

ICAP-1, a Novel β_1 Integrin Cytoplasmic Domain-associated Protein, Binds to a Conserved and Functionally Important NPXY Sequence Motif of β_1 Integrin

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Abstract. The cytoplasmic domains of integrins are essential for cell adhesion. We report identification of a novel protein, ICAP-1 (integrin cytoplasmic domain-associated protein-1), which binds to the β_1 integrin cytoplasmic domain. The interaction between ICAP-1 and β_1 integrins is highly specific, as demonstrated by the lack of interaction between ICAP-1 and the cytoplasmic domains of other β integrins, and requires a conserved and functionally important NPXY sequence motif found in the COOH-terminal region of the β_1 integrin cytoplasmic domain. Mutational studies reveal that Asn and Tyr of the NPXY motif and a Val residue located NH₂-terminal to this motif are critical for the ICAP-1 binding. Two isoforms of ICAP-1, a 200-amino

acid protein (ICAP-1 α) and a shorter 150-amino acid protein (ICAP-1 β), derived from alternatively spliced mRNA, are expressed in most cells. ICAP-1 α is a phosphoprotein and the extent of its phosphorylation is regulated by the cell-matrix interaction. First, an enhancement of ICAP-1 α phosphorylation is observed when cells were plated on fibronectin-coated but not on non-specific poly-L-lysine-coated surface. Second, the expression of a constitutively activated RhoA protein that disrupts the cell-matrix interaction results in dephosphorylation of ICAP-1 α . The regulation of ICAP-1 α phosphorylation by the cell-matrix interaction suggests an important role of ICAP-1 during integrin-dependent cell adhesion.

INTEGRINS comprise a family of heterodimeric cell adhesion receptors responsible for attachment of cells to the extracellular matrix or to specific cell surface counterreceptors (Hynes, 1992). Each subunit consists of a large extracellular domain that participates in the ligand recognition, a transmembrane region, and a short cytoplasmic domain. In adherent cells, the ligand binding induces recruitment of integrins to the focal adhesion plaques or focal contacts, where actin cytoskeletons converge onto the site of cell-extracellular matrix contact (for review see Burridge and Chrzanowska-Wodnicka, 1996). Studies have shown that the integrin-dependent cell adhesion can be regulated either by direct affinity modulation of integrins (Bennett and Vilaire, 1979; Altieri and Edgington, 1988; Faull et al., 1993; Stewart et al., 1996) or by clustering of integrins, which requires cytoskeletal rearrangement (Hermanoski-Vosatka et al., 1988; Haverstick et al., 1992; van Kooyk et al., 1994; Stewart et al., 1996). Either through recruitment of regulatory proteins

such as adaptor protein Shc or focal adhesion kinase (FAK)¹ to the focal contacts or by inducing reorganization of actin cytoskeleton, integrins function as transmembrane receptors for extracellular signals and participate in the activation of cytoplasmic signaling cascade (for review see Schwartz et al., 1995). The dependence of cell proliferation, prevention of apoptosis, and cell differentiation on the cell-matrix interaction mediated by integrins illustrates the importance of this adhesion-dependent cell signaling.

Although the cytoplasmic domains of integrins lack any known enzymatic activity or sequence motif involved in protein-protein interaction, studies have shown that the short cytoplasmic tails of α or β subunits are important for the regulation of integrin affinity and cytoskeletal interaction (Sastry and Horwitz, 1993; Schwartz et al., 1995). The cytoplasmic domains of different β subunits are similar in size and sequence. Mutational analysis of the cytoplasmic domain of integrin β_1 has identified three regions that are important for the recruitment of integrins to the focal contacts (Marcantonio et al., 1990; Reszka et al., 1992). The

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1. *Abbreviations used in this paper:* aa, amino acid; FAK, focal adhesion molecule; FN, fibronectin; GST, glutathione-S-transferase; ICAP-1, integrin cytoplasmic domain-associated protein-1; PLK, poly-L-lysine.

first region, located in the membrane-proximal region, is rich in charged residues and predicted to form an α -helical structure. The second and third region consist of short sequences Asn-Pro-X-Tyr (NPXY). The NPXY motif was initially recognized as a sequence motif required for receptor-mediated endocytosis (Chen et al., 1990) and represents a unique structural motif capable of generating a reverse turn in solution (Bansal and Gierasch, 1991). The two tandem NPXY motifs of the integrins are situated in the membrane-distal region that is known to undergo alternative splicing (Languino and Ruoslahti, 1992; Zhidkova et al., 1995). Naturally occurring splicing variants of the β_1 integrin lacking the NPXY motifs do not localize to the focal contacts (Balzac et al., 1993). The first NPXY motif (membrane-proximal), in addition to playing a role in integrin-cytoskeleton interaction (Reszka et al., 1992; Ylanne et al., 1995), is also involved in affinity regulation of integrins (O'Toole et al., 1995) and integrin-dependent endocytic processes (Van Nhieu et al., 1996). Mutational studies have shown that the second NPXY motif (membrane-distal), like the first NPXY motif, is important for the focal contact localization of β_1 integrins (Reszka et al., 1992) and cell adhesion by the β_2 integrins (Hibbs et al., 1991; Peter and O'Toole, 1995).

How the integrin β subunit cytoplasmic domain participates in the regulation of cell-matrix interaction has not been resolved. The initial molecular models for the adhesion-dependent recruitment of integrins to the focal contacts were based on the observation that talin (Horwitz et al., 1986) and α -actinin (Otey et al., 1990) bind to the β_1 integrins. As both of these proteins can bind actin, either directly as in α -actinin or through interaction with vinculin as in talin, the proposed function of talin and α -actinin in linking integrins to the cytoskeletal structures remains an attractive model. Other proteins that have been shown to bind β_1 integrins include FAK (Schaller et al., 1995), paxillin (Schaller et al., 1995), and a Ser/Thr kinase (ILK-1) (Hannigan et al., 1996). Of these proteins, FAK and ILK-1, because of their ability to affect cell adhesion and cell spreading, represent potential regulators of the integrin-matrix interaction.

A second unresolved issue is whether the adhesive function and cytoskeletal interaction of different integrins are regulated by a common mechanism or by similar but distinct processes. Despite the remarkable similarities in the amino acid sequences of different integrin β subunit cytoplasmic domains, each β subunit displays distinct differences in its ability to localize to the focal contacts (Wayner et al., 1991; LaFlamme et al., 1994), to induce tyrosine phosphorylation of cytoplasmic proteins upon surface clustering (Freedman et al., 1993), and to participate in gene induction (Yurochko et al., 1992). In particular, a direct comparison of β_1 and β_5 cytoplasmic domains using a chimeric β_1 - β_5 construct where the cytoplasmic domain of β_1 was replaced with that of β_5 has demonstrated that the β_5 cytoplasmic domain, unlike the β_1 counterpart, does not efficiently direct integrins to the focal contact or promote cell proliferation (Pasqualini and Hemler, 1994). Recently identified β_3 -endonexin (Shattil et al., 1995) and cytohesin-1 (Kolanus et al., 1996), which display restricted binding to the β_3 and β_2 cytoplasmic domain, respectively, suggest the presence of proteins that can discriminate the

subtle differences in the amino acid sequence of different β subunits. These β subunit cytoplasmic domain-specific binding proteins may allow specific regulation of individual β subunits.

In the present study, we report a novel polypeptide named ICAP-1 α (integrin cytoplasmic domain-associated protein-1) that binds to the β_1 integrin cytoplasmic domain. The interaction, which can be demonstrated both *in vitro* and *in vivo*, is specific for the β_1 integrins and requires the Asn and Tyr residues of the membrane-distal NPXY motif. The ability of ICAP-1 α to interact only with the β_1 cytoplasmic domain is attributed to an additional requirement of Val residue NH₂-terminal to the NPXY. The functional role of ICAP-1 in cell adhesion is suggested by the observation that ICAP-1 α is a phosphoprotein and that the degree of phosphorylation is regulated by integrin-dependent, cell-matrix interaction.

Materials and Methods

Cell Lines and Antibodies

293, HeLa, Jurkat, K562, and Cos-7 cells were obtained from American Type Culture Collection (Rockville, MD). SaOS, Rat-1, NIH3T3, 2f-TGH (human fibroblast cells), and UTA-6 (Englert et al., 1995) were gifts from C. Sawyers (University of California, Los Angeles, CA [UCLA]), K. Shuai (UCLA), and D. Haber (Massachusetts General Hospital, Boston, MA).

Cells expressing constitutively activated RhoA were generated using UTA-6 (Englert et al., 1995), a derivative of U2OS cells (osteosarcoma cell line) expressing tetracycline-repressible transactivator (Gossen and Bujard, 1992). An NH₂-terminal FLAG epitope-tagged RhoA(Q63L) (Coso et al., 1995) was cloned into pTPH-1 (Gossen and Bujard, 1992). The expression construct was introduced into UTA-6 cells using a Ca₂PO₄-precipitation method (Ausubel et al., 1994) and hygromycin-resistant clones were isolated in the presence of 1 μ g/ml tetracycline.

An anti- β_1 integrin mAb producing hybridoma cell line, TS2/16, was generously provided by M. Hemler (Dana Farber Cancer Institute, Boston, MA). A mouse hybridoma cell line TS1/18 producing anti- β_1 integrin mAb were obtained from ATCC. Hybridoma cell lines were cultured in DME + 10% CPSR-3 (Sigma Chemical Co., St. Louis, MO). mAb from tissue culture supernatant was purified on protein A-Sepharose (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ).

Polyclonal rabbit antisera against the α subunit of LFA-1 ($\alpha_L\beta_2$) were prepared by immunizing rabbits with bacterially expressed glutathione-S-transferase (GST) fusion protein containing the entire 58-amino acid (aa) cytoplasmic domain (aa 1,088-1,145). For the generation of rabbit antisera against ICAP-1, the entire ICAP-1 α coding sequences were cloned in pET16b (Novagen Inc., Madison, WI) and expressed as a histidine-tagged protein in *Escherichia coli* DE21 (Studier and Moffatt, 1986). Histagged ICAP-1 α was purified on nickel charged column (Novagen Inc.) under denaturing conditions following the manufacturer's recommendations and used as the immunogen.

Yeast Genetic Screening

The yeast genetic screening for the isolation of proteins interacting with the cytoplasmic domain of β_1 integrin was carried out essentially as described previously (Gyuris et al., 1993). COOH-terminal 21 aa (GENPIYKSAVTTVVNPXYEGK) of the β_1 subunit was cloned in frame into LexA coding sequence to generate a "bait" plasmid pNlex- β_1 cyto. The resulting Lex- β_1 cyto fusion protein was able to bind LexA operator in yeast, but displayed no basal transcriptional activity. A yeast expression library was generated from oligo dT-primed cDNA from JY cell (human B cell line) mRNA. The cDNA was cloned unidirectionally into the EcoRI/XhoI sites of a yeast expression vector pJG4-5. This cDNA library that had the complexity of $>10^6$ was amplified once and used to transform a yeast strain EGY48 harboring pNlex- β_1 cyto and JK103 lacZ reporter plasmid. Approximately 2×10^6 independent yeast transformants were pooled and subjected to selection as described. Eight positive clones obtained all had identical cDNA insert. Plasmid DNA from one isolate

(Clone E16-1) was rescued using *E. coli* KC8 (Gyuris et al., 1993) and amplified for further analysis. All bait constructs containing various integrin cytoplasmic domains or β_1 integrin cytoplasmic domain mutants were tested for proper expression in a "suppression assay" in yeast using a reporter construct JK101 (Gyuris et al., 1993). β -galactosidase activity measurement was carried out as described previously (Ausubel, 1994).

Northern Blot Analysis and cDNA Cloning

The cDNA insert from Clone E16-1 was used as the probe to screen a multiple tissue mRNA blot (Clontech, Palo Alto, CA). A full-length cDNA was isolated from a HeLa cell cDNA library (a gift from K. Shuai, UCLA) using Clone E16-1 as the probe. An expressed sequence tag (EST) clone corresponding to ICAP-1 β was obtained from the IMAGE Consortium (these sequence data are available EMBL/GenBank/DBJ under accession number T69975). The presence of cDNA corresponding to ICAP-1 β was independently confirmed by PCR (40 cycles: 94°C for 45 s; 55°C for 45 s; 72°C for 30 s) using primers A3 (5'-CCCAGCAAGATG-GAAAGTTGCC-3') and B2 (5'-GATCAGCATTTCACACAATCCA-3') flanking the deleted sequences, which generated a 459- (ICAP-1 α) or a shorter 308-bp fragment (ICAP-1 β).

In Vitro Interaction Assay

For in vitro GST "pull-down" experiments, the COOH-terminal cytoplasmic domains of β_1 subunit (see above), β_2 subunit (DNPLFKSATTTVM-NPKFAES), and α_L (KVGFFKRNLEKMEAGRGVNGIPAEDSE-QLASGQEAGDPGCLKPLHEKDSSEGGGD) were individually expressed in bacteria as GST fusion proteins. The EcoRI/XhoI insert of Clone E16-1 encoding ICAP-1 α (aa 54–200) was cloned into the EcoRI/XhoI site of an in vitro transcription vector pCITE-3a (Novagen, Inc.). The resulting pCITE-ICAP1 α plasmid was used as the template in cotranscription/translation reaction using T7 RNA polymerase and rabbit reticulocyte lysate (Promega Corp., Madison, WI) to generate [³⁵S]methionine-labeled polypeptides. An equal amount of labeled polypeptides was added to ~2 μ g of GST fusion proteins bound on glutathione-Sepharose beads (Pharmacia LKB Biotechnology Inc.) and incubated overnight at 4°C in NET (25 mM Tris-HCl, pH 7.6, 100 mM NaCl, 3 mM EDTA) containing 1 mM DTT, 1% BSA, and 0.1% Triton X-100. Beads were washed twice in the binding buffer and twice in 0.05% Triton X-100 in NET. Bound proteins were eluted by boiling in SDS sample buffer and analyzed by SDS gel electrophoresis.

Eukaryotic Expression Plasmids

The coding sequences of human α_L integrin (CD11a) and human β_2 (CD18) from the expression vectors previously described (Hibbs et al., 1991) were cloned into the XbaI site of the eukaryotic expression vector pcDNA3 (Invitrogen Inc., Madison, WI). To construct a hybrid $\beta_{2,1}$ subunit, two PCR-generated fragments corresponding to the amino acids 634–749 of β_2 and amino acids 778–798 of β_1 were ligated together using a XcmI site that was introduced during the PCR. This fragment was cloned into the BstBI and NotI site of pcDNA3/ β_2 to generate pcDNA/ $\beta_{2,1}$. The sequence of the cytoplasmic domain in this hybrid $\beta_{2,1}$ subunit consists of NH₃-KALIHLSLDREYRRFEKEKLSQWNGENPIYKSAVTTVV-NPXYEGK-COOH.

The full-length ICAP-1 α cDNA was cloned into the EcoRV site of pcDNA3 to generate pcDNA/ICAP-1 α . To generate the full-length ICAP-1 β coding sequence, the 5' half of the above PCR fragment and the 3' half of a second PCR fragment amplified from T69975 clone (see above) were ligated in frame using a unique HpaII restriction site. The ligation product was cloned into the EcoRV site of pcDNA3 to produce pcDNA/ICAP-1 β . The GST-tagged ICAP-1 constructs were generated using eukaryotic expression vector pEBG. The coding sequence of the partial ICAP-1 α (aa 54–200) was derived from Clone E16-1.

Eukaryotic Expression and In Vivo Interaction Assay

5–10 μ g of plasmid DNA was transfected into 293T cells by using a Ca₂PO₄ precipitation method (Ausubel et al., 1994). 48 h after transfection, cells were lysed in TBSM (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 2 mM MgCl₂) containing 0.5% NP-40, leupeptin, aprotinin, and PMSF. Detergent insoluble materials were removed by centrifugation at 12,000 g for 15 min. 500 μ g of cleared lysates were mixed with an equal volume of TBSM to reduce the final detergent concentration to 0.25% NP-40 and incubated with glutathione-Sepharose beads for 3 h at 4°C.

Beads were washed with TBSM + 0.25% NP-40 once, TBSM + 0.1% NP-40 twice, and TBSM alone twice. Coprecipitation of β_1 integrins with bound GST-ICAP1 was determined on a Western analysis using mAb TS2/16 (anti- β_1 integrin).

In Vitro Phosphatase Assay

50 μ g NP-40 detergent lysate in 40 μ l of 25 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 10 mM MgCl₂, was incubated at 30°C for 30 min, either in the presence or absence of phosphatase inhibitors (1 mM NaVO₃ and 0.5 μ M calyculin A). The reaction was terminated by adding SDS sample buffer and boiling for 5 min. 15 μ g of lysates were analyzed on Western blot using anti-ICAP1 antibody.

Adhesion Assay

Cell adhesion was carried out using six-well tissue culture plates. Each 35-mm well was coated with 1 ml of fibronectin (20 μ g/ml) or poly-L-lysine (PLK) (50 μ g/ml) in TBS (25 mM Tris-HCl, pH 8.0, 150 mM NaCl) overnight at 37°C. The coated wells were subsequently blocked with 1% BSA (Fraction V; GIBCO BRL, Gaithersburg, MD) in TBS before the addition of cells. Adhesion was carried out using 5 \times 10⁵ cells per well at 37°C for 15–30 min and bound cells were lysed in 0.2% SDS in TE (25 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Results

Cloning of ICAP-1

A genetic screening based on the protein-protein interaction in yeast (Gyuris et al., 1993) was used to identify polypeptides that interact with the β_1 integrin cytoplasmic domain. Of the 47 aa that comprise the entire β_1 integrin cytoplasmic domain, the COOH-terminal 21 aa were cloned in frame into the DNA-binding domain of bacterial protein LexA. Mutational studies and analysis of naturally occurring splicing variants lacking this 21-aa region have shown that this region is involved in the localization of β_1 integrins to the focal contacts and proper adhesive function of integrins (Balzac et al., 1993; Meredith et al., 1995).

Using the COOH-terminal 21 aa of integrin β_1 as the bait, a ~1.1-kb partial cDNA was isolated in a yeast two-hybrid screening of a human B cell cDNA library. This partial cDNA insert was used to obtain a full-length cDNA encoding a polypeptide of 200 aa. In a yeast two-hybrid assay, both the original partial cDNA (aa 154–200) and the full-length cDNA exhibited comparable interaction with the β_1 integrin cytoplasmic domain but not with unrelated baits (Table I). In addition, the partial or full-length cDNA clones interacted with baits containing either the β_1 integrin COOH-terminal 21 aa or a longer 47 aa that comprise the complete cytoplasmic domain. The interaction was specific towards only the β_1 integrin as neither the partial nor the full-length cDNA clone interacted with the β_2 , β_3 , or β_5 integrin cytoplasmic domain. The isolated clone hereon will be referred as ICAP-1 α .

The deduced amino acid sequence of ICAP-1 α was unrevealing except for the preponderance (especially in the NH₂-terminal region) of Ser and Thr, several of which represented potential phosphorylation sites (Fig. 1 A). In particular, Ser²⁰, Ser⁴⁶, and Ser¹⁹⁷ represent potential phosphorylation sites by protein kinase C (Woodgett et al., 1986), and Ser¹⁰ is present in sequence context favorable for phosphorylation by cyclic nucleotide dependent protein kinases (Glass et al., 1986). The initiation codon ATG in the sequence was preceded by an in frame termination

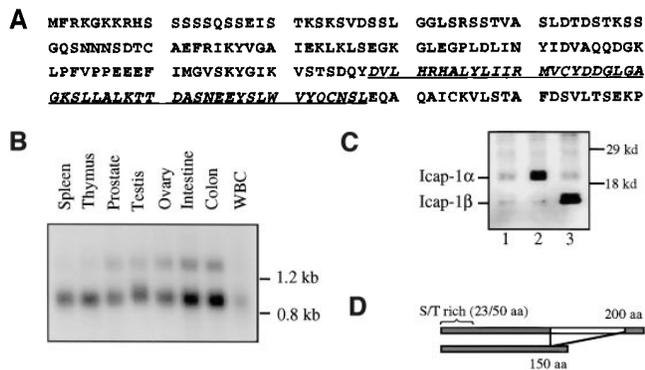


Figure 1. ICAP-1 α is a novel serine/threonine-rich 20-kD protein. (A) Sequence of ICAP-1. The translated amino acid sequence of ICAP-1 α is shown. 50 amino acids absent in ICAP-1 β are indicated in underlined italics. The nucleotide sequence data are available from GenBank/EMBL/DBJ under accession number AF012023. (B) Northern blot analysis of ICAP-1 α . Northern blots of transcripts from various human tissues (Clontech) were hybridized with ICAP-1 α cDNA probe. The 0.9-kb mRNA is expressed in all eight tissue samples. The minor 1.3-kb mRNA, derived from the use of the downstream polyadenylation site, is also expressed in varying amount in all eight samples. (C) Western blot analysis of ICAP-1. ICAP-1 α and ICAP-1 β were detected in the total cell lysate (5 μ g) from 293T cells using polyclonal anti-ICAP-1 antibody (lane 1). The assignments of ICAP-1 α and ICAP-1 β were confirmed by transiently expressing ICAP-1 α (lane 2) and ICAP-1 β cDNA (lane 3) in 293T cells. (D) The full-length 200-aa ICAP-1 α and an alternatively spliced ICAP-1 β lacking internal 50 aa are shown in a schematic diagram.

codon and was present in correct sequence context for translational initiation (Kozak, 1992). Although clones corresponding to the ICAP-1 α cDNA were represented in the National Center for Biotechnology Information (NCBI) EST database, analyses of the NCBI Non-Redundant database using the BLAST Enhanced Alignment Utility algorithm failed to identify any significant similarities to known proteins.

In Northern analyses, ICAP-1 α transcripts of 0.9 and 1.3 kb in size were detected in mRNA isolated from several tissues (Fig. 1 B). The β_1 integrins are ubiquitously expressed and ICAP-1, as a β_1 integrin-binding protein, is expected to have a similar broad expression in various tissues and cell lines. The differences in the size of two transcripts probably reflect the usage of two different polyadenylation sites observed during cDNA sequence analysis.

In Western analyses using polyclonal anti-ICAP1 antibodies, two distinct polypeptides of 20 and 16 kd were detected (Fig. 1 C, lane 1). In hypotonic cell lysis, ICAP-1 α fractionates in the cytosolic fraction suggesting ICAP-1 α is a cytoplasmic protein (data not shown). Expression of ICAP-1 α cDNA in 293T cells produced the exact 20-kD polypeptides (Fig. 1 C, lane 2). The shorter 16-kD species likely represent ICAP-1 β , an alternatively spliced variant of ICAP-1 α lacking internal 50 aa (Fig. 1 D). A cDNA corresponding to ICAP-1 β mRNA was recognized during the database search. The presence of ICAP-1 β cDNA in several human cDNA libraries was subsequently confirmed by PCR analyses (data not shown). The expression of ICAP-1 β cDNA in 293T cells, as expected, produced

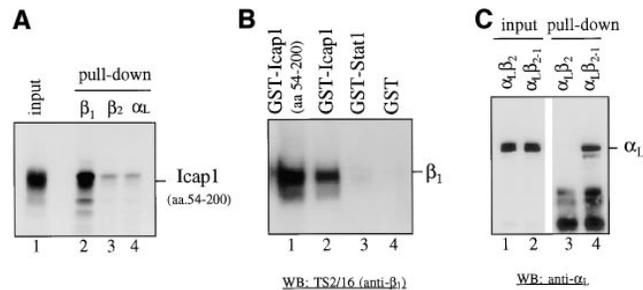


Figure 2. In vitro and in vivo interaction between ICAP-1 α and β_1 integrins. (A) Interaction between GST- β_1 and ICAP-1 α in vitro. ICAP-1 α (aa 54–200) was synthesized in vitro using reticulocyte lysate (lane 1) and incubated with 2 μ g of bacterially expressed GST fusion proteins containing the cytoplasmic domains of integrin β_1 (lane 2), β_2 (lane 3), and α_L (lane 4). (B) Interaction between GST-ICAP1 α and β_1 integrins in vivo. ICAP-1 α (aa 54–200) (lane 1) and a full-length ICAP1 α (lane 2) were expressed as GST fusion protein in 293T cells using eukaryotic expression vector pEBG. For controls, GST-Stat1 (lane 3) and GST (lane 4) were used. Coprecipitation of the endogenous β_1 integrins with the GST fusion proteins were determined on a Western blot using TS2/16 (anti-human β_1 integrin antibody). (C) Restricted binding specificity of ICAP1 α . GST-ICAP1 α was expressed in 293T cells along with expression constructs for $\alpha_L\beta_2$ or $\alpha_L\beta_{2-1}$ (chimeric β_2 subunit that has the COOH-terminal 20 aa replaced with the COOH-terminal 21 aa of β_1 integrin). Coprecipitation of transfected integrins with the GST-ICAP1 α was determined on a Western blot using anti- α_L antibody. Lanes 1 and 2, total lysates of cells expressing GST-ICAP1 α and $\alpha_L\beta_2$ (lane 1) and $\alpha_L\beta_{2-1}$ (lane 2). Lanes 3 and 4, the same samples as in lanes 1 and 2, respectively, after GST “pull-down.”

the exact 16-kD polypeptide that comigrated with the endogenous ICAP-1 β polypeptides (Fig. 1 C, lane 3). Interestingly, ICAP-1 β did not interact with the integrin β_1 cytoplasmic domain in a yeast two-hybrid assay (data not shown).

β_1 Integrin Cytoplasmic Domain Restricted Binding of ICAP-1 α

To verify the specific interaction between ICAP-1 α and the β_1 integrin cytoplasmic domain seen in the yeast genetic screening, in vitro-translated ICAP-1 α was incubated with various integrin cytoplasmic domains expressed in bacteria as GST fusion proteins (Fig. 2 A). After incubation, GST fusion proteins were isolated on glutathione-Sepharose beads and the bound ICAP-1 α polypeptides were analyzed by a SDS gel electrophoresis. As in a yeast two-hybrid assay, ICAP-1 α was bound only to the GST- β_1 fusion protein and not to the GST- β_2 or GST- α_L fusion proteins.

Reciprocal GST pull-down experiments were used to demonstrate the interaction between ICAP-1 α and endogenous β_1 integrins in vivo. GST epitope-tagged ICAP-1 α was transiently expressed in 293T cells. Glutathione-Sepharose beads were used to purified GST-ICAP1 α and copurification of endogenous β_1 integrins was tested on a Western analysis using anti- β_1 antibody. Confirming the results of the yeast two-hybrid assays and in vitro binding studies, β_1 integrins copurified with GST-ICAP1 α , but not with unrelated GST fusion proteins or GST alone (Fig. 2 B).

Table I. ICAP1: Integrin Cytoplasmic Domain Interaction in Yeast Genetic Screen

Baits	Sequence*	Interaction [‡]	β-gal. Activity [§]
β ₁ cyto	KLLMI IHDRREFAKFEKEKMNAKWD T GENPIYKSAVTTVVNPKYE G K	+	ND
β ₂ cyto	KALIHLSDDLREYRRFEKEKLSQWNN D NPL F KSA T TTVMNPK F AES	–	<1.0
β ₁ (E16)	GENPIYKSAVTTVVNPKYE G K	+	149.5 ± 18.3
β ₂ (E16)	D NPL F KSA T TTVMNPK F AES	–	<1.0
β ₃ (E16)	ANNPLYKEATSTFTNITYRGT	–	<1.0
β ₅ (E16)	ASNPLYRKPISTHTVDFTFNKSYNGTVD	–	2.41 ± 3.0
α _L	KVGF F KRN L KEK M EAGRGV P NGIP A ED S EQLAS G Q E AGDP G CL K PL H E K D S ES G GG D	–	<1.0
α ₂	KVGF F KR K Y E K M T K N P D E I D ET T EL S S	–	ND

*The conserved NPXY motifs of integrin β chains are indicated in bold letters. β₁ cyto, complete cytoplasmic domain of integrin β₁. β₁(E16), COOH-terminal 21 aa of integrin β₁. α_L, complete cytoplasmic domain of integrin α_L.

[‡]Positive interaction denotes growth of blue colonies on X-gal indicator plates lacking leucine.

[§]β-galactosidase activity was derived from a minimum four independent measurements.

Finally, to demonstrate that ICAP-1α is a specific β₁ integrin binding protein in vivo, GST epitope-tagged ICAP-1α was coexpressed in 293T cells with the integrin β₂ subunit or a chimeric β₂ subunit (β_{2.1}) constructed by replacing the COOH-terminal 20 aa region of the β₂ subunit with the corresponding sequences of the β₁ subunit. The expression of β₂ integrins is limited to cells of hematopoietic origin and 293T cells completely lack expression of β₂ integrins (Liu, J., and D. Chang, unpublished observation). To ensure a proper cell surface expression, the α_L subunit capable of forming stable heterodimer with the β₂ subunit was also expressed. A comparable surface expression of α_Lβ₂ and α_Lβ_{2.1} was seen on FACS[®] analysis (data not shown). After precipitation of GST-ICAP1α on glutathione–Sepharose beads, copurification of β₂ integrin was indirectly tested in Western analysis using anti-α_L antibody. In GST pull-down experiments, as predicted from the yeast two-hybrid assays and in vitro binding assays, only α_Lβ_{2.1} and not α_Lβ₂ copurified with ICAP-1α (Fig. 2 C). These findings demonstrate the ability of ICAP-1α to interact with integrin αβ heterodimer in a β₁ cytoplasmic domain-dependent fashion.

Conserved NPXY Motif of Integrin β₁ Cytoplasmic Domain is Required for the ICAP-1α Binding

ICAP-1α interacts with the β₁ integrins through the COOH-terminal 21 aa region. Naturally occurring, alternatively spliced variants of β₁ integrins, which lack this region or mutant β₁ integrins containing amino acid substitutions at either of the two conserved NPXY motifs do not localize to the focal contacts (Reszka et al., 1992; Balzac et al., 1993). Furthermore, mutations in the analogous region of β₂ or β₃ integrins affect the leukocyte integrin α_Lβ₂ (LFA-1)-dependent cell adhesion to intercellular cell adhesion molecules-1 or the affinity regulation of the platelet integrin α_{IIb}β₃ (Hibbs et al., 1991; O'Toole et al., 1995; Peter and O'Toole, 1995).

The exact ICAP-1α binding site within the β₁ integrin cytoplasmic domain was further delineated by testing the interaction between ICAP-1α and truncated β₁ integrin cytoplasmic domain (Table II). NH₂-terminal deletions up to 8 aa did not affect the interaction with ICAP-1α. A 3-aa deletion in the COOH terminus, however, completely abolished the interaction. Therefore, a minimum binding site for ICAP-1α on the β₁ integrin cytoplasmic must consist of this 13-aa region that included one of the two

NPXY motifs. The NPXY motif is required for the interaction as amino acid substitutions at the conserved Asn to Glu or Ala abolished the binding. Similarly, a substitution of Tyr to Ala disrupted the ICAP-1α binding whereas a more conservative replacement of Tyr to Phe had no effect.

In yeast interaction and in vitro binding assays, ICAP-1α failed to interact with the β₂ integrin cytoplasmic domain, which is closely related to the β₁ integrin cytoplasmic domain in sequence (Table III). The molecular basis of this highly discriminatory binding specificity of ICAP-1α was investigated by progressively mutating the β₂ integrin cytoplasmic domain. There are two conserved and four nonconserved amino acid differences between the β₁ and β₂ integrins within the minimum 13 aa ICAP-1α binding region. A replacement of three consecutive nonconserved residues found in the COOH terminus of β₂ integrin from Ala-Glu-Ser to Glu-Gly-Lys found in the β₁ subunit did not allow the ICAP-1α binding. Interestingly, a single amino acid replacement at the –11 position from Thr to Val allowed the mutant β₂ integrin to interact with ICAP-1α, demonstrating that in addition to the NPXY motif, Val at the –11 position is essential for the interaction between β₁ integrins and ICAP-1α.

ICAP-1α Is a Phosphoprotein

In addition to the 20- and 16-kD polypeptides corresponding to ICAP-1α and ICAP-1β, heterogeneous bands migrating slower than the 20-kD band were detected in Western analysis of 293T cell lysates using polyclonal anti-ICAP1 antisera (data not shown). Because the amino acid composition of ICAP-1 is rich in Ser and Thr, the possibility that this mobility difference is due to protein phosphorylation was raised. When total detergent lysates of several

Table II. ICAP1: β₁ Subunit Deletion Mutant Interaction

Baits	Sequence*	Interaction [‡]
β ₁ (E16)	GENPIYKSAVTTVVNPKYE G K	+
β ₁ NΔ-3	IYKSAVTTVVNPKYE G K	+
β ₁ NΔ-8	AVTTVVNPKYE G K	+
β ₁ CΔ-3	GENPIYKSAVTTVVNPKY	–
β ₁ EGK→AES	GENPIYKSAVTTVVNPKY A E S	+

*The conserved NPXY motifs of integrin β chain are indicated in bold letters.
[‡]Positive interaction denotes growth of blue colonies on X-gal indicator plates lacking leucine.

Table III. ICAP1: β_1 and β_2 Subunit Point Mutant Interaction

Baits	Sequence*	Interaction [†]
β_1 V787A	.A.....	-
β_1 N792AA.....	-
β_1 N792DD.....	-
β_1 Y792AA.....	-
β_1 Y792FF....	+
β_1 N Δ -8	AVTTVVNPKYEGK •	+
β_2 N Δ -8	ATTTVVNPKFAES	-
β_2 AES/EGKEGK	-
β_2 F766YY....	-
β_2 T758V	.V.....	+

*The amino acid replaced in each β_1 integrin mutant is indicated. Dots denote the sequences that are identical to the β_1 N Δ -8 or β_2 N Δ -8 sequences.

[†]Positive interaction denotes growth of blue colonies on X-gal indicator plates lacking leucine.

different cell lines were surveyed by Western analysis, there was a significant variation in the relative amount of slow migrating species (Fig. 3 A). In particular, two osteosarcoma cell lines, SaOS (Fig. 3 A, lane 2) and UTA-6 (Fig. 3 A, lane 3), displayed high abundance of the slow migrating species. To directly confirm that the slow migrating species represented phosphorylated forms of ICAP-1 α , UTA-6 cell lysates incubated at 30°C to activate endogenous phosphatases (Fig. 3 B). The slow migrating species disappeared with a concomitant increase in the 20 kD ICAP-1 α upon 30°C incubation (Fig. 3 B, lane 3). The addition of phosphatase inhibitors, sodium vanadate, and calyculin A, completely prevented the conversion of the slow migrating species to the 20 kD ICAP-1 α (Fig. 3 B, lanes 2 and 4), confirming the mobility difference is due to protein phosphorylation. Interestingly, during in vitro phosphatase experiment, we failed to detect any mobility shift in ICAP-1 β , suggesting that either ICAP-1 β is not phosphorylated or the mobility of ICAP-1 β does not change significantly by protein phosphorylation.

Regulation of ICAP-1 α Phosphorylation by Integrin-dependent Cell-Matrix Interaction

Many focal contact proteins, FAK, paxillin, and tensin being a few representative ones, have been shown to undergo protein phosphorylation during cell attachment (Clark and Brugge, 1995). The possibility that the phosphorylation of ICAP-1 α may be regulated in a similar manner during cell attachment was directly addressed by binding UTA-6 cells to either PLK or fibronectin (FN)-coated surface (Fig. 4). Western analyses of cell lysates revealed an enhanced phosphorylation of ICAP-1 α when cells adhere to the FN-coated surface. The maximum enhancement occurred within the minimum 30 min required for sufficient number of cells to adhere and undergo cell spreading (Fig. 4, lanes 2 and 4). A longer incubation did not further enhance ICAP-1 α phosphorylation (data not shown). This enhancement was specific for cell attachment to FN-coated surface, which requires β_1 integrins. On PLK-coated surface, cells adhere very efficiently, but fail to initiate subsequent cell spreading. Under these conditions, the phosphorylation of ICAP-1 α remained at a reduced level (Fig. 4, lanes 1 and 3).

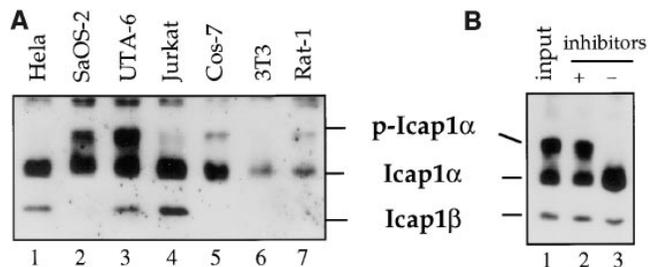


Figure 3. ICAP-1 α is a phosphoprotein. (A) Western blot analysis showing ICAP-1 expression. Total cell lysates (15 μ g) from various cell lines were analyzed on a Western blot using anti-ICAP1 antibody. The positions of ICAP-1 α and ICAP-1 β as well as the slow migrating species corresponding to the phosphorylated ICAP-1 α (*p-ICAP1 α*) are indicated. (B) In vitro phosphatase treatment experiment. 15 μ g of UTA-6 cell lysates were incubated at 30°C for 30 min. Lane 1, input; lane 2, incubation in the presence of sodium vanadate and calyculin A; lane 3, incubation at 30°C.

During integrin-dependent cell adhesion, the rearrangement of cytoskeletal actin stress fibers and assembly of focal adhesion plaques are regulated by Rho-family GTPases (Hall, 1994; Burridge and Chrzanowska-Wodnicka, 1996). In UTA-6 derivative cell lines expressing constitutively activated RhoA(Q63L) under the control of tetracycline-inducible system, the induction of RhoA(Q63L) expression results in the formation of dense stress fibers and increase in the number of focal adhesion plaques (Wong, C., and D. Chang, manuscript in preparation). These cells display a significantly delayed and incomplete cell spreading when plated on FN-coated surface (Fig. 5 A). To test whether the alteration in cell-matrix interaction induced by the expression of RhoA(Q63L) had any effect on the phosphorylation status of ICAP-1 α , a Western analysis was carried out using the lysates from RhoA(Q63L) expressing cells. The lysates prepared from cells grown in the presence of tetracycline, which represses the expression of RhoA(Q63L), were used as controls. Fig. 5 B shows that the expression of constitutively activated RhoA(Q63L) reduces the extent of ICAP-1 α phosphorylation in two independently derived cell lines. Thus, taken together, these findings suggest that phosphorylation of ICAP-1 α is regulated during integrin-dependent cell adhesion and spreading.

Discussion

Integrins, through binding to both extracellular matrix proteins and cytoskeletal structures, provide a direct link-

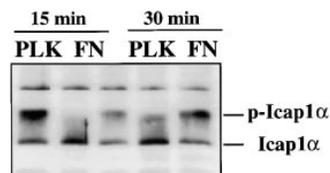


Figure 4. Regulation of ICAP-1 α phosphorylation during cell adhesion. UTA-6 cells were trypsinized and replated on plates coated with poly-L-lysine (PLK) (lanes 1 and 3) or fibronectin (FN) (lanes 2 and 4). Adherent cells were lysed in 0.5% NP-40 at $t = 15$ min (lanes 1 and 2), and $t = 30$ min (lanes 3 and 4) and the extent of ICAP-1 α phosphorylation was determined on a Western blot using anti-ICAP-1 antibody.

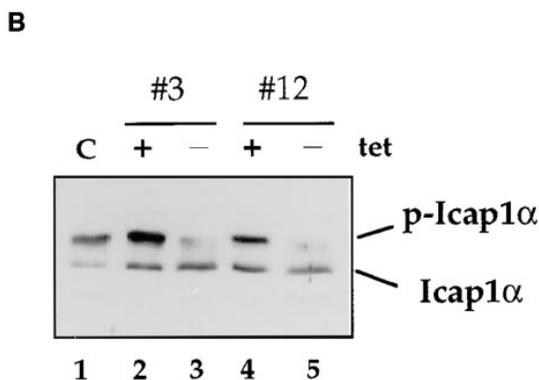
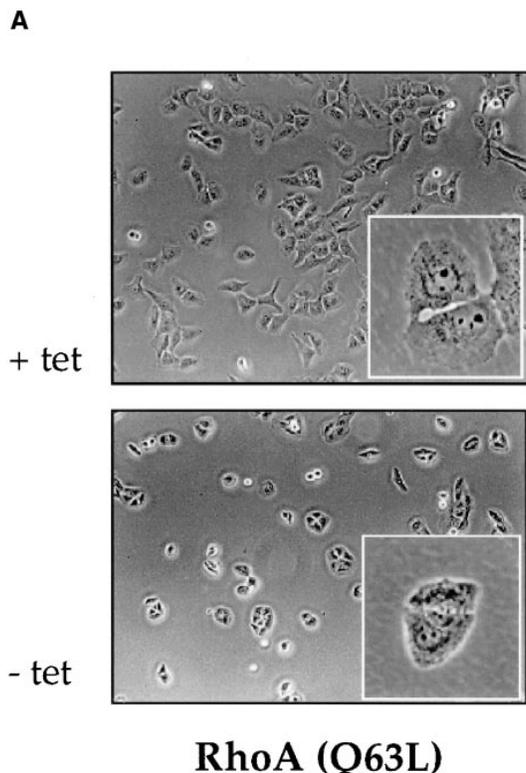


Figure 5. Control of ICAP-1 α phosphorylation by RhoA protein. (A) Expression of activated RhoA interferes with cell spreading. The morphology of UTA-6 cells expressing RhoA(Q63L) under the control of tetracycline-inducible promoter is shown. In the absence of tetracycline, which induces the expression of RhoA (Q63L), cells remain rounded and fail to spread. (B) Expression of activated RhoA results in dephosphorylation of ICAP-1 α . The extent of ICAP-1 α phosphorylation in two independently derived UTA-6 cell lines expressing RhoA (Q63L) was assessed in Western blot analysis using anti-ICAP1 antibody. In parental cells (lane 1) or in the presence of tetracycline (lanes 2 and 4), a significant portion of ICAP-1 α is phosphorylated. Induction of RhoA(Q63L) is associated with the loss of ICAP-1 α phosphorylation in both cell lines (lanes 3 and 5).

age between the extracellular environment and the cell interior. The cytoplasmic domains of integrins have been implicated in the integrin affinity regulation and localization of integrins to the focal contacts. Understanding how the short cytoplasmic tails of integrins affect the functions of integrins requires characterization of cellular proteins that bind, either directly or indirectly, to the integrin cytoplasmic

domains. To this end, we have used a yeast two-hybrid screen to identify proteins that directly bind to the β_1 integrin cytoplasmic domain. In particular, we have focused on the COOH-terminal 21-aa region of the β_1 integrin, which is required for the localization of integrins to the focal contacts. In this study, we report identification and characterization of ICAP-1 α , a novel 200-aa protein that specifically interacts with the β_1 integrin cytoplasmic domain through a conserved and functionally important NPXY sequence motif. In addition, ICAP-1 α undergoes protein phosphorylation that is subject to regulation, suggesting that ICAP-1 α plays an important role during integrin-dependent cell adhesion.

The broad tissue distribution of ICAP-1 α mRNA and the detection of ICAP-1 α protein in cell lines of various tissue origins indicate ICAP-1 α , like β_1 integrins, is ubiquitously expressed. In contrast, the expression of ICAP-1 β , an alternatively spliced variant of ICAP-1 α that does not bind to the β_1 integrin cytoplasmic domain, was more variable in cell lines we have tested. In particular, the ICAP-1 β expression was low or absent in three cell lines, SaOS (human osteosarcoma line), TPH-1 (human monocytic line), and Cos-7 (monkey kidney line). The differences in the ability to interact with the β_1 cytoplasmic domain and in the expression level provide a mechanism for regulation of ICAP-1 α function by ICAP-1 β . It is noteworthy that β_3 -endoneixin likewise has an alternatively spliced variant that does not interact with the β_3 integrin cytoplasmic domain (Shattil et al., 1995).

The amino acid sequence of ICAP-1 α is unique and displays no similarities to any known proteins. Several proteins, including known focal contact proteins, α -actinin, paxillin, talin, and FAK, as well as recently identified potential regulatory proteins such as β_3 -endoneixin, ILK-1, and cytohesin-1 have been shown to bind integrins through the β subunit cytoplasmic domain (for review see Sastry and Horwitz, 1993; Shattil et al., 1995; Hannigan et al., 1996; Kolanus et al., 1996). In contrary to α -actinin, FAK, and ILK-1, which interact with more than one type of β subunit, the interaction of ICAP-1 α is restricted to the β_1 subunit only. This property of ICAP-1 α is similar to two recently identified proteins, β_3 -endoneixin and cytohesin-1, which bind specifically to integrins β_3 and β_2 , respectively (Shattil et al., 1995; Kolanus et al., 1996). As these proteins are not related in sequence to each other or to ICAP-1 α , it remains to be seen how these unrelated proteins are coupled to the β integrin cytoplasmic domains, which are related and functionally interchangeable in some cases.

The results from our mutagenesis studies indicate that the COOH-terminal 13-aa region of the β_1 subunit, which includes a conserved NPXY motif, is sufficient to bind ICAP-1 α . Several studies have shown that this region, especially the NPXY motif, is important for the recruitment of β_1 integrins to the focal contacts (Marcantonio et al., 1990; Reszka et al., 1992; Peter and O'Toole, 1995; Ylanne et al., 1995) and the colocalization of talin, FAK, and actin with β_1 integrins (Lewis and Schwartz, 1995). Our finding that Asn to Ala (or Glu), or Tyr to Ala substitution within the NPXY motif completely abolished the interaction between ICAP-1 α and integrins, while a more conservative replacement of Tyr with Phe had no effect, demonstrates a remarkable similarity between the sequence requirement

for the binding of ICAP-1 α to integrins and localization of β_1 integrins to the focal contacts and suggests that ICAP-1 α may play a role in the recruitment of β_1 integrins to the focal contacts. Alternatively, the COOH-terminal 13-aa region may be involved in initiating signal transduction events required to trigger cell spreading and ICAP-1 α may participate in the initiation of this signaling event. As β_2 , β_3 , and β_5 integrin cytoplasmic domains (which do not bind ICAP-1 α) can also direct integrins to the focal contacts (LaFlamme et al., 1994; Peter and O'Toole, 1995), there must be parallel mechanisms for recruiting various integrins to the focal contacts.

The COOH-terminal regions of different β integrins are somewhat diverse in amino acid sequence, which may explain in part the observed specificity of ICAP-1 α interaction with the β_1 integrin cytoplasmic domain. The basis of this restricted specificity, however, was attributed to a single Val residue NH₂-terminal to the conserved NPXY motif. In β_2 or β_3 subunits, the corresponding position is occupied by Thr (Sastry and Horwitz, 1993). Confirming the importance of Val at this position, the replacement of the Thr with a Val in the β_2 subunit cytoplasmic tail allowed it to bind ICAP-1 α . The β_3 integrin cytoplasmic domain, in addition, has a less conserved NITY in place of the NPXY motif, which may also account for the lack of interaction with ICAP-1 α . A recent study on the amino acid sequence requirement for the interaction between β_3 -endoneixin and β_3 integrin cytoplasmic domain indicated that the NITY motif is critical for the binding specificity (Egenthaler et al., 1997). Altogether these findings suggest that the COOH-terminal regions of different β integrins may constitute specific binding sites for different cytoplasmic proteins. In addition, the existence of integrin β subunit specific binding proteins indicates that the functions of individual subunits can be differentially regulated.

The presence of ICAP-1 α immunoreacting species, which migrated slower than the expected molecular weight of 20 kD predicted from the ICAP-1 α reading frame suggested that ICAP-1 α undergoes posttranslational modification. The conversion of slower migrating species to the expected 20-kD species during 30°C incubation, which activates the endogenous phosphatases present in nonionic detergent lysates, and the observation that this conversion can be effectively blocked by the addition of known phosphatase inhibitors demonstrate that ICAP-1 α is a phosphoprotein.

The most intriguing property of ICAP-1 α is that its phosphorylation is regulated during cell adhesion and by RhoA protein. We suspect that the effect on ICAP-1 α phosphorylation seen during cell adhesion on FN-coated surface and in cells expressing constitutively activated RhoA(Q63L) are related events, reflective of the cytoskeletal rearrangement that occurs during cell adhesion. It is well known that cells, soon after making an initial contact on FN-coated surface, undergo cell spreading that involves the formation of focal adhesion plaques and actin stress fibers (for review see Hall, 1994). Both these events are known to be regulated by RhoA (Ridley and Hall, 1992; Nobes and Hall, 1995). Furthermore, both matrix assembly and cell spreading are integrin-dependent processes and require integrin cytoplasmic domain (for review see Burridge and Chrzanowska-Wodnicka, 1996; LaFlamme

et al., 1994). On a nonspecific PLK-coated surface, cells make initial contact but fail to promote matrix assembly and initiate spreading. Cells expressing RhoA(Q61L) also display a delayed and incomplete spreading, presumably due to the interference from dense stress fibers. According to this scenario, the diminished ICAP-1 α phosphorylation observed during cell adhesion on PLK-coated surface and in RhoA(Q63L) expressing cells is a result of inefficient cell spreading. Our findings, however, do not rule out the possibility that phosphorylation of ICAP-1 α may be a direct consequence of the interaction between integrins and fibronectin. Regardless of the exact mechanism underlying the control of ICAP-1 α phosphorylation, our data clearly demonstrate that the phosphorylation of ICAP-1 α is regulated during the cell-matrix interaction.

It remains to be seen whether the enhancement in ICAP-1 α phosphorylation during cell attachment involves Ser/Thr phosphorylation or Tyr phosphorylation. Although Ser/Thr phosphorylation is suspected based on the amino acid composition of ICAP-1 α (21% Ser/Thr) and the presence of potential protein kinase C and protein kinase A phosphorylation sites, the exact amino acid residues that are phosphorylated are not known. We have not been able to demonstrate the immunoreactivity of ICAP-1 α with PY20 anti-phosphotyrosine antibody (data not shown).

In summary, we present initial characterization of ICAP-1 α , a novel β_1 integrin cytoplasmic domain binding protein. Two observations, (a) the binding of ICAP-1 α to a conserved region of β_1 integrin cytoplasmic domain that previously has been shown to be important for the adhesive function and focal contact localization of integrins, and (b) the extent of ICAP-1 α phosphorylation is regulated during cell-matrix interaction, suggest that ICAP-1 α plays a role during integrin-dependent cell adhesion. β Integrin cytoplasmic domains likely contain overlapping binding sites for both structural and regulatory proteins that coordinate cell adhesion and subsequent cytoskeletal rearrangement. The identification of ICAP-1 α together with the characterization of the sequences on β_1 integrin cytoplasmic domain required for the ICAP-1 α binding should facilitate future studies on how specific integrin-dependent cellular events are regulated.

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