Disruption of focal adhesions by integrin cytoplasmic domain-associated protein-1 alpha.

DOI:10.1074/jbc.M211258200

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 8, Issue of February 21, pp. 6567–6574, 2003
Printed in U.S.A.

Disruption of Focal Adhesions by Integrin Cytoplasmic Domain-associated Protein-1α*

Received for publication, November 4, 2002
Published, JBC Papers in Press, December 7, 2002, DOI 10.1074/jbc.M211258200

Daniel Bouvard‡§¶, Lucile Vignoud‡, Sandra Dupé-Manet‡, Nadia Abed‡, Henri-Noël Fournier‡, Carole Vincent-Monegat‡, Saverio Francesco Retta‖, Reinhard Fässler§, and Marc R. Block‡**

From the ‡Laboratoire d'Etude de la Differenciation et de l'Adhérence Cellulaires, Unité Mixte de Recherche UJF/CNRS 5538, Institut Albert Bonniot, Faculte de Médecine de Grenoble, La Tronche F38706 cedex, France, the ‖Department of Genetics, Biology, and Biochemistry, University of Torino, Torino 10126, Italy, and the §Department of Molecular Medicine, Max Planck Institute for Biochemistry, Am Klopferspitz 18A, Martinsried D-82152, Germany

Regulation of integrin affinity and clustering plays a key role in the control of cell adhesion and migration. The protein ICAP-1α (integrin cytoplasmic domain-associated protein-1α) binds to the cytoplasmic domain of the β_{1A} integrin and controls cell spreading on fibronectin. Here, we demonstrate that, despite its ability to interact with β_{1A} integrin, ICAP-1α is not recruited in focal adhesions, whereas it is colocalized with the integrin at the ruffling edges of the cells. ICAP-1α induced a rapid disruption of focal adhesions, which may result from the ability of ICAP-1α to inhibit the association of β_{1A} integrin with talin, which is crucial for the assembly of these structures. ICAP-1α-mediated dispersion of β_{1A} integrins is not observed with β_{1D} integrins that do not bind ICAP. This strongly suggests that ICAP-1α action depends on a direct interaction between ICAP-1α and the cytoplasmic domain of the β_1 chains. Altogether, these results suggest that ICAP-1α plays a key role in cell adhesion by acting as a negative regulator of β_1 integrin avidity.

Interactions of cells with the extracellular matrix are essential for survival, differentiation, and proliferation of cells (1). They are mainly mediated by type I $\alpha\beta$ heterodimer transmembrane receptors named integrins (2). Integrin-mediated cell adhesion is a highly controlled process that can be modulated very rapidly by two mechanisms: the modulation of the receptor affinity by a conformational change and the modulation of receptor avidity by lateral diffusion and clustering into highly ordered structures named focal adhesions. As shown for the platelet integrin $\alpha_{IIb}\beta_3$, the effects of integrin clustering and affinity modulation are additive and seem to play complementary roles (3). The conformational change that modulates the affinity of some integrins is mediated by monomeric G proteins of the Ras family. R-Ras seems to prevent H-Ras-dependent decrease in integrin affinity (4–6). However, proteins involved in this signaling pathway are still largely unknown (6, 7).

On the other hand, it has been reported that intracellular calcium plays a key role in cell adhesion (8). Calcium-dependent cycles between high and low affinity states of integrins seem to be crucial for cell migration (9–12). More recently, we found that the affinity state of the $\alpha_5\beta_1$ integrin in CHO[1] cells may be switched by the balance between two antagonistic enzymatic activities: calcineurin and calcium/calmodulin-dependent protein kinase of type II (CaMKII) (13, 14). A CaMKII-dependent inside-out signaling was also described as the molecular basis of the cross-talk between $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (15). Although this regulatory pathway remains to be unraveled, calcineurin has been shown to control $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrin affinity in neutrophils and CHO cells, respectively (16, 17). Finally a complex between β_1 integrin and CaMKII was observed in breast cancer MCF-7 cells (18). Although the regulation of integrin function may involve phosphorylation events on the threonine doublet TT788–789 of the β_{1A} chain (19) or on the threonine triplet TTT758–760 of the β_2 chain (20), these phosphorylation sites do not seem to be directly linked to the CaMKII-dependent control of integrin affinity. Therefore, it is likely that this latter signaling pathway occurs via an intermediate regulatory protein. This hypothesis was further supported by the fact that ectopically expressed β cytoplasmic domains have a dominant negative effect on integrin function, suggesting that some control proteins are titrated by the overexpression of β cytoplasmic tails (21, 22).

Integrin cytoplasmic domain-associated protein-1α (ICAP-1α) was identified in a yeast two-hybrid screen as a protein specifically associated with the cytoplasmic domain of β_{1A} integrins (23). This protein has two isoforms named α and β of 200 and 150 amino acids, respectively. ICAP-1 is expressed throughout development and also in adult tissues (24). ICAP-1α but not ICAP-1β interacts with the cytoplasmic tail of the β_{1A} chain in a manner that depends on the conserved NP*X*Y integrin motif (25). ICAP-1α contains a number of putative phosphorylation sites, including a phosphorylation motif for the CaMKII around threonine 38. We could show that a point mutation T38D (that mimics the phosphorylated form) or T38A (which cannot be phosphorylated) in ICAP-1α and expression of the corresponding recombinant proteins reduced or increased cell spreading on fibronectin, respectively. These data suggest that phosphorylation of ICAP-1α on threonine 38 by CaMKII modulates $\alpha_5\beta_1$ integrin function (13). A further involvement of ICAP-1α in the regulation of β_1 integrin function was suggested by experiments indicating that its overex-

---

* This work was supported in part by the Fédération Nationale des Ligues Contre le Cancer, the CNRS (Program Biologie Cellulaire: du Normal au Pathologique), and the Association pour la Recherche contre le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *"advertisement"* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of a fellowship from the Ministère de la Recherche and the Association pour la Recherche contre le Cancer and presently supported by a Marie-Curie fellowship.

** To whom correspondence should be addressed. Tel.: 33-476-54-95-70; Fax: 33-476-54-94-25; E-mail: marc.block@ujf-grenoble.fr.

[1] The abbreviations used are: CHO, Chinese hamster ovary; CaMKII, calmodulin-dependent protein kinase of type II; ICAP-1α, integrin cytoplasmic domain-associated protein-1α; PBS, phosphate-buffered saline; BSA, bovine serum albumin; VPM, ventral plasma membrane.

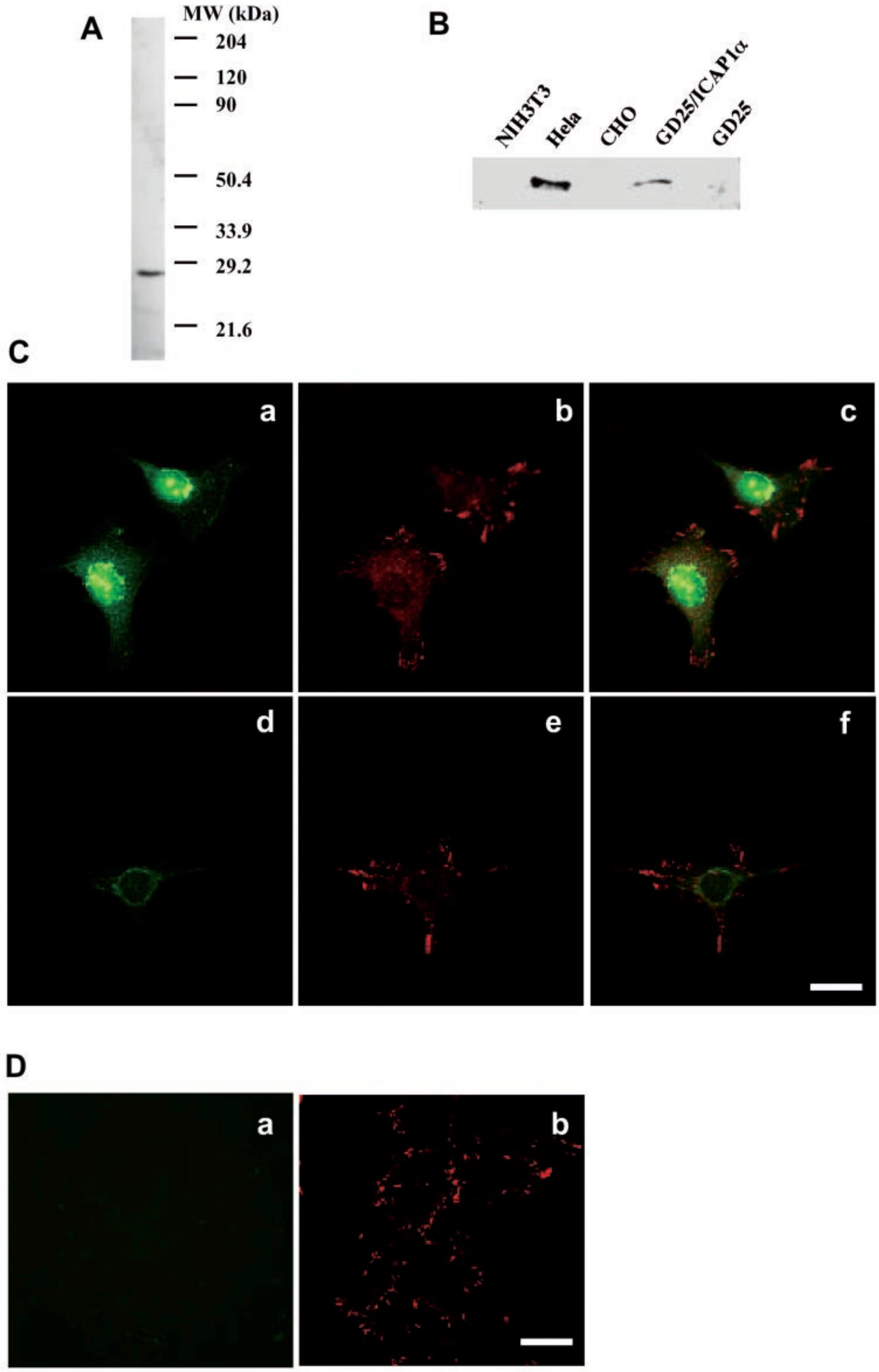

FIG. 1. **Antibodies characterization and cellular localization of the protein ICAP-1α.** *A*, the proteins of a HeLa cell lysate in radioimmune precipitation assay buffer were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The protein ICAP-1α was detected with polyclonal antibodies. *B*, Western blots of ICAP-1α protein in NIH3T3 cells, HeLa cells, CHO cells, GD-25 cells, and GD-25 cells transfected with ICAP-1α cDNA. *C*, HeLa cells were cultured overnight on fibronectin, fixed, permeabilized, and processed for double immunofluorescence labeling. In *a*, HeLa cells are stained using polyclonal antibodies directed against ICAP-1α. In *d*, HeLa cells are stained with the same polyclonal antibodies directed against ICAP-1α, which has been incubated with the recombinant ICAP-1α protein to compete with the ICAP-1α-specific labeling. In *b* and *e*, HeLa cells are stained using a monoclonal antibody directed against vinculin. In *c* and *f* is shown the merged images of *a* with *b* and *d* with *e*, respectively. *D*, ventral plasma membranes (*VPM*) from HeLa cells were isolated, and double labeling of ICAP-1α (*a*) and vinculin (*b*) was carried out with specific primary antibodies. These results are representative of three independent experiments. *Bar*, 10 μm.

pression increases cell motility on a β_1-dependent substrate such as fibronectin (26).

In this report we show that ICAP-1α, despite its ability to interact directly and specifically with the β_1 integrin cytoplasmic domain *in vitro*, was never observed in focal adhesions. In addition, ICAP-1α could inhibit the interaction between talin and the β_1 cytoplasmic tail *in vitro*. Because talin recruitment is a prerequisite for focal adhesion assembly (27, 28), we have analyzed the effect of ICAP-1α on the organization of these structures and showed that this protein was able to disorganize focal adhesions in a manner dependent on its direct interaction with the β_1 cytoplasmic tail. These results strongly suggest that ICAP-1α is a key regulator of cell adhesion mediated through β_1 integrin and focal adhesion dynamic by weakening talin binding to the β_1 integrin.

EXPERIMENTAL PROCEDURES

Antibodies—The anti-β_1 tail serum (anti cyto-β_1) was raised against a synthetic peptide corresponding to the cytoplasmic domain of the β_1 chain covalently coupled to keyhole limpet hemocyanin. Anti-talin monoclonal antibody (8d4) was purchased from Sigma (St. Louis, MO). The monoclonal antibody 9EG7 directed against the β_1 subunit was kindly supplied by Dr. D. Vestweber (Muenster, Germany). The monoclonal antibody 7E2 directed against the hamster β_1 subunit was a generous gift of Dr. R. Juliano (Chapel Hill, NC). Polyclonal antibody directed against the human ICAP-1α protein was previously described (13). Cyanin3-, Alexa-, or rhodamine-conjugated goat anti-mouse or anti-rabbit from Molecular Probes (Eugene, OR) or Immunotech (Marseille, France) were used as secondary antibodies.

Cells and Cell Culture—The murine NIH3T3, the hamster CHO, and the human HeLa cell lines were grown in α-minimal essential medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. The murine GD25, GD25-β_{1A}, and GD25-β_{1D} were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. GD25 cells do not express the β_1 integrin chain due to a null mutation in both alleles (29). GD25 cells transfected with either the murine β_{1A}, or the human β_{1A} and β_{1D} full-length cDNA are called GD25-β_{1A} and GD-β_{1D}, respectively, and have been described earlier (30, 31). All transfected cells were grown in complete medium supplemented with the appropriate antibiotics for the selection of the transfected cells.

Protein Purifications—ICAP-1α and ICAP-1α fragments fused to a polyhistidine tag at the N-terminal position were purified from the BL21(DE3) *Escherichia coli* strain containing the vector pET19b-ICAP-1α. Briefly, human ICAP-1α cDNA cloned in pBluescript was used as a template in a PCR reaction using primers with an *Xho*I site in the 5′ position. In the sense primer the *Xho*I site is in-frame with the first methionine of ICAP-1α. Then the *Xho*I-digested PCR product was cloned into the *Xho*I site of pET-19b vector (Novagen). Fragments were obtained by insertion of stop codons at different positions using the QuikChange mutagenesis kit (Stratagene). All constructs used in this study have been sequenced by the Eurogentec direct sequencing department (Belgium). Purification was carried out using the nickel-charged resin nickel-nitrilotriacetic acid from Qiagen. Inclusion bodies were solubilized in urea. Protein refolding was performed directly on the column by progressive removal of the chaotropic agent. The purity of the protein was checked by SDS-PAGE and Coomassie Blue staining and was greater than 90–95%. All experiments were carried out with freshly purified proteins. Before each experiment, the capacity of each batch of the purified protein to interact with the β_1 cytoplasmic domain was estimated in a solid-phase assay.

The polypeptide corresponding to the β_1 integrin cytoplasmic domain was produced from the BLR(DE3)pLysS *E. coli* strain containing the vector pET19b-cytoβ1. This construct allows the production of the fragment 752–798 of the β_1 integrin cytoplasmic domain. This peptide was recognized by a polyclonal antibody raised against a synthetic β_1 cytoplasmic peptide coupled to keyhole limpet hemocyanin. Talin and α-actinin were purified as previously described (32), and fibronectin was purified according to a previous study (33).

Transfection in Mammalian Cells and Selection of Stable Clones—Full-length human ICAP-1α was excised from the pBS-ICAP-1α vector as an *Eco*RI/*Xba*I fragment and inserted into the pcDNA3.1(+) vector (Invitrogen, The Netherlands). Stable GD25-β_{1A} cell lines expressing ICAP-1α were obtained by electroporation of 4×10^6 cells in 400 µl of PBS at 280 V with 15 µg of pcDNA3.1(+)-ICAP-1α vector. Transfected cells were selected in complete medium with Zeocin (Invitrogen, The Netherlands) at a final concentration of 300 µg/ml. The expression of ICAP-1α was monitored by indirect immunofluorescence and Western blot analysis using the ICAP-1α polyclonal antibodies.

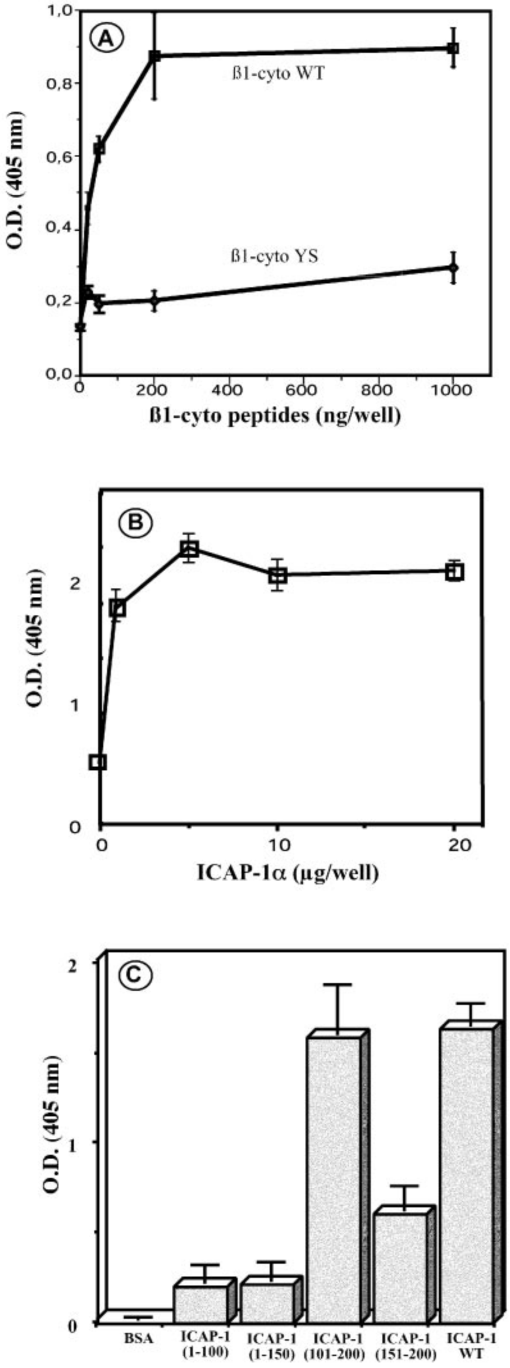

FIG. 2. **ICAP-1α interacts specifically and directly with β_1 integrins.** *A*, the capacity of ICAP-1α to interact with a peptide corresponding to the β_1 integrin cytoplasmic domain was checked in a solid-phase binding assay. A constant amount of purified recombinant ICAP-1α (10 µg/well) or BSA (3% w:v) was used to coat a 96-well tray overnight at 4 °C. After a blocking step, increasing amounts of the wild type β_1-cyto peptide or YS β_1-cyto mutant were added into the wells and detected with a specific polyclonal antibodies. Each experimental point was obtained from triplicate experiments, and background values of BSA coating have been subtracted. These results are representative of three independent experiments using different preparations of the purified ICAP-1α protein and cyto-β_1 peptides. *B*, increasing amounts of the recombinant ICAP-1α protein were used to coat plastic wells of a 96-well tray. Subsequently, a constant amount (300 µg/well) of a CHO cell lysate was added. The β_1 integrin receptors bound to ICAP-1α were detected using the non-blocking monoclonal antibody 7E2 (raised against the hamster β_1 chain). The results from three independent experiments using different preparations of the purified ICAP-1α were averaged, and standard deviations are shown. *C*, polyhistidine-tagged ICAP-1α fragments were used in the β_1 binding assay described above. The wells were coated with 10 µg of the ICAP-1α recombinant fragments and then incubated with 300 µg of CHO cell lysate proteins. The bound $\alpha_5\beta_1$ was immunodetected by the 7E2 anti-hamster β_1 monoclonal antibody. Each histogram represents mean ± S.D. of three independent experiments.

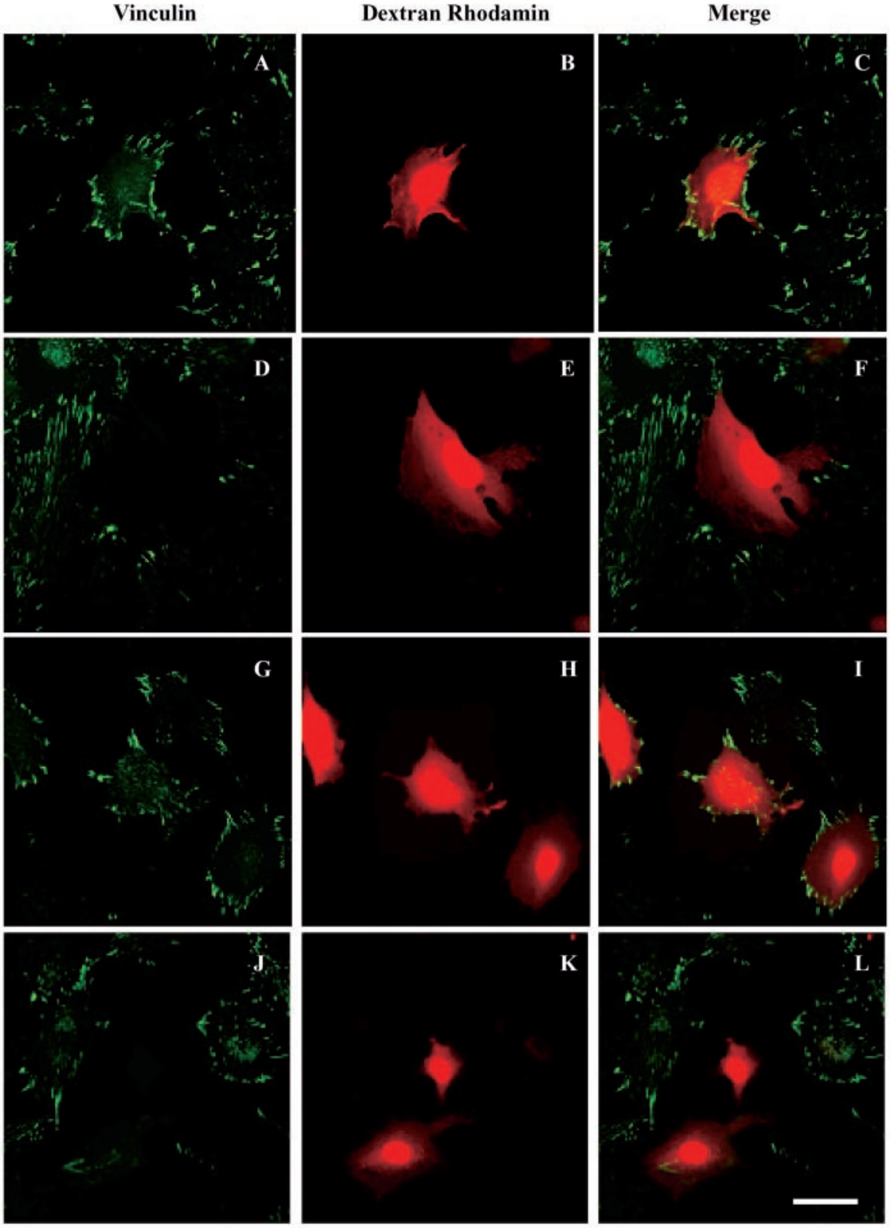

FIG. 3. **Microinjection of purified ICAP-1α causes focal adhesion disassembly.** NIH3T3 cells were seeded onto fibronectin-coated coverslips and allowed to spread overnight at 37 °C. Then a PBS solution of dextran-rhodamine alone (*A–C*) or supplemented with the purified recombinant ICAP-1α protein at 1 mg/ml (*D–F*), ICAP-1α 1–100 fragment (*G–I*), or ICAP-1α 100–200 fragment (*J–L*), was microinjected into the cells. After microinjection, the cells were fixed, permeabilized as described under "Experimental Procedures" and immunostained for vinculin. These panels are representative of four independent experiments using different preparations of purified recombinant ICAP-1α protein and fragments.

Immunofluorescence Microscopy—Immunofluorescence was carried out using standard procedures. Stained cells were analyzed with an inverted fluorescence microscope (Olympus Provis AX70) equipped with a Plan Apo ×63 oil immersion, numerical aperture 1.40 objective lens. For all double-staining experiments, the appropriate controls were performed to ensure that no undesired cross-reactivity occurred between the primary and secondary antibodies.

Purification of Ventral Plasma Membranes—The purification of HeLa, GD25-β_1, or NIH3T3 ventral plasma membranes was performed as previously described by Cattelino *et al.* (34). The cells were grown overnight on fibronectin-coated coverslips in complete medium. After two washes in PBS, the cells were incubated with cold water for 2 min and then flushed with a 1000-μl tip. Cell disruption was confirmed by microscopy. Ventral plasma membranes were either immediately fixed with paraformaldehyde or were preincubated for 30 min at 4 °C with ICAP-1α or ICAP-1α fragments at the concentration of 5 μM in a VPM buffer containing 125 mM potassium acetate, 2.5 mM $MgCl_2$, 12 mM glucose, and 25 mM HEPES, pH 7.5, prior to fixation.

Solid-phase Assays—The interaction between ICAP-1α and the cyto-β_1 peptide or the whole $\alpha_5\beta_1$ integrin was carried out using a solid-phase assay. Briefly, a 96-well tray (MaxiSorp, Nunc) was coated with the whole ICAP-1α protein or ICAP-1α fragments for 16 h at 4 °C and blocked with a 3% BSA/PBS solution for 1 h at room temperature. A Triton X-100 CHO cell lysate made in PBS supplemented with 1% Triton X-100 (w:v) or the cyto-β_1 peptide were incubated for 1 h at 37 °C. After three washes in PBS containing 3% BSA and 0.01% Tween-20, detection of the $\alpha_5\beta_1$ integrin from the CHO cell lysate was performed using the 7E2 monoclonal antibody, whereas the detection of the cyto-β_1 peptide was achieved with a polyclonal antibody directed against a synthetic peptide corresponding to the β_1 tail.

Microinjection into NIH3T3 Cells—NIH3T3 cells were seeded onto fibronectin-coated glass coverslips overnight at 37 °C. All injections were carried out with the aid of a micromanipulator 5171 connected to an Eppendorf microinjector unit (Transjector 5246). The cells were microinjected with PBS containing a final concentration of 1 mg/ml of the freshly purified recombinant ICAP-1α protein, or the N-terminal (1–100) or C-terminal (101–200) fragments, in the presence of 100 μM tetramethylrhodamine-dextran amine (M_r 3000, Molecular Probes, Interchim, France) to view the injected cells. Three hours (whole ICAP-1α protein) or 30 min (ICAP-1α fragments) after microinjection, the cells were fixed with 3% paraformaldehyde and 2% sucrose in PBS for 10 min at 37 °C and then immunostained for vinculin localization.

RESULTS

ICAP-1α Does Not Localize in Focal Adhesions—The protein ICAP-1α was isolated as a β_{1A}-interacting protein in a yeast two-hybrid screen (23) and was shown to modulate CHO cell

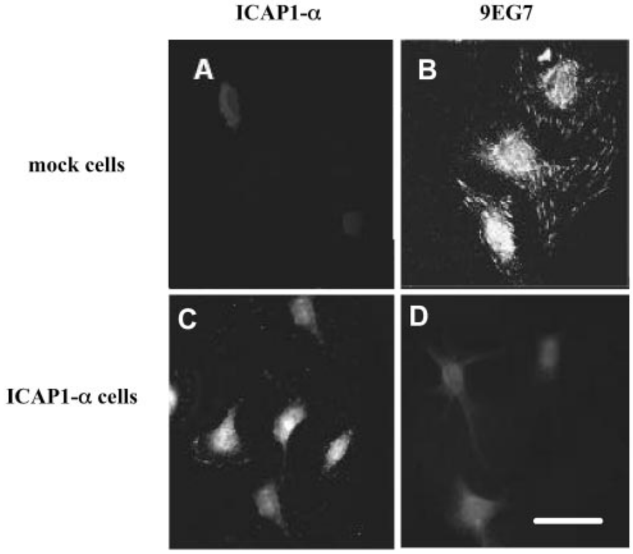

FIG. 4. **ICAP-1α expression disrupts β_1 integrin-containing focal adhesions.** GD25-β_{1A} cells were stably transfected either with vector alone (*A* and *B*) or with a cDNA coding for the full-length ICAP-1α protein (*C* and *D*). Transfected cells were spread overnight at 37 °C on fibronectin-coated coverslips. The expression of ICAP-1α was visualized with polyclonal antibodies (*A* and C) and the high affinity conformational state of the β_1 integrin with the 9EG7 monoclonal antibody (*B* and *D*). Note that the reduction of 9EG7 staining correlated with the expression of ICAP-1α. *Bar*, 10 μm.

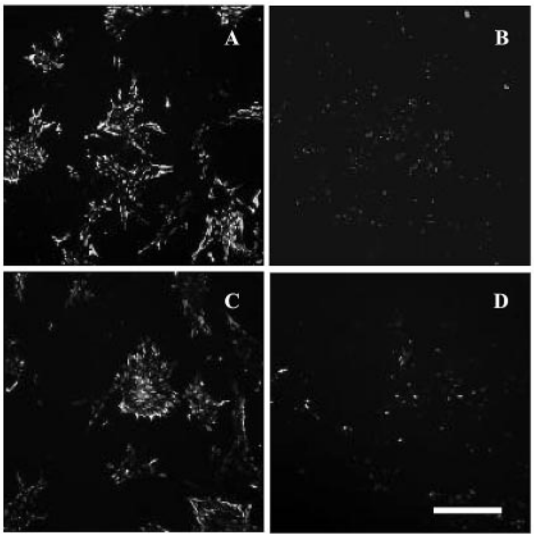

FIG. 5. **Purified ICAP-1α disrupts focal adhesions *in vitro*.** Ventral plasma membranes from NIH3T3 cells were prepared as described under "Experimental Procedures." The membranes were incubated at 4 °C for 30 min in the absence (*A*) or in the presence (*B*) of purified recombinant ICAP-1α (5 μM). Alternatively, the purified N-terminal moiety of ICAP-1α (amino acids 1–100) shown in *C* or the C-terminal moiety of ICAP-1α (amino acids 101–200) shown in *D* were added at a concentration of 5 μM. The membranes were subsequently fixed and stained for vinculin. Note the dramatic reduction of vinculin staining upon the addition of recombinant ICAP-1α or the C-terminal domain (*B* and *D*). Photographs were taken with identical exposure times. These observations are representative of four independent experiments using different preparations of purified recombinant ICAP-1α. *Bar*, 10 μm.

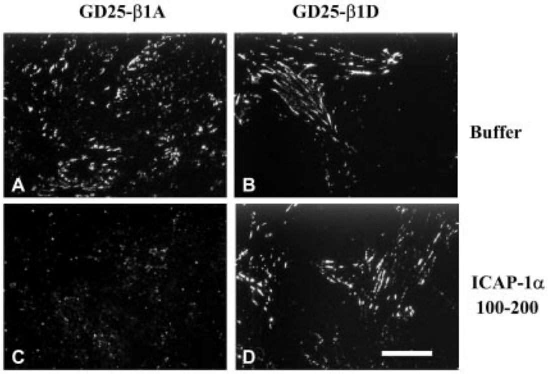

FIG. 6. **ICAP-1α integrin binding domain displaces β_1A but not β_1D integrins from focal adhesions.** Ventral plasma membranes from GD25-β_{1A} (*A* and *C*) and GD25-β_{1D} (*B* and *D*) cells were prepared as described under "Experimental Procedures." The membranes were incubated at 4 °C for 30 min in the absence (*A* and *B*) or in the presence (*C* and *D*) of the C-terminal moiety of ICAP-1α (amino acids 101–200) added at a concentration of 5 μM. The membranes were subsequently fixed and stained for β_1 integrins using the monoclonal antibody 4B7R. Photographs were taken with identical exposure times. These observations are representative of four independent experiments using different preparations of purified recombinant ICAP-1α. *Bar*, 10 μm.

adhesion (13) and to promote cell motility (26). In epithelial cells or in cell lines derived from epithelial cells such as HeLa, ICAP-1α could be detected in a cell lysate by Western blot using a polyclonal antibody raised against the full-length recombinant protein (Fig. 1*A*). The endogenous human ICAP-1α protein in HeLa cells migrates in SDS-PAGE like the ectopically expressed protein in rodent fibroblast-like GD25 cells (Fig. 1*B*). GD25, CHO, and NIH3T3 cells showed no detectable ICAP-1α expression as monitored by Western blot analysis. To determine the physiological relevance of the interaction between ICAP-1α and the β_1 integrin, we carried out immunomicroscopy experiments of ICAP-1α in different cell lines. In HeLa cells, ICAP-1α showed a diffuse expression pattern and often some nuclear localization (Fig. 1*C*, *panel a*). Surprisingly, no accumulation of ICAP-1α was observed in focal adhesions visualized by vinculin staining (Fig. 1*C*, *panels a–c*). Similarly, we reported previously that in the Hs68 cell line, ICAP-1α and β_1 colocalize in ruffles but not in focal adhesions (35). A direct competition of endogenous ICAP-1α with the purified recombinant protein revealed a dramatic decrease in ICAP-1α immunostaining and confirmed the specificity of the immunolabeling (Fig. 1*C*, *panels d–f*). Despite the diffuse ICAP-1α localization, these cells were able to form well-organized focal adhesions connected to stress fibers as judged by double labeling using a monoclonal antibody directed against vinculin and phalloidin-rhodamine-stained stress fibers (not shown).

To have direct access to focal adhesion proteins, ventral plasma membranes were obtained from HeLa cells grown overnight on fibronectin. Double immunostaining was performed with an anti-vinculin antibody and anti-ICAP-1α polyclonal antibodies. In these membrane preparations, focal adhesions could be viewed by vinculin staining (Fig. 1*D*, *panel b*) or by talin or β_1 staining (not shown), whereas anti-ICAP-1α antibodies showed a faint background staining that was barely detectable (Fig. 1*D*, *panel a*). Altogether these results suggest that ICAP-1α is not present in focal adhesions.

Interaction of ICAP-1α with the $\alpha_5\beta_1$ Integrin—The absence of ICAP-1α in focal adhesions prompted us to study the interaction of ICAP-1α with β_1 integrins in more detail. ICAP-1α and the β_1 cytoplasmic domains were expressed in bacteria as polyhistidine fusion proteins. Fig. 2*A* shows that the purified ICAP-1α protein interacted specifically with the purified β_1 cytoplasmic domain in a solid-phase assay, which is consistent with previous reports (23, 26). As a control, we used a β_1 cytoplasmic domain bearing the point mutation Y to S in the NP*X*Y membrane distal (cyto3) domain. In full agreement with a previous report (23), this mutation abolished the interaction between ICAP-1α and the β_1 cytoplasmic tail (Fig. 2*A*).

Next, we tested whether ICAP-1α was able to interact with

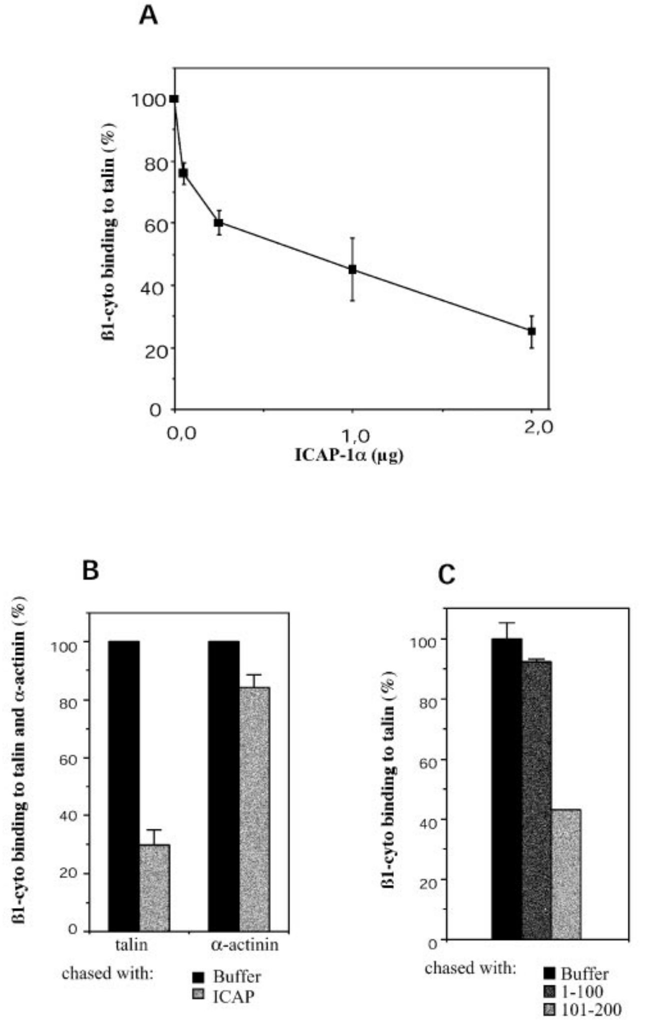

FIG. 7. **ICAP-1α competes with talin but not with α-actinin binding to the β_1 cytoplasmic domain.** *A*, increasing amounts of purified recombinant ICAP-1α were preincubated with 1 μg of the cyto-β_1 peptide and then incubated in a 96-well tray coated with equal amounts (10 μg/well) of talin purified from human platelets. The binding of the cyto-β_1 peptide to talin was detected by polyclonal antibodies raised against the cytoplasmic domain of the β_1 integrin chain and a biotin-conjugated anti rabbit secondary antibody. *B*, an amount of 2 μg of the recombinant protein ICAP-1α was preincubated with 1 μg of the cyto-β_1 peptide and incubated in 96-well plastic trays coated with 10 μg of purified talin (from human platelets) or α-actinin (from chicken gizzard). The binding of the cyto-β_1 peptide to talin or α-actinin was detected by polyclonal antibodies raised against the cytoplasmic domain of the β_1 integrin chain and a biotin-conjugated anti-rabbit secondary antibody. *C*, a concentration of 1.5 μg of ICAP-1α fragments 1–100 and 101–200 was preincubated with 1 μg of the cyto-β_1 peptide and incubated in 96-well plastic trays coated with 10 μl of purified talin. The binding of the cyto-β_1 peptide to talin was detected by polyclonal antibodies raised against the cytoplasmic domain of the β_1 integrin chain and a biotin-conjugated anti rabbit secondary antibody. Each experiment was performed in triplicate.

the whole $\alpha_5\beta_1$ integrin from a CHO cell lysate. This was crucial, because beta subunits do not exist in isolation in cells, and therefore, two hybrid experiments with integrins may be prone to artifacts. Increasing amounts of the recombinant ICAP-1α protein were used to coat 96-well trays. The protein concentration during coating was maintained constant by adding BSA. An equal amount of a CHO cell lysate in Triton X-100 was subsequently incubated in each coated well. A dose-dependent and -specific binding of the β_1 integrin was detected by a specific antibody (Fig. 2*B*). These data indicate that ICAP-1α expressed in bacteria is able to interact with the β_1A cytoplasmic domain, and that the cytoplasmic domain of the α subunit did not impair the interaction with ICAP-1α.

Finally, we expressed ICAP-1α fragments in bacteria and used them in a solid-phase binding assay to map the β_1 binding site. Only the C-terminal moiety (amino acids 100–200) of the protein was able to bind to the β_1 integrin (Fig. 2*C*). But neither the fragment corresponding to amino acids 1–150 nor the fragment corresponding to amino acids 151–200 of ICAP-1α were found to interact strongly with the $\alpha_5\beta_1$ integrin from cell lysate (Fig. 2*C*).

ICAP-1α Disorganizes Focal Adhesions ex Vivo—Despite its specific and direct association with the β_1 integrin, ICAP-1α was not localized in focal adhesions. One possible explanation for these contradictory results could be that ICAP-1α might act as a negative regulator of the recruitment of focal adhesion components. To investigate this possibility we microinjected ICAP-1α recombinant protein into the cytoplasm of NIH3T3 cells and monitored focal adhesion organization by staining for vinculin. Although microinjection of dextran-coupled rhodamine alone had no significant effect on the localization of vinculin (Fig. 3, *A–C*), talin, and α-actinin (not shown), microinjection of the full-length ICAP-1α in the dextran-coupled rhodamine buffer induced a rapid delocalization of vinculin (Fig. 3, *D–F*) or talin and α-actinin (not shown) observed in 70% of the cells. Microinjection of the C-terminal moiety of ICAP-1α (amino acids 101–200) that encompasses the β_1 binding site had similar effects (Fig. 3, *J–L*) in 77% of the injected cells. Because the N-terminal fragment (amino acids 1–100) does not bind the β_1 integrin domain (Fig. 2*C*), we made use of this recombinant fragment as a control. Indeed, the microinjection of this part of ICAP-1α did not interfere with vinculin staining (Fig. 3, *G–I*).

Finally, disruption of focal adhesions by ICAP-1α was also investigated in a cellular context after stable transfection into GD25-β_{1A} cells of a vector containing human ICAP-1α cDNA. This cell line expresses functional β_1 integrins at the cell surface (19) that can be monitored by the 9EG7 monoclonal antibody, which recognizes a ligand-induced binding site epitope correlating with the occupied conformational state of β_1 integrins (36, 37). Under our experimental conditions, immunofluorescence microscopy did not reveal any detectable staining for endogenous ICAP-1α in GD25-β_{1A} cells (Fig. 4*A*). On the other hand, these cells exhibited surface expression of β_{1A} integrins confined to focal adhesions that could be monitored by the 9EG7 antibody (Fig. 4*B*). In a non-clonal population of GD25-β_{1A} cells transfected with a cDNA encoding the human ICAP-1α, a positive immunofluorescence signal for ICAP-1α was diffusely present within the cytoplasm (Fig. 4*C*). Simultaneously, a diminution of cell spreading and loss of 9EG7 monoclonal antibody staining was observed, suggesting that β_1 integrins were no longer occupied and involved in focal adhesions (Fig. 4*D*).

Disruption of Focal Adhesions by ICAP-1α Requires Direct Interaction with the β_1 Integrin Chain—The action of ICAP-1α on focal adhesions might be indirect, for instance due to the interference with some regulatory pathways. Therefore, the purified recombinant ICAP-1α was also tested for its ability to disassemble focal adhesions *in vitro* in a cytosol-free ventral plasma membrane preparation (VPM). These preparations are depleted in nucleotide triphosphate and soluble signaling enzymes. The cell membranes were incubated for 30 min at 4 °C with a solution of purified ICAP-1α in acetate buffer and glucose. Although buffer alone did not interfere with the detection of focal adhesion proteins such as vinculin (Fig. 5*A*), the incubation with ICAP-1α efficiently displaced vinculin from focal adhesions (Fig. 5*B*). A similar result was also observed for talin and α-actinin (not shown). The same result was obtained by the incubation of the C-terminal part (amino acids 101–200) of ICAP-1α (Fig. 5*D*). Finally, incubation of these ventral membranes with the N-terminal purified fragment (amino acids 1–100) had no effect on focal adhesion organization (Fig. 5*C*).

ICAP-1α was suggested to have a GDP dissociating inhibitor activity for Rac and Cdc42 (38), two monomeric G proteins of the Rho family involved in the regulation of cytoskeleton organization. This activity might account for ICAP-1α destabilizing action on focal adhesions of ventral plasma membranes. To assess whether ICAP-1α action on focal adhesions was due to its direct binding on β_1 integrin chains or to some interference with Rho signaling pathways, we performed similar experiment on VPM from GD-25β_{1A} and GD-25β_{1D} cells lines. The β_{1D} and β_{1A} isoforms are functionally similar with regard to integrin-mediated signaling (39), but the former strongly binds talin (31) and does not bind ICAP-1α (38). Upon addition of the ICAP-1α fragment 100–200, the dispersion of β_{1A} integrins initially clustered into focal adhesions was observed (Fig. 6, *A* and *C*), whereas β_{1D}-containing focal adhesions remained unaffected (Fig. 6, *B* and *D*). This result strongly suggests that a direct interaction between ICAP-1α and the β_1 chain is a prerequisite for focal adhesion disassembly.

Talin and ICAP-1α Compete for Binding on the Cytosolic Domain of the β_1 Integrin Chain—Because talin interacts directly with the β_1 integrin cytoplasmic domain and is crucial for focal adhesion assembly, one attractive hypothesis is that ICAP-1α is involved in the control of talin-integrin interaction. Therefore, we tested whether ICAP-1α could modulate the binding of talin to the integrin β_1 cytoplasmic domain. In an *in vitro* solid-phase assay, ICAP-1α could inhibit talin binding to the cytoplasmic tail of the β_{1A} chain in a dose-dependent manner (Fig. 7*A*). These data suggest that the displacement of talin from its binding site on β_{1A} may be sufficient for focal adhesion disruption and, consequently, for a decrease in the integrin avidity. Moreover, the competition of ICAP-1α and talin for the binding to β_1 was specific, because it could not be observed either with α-actinin, another β_1 interacting protein (Fig. 7*B*), or with the 1–100 ICAP-1α moiety (Fig. 7*C*).

DISCUSSION

We examined the cellular localization of the endogenous ICAP-1α protein. Surprisingly, this protein was never detected in focal adhesions, but instead, exhibited a diffuse pattern within the cell, although a significant amount of the protein was associated within the Triton X-100-insoluble fraction (not shown) and often, a nuclear staining was observed. Using purified ventral membrane preparation from HeLa cells, we never observed ICAP-1α colocalized with vinculin or talin, which were used as markers of focal adhesions.

Even though ICAP-1α was not detected in focal adhesions, the purified recombinant protein interacted strongly with the cytoplasmic domain of the β_{1A} integrin chain as reported previously (23, 26). Additionally, this interaction also occurred with the whole integrin receptors purified from a cell lysate. The strong binding of ICAP-1α to the cytoplasmic domain of the β_1 integrin and its complete absence from focal adhesions suggested that this interaction may disrupt focal adhesion structures. To confirm this hypothesis we microinjected ICAP-1α in NIH3T3 cells, and we indeed observed a rapid disorganization of focal adhesions. In addition, recombinant ICAP-1α was able to disaggregate focal adhesions when added to purified ventral plasma membranes from NIH3T3 and GD-25β_{1A} cells. Conversely, the β_{1D}-containing integrins were resistant to ICAP-1α. This latter experiment strongly suggests that the disassembly of focal adhesions is due to a direct interaction with the β_{1A} integrin subunit and is independent of a cellular signaling pathway. Furthermore, the focal adhesion disruption mediated by ICAP-1α is in good correlation with our previous data, which have shown that ectopic expression of ICAP-1α-regulated CHO cell spreading (13).

Several reports have shown that talin is crucial for the formation of focal adhesions (27, 28, 40). A simple explanation for the negative effect of ICAP-1α on focal adhesion structure could be its ability to disrupt the direct association between the integrin and talin. To investigate this hypothesis we performed an *in vitro* assay and found that talin and ICAP-1α compete for binding to the β_{1A} cytoplasmic domain. On the other hand, we found that the interaction between α-actinin and the β_1 integrin is not inhibited by the presence of ICAP-1α. This shows that ICAP-1α inhibits the interaction between β_{1A} integrins and talin in a specific manner and confirms previous reports showing that the interaction of α-actinin with the β_1 cytoplasmic domain is not sufficient to stabilize focal adhesion sites (40). The lack of effect of ICAP-1α on β_{1D} localization suggests that, under our experimental conditions, this action is direct and not dependent on the GDP dissociating inhibitor activity recently suggested (38). Based on these findings we propose that ICAP-1α and talin compete for integrin β_{1A} binding and thereby modulate focal adhesion assembly and/or dynamic. How ICAP-1α interferes with talin binding on the β_1 integrin needs further investigation. The talin binding site is not unambiguously defined. Recent reports have demonstrated that the talin N-terminal head binds to the β_3, β_{1A}, and β_{1D} cytoplasmic domains (41, 42). Some data indicated that the binding site of the talin head could be located on the proximal membrane region of the integrin β chain (41). Conversely, other reports indicate that a phosphotyrosine binding-like subdomain of the FERM domain of talin head is the major binding site that triggers the activation of the $\alpha_{IIb}\beta_3$ integrin (43). This finding is very interesting, because it offers some molecular basis of ICAP-1α and talin competition. Indeed, sequence homology and molecular modeling favor the view that ICAP-1α is a phosphotyrosine binding domain protein. It was suggested that the interaction specificity with the β_{1A} cytosolic tail was due to the interaction of Val-787 on the integrin and an hydrophobic pocket created by Leu-82 and Tyr-144 of ICAP-1α (25). This is fairly consistent with the lack of interaction of ICAP-1α with the β_{1D} isoform that do not have a valine at this position. This latter residue is very close to the tyrosine 783 on the human β_{1A} chain. The tyrosine at this position on the β_1 chain or on the homologous position 747 on the β_3 chain seems to be crucial for integrin conformational switch and talin head binding. Moreover, talin C-terminal rod domain contains another binding site located within the residues 1984–2541 (44). Because the talin-active form is an anti-parallel homodimer (32, 45), the head and tail integrin binding sites in the adjacent talin molecules would be in close proximity with each other. Therefore, it is likely that talin and ICAP-1α binding sites on the integrin β_{1A} tail overlap.

The distribution of ICAP-1α in ruffles and its absence from focal adhesions suggest that the interaction between ICAP-1α and the β_1 integrin cytoplasmic domain is regulated. It is possible that ICAP-1α is sequestered inside the cell and that the interaction between a sequestering protein and ICAP-1α may be the regulated event. Alternatively, the interaction of ICAP-1α with the cytoplasmic domain of the β_1 integrin may be modulated by post-translational modifications (like phosphorylation). Indeed we have previously shown that a point mutation into the CaMKII putative phosphorylation site dramatically affected cell spreading (13). Moreover, pull-down assays showed that only a small fraction of ICAP-1α was able to interact with β_{1A} (26). How the interaction of ICAP-1α and the integrin is regulated is not yet understood and requires further investigations.

Recently, a 20-kDa protein named TAP-20 (with marked homology with β_3-endonexin) was shown to interact specifically with the β_5 cytosolic domain of the $\alpha_v\beta_5$ integrin (46). Overexpression of this protein leads to decreased adhesion and focal adhesion formation, and enhances migration. These properties are quite reminiscent of those of ICAP-1α, suggesting that a family of negative regulators may control specific integrin classes in a similar fashion.

Acknowledgments—We thank Dr. Frank Gertler for suggestions and critical reading of the manuscript and Dr. R. Juliano and Dr. D. Vestweber for kindly providing monoclonal antibodies.

REFERENCES

1. Giancotti, F. G., and Ruoslahti, E. (1999) *Science* **285,** 1028–1032
2. Hynes, R. O. (1992) *Cell* **69,** 11–25
3. Hato, T., Pampori, N., and Shattil, S. J. (1998) *J. Cell Biol.* **141,** 1685–1695
4. Hughes, P. E., Renshaw, M. W., Pfaff, M., Forsyth, J., Keivens, V. M., Schwartz, M. A., and Ginsberg, M. H. (1997) *Cell* **88,** 521–530
5. Sethi, T., Ginsberg, M. H., Downward, J., and Hughes, P. E. (1999) *Mol. Biol. Cell* **10,** 1799–1809
6. Zhang, Z., Vuori, K., Wang, H., Reed, J. C., and Ruoslahti, E. (1996) *Cell* **85,** 61–69
7. Keely, P. J., Rusyn, E. V., Cox, A. D., and Parise, L. V. (1999) *J. Cell Biol.* **145,** 1077–1088
8. Sjaastad, M. D., and Nelson, W. J. (1997) *Bioessays* **19,** 47–55
9. Bilato, C., Curto, K. A., Monticone, R. E., Pauly, R. R., White, A. J., and Crow, M. T. (1997) *J. Clin. Invest.* **100,** 693–704
10. Hendey, B., Klee, C. B., and Maxfield, F. R. (1992) *Science* **258,** 296–299
11. Lawson, M. A., and Maxfield, F. R. (1995) *Nature* **377,** 75–79
12. Pauly, R. R., Bilato, C., Sollott, S. J., Monticone, R., Kelly, P. T., Lakatta, E. G., and Crow, M. T. (1995) *Circulation* **91,** 1107–1115
13. Bouvard, D., and Block, M. R. (1998) *Biochem. Biophys. Res. Commun.* **252,** 46–50
14. Bouvard, D., Molla, A., and Block, M. R. (1998) *J. Cell Sci.* **111,** 657–665
15. Blystone, S. D., Slater, S. E., Williams, M. P., Crow, M. T., and Brown, E. J. (1999) *J. Cell Biol.* **145,** 889–897
16. Hendey, B., Lawson, M., Marcantonio, E. E., and Maxfield, F. R. (1996) *Blood* **87,** 2038–2048
17. Pomies, P., Frachet, P., and Block, M. R. (1995) *Biochemistry* **34,** 5104–5112
18. Takahashi, K. (2001) *BMC Cell Biol.* **2,** 23
19. Wennerberg, K., Fassler, R., Warmegard, B., and Johansson, S. (1998) *J. Cell Sci.* **111,** 1117–1126
20. Valmu, L., Hilden, T. J., van Willigen, G., and Gahmberg, C. G. (1999) *Biochem. J.* **339,** 119–125
21. Chen, Y. P., O'Toole, T. E., Shipley, T., Forsyth, J., LaFlamme, S. E., Yamada, K. M., Shattil, S. J., and Ginsberg, M. H. (1994) *J. Biol. Chem.* **269,** 18307–18310
22. Lukashev, M. E., Sheppard, D., and Pytela, R. (1994) *J. Biol. Chem.* **269,** 18311–18314
23. Chang, D. D., Wong, C., Smith, H., and Liu, J. (1997) *J. Cell Biol.* **138,** 1149–1157
24. Faisst, A. M., and Gruss, P. (1998) *Dev. Dyn.* **212,** 293–303
25. Chang, D. D., Hoang, B. Q., Liu, J., and Springer, T. A. (2002) *J. Biol. Chem.* **277,** 8140–8145
26. Zhang, X. A., and Hemler, M. E. (1999) *J. Biol. Chem.* **274,** 11–19
27. Albiges-Rizo, C., Frachet, P., and Block, M. R. (1995) *J. Cell Sci.* **108,** 3317–3329
28. Nuckolls, G. H., Romer, L. H., and Burridge, K. (1992) *J. Cell Sci.* **102,** 753–762
29. Fassler, R., Pfaff, M., Murphy, J., Noegel, A. A., Johansson, S., Timpl, R., and Albrecht, R. (1995) *J. Cell Biol.* **128,** 979–988
30. Wennerberg, K., Lohikangas, L., Gullberg, D., Pfaff, M., Johansson, S., and Fassler, R. (1996) *J. Cell Biol.* **132,** 227–238
31. Belkin, A. M., Retta, S. F., Pletjushkina, O. Y., Balzac, F., Silengo, L., Fassler, R., Koteliansky, V. E., Burridge, K., and Tarone, G. (1997) *J. Cell Biol.* **139,** 1583–1595
32. Vignoud, L., Albiges-Rizo, C., Frachet, P., and Block, M. R. (1997) *J. Cell Sci.* **110,** 1421–1430
33. Engvall, E., and Ruoslhati, E. (1977) *Int. J. Cancer* **20,** 1–5
34. Cattelino, A., Longhi, R., and de Curtis, I. (1995) *J. Cell Sci.* **108,** 3067–3078
35. Fournier, H. N., Dupe-Manet, S., Bouvard, D., Lacombe, M. L., Marie, C., Block, M. R., and Albiges-Rizo, C. (2002) *J. Biol. Chem.* **277,** 20895–20902
36. Bazzoni, G., Shih, D. T., Buck, C. A., and Hemler, M. E. (1995) *J. Biol. Chem.* **270,** 25570–25577
37. Lenter, M., Uhlig, H., Hamann, A., Jeno, P., Imhof, B., and Vestweber, D. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 9051–9055
38. Degani, S., Balzac, F., Brancaccio, M., Guazzone, S., Retta, S. F., Silengo, L., Eva, A., and Tarone, G. (2002) *J. Cell Biol.* **156,** 377–387
39. Belkin, A. M., Zhidkova, N. I., Balzac, F., Altruda, F., Tomatis, D., Maier, A., Tarone, G., Koteliansky, V. E., and Burridge, K. (1996) *J. Cell Biol.* **132,** 211–226
40. Priddle, H., Hemmings, L., Monkley, S., Woods, A., Patel, B., Sutton, D., Dunn, G. A., Zicha, D., and Critchley, D. R. (1998) *J. Cell Biol.* **142,** 1121–1133
41. Patil, S., Jedsadayanmata, A., Wencel-Drake, J. D., Wang, W., Knezevic, I., and Lam, S. C. (1999) *J. Biol. Chem.* **274,** 28575–28583
42. Calderwood, D. A., Zent, R., Grant, R., Rees, D. J., Hynes, R. O., and Ginsberg, M. H. (1999) *J. Biol. Chem.* **274,** 28071–28074
43. Calderwood, D. A., Yan, B., de Pereda, J. M., Alvarez, B. G., Fujioka, Y., Liddington, R. C., and Ginsberg, M. H. (2002) *J. Biol. Chem.* **277,** 21749–21758
44. Xing, B., Jedsadayanmata, A., and Lam, S. C. (2001) *J. Biol. Chem.* **276,** 44373–44378
45. Goldmann, W. H., Bremer, A., Haner, M., Aebi, U., and Isenberg, G. (1994) *J. Struct. Biol.* **112,** 3–10
46. Tang, S., Gao, Y., and Ware, J. A. (1999) *J. Cell Biol.* **147,** 1073–1084