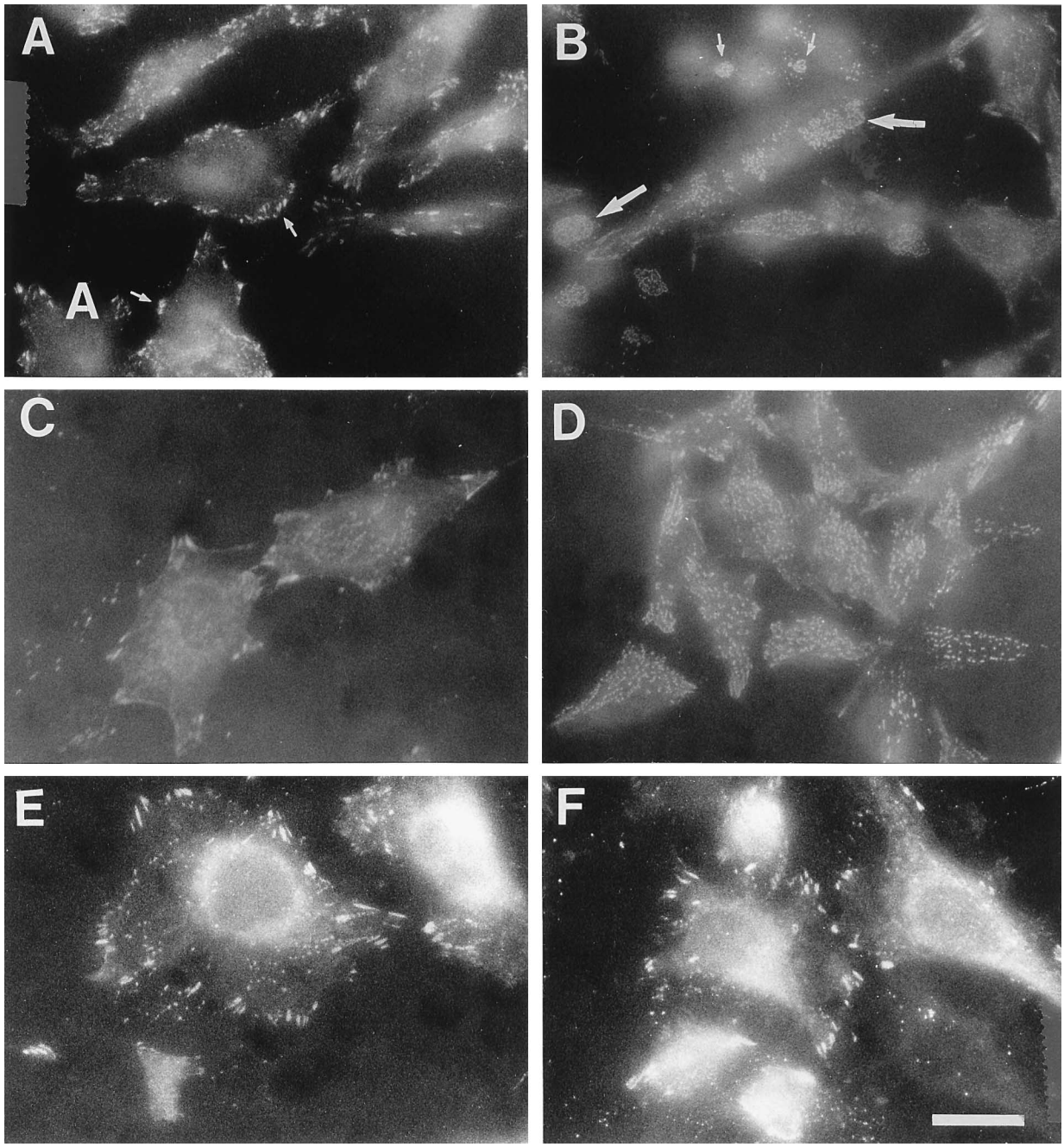

FIG. 8. Localization of α_v and β_5 integrins in Csk-overexpressing cells. (A) Anti-α_v immunostaining of uninduced HtCW2 cells showing the antigen in focal adhesions (arrows). (B) Anti-α_v staining of wt Csk-overexpressing cells. The staining of focal adhesions is totally abolished, and the α_v integrin is rearranged into round punctate plaques (large arrows). Note that the most spherical cells show a dense round plaque adjacent to the culture substratum (small arrows). (C) Anti-β_5 staining of uninduced HtCW2 cells. (D) Anti-β_5 staining of wt Csk-overexpressing cells. (E) Anti-α_v staining of uninduced HtCR1 cells. (F) Anti-α_v staining of cells overexpressing the R-222 Csk mutant. Note that the staining pattern of focal adhesions is retained when the inactive form of Csk is overexpressed. Scale bar = 10 μm.

tate, ventrally located plaques (Fig. 8B). A similar pattern was obtained with the MAb specific for the $\alpha_v\beta_5$ integrin complex (Fig. 8C and D), suggesting that α_v was complexed with the β_5 subunit in these cells, forming vitronectin receptors (23). The α_v and $\alpha_v\beta_5$ staining of the HtCR1 cells overexpressing the R-222 Csk mutant was indistinguishable from the uninduced cells (Fig. 8E and F and data not shown). Furthermore, the formation of punctate α_v integrin-containing plaques occurred in concert with the increase of the amount of $p50^{csk}$ and its activity (Fig. 9A to C; see also Fig. 2). Inspection of the spherical, brightly anti-α_v-stained cells shown in Fig. 9C at different focal planes suggested that several of these cells were mushroom shaped and attached to the substratum through a stalk-like structure, made up by the punctate plaques. Both the morphological changes of the cells and the reorganization of the vitronectin receptor were independent of the nature of the growth substratum of the cells, since no significant differences were seen when the cells were grown on uncoated

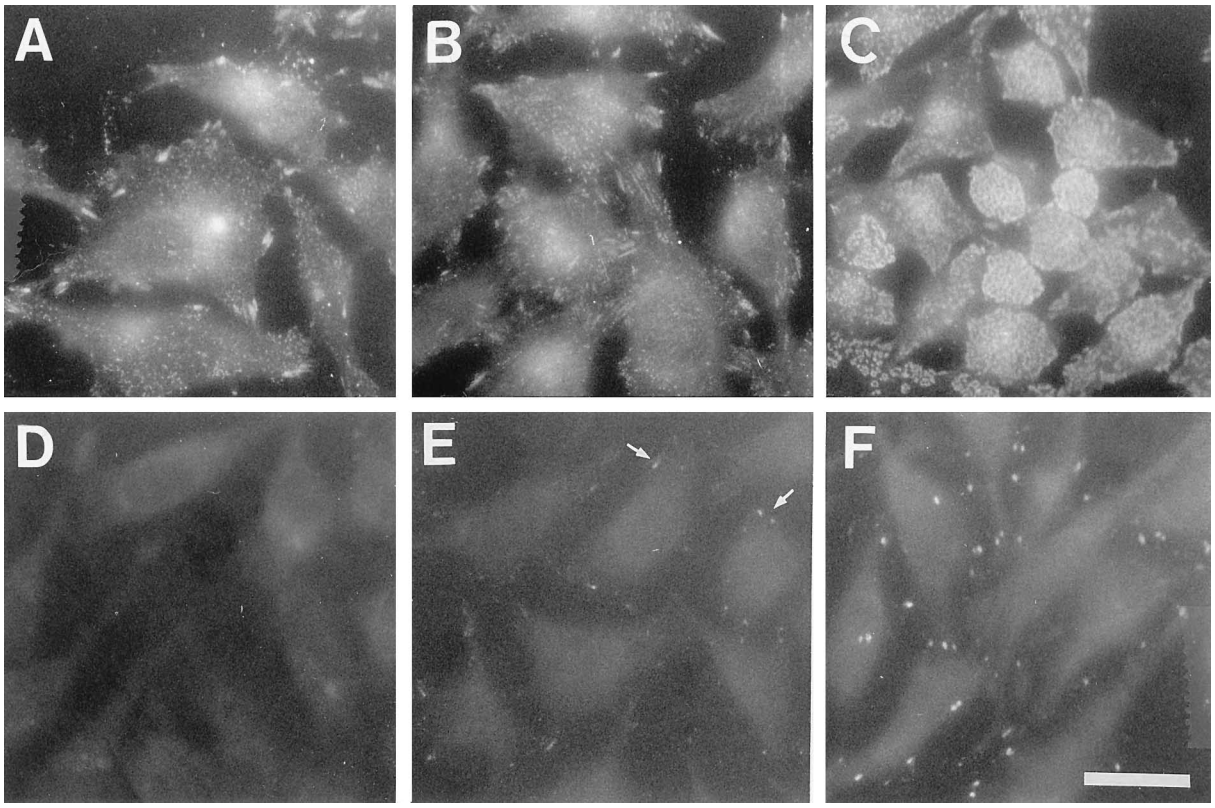

FIG. 9. Distribution of α_v integrin and PTyr during induction of wt Csk. (A) Anti-α_v immunostaining of uninduced HtCW2 cells. The majority of α_v integrin is found in focal adhesions. (B) Localization of α_v integrin 24 h after removal of tetracycline. The focal adhesions are less brightly stained, while punctate plaques of α_v integrin are being formed. (C) Anti-α_v integrin-stained cells 96 h after removal of tetracycline. The spherical, brightly stained cells in the middle of the photomicrograph represent mushroom-shaped cells (see the text). (D) Anti-PTyr immunostaining of uninduced HtCW2 cells. (E) Anti-PTyr staining at 24 h of Csk induction. Note that a weak fluorescence is detected in focal adhesions (arrows). (F) The cells at 96 h after removal of tetracycline. The anti-PTyr staining becomes very bright in the well-spread cells. Scale bar = 10 μm.

coverslips or on coverslips coated with fibronectin, laminin, collagen type I, or vitronectin (data not shown). The other wt- and R-222 Csk-overexpressing clones showed properties very similar to those of the two clones described above (Table 1).

Analysis of tyrosyl phosphorylation in HeLa cells overexpressing Csk. Anti-PTyr immunoblotting or immunoprecipitation of whole-cell lysates obtained at different time points after induction did not reveal significant differences between the uninduced and induced cells (data not shown). These results support the view that $p50^{csk}$ has a very specific substrate spectrum. On the other hand, immunofluorescence analysis using anti-PTyr antibodies revealed a strong but very local increase of the PTyr content in focal adhesions during induction of wt Csk (Fig. 9D and F; Fig. 10). Tyrosyl phosphorylation of immunoprecipitated $pp125^{FAK}$, talin, vinculin, paxillin, or tensin was not significantly changed upon Csk induction (data not shown). Also, anti-PTyr immunoblotting of anti-α_v and anti-β_5

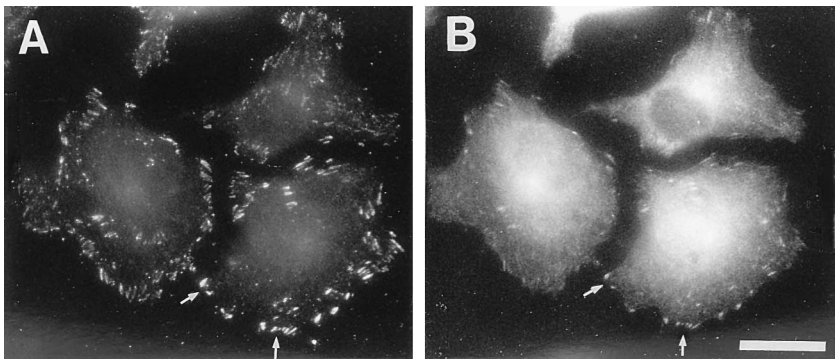

FIG. 10. Colocalization of anti-PTyr and antivinculin immunofluorescence in cells induced to overexpress wt Csk. (A) Anti-PTyr immunostaining of induced cells; (B) antivinculin staining of the same cells. Note the overlapping pattern of fluorescence (arrows). Scale bar = 10 μm.

immunoprecipitates gave negative results, suggesting that the PTyr immunostaining did not originate from these components of the adhesion plaques.

DISCUSSION

To obtain a model system in which Csk overexpression could be controlled, we have produced cell lines overexpressing different forms of Csk under the regulation of a tetracycline-sensitive promoter. This expression system is very efficient; for $p50^{csk}$, over 10-fold induction was achieved, and the elevated amount of $p50^{csk}$ in the cells was accompanied by a similar increase of Csk activity. This suggests that the specific activity of $p50^{csk}$ was not altered.

The morphological changes in the cells overexpressing wt Csk were quite dramatic. Time-lapse video monitoring indicated that after mitosis, cell spreading and reattachment to the culture substratum were most severely affected. Csk immunostaining showed that a major part of both wt and inactive $p50^{csk}$ accumulated in the cytoplasm. However, a portion of the Csk protein was concentrated in focal adhesions, colocalizing with $pp125^{FAK}$ and talin. The experiments using mutant forms of Csk showed that the SH2 and SH3 domains were responsible for targeting of Csk to focal adhesions. Exogenously overexpressed Csk has recently been shown to be localized in focal adhesions in $Csk^{-/-}$ cells, in which Src family kinases are activated (24, 31), and in cells overexpressing activated Src (21). However, here we show that Csk is localized in focal adhesions in cells expressing only endogenous c-Src. Thus, the focal adhesion localization of Csk may not require Src tyrosine kinase activity. This suggests that in normal cells, a portion of the endogenous $p50^{csk}$ is also associated with focal adhesions, but such amounts would be below our detection limit. Targeting of Csk to focal adhesion plaques of the HeLa cells did not require Csk activity and appeared to occur through association of the SH2 domain of Csk with the focal adhesion proteins paxillin and $pp125^{FAK}$. These results are in good agreement with recent data obtained from chicken, rat, and mouse fibroblasts (40).

The increased Csk activity and the enhanced PTyr content of the focal adhesions of the induced wt Csk-expressing cells did not appear to affect any of the focal adhesion-associated proteins talin, vinculin, and $pp125^{FAK}$. A striking exception was the exclusion of the α_v integrin from the focal adhesions upon induction of wt Csk. Instead of adhesion plaques, α_v was now mostly found in novel types of punctate plaques located on the ventral cell surface. In the most rounded cells, these plaques appeared to form a stalk anchoring the cells to the substratum. The morphological changes were dependent on Csk activity, since they were not seen upon induction of the mutant form of Csk. Such distinct punctate plaques have not been described before, but in certain carcinoma and melanoma cells, only the $\alpha_v\beta_3$ integrin complex was located in focal adhesions, while the $\alpha_v\beta_5$ complex showed a different, punctate, nonfocal localization (51). The α_v integrin, together with either the β_3 or β_5 integrin subunit, forms vitronectin receptors, and together with the β_1 integrin, it can form a fibronectin receptor. The α_v integrin has been suggested to play a role in the regulation of adhesive properties and invasiveness of carcinoma and melanoma cells (1, 9, 46, 51). The correlation of Csk-induced reorganization of the $\alpha_v\beta_5$ complex and the morphological changes seen in the induced cells suggests that this integrin is crucial for the regulation of the morphological properties of HeLa cells. However, despite their changed morphology, the induced cells did not show increased DNA synthesis, growth in serum-starved conditions, or growth in soft agar.

The fact that the strong overexpression of $p50^{csk}$ caused very minor, if any, changes in the overall pattern of PTyr proteins in the cells indicates that the Csk kinase has a narrow substrate specificity. This is in agreement with data for Csk expression in yeasts (29, 33, 49) and with in vitro data showing that immunoprecipitated or purified $p50^{csk}$ specifically phosphorylates the conserved C-terminal Tyr residue of Src-type kinases (5, 30, 34; our unpublished observations). Also, the immunoprecipitated α_v and β_5 integrin subunits or the punctate plaques in the induced cells were not detected by anti-PTyr antibodies. However, the very distinct anti-PTyr staining of focal adhesions detected upon Csk induction indicates the presence of Csk substrates in these structures. Whatever these proteins, they may be efficiently dephosphorylated upon cell lysis, since they could be detected only in intact cells. Alternatively, overexpression of Csk might not affect the level of tyrosyl phosphorylation of these proteins but rather cause their accumulation in adhesion plaques.

We detected some clonal variation in basal c-Src activity in the uninduced wt Csk-transfected cells. Also, Csk was able to suppress $pp60^{c\text{-}src}$ predominantly in the clones showing elevated c-Src activity. This finding is in agreement with studies showing suppression of c-Src activity by Csk in cells in which $pp60^{c\text{-}src}$ is activated by v-*crk* expression (42) or as a consequence of hypophosphorylation of Tyr-527 due to targeted mutagenesis of the *csk* gene (21). A strong down-regulation of endogenous c-Src activity by overexpression of Csk probably cannot occur, since a majority of the c-Src molecules are phosphorylated at Tyr-527 by endogenous Csk and consequently inactive. However, despite the variation of c-Src activity observed in the present study, the morphological changes were identical in all induced wt Csk clones, indicating that these changes were not caused by changes of c-Src activity.

Whatever the mechanism of the morphological and cytoskeletal changes, it is clear that they were caused by the increased activity of Csk during induction. It is not clear, however, whether the reorganization of the α_v integrin subunit caused the changes in cell shape or vice versa. Since the vitronectin receptor subunits were not tyrosyl phosphorylated, phosphorylation of other components of focal adhesions may be responsible for the exclusion of α_v from the adhesion plaques. Thus, Csk may have some unidentified substrates in focal adhesions. However, anti-PTyr immunoblotting of several known focal adhesion proteins ($pp125^{FAK}$, paxillin, talin, and vinculin) revealed no increase of phosphorylation upon induction of Csk, despite the fact that the SH2 domain, which was required for Csk interaction with the focal adhesions, formed complexes with $pp125^{FAK}$ and paxillin. Since most Csk molecules in the induced cells reside in the cytoplasm, it is also possible that the observed effects are mediated through cytoplasmic proteins which control the structure and function of focal adhesions. Such possible changes should concern only a minor population of cellular PTyr-containing proteins, because no significant differences between induced and noninduced cells were seen in Western blotting analysis of cellular phosphoproteins.

In conclusion, we show that Csk overexpression causes dramatic changes in HeLa cell morphology. In these cells and in rat myoblasts, a fraction of the Csk molecules are targeted to focal adhesions, via PTyr-dependent interactions of their SH2 domains with $pp125^{FAK}$ and paxillin and via so far unknown interactions of the SH3 domain. The effects of Csk overexpression involve specifically the organization of integrins forming the vitronectin receptor, and they do not seem to be mediated via the activity of Src family kinases. In this context, one must consider the possibility that Csk has substrates outside of the Src TK family.

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