Phosphorylation of p125^{FAK} and paxillin focal adhesion proteins in src-transformed cells with different metastatic capacity

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Abstract Hamster fibroblasts transformed by Rous sarcoma virus (RSV) display different metastatic potentials that are associated with specific structural features of the v-src oncoprotein. This diverse metastatic activity could be due to various tyrosine phosphorylation levels of specific src protein substrates. To check this hypothesis, phosphorylation of the FAK and paxillin proteins, involved in signal transduction pathways and known as src protein substrates, was tested. It was shown that FAK and paxillin are hyperphosphorylated in the high metastatic cell lines as compared with the phosphotyrosine level of these proteins found in the low metastatic cell lines. In addition, our data confirm that v-src protein plays a direct role in paxillin phosphorylation.

Key words: V-src; Metastasis; Focal adhesion kinase; Paxillin; Hamster cell; Tyrosine phosphorylation

1. Introduction

Syrian hamster fibroblast cell lines transformed in vitro by different stocks of the Schmidt-Ruppin D strain of Rous sarcoma virus were shown to possess diverse metastatic activity in vivo [1]. V-src genes both from high (HM) and low (LM) metastatic cells had significant dissimilarities with the known alleles of the v-src gene. In addition, certain point mutations were revealed between v-src HM and v-src LM [2]. As a consequence of these changes, the tyrosine phosphorylation of specific src substrates could be altered. To verify this possibility, phosphorylation of proteins involved in integrin signal transduction pathways and known as src targets was studied. The comparative phosphorylation analysis of the two focal adhesion proteins (p125^{FAK} and paxillin), in a panel of src-transformed cell lines displaying different metastatic potentials is presented here.

Focal adhesion kinase p125^{FAK} is localized in the areas of cell attachment to components of the extracellular matrix [3,4] and plays a role in the regulation of mature focal adhesion contacts [5].

Paxillin is a 68-kDa protein that interacts with members of the src family of tyrosine kinases, with several cytoskeletal proteins and with focal adhesion kinase. This suggests a function for paxillin as a molecular adapter, responsible for the recruitment of structural and signaling molecules to focal adhesions [6,7].

FAK and paxillin were first revealed in cells transformed by the v-src oncogene as proteins that contain a high level of phosphotyrosine [8,9]. This finding suggested that FAK and paxillin act as substrates of v-src protein and that they might be connected with the transforming capacity of v-src.

The initial activation of FAK is caused by its autophosphorylation at Tyr-397 [10]. pTyr-397 is the interaction site between FAK and the SH2 domain of src [10,11]. Src protein phosphorylates several tyrosine residues of FAK [12]. Consistently, phosphorylation of these tyrosines results in activation of the enzymatic activity of focal adhesion kinase [12].

Paxillin cooperates with three tyrosine kinases: FAK, src and Csk [13–15], but the precise mechanisms responsible for paxillin phosphorylation are not clear.

Here, we show that FAK protein and paxillin are hyperphosphorylated in the HM cell lines in comparison with the phosphotyrosine level of these proteins in the LM cell lines. Our data also suggest that the v-src protein plays a role in paxillin phosphorylation and FAK does not seem to phosphorylate paxillin.

2. Materials and methods

2.1. Cell cultures

The following Syrian hamster cell lines were used: spontaneously transformed embryo fibroblast cell line, STHE; embryo fibroblast cell lines transformed in vitro with different stocks of the Schmidt-Ruppin D strain of RSV from the Russian Cancer Research Center viral collection: HET-SR, HET-SR1, HET-SR8; RSV-transformed embryo fibroblast cell lines, obtained after selection in vivo of HET-SR cells – HET-SR-2SC [1], HET-SR-N-ras cl.6 and HET-SR-2SC-N-ras cl.34 – are HET-SR and HET-SR-2SC parental lines transfected by activated N-ras oncogene [16].

Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 80 μg/ml of gentamicin at 37°C in 5% CO_2.

2.2. Cell lysate preparation and immunoblotting

Cells were lysed by scraping in the following buffer: 20 mM Tris-HCl, pH 7.5; 150 mM NaCl, 10 mM EDTA, 1% NP-40, 1 mM Na_3VO_4, 1 mM DTT, 50 μg/ml of PMSF. The lysates were clarified by centrifugation at 14000×*g* for 15 min at +4°C. Protein quantification was done as described [17]. 20 μg of total cell protein was resuspended in SDS-containing sample buffer, separated by SDS-10% PAGE and transferred onto nitrocellulose (BA 83, Schleicher and Schuell). Monoclonal antibodies (mAbs) anti-p125^{FAK} (UBI) or anti-paxillin (Transduction Laboratories) were used to detect p125^{FAK} or paxillin proteins, respectively. Primary antibodies were detected by

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Abbreviations: FAK, focal adhesion kinase; Csk, carboxy-terminus src kinase; HM, high metastatic; LM, low metastatic; RSV, Rous sarcoma virus; PMSF, phenylmethylsulfonyl fluoride; mAbs, monoclonal antibodies

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horseradish peroxidase-conjugated anti-mouse IgG and subsequent enhanced chemiluminescence (Amersham).

2.3. Immunoprecipitation and immunoblotting

Anti-p125^{FAK} or anti-paxillin mAbs mentioned above were used for the immunoprecipitation. Purified proteins were immunoprecipitated from 0.5 mg of cell lysate and the immune complexes were collected with protein G-Sepharose (Sigma) and washed five times with the lysis buffer. The samples were boiled in the sample buffer and resolved by SDS-10% PAGE. Proteins were detected by immunoblotting with various mAbs and phosphotyrosine was detected by immunoblotting with the anti-phosphotyrosine-specific mAb PY20 (Santa Cruz) non-cross-reactive with phosphoserine or phosphothreonine.

2.4. Quantitative evaluation of the data

Quantification of the data was carried out with computer program Image Quant for Windows, version 3.3. Data were analyzed with Excel for Windows, version 7.0.

3. Results

3.1. p125^{FAK} level and phosphorylation in high and low metastatic cell lines

In this study, cell lines with different metastatic abilities and various productions of v-src protein were used: low metastatic cell lines STHE, HET-SR, HET-SR-N-ras cl.6, HET-SR-2SC-N-ras cl.34; high metastatic cell lines HET-SR1, HET-SR8 and HET-SR-2SC. HET-SR, HET-SR1 and HET-SR8 cell lines contained one copy of integrated RSV provirus and synthesized comparable levels of v-src-specific protein. HET-SR-2SC cell line contains two copies of provirus. HET-SR-N-ras cl.6 and HET-SR-2SC-N-ras cl.34 cell lines had reduced or no expression of v-src gene, respectively.

It is known that migration capacity is one of the general properties of metastatic cells. There are some reports concerning p125^{FAK} function in cell migration [18,19]. In the light of these data, we checked the steady-state level of FAK protein in RSV-transformed hamster fibroblast cell lines with different metastatic activities. It was established that these cells produce slightly different amounts of FAK protein but we did not observe any correlation between this protein production and the metastatic potential of these cell lines (Fig. 1a, Table 1).

The phosphorylation level of the FAK protein, in the same panel of src-transformed cell lines, was tested (Fig. 1b, Table 1). HET-SR1, HET-SR8 and HET-SR-2SC (Fig. 1b, lanes 3–5, respectively) had hyperphosphorylated p125^{FAK} on tyrosine in comparison with the FAK phosphorylation level in the HET-SR cell line (Fig. 1b, lane 2). Spontaneously transformed cells STHE (Fig. 1b, lane 1), which do not express v-src, had some FAK phosphorylation, probably as a result of FAK autophosphorylation. Interestingly, in HET-SR-N-ras cl.6 and HET-SR-2SC-N-ras cl.34 (Fig. 1b, lanes 7 and 6, respectively), where there is low or no expression of v-src gene, FAK phosphorylation was higher than in HET-SR where the synthesis of v-src protein is at a normal level. The amount of immunoprecipitated antigen was not dependent on phosphorylation level of the protein (Fig. 1c).

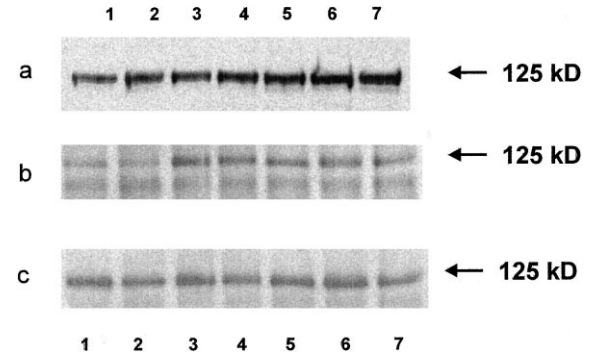

Fig. 1. Immunoblot analysis of FAK protein (a) and its phosphorylation (b) in src-transformed hamster cell lines. a: 20 μg of total cell lysate was immunoblotted with anti-FAK antibodies. b: FAK was immunoprecipitated from total cell lysates, and the immune complexes were immunoblotted with antibodies recognizing phosphotyrosine. c: Phosphotyrosine blot was stripped and reprobed with anti-FAK antibodies to visualize the quantity of FAK protein. The panel of cell lines was: STHE (1); HET-SR (2); HET-SR1 (3); HET-SR8 (4); HET-SR-2SC (5); HET-SR-2SC-N-ras cl.34 (6); and HET-SR-N-ras cl.6 (7).

3.2. Paxillin level and phosphorylation in high and low metastatic cell lines

Paxillin is a focal adhesion component. It interacts with many proteins, some of which are tyrosine kinases but, until now, the tyrosine kinase responsible for phosphorylation of paxillin has remained unknown.

According to Western blot analysis, paxillin was produced in all the v-src-transformed cell lines at approximately the same levels. In spontaneously transformed STHE cells, the amount of this protein was reduced (Fig. 2a, Table 1).

It was suggested earlier that src protein kinase is essential for paxillin phosphorylation [21]. The tyrosine phosphorylation of paxillin in v-src-transformed cell lines with different levels of oncoprotein production and different metastatic properties was studied (Fig. 2b, Table 1).

In the high metastatic cell lines HET-SR1, HET-SR8 and HET-SR-2SC, we found that paxillin was hyperphosphoryl-

Table 1
Relative amount of Fak and paxillin proteins and level of its phosphorylation in the analyzed cell lines

Cell line	Spontaneous metastatic activity[a]	Relative amount of v-src protein [20]	Relative amount of FAK protein	FAK phosphorylation[b]	Relative amount of paxillin protein	Paxillin phosphorylation[b]
HSR	low	1.00	1.00	1.00	1.00	1.00
HSR1	high	1.50	0.85	1.77	0.90	3.08
HSR8	high	1.37	1.13	2.28	1.10	6.30
HSR-2SC	high	1.30	1.35	1.60	1.39	3.25
HET-SR-N-ras cl.6	low	0.22	1.47	1.29	1.36	1.43
HSR-2SC-N-ras cl.34	low	0.00	1.57	1.17	1.39	0.00
STHE	low	0.00	0.74	0.84	0.55	0.00

[a]Low: metastatic nodules were usually not identified after inoculation of hamsters; high: between 70 and 200 metastatic nodules in the lung of inoculated hamsters.
[b]Relative phosphorylation of FAK or paxillin normalized to the production of these proteins in corresponding cell lines.

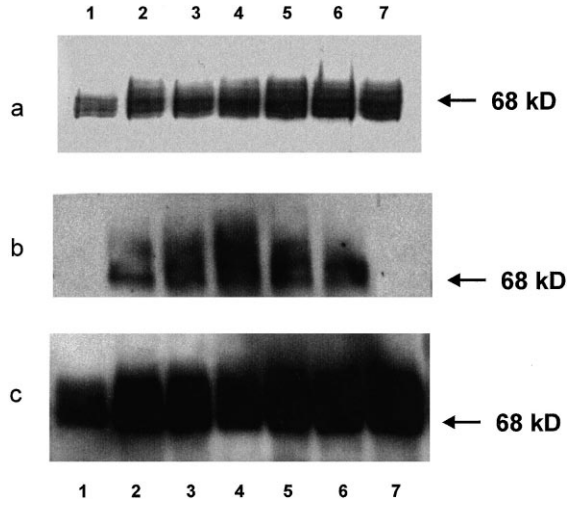

Fig. 2. Immunoblot analysis of paxillin protein (a) and its phosphorylation (b) in src-transformed hamster cell lines. a: 20 μg of total cell lysate was immunoblotted with anti-paxillin antibodies. b: Paxillin was immunoprecipitated from total cell lysates, and the immune complexes were immunoblotted with antibodies recognizing phosphotyrosine. c: Phosphotyrosine blot was stripped and reprobed with anti-paxillin antibodies to visualize the quantity of paxillin protein. The panel of cell lines was: STHE (1); HET-SR (2); HET-SR1 (3); HET-SR8 (4); HET-SR-2SC (5); HET-SR-N-ras cl.6 (6); and HET-SR-2SC-N-ras cl.34 (7).

ated on tyrosine (Fig. 2b, lanes 3–5, respectively). Paxillin phosphorylation was not detectable in STHE (Fig. 2b, lane 1) and HET-SR-2SC-N-ras cl.34 (Fig. 2b, lane 7) cell lines, although these cells contain substantial amounts of paxillin protein (Fig. 2a).

Thus, in the majority of src-transformed cells, the phosphorylation of paxillin is correlated with the level of v-src protein.

4. Discussion

Transformation of cells by active variants of src protein leads to a pronounced increase in tyrosine phosphorylation of a number of cellular proteins, including several signal transducing factors. Here, we report a novel attempt to study the production and phosphorylation of $p125^{FAK}$ and paxillin, involved in the integrin signal transducing pathway. We took advantage of the described model system of hamster cells transformed by two isoforms of v-src oncogene [2]. The panel of analyzed cells includes lines with different metastatic potentials in vivo and various levels of v-src protein.

It is known that FAK plays a role in cell motility and that overexpression of this kinase increases cell migration on fibronectin [18,22]. We have studied the steady-state level of FAK in RSV-transformed hamster fibroblasts to reveal any possible correlation existing between FAK synthesis and metastatic properties of the cells. It has been established that these cells produce different amounts of FAK protein. However, an association of this parameter with the metastatic status of the cell lines could not be detected (Table 1).

A correlation was found between the metastatic phenotype of cells and the tyrosine phosphorylation level of $p125^{FAK}$. In highly metastatic cell lines, this protein was 1.6–2.2 times more phosphorylated than in the low metastatic cell lines (Table 1).

There is strong evidence concerning the role of src in the phosphorylation of FAK [12]. $p125^{FAK}$ phosphotyrosine-397 (the site of autophosphorylation) is the interaction site between FAK and the SH2 domain of src [10,11]. Src phosphorylates FAK on Tyr-576 and Tyr-577, localized in the FAK kinase domain, and on Tyr-407 [12] and Tyr-925 [23]. According to our data, v-src is probably not the main factor responsible for FAK phosphorylation, at least in the transformed cells we studied. For instance, in the cells over-transfected by N-ras, leading to a reduction or an elimination of v-src expression (HET-SR-N-ras cl.6 and HET-SR-2SC-N-ras cl.34), there is no association with significant loss of FAK phosphotyrosine. We suggest that in these clones the phosphorylation of FAK is associated with an increased steady-state level of cellular src [24] and/or appears to be associated with the effect of constitutively activated N-ras.

The amount of paxillin in the analyzed cells was not connected with their metastatic potential in vivo (Table 1), but a significant correlation was found between the level of tyrosine phosphorylation of this protein and the biological properties of cell lines. In all highly metastatic cell lines, paxillin was 3–6 times more phosphorylated than in the low metastatic HET-SR cells (Table 1).

The mechanisms involved in paxillin phosphorylation are not clear. Previously, it was demonstrated that at least three tyrosine kinases are able to phosphorylate paxillin, namely $p125^{FAK}$, $pp60^{src}$ and Csk [25]. Our data suggest that in the cell lines we studied, the phosphorylation of paxillin is due to v-src (this is not the case for FAK in these cell lines). In cells with suppressed v-src gene expression (HET-SR-2SC-N-ras cl.34) tyrosine phosphorylation of paxillin was not detected (Fig. 2b, Table 1). We suggest that in the analyzed cell lines, FAK is not essential for paxillin phosphorylation, because this protein remains unphosphorylated in cells containing a significant amount of $p125^{FAK}$.

In the study presented in this paper, we surmised that alterations in the phosphorylation level of target proteins, modulating some signal transduction processes, might exert influence on many different biological properties of the cells, such as metastatic activity.

The comparative analysis of downstream components, interacting with src in signal transducing pathways, might help to understand some of the molecular events underlying metastatic processes.

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