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Calcium/Calmodulin-Dependent Protein Kinase II Controls Integrin $\alpha_5\beta_1$-Mediated Cell Adhesion through the Integrin Cytoplasmic Domain Associated Protein-1α

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This paper provided evidence that the regulation of CHO cell adhesion on fibronectin by calcium/calmodulin-dependent protein kinase II (CaMKII) is mediated through the recently described integrin cytoplasmic domain associated protein-1α (ICAP-1α). The point mutation T38D localized within the optimal CaMKII recognition motif of ICAP-1α results in a strong defect in cell spreading which cannot be overcome by the inhibition of the endogenous CaMKII. This fact strongly suggests that the phosphorylation of Threonine 38 by CaMKII modulates the $\alpha_5\beta_1$ integrin function. Conversely, the mutation T38A produces an analog of ICAP-1α that cannot be phosphorylated and that stimulates cell spreading on fibronectin to a similar extent when CaMKII is inhibited. © 1998 Academic Press

The integrin receptors family play a prominent role in cell/extracellular matrix interactions and matrix assembly. The integrin affinity can be modulated by a conformational change of its extracellular moiety that is generated by the cytoplasmic domain. This control was named inside-out integrin signaling (1). It is likely due to a cytoplasmic protein regulator that can be disclosed by the dominant negative effect of autonomously expressed β cytoplasmic domains suggesting that some effector is titrated by the overexpression of the β cytosolic tails (2, 3). Recently, ICAP-1 (integrin cytoplasmic domain associated protein-1) was identified as a protein specifically associated with the β_1 cytoplasmic domain of the integrins (4). This protein has two isoforms named α and β corresponding of 200 and 150 amino-acids chains, respectively and is ubiquitously expressed in various cell lines and tissues. ICAP-1α but not ICAP-1β interacts with the cytoplasmic tail of the β integrin chain in a manner that depends on the conserved NPXY integrin motif. ICAP-1α contains a number of putative phosphorylation sites and shares homologies with β_3-endonexin, a protein that interacts specifically with β_3 integrin cytoplasmic domains (5). This latter protein has been shown to positively control the affinity state of the platelet integrin $\alpha_{IIb}\beta_3$ (6). We postulated that ICAP-1 could play a similar role in the β_1 integrin family. Moreover, we have recently shown that the affinity of $\alpha_5\beta_1$ was controlled by the balance between the activities of calcium and calmodulin dependent protein kinase II (CaMKII) (7) and the calcium and calmodulin dependent protein phosphatase 2B or calcineurin (8). Since the integrin receptor did not undergo cycle of phosphorylation and dephosphorylation under our experimental conditions, the conformational change of integrin may be mediated by an intermediate effector such as ICAP-1α. To test this hypothesis, we analyzed the putative CaMKII phosphorylation site on the protein and performed site directed mutagenesis to produce a non phosphorylatable protein (mutant T38A) or a mutant that mimicked a constitutively phosphorylated protein (mutant T38D). Adhesion on fibronectin of CHO cells is mediated solely by $\alpha_5\beta_1$ integrin (9). Then, we expressed in CHO cells wild type and ICAP-1α mutants and analyzed the adhesion of the transfected cells on fibronectin. Our results indicated that ICAP-1α is an essential component of the CaMKII inside-out $\alpha_5\beta_1$ integrin signaling pathway.

MATERIALS AND METHODS

ICAP-1α cDNA. The nucleotide sequence of ICAP-1α cDNA (GenBank accession number aa148053) was used to perform a BLASTn search of the world wide web dbEST database. The cDNA clone (ID#590468) was subsequently obtained from an I.M.A.G.E. Consortium (LLNL) cDNA (10). Sequence determination of this cDNA clone revealed the full length human cDNA coding for ICAP-1α.

Cell culture. Chinese Hamster Ovary (CHO) cells stably transfected with a modified ecdysone receptor (Ecr CHO cells) were purchased from Invitrogen (Netherlands). Ecr CHO cells were grown on plates in Minimum Essential Medium with alpha modification (α MEM) without nucleoside and supplemented with 7.5% fetal calf

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serum (v:v) at 37°C, in a humidified 5% CO2/95% air atmosphere. Transfected Ecr CHO cells were selected with zeocin antibiotic according to manufacturer's recommendations. Cells were harvested with Phosphate Buffer Saline (PBS) supplemented with 1mM EDTA and 0.05% trypsin (w:v).

Site directed mutagenesis. Site directed mutagenesis was performed with the "Quick Change" mutagenesis kit (Stratagene) using the human ICAP-1α cDNA in the pBluescript SK- vector (pBS-ICAP-1α) as a template and following the manufacturer's instructions. The Threonine 38 of ICAP-1a was replaced either by an aspartic acid (T38D) in the goal to mimic a phosphorylated amino acid or by an alanine (T38A) to impair the phosphorylation of the protein. The T38D mutant was generated using the CCA GCG ATG TGG CCA GC and GCT GGC CAC ATC GCT GG primers while T38A mutant was generated using the CCA GCG CTG TGG CCA GC and GCT GGC CAC AGC GCT GG primers. Both mutations were controlled by DNA sequencing analysis.

Transfection of ICAP-1α in CHO cells. The cDNA of wild type, T38D and T38A ICAP-1α mutants were excised from pBS-ICAP-1α vector as EcoRI/XbaI fragments and inserted into the pIND ecdysone inducible vector (Invitrogen, Netherlands). Stable Ecr CHO transfectants were obtained by electroporation of 4×10^6 cells in 400 μl of PBS at 340 V with 10 μg of pIND-ICAP-1α either WT or T38D or T38A) vector or with 10 μg of pIND vector, and were subsequently cultured in αMEM medium supplemented with 7.5% fetal calf serum (v:v), 100 units/ml penicillin and 100 μg/ml streptomycin. After 48 hours, Zeocin was added to the culture medium. The time frame during which Zeocin was applied was 3 weeks, and the selected cells were cultured in medium supplemented with Zeocin excepted during the induction steps.

For the induction of ICAP WT, T38A and T38D expression, Ecr CHO cells were incubated with 2-5 μM Muristerone A for 20 hours at 37°C or in the control with an equal amount of ethanol 100% in the case of control. The induction of expression was confirmed by indirect immunofluorescence on induced Ecr CHO cells plated onto 25 μg/ml of fibronectin using a polyclonal antibody against ICAP-1.

For the generation of rabbit antisera against ICAP-1, the full length coding sequence was cloned in pET19b vector and expressed as a histidine tagged protein in E. Coli BL21(DE3) strain. The protein was purified using nickel charged column (Novagen, Inc) under denaturing conditions following the manufacturer's instructions.

Cell spreading assays. Ecr CHO cells were harvested from 100 mm culture dishes with Trypsin/EDTA in PBS, then washed three times with αMEM containing 7.5% fetal calf serum (v:v) depleted in fibronectin. The cells were incubated for 30 minutes at 37°C with or without inhibitors. After incubation, the cells were allowed to spread on fibronectin for 4 hours at 37°C on glass coverslips coated with a fibronectin solution at different concentrations ranging from 0.25 to 2 μg/ml for 4 hours at 37°C and post coated with BSA 1% (w:v) for 30 min at 37°C). Then the cells were fixed with 3% paraformaldehyde and 2% sucrose and stained with a solution of 0.25% (w:v) Coomassie Blue R250 in 10% and 20% (v:v) acetic and isopropanol solution. The cells were observed at a magnification of 100× using an inverted microscope (Zeiss Axiovert 135) equipped with a X32 Ph1, NA 0.60 Achrostigmat objective lens. The quantification of the results was performed by analyzing four different dish areas and about 600 cells scored as round or attached and spread. These results are representative of at least three different experiments.

Reagents. Fibronectin was purified from bovine plasma as described by Engvall and Ruoslahti (13). KN-62 was from Seikagaku, Muristerone A from Invitrogen. All other reagents were from Sigma.

RESULTS AND DISCUSSION

Calcium and calmodulin-dependent protein kinase II was recently described as a crucial mediator of the affinity of the $\alpha_5\beta_1$ integrin (7). Moreover, it did not phosphorylate the integrin chains, thus, it must act indirectly through a cellular integrin effector. CaMKII and CaMKIV are distinguished by their abilities to phosphorylate a wide range of phosphoaccepting substrates both *in vivo* and *in vitro,* leading to their designation as multifunctional CaM kinases. To identify the CaMKII potential targets, the use of synthetic phosphoaccepting peptides allowed the definition of its substrate recognition motif (consensus sequence) (11). CaMKII was shown to preferentially phosphorylate substrates with the motif Hyd-X-Arg-NB-X-Ser*/Thr*-Hyd where Hyd represents a hydrophobic amino acid, X any amino acid, and NB a non basic amino acid residue. Since ICAP-1α interacts with β1 integrin and shares homologies with β_3-endonexin, a cytoplasmic regulator of $\alpha_{IIb}\beta_3$, it may play a similar role among the β_1 integrins and is a potential phosphorylation site of the CaMKII inside-out integrin pathway. We analyzed the putative phosphorylation sites of the protein. ICAP-1 has multiple potential phosphorylation sites positioned primarily within the N-terminus domain of the protein and one putative PKC site next to the C-terminus (Fig. 1). Among those sites, a CaMKII consensus sequence matched the optimal substrate recognition motif for the kinase as described by White *et al* (1998). It corresponded to a phosphorylation on the Threonine 38. Then we decided to generate point mutation of this amino acid. Indeed, if ICAP-1 was the integrin effector regulated by CaMKII phosphorylation, one can expect the following results: the expression in CHO cells of mutant T38A that cannot be phosphorylated by CaMKII, should result in a dominant negative phenotype (due to the competition of T38A with the endogenous ICAP-1) similar to that observed when the CaMKII activity was inhibited by KN-62. Conversely, the expression of mutant T38D that mimicked a constitutively phosphorylated protein, should result in a dominant positive phenotype identical to that observed when a constitutively active CaMKII was expressed.

Overexpression of ICAP-1α has a dramatic effect on cell growth and survival (data not shown), therefrom we expressed the protein in an ecdysone inducible mammalian expression system. After induction with 5 μM of Muristerone A, (a stable ecdysone analog), for 20 hours at 37°C, the transfected cells were harvested and plated on glass coverslips coated with 2 μg/ml of fibronectin, a concentration that supports only a partial spreading of non or mock transfected Ecr CHO cells (7). As shown in Fig. 2A, the expression of wild type ICAP-1α in CHO cells did not result in significant cell spreading alteration as compared to the control mock

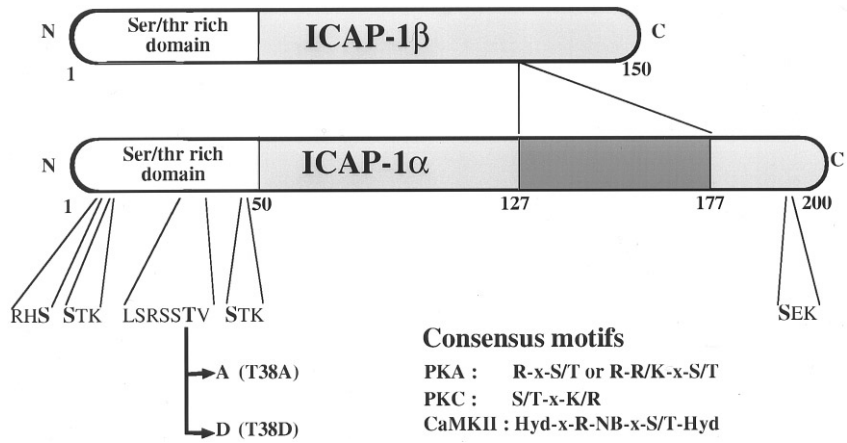


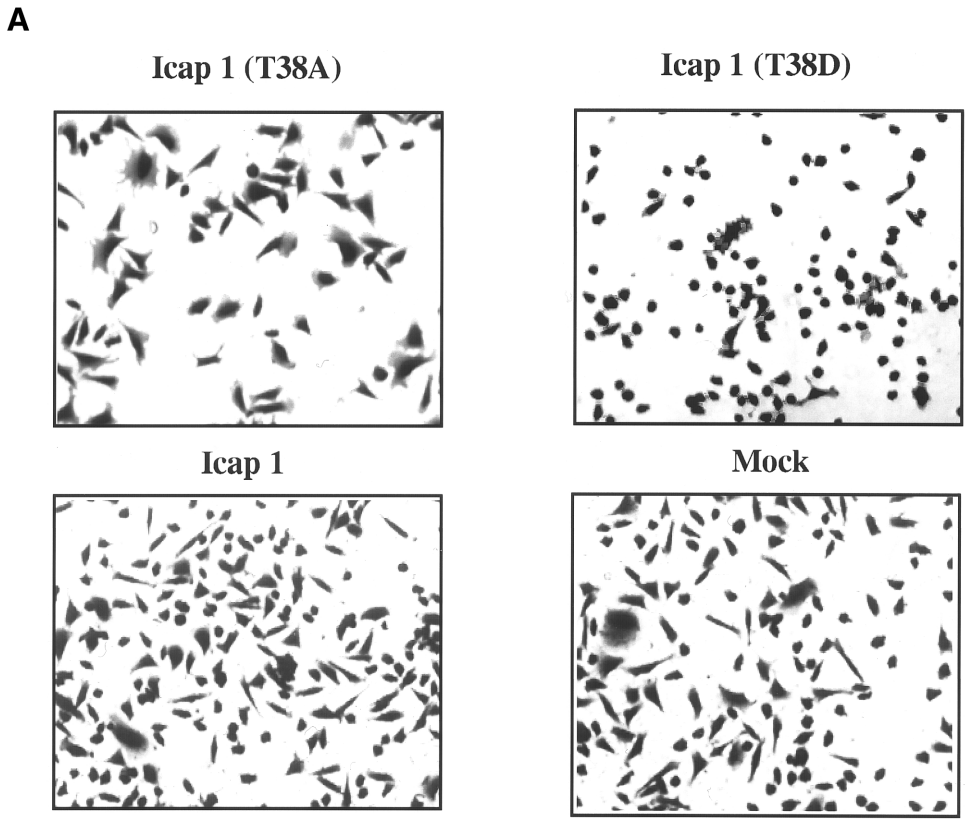
FIG. 1. Schematic representation of ICAP-1 variants α and β. Three putative phosphorylation site for PKC and one putative phosphorylation site for PKA and CaMKII, respectively, are localized within the polypeptide chain. Most of the sites are clustered within the first 50 amino acids in a serine/threonine rich domain. Only ICAP-1α binds to the β1 integrin chain, likely on its 50 amino acid long additional domain (127-177). The ICAP-1α mutants used in this work are represented; the mutated threonine in position 38 is in bold type.

transfected cells (without insert in the plasmid). However, quantification of cell spreading as described under Materials and Methods, revealed that ICAP-1α slightly improved cell spreading at this limiting concentration of fibronectin. Conversely, the expression of the ICAP-1α mutant T38A strongly stimulated cell spreading (Figs. 2A and 2B). It was noteworthy that the effect obtained with this mutant was similar to that observed when KN-62 (1-[*N*,*O*-Bis(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenylpiperazine), a highly specific permeant inhibitor of CaMKII was added directly to the cells (Fig. 3 and (7)). Moreover, the expression of the ICAP-1α mutant T38D that should adopt a conformation similar to the phosphorylated protein resulted in a dramatic impairment of cell spreading on fibronectin matrix (Figs. 2A and 2B). Moreover, this phenotype was identical to that observed upon the expression of a constitutively active T286D CaMKII mutant (7, 12). It is noteworthy that Muristerone A did not modify cell surface expression of the integrin $\alpha_5\beta_1$ (not shown). These results are in agreement with the hypothesis that ICAP-1α is a modulator of $\alpha_5\beta_1$ mediated cell spreading, depending on its phosphorylation by CaMKII on Threonine 38.

To further demonstrate that CaMKII regulates the integrin $\alpha_5\beta_1$ by phosphorylating ICAP-1α, we investigated the effect of KN-62 on Ecr CHO cell spreading on fibronectin coated at 0.25 μg/ml. At this fibronectin concentration, non or mock transfected Ecr CHO cells remained round, but full attachment and spreading was observed when KN-62 was added (Fig. 3). Induction of cell spreading by KN-62 inhibitor was not observed in the case of ICAP-1α mutant T38D. Since this mutated protein should be permanently in a conformational state similar to the phosphorylated wild type protein, it should result in adhesion defect independently of the kinase activity. Quantification of spreading experiments in either the presence or the absence of KN-62 are presented in Fig. 3. As expected the expression of ICAP-1α mutant T38A resulted in an already maximal stimulation of cell spreading that cannot be enhanced by addition of KN-62. Altogether these results strongly suggested that the action of CaMKII is mediated by the phosphorylation of ICAP-1α on the Threonine 38.

At such a fibronectin concentration CHO cells spreading was only mediated by $\alpha_5\beta_1$ integrin since it could be inhibited by the blocking anti-Integrin antibody PB1(not shown). Moreover, our data strongly suggest that the biological activity of ICAP-1α is regulated by CaMKII. Indeed, it contains an optimal CaMKII phosphorylation consensus motif. Replacement of the Threonine 38 within this sequence by an aspartic acid and the expression of the mutant protein results in a defect in cell spreading identical to that observed when constitutively active CaMKII is expressed (7). Finally, the expression of the mutant T38D blocked cell spreading induced by KN-62, a molecule that inhibits endogenous CaMKII, indicating that the effect of the kinase involved ICAP-1α. We have previously shown that

FIG. 2. Effect of the expression of ICAP-1α and its mutants on Ecr CHO cells spreading on fibronectin. A) The cells were allowed to spread on fibronectin coated glass coverslips (2 μg/ml) for 4 hours at 37°C. Then they were fixed and stained with Coomassie Blue. Images were obtained at a magnification of 100× with an Axiovert 135 Zeiss microscope equipped with an achrostigmat X10, NA 0.25 objective lens. B) Quantification of cell adhesion assays according to the experimental procedure described under Materials and Methods.


A
Icap 1 (T38A)
Icap 1 (T38D)
Icap 1
Mock
B
Spread Cells (% of total)
100
80
60
40
20
0
MOCK
ICAP-1α WT
ICAP-1α (T38A)
ICAP-1α (T38D)

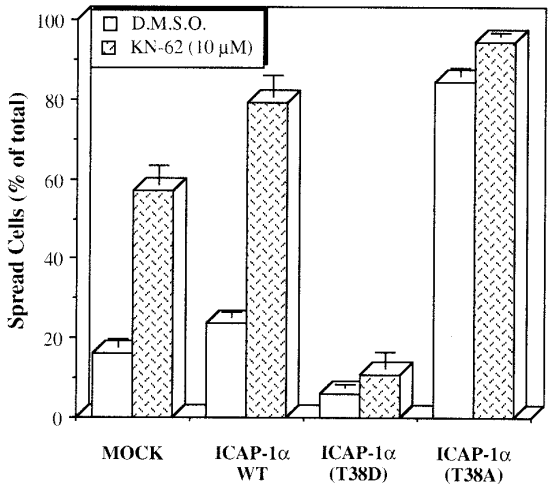


FIG. 3. Effect of endogenous CaMKII inhibition in Ecr CHO transfected by ICAP-1α and its mutants on fibronectin cells spreading. The cells were allowed to spread on fibronectin coated glass coverslips (0.25 μg/ml) for 4 hours at 37°C. They were fixed and stained with Coomassie Blue. Images were obtained at a magnification of 100× with an Axiovert 135 Zeiss microscope equipped with an achrostigmat X10, NA 0.25 objective lens. Quantification of cell adhesion assays was carried out using the experimental procedure described under Materials and Methods. This very low fibronectin concentration does not sustain cell spreading of wild type or mock transfected cells when CaMKII is not inhibited by KN-62; conversely KN-62 (10 μM) strongly stimulates cell spreading. No effect is observed upon transfection of ICAP-1α mutant (T38D).

CHO cell spreading on a low fibronectin concentration was necessary mediated by $\alpha_5\beta_1$ integrins in high affinity state, and that CaMKII is largely involved in this signaling pathway (7). Altogether these results suggested that CaMKII could phosphorylated ICAP-1α at position 38, once phosphorylated this protein should shift integrins in a low affinity state. It is noteworthy that exogenous expression of wild type ICAP-1α significantly stimulates CHO cell spreading on fibronectin coated at 2 μg/ml. This effect was only minor when the coating was performed at 0.25 μg/ml. A plausible explanation would be that the interaction between concentrated fibronectin and low affinity integrins would results in the activation of both CaMKII and the antagonist phosphatase PP2B (8). The balance between both enzyme activities would result in a partial switch of the integrins in their high affinity state. This switch would be more complete upon overexpression of ICAP-1α. Alternatively, at low fibronectin concentration, there is a prevalent role of the integrin affinity in the control of cell adhesion. By increasing the coat concentration, one can allow the integrin receptors in a low affinity state to interact with the matrix. Under these experimental conditions, any action on cell spreading will be more pronounced. Therefore, one cannot exclude at this stage that ICAP-1α can not only control integrin affinity, but also affect cell spreading. Indeed, the well conserved NPXY motif in β integrin subunit have been shown to be involved in the recruitment of integrins into focal adhesion, probably by anchoring some regulators proteins. Since ICAP-1α interacted specifically with this sequence on β_1 subunit (4), this protein appeared to be a good candidate for this purpose. The precise interaction between ICAP-1α and its mutants with the cytoplasmic domain of β_1 subunit are under investigation.

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