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QUARTERLY

Signal transduction in confluent C3H 10T1/2 cells. The role of focal adhesion kinase★✪

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The activities of extracellular signal-regulated kinases (ERK1/ERK2) is required for proliferation of several types of cells. The performed analysis showed stimulation of ERK's by fetal calf serum (FCS) or fibronectin in the C3H 10T1/2 cell cultures at logarithmic phase of growth. The ERKs activity was not stimulated in confluent cells. This could not be accounted for a partial down regulation of ERK since its level was stable in both types of cells regardless of their density and kind of stimulation. Searching for ERK upstream elements we studied the integrin receptor gene transcript by RT-PCR and focal adhesion kinase (FAK) by Western blotting and phosphorylation assays.

It was found that FCS and fibronectin stimulated phosphorylating activity of FAK in the cells at the logarithmic phase of growth, but were inefficient in the confluent cells. RT-PCR showed the presence of $\alpha 5$ and $\beta 1$ integrin transcripts, and $p125^{FAK}$ was at the same level regardless of the type of stimulation.

These data indicate that the ability of FAK to be activated plays an important role in ERK regulation and, in consequence in proliferation and growth inhibition during confluence.

Non-transformed cell lines become contact inhibited when they reach confluence in monolayer tissue cultures even in the presence of growth factors. The release from this contact inhibition is associated with cellular transformation. Since the contact inhibition of growth occurs even in the presence of serum, it may be expected that either a growth factor dependent signal is discriminated or the cell surface molecules transmit a growth inhibiting signal, as it has been suggested for cadherins in epithelial cells (St. Croix *et al.*, 1998), and contactinhibin in human diploid fibroblasts (Gradl *et al.*, 1995). In turn, interactions between integrins and extracellular matrix promote either cell proliferation or growth inhibition (Assoian, 1997; Ilić *et al.*, 1997; 1998). These interactions take place at sites called focal adhesion sites, which contain many tyrosine kinases including fo-

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Abbreviations: FAK, focal adhesion kinase; FCS, fetal calf serum; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; NaCl/P_i buffer, phosphate-buffered saline.

cal adhesion kinase (FAK), which is activated upon cell binding to the extracellular matrix. In addition to FAK activation, cell adhesion to fibronectin enhances phosphorylation by mitogen-activated protein kinase–ERK (MAP kinase–ERK), suggesting an anchorage-dependent step in the MAP pathway (Lin *et al.*, 1997; Schwartz, 1997).

Recently, two distinct pathways regulating cell migration have been documented; one involving stimulation of extracellular signal-regulated kinase (ERK) and the other involving activation of FAK and $p130^{Cas}$ (Gu *et al.*, 1999). This strongly suggests cooperation between the two pathways: one beginning with integrin and the other with growth factor receptors.

It was previously shown that protein kinase C in the contact inhibited 10T1/2 cells did not translocate to the membrane following fetal calf serum (FCS) stimulation, whereas it did so in sparse cultures (Miłoszewska *et al.*, 1986). This finding implied inefficient signal transduction in the contact inhibited cells. The study of Viñals & Pouysségeur (1999) on confluent vascular endothelial cells documented decreased p42/p44 MAP kinase activation following FCS stimulation. The authors concluded that phosphatases play a role in decreasing ERK1/ERK2 activity. In turn, Derijard *et al.* (1995) proposed that for activation of ERK a double phosphorylation by MEK 1 and MEK 2 is needed at the threonine and tyrosine sequences. The requirement for growth factors and integrin receptor activation for proliferation led us to the assumption that at least one of these pathways is inefficient in confluent contact inhibited cells.

In the present work we studied ERK1/ERK2 activation in confluent contact inhibited and sparse fast growing C3H 10T1/2 cells following FCS and fibronectin stimulation, assuming that the extent of ERK's activation or inhibition may be a consequence of FAK activity.

MATERIALS AND METHODS

Cells and culture conditions. C3H 10T1/2 fetal mouse fibroblasts from the 10^{th} passage were used. The cells were propagated in MEM supplemented with 10% FCS and antibiotics in Assab incubator (37°C, 5% CO_2). These cells exhibited post confluence inhibition of growth as described by Reznikoff *et al.* (1973). For experiments 2000 cells were seeded into each Petri dish. Cells were collected at day 5 (logarithmic phase of growth, for simplification called sparse) or day 10 after seeding (confluent cultures). Before the experiment the cultures were cultivated for 20 h in serum-free medium, and then stimulated with fibronectin solution (20 μg/ml) or 10% serum for 4 h for Western blotting analysis and 15 min for phosphorylation reactions.

Determination of integrins α5 and β1 by RT PCR. Total RNA was extracted from cultured cells as described by Chomczynski & Sacchi (1987), using TRIzol reagent (Gibco BRL). cDNA was synthesized by reverse transcription from 5 ng of RNA with Superscript Amplification System (Gibco). PCR amplification of cDNA was performed using specific primers: for integrin α5, 5′-CATTTCCGAGTCTGGGCCAA-3′ and 5′-GGAGGCTTGAGCTGCTTT-3′ (323 bp fragment), for integrin β1, 5′-TGTTCAGTGCAGAGCCTTCA-3′ and 5′-CCTCATACTTCGATTGACC-3′ (452 bp fragment), as described by Tang *et al.* (1995). Bands were visualized by ethidium bromide staining.

Western blotting. Dishes with cells were washed with $NaCl/P_i$, harvested and lysed in modified RIPA buffer. Total cell protein in lysates was standardized with the use of the Lowry *et al.* (1951) protein assay kit (Bio-Rad) before immunoprecipitation. About 2 mg of total protein were incubated for 4 h at 4°C with specific antibodies against FAK (10 μl/ml, Santa Cruz Biotechnology, Inc.). Next, 100 μl/ml protein A/Sepharose (Pharmacia) was added to each sample and left overnight. After incubation with protein A/Sepharose, the samples were washed, boiled with electrophoresis sample buffer and subjected to electrophoresis in 7% polyacrylamide gel. After being electrotransferred the immunoprecipitated samples were assayed by Western blotting, using antibodies against FAK (Santa Cruz Biotechnology, Inc.), the same as the antibodies used for immunoprecipitation. Specific

bands were visualized by the enhanced chemoluminescence (ECL) method (Amersham). ERK proteins were assayed in the total cell extract by the method of Western blotting using specific antibodies against ERK1 (Santa Cruz Biotechnology, Inc.).

ERK phosphorylation assay. The C3H 10T1/2 cells were lysed on ice in 0.5 ml of ice-cold immunoprecipitation buffer containing: 150 mM NaCl, 50 mM Tris/HCl (pH 7.5), 5 mM EGTA, 1% Triton X-100, 0.5% NP-40, 25 mM *p*-nitrophenyl phosphate, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 20 mM sodium fluoride, 0.2 mM sodium orthovanadate, and 10 μg/ml leupeptin, for 0.5 h at 4°C. Next, the homogenates were centrifuged at 13 000 r.p.m. for 15 min at 4°C. Cytoplasmic extracts containing 2 mg of total protein were immunoprecipitated with 5 μg of polyclonal anti-ERK1 antibody and 5 μg of anti-ERK2 antibody in the immunoprecipitation buffer for 3 h at 4°C on a rotator. Then, 50 μl of protein A beads, which had been preincubated with the same buffer, were added and the samples were incubated overnight at 4°C on the rotator. The probes were then centrifuged at 13 000 r.p.m. for 5 min at 4°C. Next, the pellets were washed three times with immunoprecipitation buffer and twice with the MAP kinase assay buffer (50 mM Hepes/NaOH, pH 7.5, 20 mM β-glycerophosphate, 40 mM $MgCl_2$, 4 mM $MnCl_2$, 0.2% Triton X-100, 1 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4). Next, the beads were mixed with 40 μl of MAP kinase assay buffer containing 20 μM ATP, 0.3 μCi [γ-^{32}P]ATP and 15 μg of MBP peptide (APRTPGGRR). Phosphorylation reactions were carried out for 10 min at 30°C and terminated by adding 10 μl of 25% trichloroacetic acid. After centrifugation, the supernatants were spotted onto Whatman P-81 papers. The papers were washed several times in 0.1% phosphoric acid, rinsed with acetone, dried, and radioactivity was counted. The results were expressed in c.p.m. as radioactivity incorporated into the peptide substrate.

FAK phosphorylation assay. The C3H 10T1/2 cells were lysed on ice with lysis buffer (20 mM Tris/HCl, pH 7.6, 140 mM NaCl, 2.6 mM $CaCl_2$, 1 mM $MgCl_2$, 1% Nonidet P-40, 10% (v/v) glycerol also containing 1 mM PMSF and 1 mM sodium vanadate). The lysates were centrifuged at 10 000 $\times$ ***g*** for 15 min at 4°C, and the resulting supernatants, 2 mg protein/probe, were subjected to immunoprecipitation. This was performed by incubating cell lysates for 3 h at 4°C with 10 μl of anti-FAK polyclonal antibody and overnight with protein A/Sepharose beads. The immunoprecipitates were washed twice with kinase assay buffer (50 mM Hepes/NaOH, pH 7.6, 3 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM DTT, 10 μM ATP) and incubated for 30 min at 24°C in kinase buffer (50 μl) in the presence of [γ-^{32}P]ATP (0.1 mCi/ml), with poly(Glu,Tyr), 1 mg/ml, as substrate. The phosphorylation reactions were terminated by adding 10 μl of 25% trichloroacetic acid. Then the samples were centrifuged at 10 000 $\times$ ***g*** for 5 min at 4°C and the supernatants were spotted onto P-81 paper (Whatman), which was washed several times with 0.1% phosphoric acid. The associated radioactivity was determined by liquid scintillation counting (Liquid Scintillation Analyzer, Packard).

RESULTS

Integrins α5 and β1

RT-PCR experiments showed that C3H 10T1/2 cells express transcripts for α5 and β1 elements of integrin receptor independently of experimental conditions. The amounts of α5 as well as β1 transcripts were almost the same in the sparse and confluent cells and they did not depend on the stimulators applied (serum, fibronectin). These results (Fig. 1) may indicate that neither the state of proliferation nor treatment conditions do affect the presence of integrin receptor.

Effect of fibronectin or serum treatment of 10T1/2 cells on ERK's

ERK's protein study

Figure 2 shows the presence of proteins ERK1/ERK2 in fibronectin- or serum-treated con-

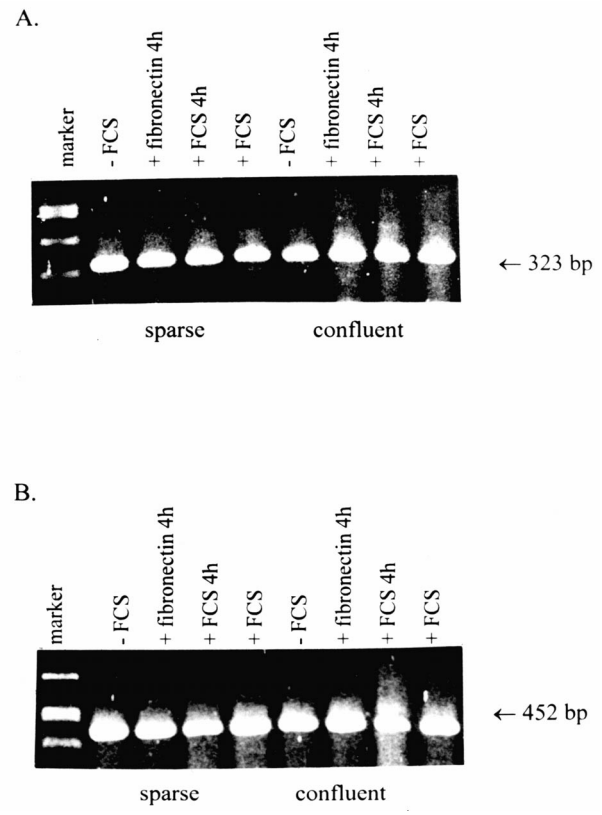

Figure 1. RT-PCR products obtained from RNA of C3H 10T1/2 cells using oligonucleotide primers for murine integrin $\alpha 5$ (upper panel) and integrin $\beta 1$ (lower panel).

The probes were analyzed on 2% agarose gel and visualized using ethidium bromide staining. Arrows point to PCR products of expected sizes of 323 and 452 base pairs.

fluent (contact inhibited) and C3H 10T1/2 cells at the logarithmic phase of growth. The levels of ERK1/ERK2 proteins were the same in confluent and sparse cells treated for 4 h with FCS or fibronectin, as compared to the non-stimulated controls. The much higher levels of ERK1/ERK2 were observed only in the cells kept in a medium supplemented with 10% serum throughout their propagation.

ERK's phosphorylation

Table 1 demonstrates that phosphorylation of the peptide substrate (APRTPGGRR) by ERK1/ERK2 from fast growing C3H 10T1/2 cells after FCS or fibronectin stimulation was significantly higher (by Student's *t*-test) as compared to the non-stimulated control.

In the case of confluent cells FCS or fibronectin did not increase of ERK activity. However, the basal level of ERK1/ERK2 phosphorylation was found to be higher in confluent than in fast growing cells.

Effect of fibronectin or serum treatment of 10T1/2 cells on FAK

$p125^{FAK}$ protein

Figure 3 shows the presence of FAK protein in both confluent and sparse C3H 10T1/2 cells non-treated, and serum or fibronectin stimulated cells. The level of FAK was the same in all experimental groups.

FAK phosphorylation

Table 2 shows FAK activity in cells from logarithmic phase of growth, as well as, in confluent contact inhibited. The activity of FAK in fast growing cells was stimulated by fibronectin or

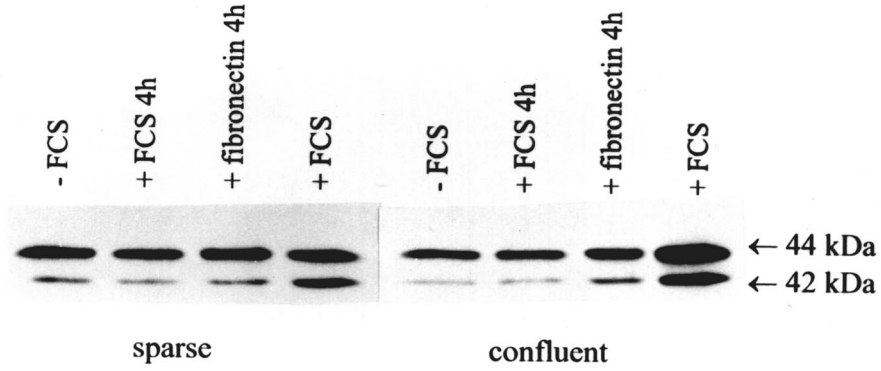

Figure 2. The effect of FCS and fibronectin treatment of C3H 10T1/2 cells on ERK1/ERK2 (band 44 kDa→ERK1, band 42 kDa→ERK2).

Equal probes of lysates were subjected to Western blot analysis. The blots were immunoblotted with anti-ERK1 antibody reactive with ERK1 protein and, to lesser extent, with ERK2 protein.

Table 1. Activity of ERK1/ERK2 in the fast growing and confluent (contact inhibited) C3H 10T1/2 cells

	Non-stimulated	FCS-stimulated	Fibronectin-stimulated
Fast growing (sparse)	3258 ± 1942 n=10	5724 ± 1420* n=10	6923 ± 1593* n=10
Confluent	9765 ± 1326 n=9	6123 ± 1503 n=6	6491 ± 1791 n=7

Proteins from cytosolic lysates were immunoprecipitated with anti-ERK1/ERK2 antibodies, assayed for phosphorylation of MBP peptide, and expressed as c.p.m. ±S.D. *Statistically significant difference as compared to non-stimulated cells (Student's *t*-test), $P \leq 0.01$, n = number of experiments.

FCS. The difference in FAK activity between proteins from stimulated cells and non-stimulated control, were statistically significant (by Student's *t*-test) whereas we have not observed any stimulation of FAK activity in confluent contact inhibited cells after fibronectin or FCS treatment. Similarly as in the case of ERK, the basal phosphorylation by FAK was higher in confluent than in sparse cultures.

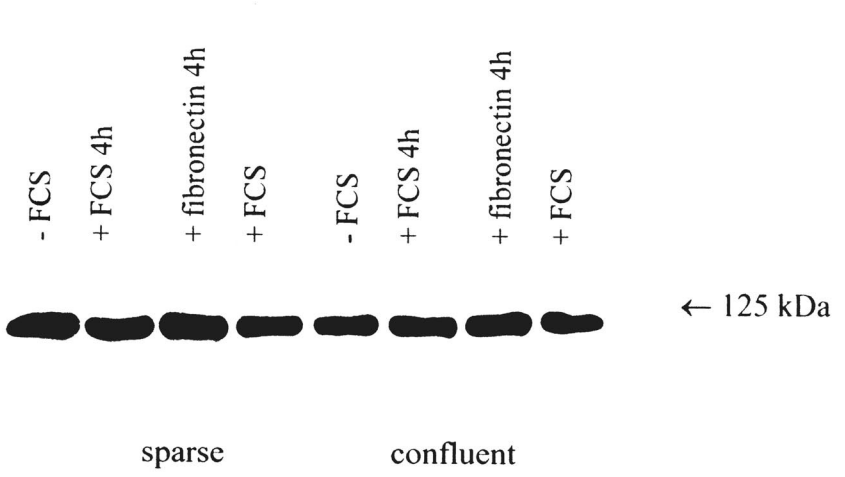

Figure 3. The effect of FCS and fibronectin treatment of C3H 10T1/2 cells on focal adhesion kinase (FAK).

Lysates from each culture were analysed for FAK by Western blotting. Lysates were both immunoprecipitated and then immunoblotted with the same anti-FAK antibody.

DISCUSSION

In this work we focused our attention on the mechanism of ERK/MAP kinase activation or inhibition in the fast growing and confluent C3H 10T1/2 cells. It has been assumed that MAP kinase plays a special role in the mitogenic response and its inhibition is sufficient to account for the arrest of growth as shown before (Viñals & Pouysségeur, 1999; Kerhoff & Rapp, 1997).

We have been able to show that, following stimulation of either sparse or confluent C3H 10T1/2 cells with FCS or fibronectin the sparse cells displayed an increase in the phosphorylation activity of ERK's, whereas the confluent cells did not. This phenomenon could not be accounted for by down or up-regulation of MAP kinase as its presence has been documented by Western blotting both in confluent and fast growing cells regardless of the kind of stimulation.

In a search for the explanation of the lack of stimulated phosphorylating activity of ERK in confluent cell cultures, at least two mechanisms could be considered. The first, as postulated by Viñals & Pouysségeur (1999) implies the action of specific phosphatases in confluent cells. The second, worth considering, is that ERK activity results from the cooperation of signaling pathways from growth factor and integrin receptors as shown before (Assoian, 1997; Chrzanowska-Wodnicka & Burridge, 1996).

Following this assumption the focal adhesion kinase has been assayed by two independent methods following stimulation with either FCS or a soluble form of fibronectin. It is worth to men-

tion that fetal calf serum contains a broad repertoire of receptor stimulating factors including the components of extracellular matrix, whereas fibronectin binds to the integrin receptor.

ally impaired in the contact inhibited C3H 10T1/2 cells, which, in turn, affected the MAP kinase activity, and resulted in growth arrest. Our experimental data support the hypothesis that the per-

Table 2. Activity of FAK in the fast growing and confluent (contact inhibited) C3H 10T1/2 cells

	Non stimulated	FCS stimulated	Fibronectin stimulated
Fast growing (sparse)	316 ± 156 n=5	895 ± 282* n=5	795 ± 198* n=5
Confluent	745 ± 323 n=5	575 ± 292 n=5	738 ± 205 n=5

Proteins from total cell lysates were immunoprecipitated with anti-FAK antibodies, assayed for phosphorylation of (Glu-Tyr)n peptide, and expressed as c.p.m. ± S.D. *Statistical difference as compared to non stimulated (Student's *t*-test), $P \leq 0.01$, n = number of experiments.

The performed experiments showed that FAK phosphorylating ability increased after FCS or fibronectin stimulation only in the fast growing C3H 10T1/2 cells. In confluent cells, substrate phosphorylation by FAK was found at a level similar to that in control non stimulated cells.

Similarly as in the case of ERK, the presence of FAK was found in all tested experimental groups independently of the type of stimulation and the density of the cells, as could be judged from Western blotting analysis. Also transcripts for α_5 and β_1 fragments of integrin receptor genes were seen in all experimental groups, which would suggest the presence of $\alpha_5\beta_1$ receptor for fibronectin in the cells studied.

From the described experiments it appears that all studied elements namely ERK, FAK and integrin receptor are present in confluent C3H 10T1/2 cells, but the signal leading to ERK activation is inefficient. Similar results were obtained by Viñals & Pouysségeur (1999), who found inhibition of p42/p44 MAP kinase activity in vascular endothelial cells following stimulation with FCS or a fibroblast growth factor although Ras and MEK upstream activators of MAP kinase were still active. These authors postulated that inhibition of MAP kinase activation in confluent cells might be mediated by a specific phosphatase.

In view of a link between FAK and MAP kinase and the fact that MAP kinase must be double phosphorylated to be active, we assumed that the pathway from integrin receptor could be functionmanent high FAK activity in confluent C3H 10T1/2 cells affects the MAP kinase function and that, in order to be functionally active, MAP kinase has to be subsequently phosphorylated/dephosphorylated. Therefore, it looks that, in our experiments, the step of dephosphorylation was impaired. Accepting that the escape from contact inhibition is a phenomenon associated with cellular transformation, our results showing that fibronectin or FCS do not stimulate FAK activity in contact inhibited cells, may be an argument in discussion on neoplastic transformation.

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