Interaction of the Integrin \( \beta_1 \) Cytoplasmic Domain with ICAP-1 Protein*

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In a yeast two-hybrid screen, a protein named ICAP-1 (\( \beta_1 \) integrin cytoplasmic domain associated protein) associated with the integrin \( \beta_1 \) cytoplasmic tail but not with tails from three other integrin \( \beta \) subunits (\( \beta_2 \), \( \beta_3 \), and \( \beta_5 \)) or from seven different \( \alpha \) subunits. Likewise in human cells, ICAP-1 associated specifically with the \( \beta_1 \) but not \( \beta_2 \), \( \beta_3 \), or \( \beta_5 \) tails. The carboxy-terminal 14 amino acids of \( \beta_1 \) were critical for ICAP-1 interaction. ICAP-1 is a ubiquitously expressed protein of 27 and 31 kDa, with the smaller form being preferentially solubilized by Triton X-100. Phosphorylation of both 27- and 31-kDa forms was constitutive but was increased by 1.5–2-fold upon cell spreading on fibronectin, compared with poly-L-lysine. Also, ICAP-1 contributes to \( \beta_1 \) integrin-dependent migration because (i) ICAP-1 transfection markedly increased chemotactic migration of COS7 cells through fibronectin-coated but not vitronectin-coated porous filters, and (ii) support of \( \beta_1 \)-dependent cell migration (in Chinese hamster ovary cells transfected with various wild type and mutant \( \beta_1 \) forms) correlated with ICAP-1 association. In summary, ICAP-1 (i) associates specifically with \( \beta_1 \) integrins, (ii) is phosphorylated upon \( \beta_1 \) integrin-mediated adhesion, and (iii) may regulate \( \beta_1 \)-dependent cell migration.

Integrin-dependent cell adhesion helps to control cell proliferation and apoptosis, as well as cell spreading, migration, morphogenesis, and differentiation (1–5). Upon cell adhesion, integrin engagement leads to downstream activation of focal adhesion kinase, mitogen-activated protein kinase, and many other key signaling molecules (6). At the same time, integrin-dependent reorganization of cytoskeletal proteins and signaling complexes facilitates growth factor signaling (7, 8). A distinctive property of integrins is that they not only deliver “outside-in” signals upon engagement with ligand but also their function is regulated by “inside-out” signals (9–12). In this regard, integrin function can be strongly modulated upon overexpression of various oncogenes (13–15) or upon engagement of various cell-surface receptors with ligands or antibodies (10, 16, 17).

The cytoplasmic tails of integrin \( \alpha \) and \( \beta \) subunits play critical roles in two-way signaling through integrins (18–20). For the large subgroup of integrins containing the \( \beta_1 \) subunit, the \( \beta_1 \) tail is particularly important. For example, each of the four naturally occurring alternatively spliced forms of the \( \beta_1 \) tail has distinctive functions (21–24). In experimental systems, perturbing the \( \beta_1 \) tail can markedly alter \( \beta_1 \) integrin-mediated function. For example, exchange of the \( \beta_1 \) and \( \beta_5 \) tails altered integrin localization into focal adhesions and support of cell migration and proliferation (25). Also, overexpression of single chain \( \beta \) tail chimeras severely impaired cell adhesion and spreading mediated by endogenous \( \beta_1 \) integrins (26–29). In addition, mutations within the \( \beta_1 \) tail can alter integrin localization (30–32), conformation, and/or ligand binding (33–35) and \( \beta_1 \) integrin-mediated endocytosis (36). Amino acids particularly important for \( \beta_1 \) localization into focal adhesions have been mapped to three \( \beta_1 \) tail subregions called cyto-1, cyto-2, and cyto-3 (32).

In vitro biochemical studies have suggested that the \( \beta_1 \) tail may directly interact with focal adhesion kinase (37) and with cytoskeletal proteins \( \alpha \)-actinin (38), talin (39, 40), paxillin (37, 41), and filamin (40). Additionally, yeast two-hybrid screening has implicated integrin-linked kinase (42), receptor for activated protein kinase C (RACK1) (43), and integrin cytoplasmic domain associated protein (ICAP-1) (44) as proteins that may interact directly with the \( \beta_1 \) tail. Many of these proposed direct interactions still need to be further explored in terms of biochemical and functional relevance.

We also have undertaken a yeast two-hybrid screen to identify \( \beta_1 \) tail-associated proteins. In the yeast, we initially identified two candidate \( \beta_1 \) tail-interacting proteins. These were (i) a fragment of RACK1 and (ii) a protein called ICAP-1 (integrin cytoplasmic tail associated protein). Additional yeast two-hybrid studies suggested that the RACK1 interaction was non-specific. However, the ICAP-1 protein did show specific interaction with the \( \beta_1 \) tail, both in yeast and in human cell lines. Furthermore, the site of ICAP-1 association was mapped to the 14 carboxy-terminal amino acids of \( \beta_1 \) (which includes an NPXY motif); ICAP-1 phosphorylation was found to be regulated upon cell spreading on fibronectin, and ICAP-1 appeared to play a role in \( \beta_1 \)-dependent cell migration.

**EXPERIMENTAL PROCEDURES**

Cell Lines and Antibodies—Human embryonic kidney 293 (HEK293), monkey kidney (COS7), and Chinese hamster ovary (CHO) cell lines were obtained from the American Type Culture Collection (Bethesda, MD). Anti-hemagglutinin (HA) monoclonal antibody (mAb) 12CA5 was from Dr. J. DeCaprio (Dana-Farber Cancer Institute, Boston), and rabbit anti-ICAP-1 polyclonal antibodies were either produced using purified full-length ICAP-1 polypeptide as immunogen or provided by

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ICAP-1 Protein Association Integrin β1 Tail

Table I

| Amino acid sequences of integrin cytoplasmic and integrin β1/β2 cytoplasmic tail mutants studied in the yeast two-hybrid system |
|---|
| **Integrin tails** | **Sequences** |
| β1, β2 | IWKLLMI1HDDRFKFEKEMKA0KD/GENPIYKSAYTTFVNPKYE0G |
| | KALIIHLS0LDBEVRFEKKEKLQSW0NNDNLFLF0SI0ATTMNFKPAES |
| β1, β2 | KLLIIH1DDRFKFEKEMKARAKFW1TANPNL1KEA1STTF11NITY1RT |
| | IWKLLVT1HDDRFKFEKQSERSARYEMASNLPNL1PYR1ST110VDTFTNF11F1NKS11SY1GTVD |
| a1 | LWKGGFLRK1KK11KMTK1NPO01D1ETTE1SS |
| | LWKCGFFKR1111RT1111AL1111KEK11Q111;1111SE1111TER1111LTDY1111 |
| a5 | V0WKGFFKR111101Q1111Q111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111111
For GST fusion protein assays, HEK293 and CHO cell lysates (prepared as above) were incubated with glutathione-conjugated Sepharose beads (Amersham Pharmacia Biotech) for 1–7 days at 270 °C.

For GST fusion protein assays, HEK293 and CHO cell lysates (pre-prepared as above) were incubated with glutathione-conjugated Sepharose beads. Beads were then washed three times with lysis buffer, and bound proteins were eluted in Laemmli sample buffer and subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. Separated proteins were electrotransferred to nitrocellulose membranes (Schleicher & Schuell) at 4 °C overnight. Membranes were blocked with 5% fat-free dried milk in PBS/Tween 20 buffer at 25 °C for 1 h and then sequentially blotted with specific mAb and horseradish peroxidase-conjugated goat anti-mouse IgG antibody, followed by four washes (15 min each) with PBS/Tween 20 buffer after each blot. Proteins were visualized using Renaissance chemiluminescent assay (NEN Life Science Products).

Cell Migration Assay—Migration assays were performed essentially as described (25), using 96-well chambers and framed polycarbonate filters with 8-μm pores (Neuroprobe, Cabin John, MD). Filters were spotted with fibronectin, vitronectin, or poly-L-lysine diluted in 0.1 M NaHCO₃, allowed to dry, rinsed with PBS, and assembled with matrix-side down in the chamber. Lower wells of the chamber contained 33 μl of MEM a medium (for CHO cells) or DMEM (for COS7 cells) with 10% FCS, unless indicated otherwise. Cells harvested in PBS with 2 mM EDTA were labeled using BCECF-AM (2',9'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; Molecular Probes, Eugene, OR) for 30 min, pelleted, and resuspended at 3 × 10⁵ cells/ml in 1% FCS (for CHO cells) or 0.1% FCS (for COS7 cells). After no preincubation (COS7 cells) or with anti-hamster α₅β₁ mAb PB1 for 30 min on ice (CHO cells), cells (suspended in 100 μl) were added to upper wells of the chamber and allowed to migrate at 37 °C for 4 h. After migration, cells attached to the upper side of the filter were mechanically removed by scraping, and cells on the lower side were quantitated using a Cytofluor 2300 fluorescence measurement system (Millipore Corp., Bedford, MA). Percent cell migration equals: (cell fluorescence on filter with matrix coating − control cell fluorescence on filter without matrix)/(total fluorescence of input cells) × 100.

RESULTS

Yeast 2-Hybrid Selection and Cloning of β₁ Integrin Tail-binding Proteins—A HeLa cell library was expressed in 2 × 10⁶ yeast transformants, selection for interaction with the integrin β₁ cytoplasmic tail protein was carried out, and 25 positive clones were obtained. Among these clones, 9 coded for protein fragments that included the carboxyl-terminal half of the receptor of activated protein kinase C (RACK1) protein. The carboxyl-terminal half of RACK1 interacted strongly with β₁, weakly with β₅, and not at all with β₂ or β₃ integrin tail bait proteins. However, yeast 2-hybrid analyses also revealed interactions between the RACK1 carboxyl-terminal fragment and integrin α₅ and α₄ cytoplasmic tail bait proteins (Table II). Because the α₅ and α₄ tail sequences show no obvious similarity to the β₁ tail (Table I), the RACK1 interactions appeared to be nonspecific and were not pursued further.

![Fig. 1. Schematic representation of related “ICAP-1” polypeptides interacting with integrin β₁ cytoplasmic tail in yeast.](image)

![Fig. 2. Sequences of ICAP-1 cDNA and protein.](image)
Another 7 of the initial 25 positive clones coded for a related group of polypeptides, with identical carboxyl termini but variable amino termini (Fig. 1). These results suggest that regions essential for interaction with the β1 tail reside within the 162 residues present in the shortest clones (clones 4 and 5). Two of the polypeptide sequences contained divergent amino termini (clones 6 and 7), which did not appear in full-length clones (as obtained below), and thus may be cloning artifacts.

Open reading frames coding for the longest polypeptides did not include a methionine start site. Thus, to obtain a full-length sequence for the ICAP-1 protein, we used a cDNA probe corresponding to clone 4 to screen a bacteriophage lambda gt11-cDNA library. The resulting sequence contained an ATG start codon, just upstream of the sequence represented in clone 1. This methionine is present in a near consensus translation initiation sequence (57) and is located downstream of stop codons in all three frames, suggestive of an authentic start codon (Fig. 2).

The full-length ICAP-1 consists of 200 amino acids and is rich in serine (16%), with the amino-terminal 50 amino acids containing 19 serine residues. There are three possible protein kinase C phosphorylation sites at Ser-20 and Ser-46 and Ser-197 (58), and one cAMP or cGMP-dependent protein kinase phosphorylation site at Ser-10 (59). No signaling motifs or domains, such as SH2 or SH3, were found in ICAP-1. Subsequent to our isolation of ICAP-1, an identical protein was described and named “ICAP-1” (44). Also, an unpublished sequence coding for the mouse homologue of ICAP-1 has appeared in GenBank™ (accession number AJ001373).

**Interaction of ICAP-1 with the Integrin β1, Cytoplasmic Tail Is Highly Specific**—In the context of the yeast two-hybrid system, ICAP-1 (as a pJG4.5-ICAP-1 prey construct) interacted strongly with the β1 tail but failed to interact with the integrin β2, β3, or β5 tails (Table II). Also ICAP-1 did not associate with 7 different integrin α chain tails (Table II). All of the pEG202-encoded bait proteins containing integrin α or β chain cytoplasmic domains were able to bind to LexA but by themselves were transcriptionally inert, thus they meet the criteria for bait constructs suitable for study in the two-hybrid system. In other yeast two-hybrid experiments, ICAP-1 failed to interact with additional bait proteins including phosphatidylinositol 3-kinase 85-kDa subunit, Max, v-Myc, p300 CH3 domain, CD2 cytoplasmic domain, and the LAR phosphatase cytoplasmic domain.

To determine the subregion of the β1 cytoplasmic domain that is critical for ICAP-1 association, we utilized bait plasmid pEG202 to synthesize chimeric β1/β5 cytoplasmic tail mutants (listed in Table I, bottom). In yeast, both the wild type β1 tail (cyto.11) and the cyto.51 chimera showed strong interaction with ICAP-1, whereas cyto.15 and cyto.55 did not.

**Tissue Expression and Biochemical Features of ICAP-1**—Northern blotting showed that ICAP-1 mRNA is present in nearly all human tissues (Fig. 3). It was highly expressed in heart, colon (mucosal lining), skeletal muscle, and small intestine, barely detectable in liver, and present at intermediate levels in all other tissues. The major ICAP-1 transcript was 1.2 kilobase pairs, with variable amounts of another form at ~1.8 kilobase pairs.

Anti-ICAP-1 antiserum immunoprecipitated a protein of

**TABLE II**

| Baits          | ICAP-1 Interaction | β-Gal activity | RACK1 Interaction
|---------------|-------------------|----------------|------------------|
| β1_cyto (Cyto.11) | +                 | 1.67 ± 32      | +                |
| Cyto.15       | -                 | 6 ± 3          | +                |
| Cyto.51       | -                 | 197 ± 13       | -                |
| β1_cyto (Cyto.55) | -                 | 3 ± 2          | +/−              |
| β2_cyto      | -                 | 4 ± 3          | -                |
| β3_cyto      | -                 | 4 ± 1          | -                |
| α1_cyto      | -                 | 3 ± 3          | -                |
| α2_cyto      | -                 | 4 ± 4          | -                |
| α3_cyto      | -                 | 9 ± 6          | +                |
| α5_cyto      | -                 | 3 ± 3          | -                |
| α6_cyto      | -                 | 1 ± 1          | -                |
| α7_cyto      | -                 | 7 ± 5          | +                |
| α8_cyto      | -                 | 4 ± 3          | -                |

* The clone B1–7 encoding RACK1 141–317 residues was used as a prey.
* Positive interaction denotes growth of blue colonies only on X-gal indicator plates lacking leucine.
* β-Galactosidase (β-Gal) activity is the mean of four independent measurements.

**Fig. 3. Tissue expression of ICAP-1 mRNA.** Filters containing mRNA from multiple human tissues (CLONTECH) were used for Northern blotting according to manufacturer’s instructions. ICAP-1 cDNA probe was prepared by EcoRI/XhoI digestion from pJG4.5 vector and labeling with [α-32P]dCTP using RadPrime DNA kit (Life Technologies, Inc.). After stripping of the ICAP-1 probe, filters were rehybridized with 32P-labeled human actin cDNA. kb, kilobase pair; PBL, peripheral blood lymphocyte.
were further solubilized in Laemmli sample buffer (lanes 10) at 4 °C for 30 min (lanes 7–10). Alternatively, HA-ICAP-1-HEK293 cells were lysed (with Triton or RIPA) in lysis buffer (Fig. 4, lanes 1–4, 11). As indicated by blotting with anti-ICAP-1 antiserum, the majority of ICAP-1 in the cell lysate bound to the GST-ICAP-1 fusion protein, selective binding of wild type β1 (β1cyto.15, was observed (Fig. 5B), as detected by Western blotting with anti-human β1 mAb A-1A5. No β1 was found to associate with immobilized GST control protein (not shown). Wild type human β1 and various tail mutants were present in CHO cells at comparable levels as seen by cell-surface flow cytometry (Fig. 6) and also as indicated by blotting with anti-human β1 mAb A-1A5 (not shown).

Reciprocal Demonstration of β1 Tail-ICAP-1 Interaction in Mammalian Cells—To investigate the interaction of the β1 tail with mammalian cell ICAP-1, immobilized β1 tail GST fusion proteins were incubated with soluble HA-ICAP-1 from lysates of transfected HEK293 cells. As indicated by Western blotting with anti-HA mAb (Fig. 5A), HA-tagged ICAP-1 bound selectively to GST-β1 (lane 2) but not to GST itself, GST-β2, GST-β3, or GST-β4 fusion proteins (lanes 1–5). We estimate that 1–2% of the total ICAP-1 in the cell lysate bound to the GST-β1 beads. In control experiments, ICAP-1 was readily visualized from a whole cell lysate of ICAP-1-transfected HEK293 cells (lane 11) but not from mock-transfected HEK293 cells (Fig. 5A, lanes 6–10 and 12).

In a reciprocal experiment we next analyzed binding of solubilized β1 integrin to immobilized GST-ICAP-1. First, CHO cells were transfected to stably express wild type or mutant human β1 subunits. In each case, the β1 extracellular and transmembrane domains were present, whereas the cytoplasmic tail was either unaltered or fully or partly exchanged with regions of the β1 tail (See Table I, bottom, for sequences). Upon incubation with GST-ICAP-1 fusion protein, selective binding of wild type β1 (β1cyto.11) and β1cyto.55, but not β1cyto.55 or β1cyto.15, was observed (Fig. 5B), as detected by Western blotting with anti-human β1 mAb A-1A5. No β1 was found to associate with immobilized GST control protein (not shown). Wild type human β1 and various tail mutants were present in CHO cells at comparable levels as seen by cell-surface flow cytometry (Fig. 6) and also as indicated by blotting with anti-human β1 mAb A-1A5 (not shown).

Regulation of ICAP-1 Phosphorylation—Because of the high serine composition and putative protein kinase C phosphorylation sites, we tested whether ICAP-1 might be phosphorylated. First, ICAP-1-transfected HEK293 cells were incubated with [32P]orthophosphate for 2 h while in suspension, and for another 1 h while spreading, prior to lysis using RIPA buffer. Then, anti-ICAP-1 antibody was used to immunoprecipitate phosphorylated proteins of 27 and 31 kDa from 32P-labeled

Fig. 4. Characterization of ICAP-1 protein. Unlabeled HA-ICAP-1-HEK293 and mock-HEK293 cells were either allowed to adhere to fibronectin for 2 h at 37 °C (Adhe.) or held in suspension for 2 h (Susp.) prior to lysis with 1% Triton X-100 or RIPA buffer (lanes 1–8). Alternatively, HA-ICAP-1-HEK293 cells were lysed (with Triton or RIPA) in suspension at 4 °C for 30 min (lanes 9 and 10), and insoluble materials were further solubilized in Laemmli sample buffer (lanes 10 and 12). After separation by SDS-polyacrylamide gel electrophoresis, Western blotting was carried out using anti-HA mAb 12CA5. After separation by SDS-polyacrylamide gel electrophoresis, Western blotting was carried out using anti-HA mAb 12CA5.

Fig. 5. Reciprocal interactions between ICAP-1 and β1 integrin from mammalian cell lysates. A, lysates from ICAP-1-transfected HEK293 cells (lanes 1–5) or mock-transfected cells (lanes 6–10) were incubated with GST fusion proteins immobilized on beads, and then bound proteins were eluted and detected by Western blotting with anti-ICAP-1 antiserum. Also shown are whole cell lysates from ICAP-1 transfected (lane 11) and Mock (lane 12)-transfected HEK293 cells. B, lysates from the indicated CHO cell transfectants were incubated with immobilized GST-ICAP-1 fusion protein, and bound proteins were detected by Western blotting with anti-β1 mAb A-1A5.
HEK293 cells (Fig. 7A, lanes 3 and 4). These proteins were not precipitated using preimmune serum or from mock-transfected cells (lanes 1, 2, and 5–8). Notably, phosphorylation was enhanced by ~2-fold for the 27-kDa protein, and 1.5-fold for the 31-kDa protein when ICAP-1-transfected HEK293 cells were spread on FN (lane 4) compared with poly-L-lysine (lane 2). In contrast, the level of phosphorylation of background proteins was unchanged as determined by comparison of protein band densities (FN/PLL ratios = 1.0). A long exposure of Fig. 7A confirmed that none of the many phosphorylated non-ICAP-1 proteins (including Control Band 1 and Control Band 2) were altered. In a separate experiment (not shown), phosphorylation of 27- and 31-kDa ICAP-1 proteins was again increased (by 1.8- and 2.0-fold), respectively, upon adhesion to fibronectin compared with PLL. Again, phosphorylation of all other (non-ICAP-1) proteins was unchanged.

A report elsewhere (44) has suggested that the more slowly migrating form of ICAP-1 may represent a phosphorylated form of the protein that may appear at elevated levels upon cell adhesion and spreading on fibronectin for 15 or 30 min (44). Thus, to supplement our results obtained upon adhesion to fibronectin for 1 h (Fig. 7A), we analyzed additional time points (Fig. 7B). At no time point from 15 to 120 min did we observe that the slowly migrating form of ICAP-1 (~31 kDa) was highly phosphorylated relative to the 27-kDa protein, even though the 31-kDa protein was well represented (e.g., see Fig. 4). Indeed, under identical extraction conditions, the 31/27-kDa ratio was 0.42 in terms of total protein but only 0.13 in terms of phosphorylated protein.

ICAP-1 May Contribute to Cell Migration—In further experiments, COS7 cells transiently transfected with ICAP-1 were found to undergo increased transwell migration, when the FCS chemoattractant was held constant at 10% and different FN levels were coated onto the underside of the filter (Fig. 8A, left panel). Also, ICAP-COS7 cells showed preferential migration compared with Mock-COS7 cells when FN coating was held constant at 10 μg/ml and different FCS chemoattractant levels were used (Fig. 8A, right panel). Although ICAP-1 caused an elevation of β1-dependent migration on fibronectin, it did not alter β1-independent migration on vitronectin, as seen in two separate experiments (Fig. 8B, right and left panels). Because COS cells express moderate to high amounts of α5 and β1, but little β3, we suspect that vitronectin-dependent migration is largely mediated by α5β3.

CHO transfectants stably expressing comparable surface levels of human wild type or chimeric β1 (see Fig. 6) were also tested for migration. The assay was performed in the presence of anti-hamster αβ1 mAb PB1 to block the contribution of endogenous hamster αβ1 (Fig. 8C). The CHO-β1.cyto11 and -β1.cyto51 transfectants showed substantially more migration than either the CHO-β1.cyto55 or -β1cyto1.5 transfectants. This differential migratory behavior precisely coincides with the differential abilities of these mutants to bind to ICAP-1 (e.g., as seen in Table I and Fig. 5B). In the absence of 10% FCS as a chemoattractant, none of the cells showed very much migration (not shown).
DISCUSSION

Specific Association of ICAP-1 with Integrin \(\beta_1\) Tail—Here we have identified and characterized ICAP-1, a 200 amino acid phosphoprotein specifically associating with the \(\beta_1\) integrin tail. Interaction seen in a yeast two-hybrid assay was confirmed in reciprocal experiments using ICAP-1- and \(\beta_1\) integrin-transfected human and hamster cell lysates. In both systems the association was highly specific. In both mammalian cell lysates and in yeast, replacement of the carboxyl-terminal 14 amino acids of \(\beta_1\) with the carboxyl-terminal 24 amino acids of \(\beta_5\) resulted in loss of ICAP-1 association. Conversely, the reciprocal exchange (\(\beta_5\) tail with terminal 14 residues from \(\beta_1\)) allowed strong association. Thus the carboxyl-terminal “SAVT-TVVNPKYEGK” sequence in \(\beta_1\) is required for ICAP-1 interaction. While this work was in progress, another group described ICAP-1 as a protein that associated selectively with the integrin \(\beta_1\) tail (44). Consistent with results shown here, residues critical for ICAP-1 association resided within the carboxyl-terminal 13 residues of the \(\beta_1\) tail (44).

Association of \(\beta_1\) Tail with RACK1?—In another report, the carboxyl-terminal portion of RACK1 was isolated by a yeast two-hybrid approach and suggested to interact specifically with integrin \(\beta_1\), \(\beta_2\), and \(\beta_5\) tails (43). We also obtained a carboxyl-terminal fragment of RACK1 upon yeast two-hybrid screening but did not study it further due to an apparent lack of interaction specificity. Although it is still possible that RACK1 could specifically participate in integrin functions, future studies will need to explain its ability to bind to multiple peptide sequences that are seemingly unrelated.

Distribution and Size of ICAP-1—Northern blotting showed
that the ICAP-1 protein is widely expressed in many human tissues, as previously shown for the β₁ integrin subunit. Also by Western blotting, ICAP-1 was ubiquitously expressed in most cultured cell lines. It is not yet clear whether the appearance of RNA of two different sizes (1.8 and 1.2 kilobase pairs) represents alternative splicing or different polyadenylation sites as previously suggested (44). Chang et al. (44) described an apparent alternatively spliced 16-kDa form of ICAP-1 (ICAP-1β)

In analyses of several cell lines, and cells transfected with ICAP-1 cDNA, we detected major (~27 kDa) and minor (~31 kDa) ICAP-1 proteins, with the latter only being seen using stringent detergent conditions. Both the ~27 and ~31-kDa proteins incorporated 32P label, with phosphorylation of the more rapidly migrating ~27-kDa form being particularly prominent. Elsewhere, it was suggested that the more slowly migrating form of ICAP-1 may be preferentially phosphorylated, because it disappeared upon incubation of lysate in the absence of phosphatase inhibitors (44). Our direct phosphorylation results contradict that conclusion. We cannot explain why phosphatase inhibitors may have facilitated the maintenance of the more slowly migrating form, except to suggest that this effect may be indirect and possibly involve other components in the cell lysate. At present, the biochemical basis for the larger size of the 31-kDa ICAP-1 protein and its relative resistance to detergent extraction (compared with the 27-kDa protein) are not clear.

Elsewhere it was also shown that appearance of the larger ICAP protein form was favored upon cell adhesion to fibronectin, whereas it was greatly diminished when cell matrix interaction was disrupted (44). We did not observe an adhesion-dependent change in levels of either the 27- or 31-kDa form of ICAP-1 (e.g., see Fig. 4). This disparity possibly could be explained by our use of a human embryonic kidney cell line (HEK293) instead of the osteosarcoma cell line (UTA-6) used in the other study.

**Functional Relevance of β₁ Tail Association with ICAP-1**—Association of the β₁ tail with ICAP-1 may be relevant for multiple reasons. First, phosphorylation of both 27- and 31-kDa forms of ICAP-1 was selectively promoted upon cell adhesion and spreading on fibronectin but not on poly-L-lysine. Thus, ICAP-1 phosphorylation appears to be regulated during the outside-in signaling that occurs upon integrin engagement with ligand. In future studies, it will be important to place ICAP-1 phosphorylation into the context of established integrin-dependent signaling events, such as the phosphorylation of focal adhesion kinase, paxillin, and other downstream targets (6). A previous report suggested that constitutively activated RhoA might down-regulate ICAP-1 phosphorylation (44). In contrast, we found that RhoAV14 transfection into NIH3T3 cells caused no elevation in ICAP-1 phosphorylation (not shown). This discrepancy is perhaps easily explained, considering (as discussed above) that Chang et al. (44) appear not to have been actually measuring ICAP-1 phosphorylation.

Second, ICAP-1 interactions with the integrin β₁ tail may support cell migration. In one set of experiments, expression of ICAP-1 in COS7 cells was associated with increased β₁-dependent cell migration on fibronectin but not β₁-independent migration on vitronectin. In another set of experiments, the carboxy-terminal amino acids within the β₁ tail that were needed for ICAP-1 association were also required for enhanced cell migration. The carboxyl terminus of β₁ could not substitute for β₁ to support migration. These results may help to explain previously noted differences between the integrin β₁ and β₂ tails in terms of supporting cell migration (25).

Other functions known to require the carboxy-terminal 14 amino acids of β₁ could potentially also involve ICAP-1. For example, the carboxyl-terminal “SAVTVVNPKYEYK” sequence in the β₁ tail includes amino acids (Thr-788, Thr-789, Asn-792, and Tyr-795 in human β₁) that help to regulate integrin affinity for ligand (33) and amino acids (Asn-792 and
Phosphorylation of ICAP-1 could play a role in these events. The functions of carboxyl-terminal 14 amino acids of the ICAP-1 protein show no similarity to any of these other proteins, and as far as we are aware the ICAP-1 interaction between the widely expressed ICAP-1 protein and cytoplasmic tail-associated proteins detected by yeast two-hybrid genetic screening. We also thank Dr. D. Chang (Massachusetts General Hospital, Boston) for providing the yeast strains and plasmids for the yeast two-hybrid genetic screening. We thank Dr. R. Brent (Massachusetts General Hospital, Boston) for sharing information about ICAP-1 before publication and for antibody to ICAP-1.

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