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Research Article

Protein kinase-dependent overexpression of the nuclear protein pirin in c-*JUN* and *RAS* transformed fibroblasts

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Abstract. Signalling via the protein kinase Raf-MEK-ERK pathway is of major importance for transformation by oncogenes. To identify genes affected by inhibition of this pathway, c-*JUN* transformed rat fibroblasts were treated with a MEK1 inhibitor (PD98059) and subjected to two-dimensional gel electrophoresis after cell lysis. Gene products with expression influenced by MEK1 inhibition were determined by mass spectrometry of fragments from in-gel tryptic digestions. The expression of pirin, a nuclear factor I-interacting protein, was lowered after inhibition of MEK1. Western blot analysis revealed increased expression of pirin in *RAS* and c-*JUN* transformed cells in the absence of PD98059. Inhibition of MEK1 also led to reduced expression of α-enolase, phosphoglycerate kinase, elongation factor 2 and heterogeneous nuclear ribonucleoprotein A3, the latter two being detected as truncated proteins. In contrast, the level of ornithine aminotransferase was increased. We conclude that inhibition of MEK1 results in major alterations of protein expression in c-*JUN* transformed cells, suggesting that this pathway is important for oncogene-induced phenotypic changes.

Key words. Two-dimensional gel electrophoresis; mass spectrometry; cell transformation.

Most oncogenes encode signal transduction molecules which induce the activity of intracellular signalling pathways [1]. One route is the Raf-MEK-ERK pathway [2] for which inhibition stops transformation of NIH 3T3 cells by *RAS* [3].

It is assumed that activation/deactivation of signal transduction pathways leads to alterations in the transcriptional activity, but little is known about which genes are affected. One approach to solving this problem is to screen for changes in gene expression. The protooncogene c-*JUN* has transforming potential in mammalian cells, either in cooperation with activated *RAS* in primary embryonic fibroblasts or alone in es-

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tablished rodent cell lines [4]. Some of the genes affected by transformation with c-*JUN* have been identified via screening of complementary DNA libraries [5].

We now report on two-dimensional (2D) gel electrophoresis as a tool to examine if inhibition of MEK1 in c-*JUN* transformed cells leads to major alterations in protein expression. We have used mass spectrometry to identify relevant proteins, and we observe specific patterns.

Materials and methods

Cell lines and reagents. FR3T3 is a clonal, immortalized fibroblast cell line [6] from which Jun3 was derived by transfection with a human c-*JUN* cDNA expression vector [5]. The FR3T3-RAS cell line [7] was isolated after transfection with the T24-H-*ras* oncogene and shows the characteristics of transformed cells. Cells were grown in Dulbecco's modified Eagle's Medium supplemented with 5% fetal calf serum. The MEK1 inhibitor PD98059 (New England Biolabs) was used at 40 μM.

Preparation of cell extracts. The cell pellet was dissolved in 8 M urea containing 4% w/v CHAPS, 40 mM Tris, 65 mM DTT and a trace of bromophenol blue.

Preparative 2D electrophoresis with immobilized pH-gradient strips. 2D gel electrophoresis was performed [8] using precast immobilized pH-gradient (IPG) strips with a nonlinear gradient (pH 3–10, Pharmacia) in the first dimension of isoelectric focusing (IEF). Samples were applied via rehydration of the strips in sample solution overnight. Before application, samples were diluted to 350 μl with 8 M urea containing 2% CHAPS, 2% IPG buffer (pH 3–10, nonlinear), 0.3% DTT and a trace of bromophenol blue. Typically, 500 μg of protein was loaded on each IPG strip, and focusing was carried out for 48000 Vh. After IEF separation, the strips were immediately equilibrated for 2×15 min in 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% glycerol and 2% SDS. DTT (2%) was included in the first equilibration solution, and 2.5% (w/v) iodoacetamide was added in the second equilibration step to alkylate thiols. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 0.75-mm thick, 10–13% SDS/polyacrylamide gradient gels with piperizine diacrylamide as cross-linker. The strips were held in position by 0.5% agarose dissolved in SDS/Tris running buffer, and electrophoresis was carried out at constant current (40 mA/gel) and temperature (10 °C). After electrophoresis, gels were stained with Coomassie brilliant blue R-250.

In-gel proteolytic digestion. Polypeptide spots were excised, destained by end-over-end mixing in 12 ml of 30% methanol for 72 h (room temperature) and washed twice for 30 min with 150 μl of acetonitrile/20 mM ammonium bicarbonate (1:1), pH 8. Each gel spot was sliced into four pieces and rehydrated in 10 μl of 20 mM ammonium bicarbonate, pH 8, containing trypsin (1 pmol/μl) (Promega, modified). An additional 10 μl of digestion buffer without trypsin was added. After incubation at 37 °C for 20 h, the condensate was collected by centrifugation, and the liquid phase was transferred into a new tube. Tryptic peptides remaining in the gel matrix were extracted 2×30 min at 30 °C with 150 μl of 60% aqueous acetonitrile containing 0.1% trifluoroacetic acid (TFA). The combined extracts were concentrated to about 5 μl using an Eppendorf speed-vac centrifuge [9].

Electrospray mass spectrometry. Mass spectrometric data for tryptic peptides from in-gel digestions were obtained in collision-induced dissociation (CID) experiments using a Finnigan-MAT LCQ ion-trap instrument. Samples were introduced via a microcapillary reverse phase high performance liquid chromatography (RP-HPLC) system [10]. The program SEQUEST [11] was used to correlate the experimental CID spectrum with theoretical CID spectra of tryptic peptides derived from proteins present in different databases.

Matrix-assisted laser description ionization (MALDI) mass spectrometry. Tryptic peptide mass mapping was carried out with a Perseptive Voyager-DE STR workstation operated in reflectron mode with delayed extraction. A 10 mg/ml solution of 2,5-dihydroxybenzoic acid in aqueous 70% acetonitrile was employed as matrix.

Western blot analysis. Rinsed cells were solubilized in SDS sample buffer (2.3% SDS, 0.065 M Tris-HCl, pH 6.8, 5% 2-mercaptoethanol and 10% glycerol) and boiled for 3 min. After SDS-PAGE, proteins were electroblotted onto polyvinylidene difluouride (PVDF) membranes and probed with human anti-pirin antibody [12] at 1:2000 dilution. Enhanced chemoluminescence substrate (Amersham) was used for the detection.

Results

Identification of PD98059 affected polypeptides in Jun3 cells. 2D gel electrophoresis was used to screen for differences in protein expression in c-*JUN* transformed cells grown in the presence and absence of a MEK1 inhibitor, PD98059 [13]. Polypeptides affected by inhibition of MEK1 are indicated in figure 1. Fragment sequences were determined for six polypeptides after in-gel digestion with trypsin and analysis with electrospray ionization (ESI) CID mass spectrometry (table 1). The levels of α-enolase [7], pirin, phosphoglycerate kinase, elongation factor 2 (EF-2) and heterogeneous nuclear ribonucleoprotein (hnRNP) A3 decreased (fig. 1, left) after treatment with PD98059, whereas the level of ornithine aminotransferase increased (fig. 1, right).

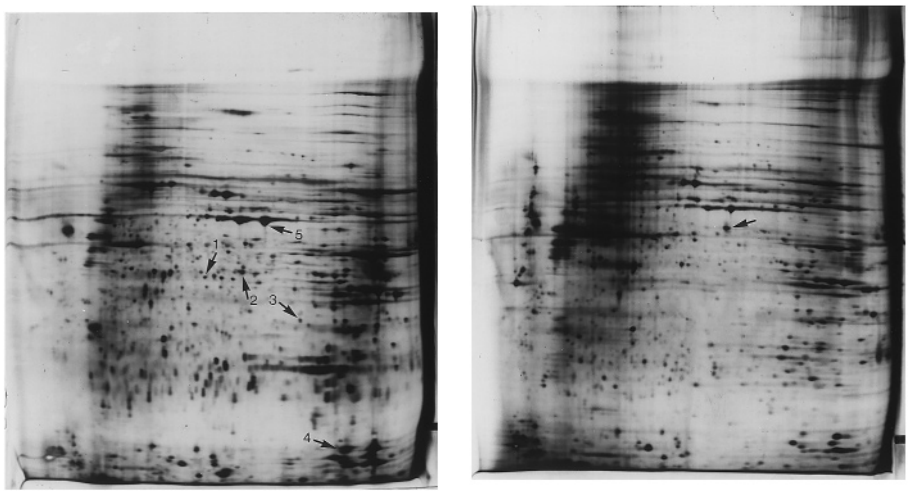

Figure 1. 2D electrophoresis pattern of c-*JUN* transformed FR3T3 cells grown in the absence (left) and presence (right) of the MEK1 inhibitor PD98059. Protein (150 µg) was loaded, IEF at pH 3–10 nonlinear, SDS-PAGE 10–13% and silver staining. For spot identification, cf. table 1.

Characterization of truncated forms of EF-2 and hnRNP A3. The molecular weight of the protein in spot 2, based on migration in SDS-PAGE, was 39 kDa (fig. 1). Seven fragments from this material were analyzed for amino acid sequence, and they were all found to belong to EF-2. The reported molecular weight of EF-2 is 95.3 kDa, and the fragments identified in the region spanning positions 573–858 (table 1) correspond to the C-terminal portion of the parent molecule. The protein in spot 6 (fig. 1) was identified as hnRNP A3, but the molecular weight was approximately 20 kDa in contrast to the expected 39.7 kDa. The sequence of two tryptic peptides was determined and found to match segments in the N-terminal portion of the hnRNP A3 structure (residues 37–52 and 152–161, table 1). The mass of additional peptides match tryptic fragments within the first 216 N-terminal residues, whereas no peptides corresponding to the C-terminal part of the protein were found (table 1). This is in agreement with a molecular weight of 20 kDa, showing spot 6 to correspond to a C-terminally truncated form of hnRNP A3.

Expression of pirin in normal and transformed rat fibroblasts. One of the polypeptides identified using 2D gel electrophoresis and mass spectrometry was pirin, a recently described nuclear factor 1 (NF-1) interacting protein [12]. Pirin expression was analyzed by Western blots of normal and transformed rat fibroblasts (fig. 2). Pirin expression is stronger in the transformed cells, and in addition to the 33-kDa form [12], a larger polypeptide of approximately 41 kDa was also detected after long exposure of the film (fig. 2). This 41-kDa form was also more strongly expressed in the transformed cells.

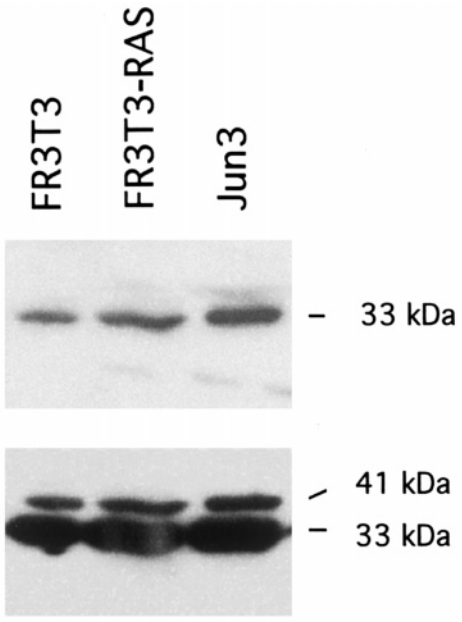

Figure 2. Western blot analysis of pirin expression in FR3T3 cells and *RAS* and c-*JUN* transformants of FR3T3, bottom panel after prolonged exposure.

Table 1. Proteins identified in c-*jun* transformed cells.

Spot (cf. Fig. 1)	Protein identified	Residues identified by			
		MALDI-MS		ESI-MS	
1	**phosphoglycerate kinase**	6–14 15–21 75–85 91–96 156–170 171–183	184–191 192–198 199–215 267–278 279–296	75–85 156–170 171–183 192–198 267–278	
2	**elongation factor 2**	1–16 151–162 401–407 573–580 595–601 606–625	639–647 668–676 677–688 717–726 768–785 786–801	573–580 606–625 639–647 668–676 717–726 786–801 846–858	
3	**pirin**	1–14 126–135 145–160 236–247		6–14 126–135 145–160 236–247	
4	**hnRNP A3**	1–17 36–57 110–127	144–161 166–182 201–216	37–52 152–161	
5	α-enolase	9–14 15–27 32–49 54–63 80–102 81–104 120–131 162–178 202–220	221–227 233–252 269–280 285–305 306–325 335–342 358–371 406–411	202–220 221–227 269–280 406–411	
	ornithine aminotransferase	1–16 50–64 114–129 170–180	332–351 363–372 427–434	1–16 50–64 114–129 170–180	332–351 363–372 427–434

Bold type: novel identification. Regular type: previously identified protein [7]. Spots 1–5 are indicated in figure 1, left panel. Ornithine aminotransferase position, right panel (arrow).

Discussion

2D gel electrophoresis is efficient in studies of changes in cellular protein expression and can detect differences in expression levels, truncated forms and posttranslational modifications of proteins. We have employed 2D gels to study protein expression in c-*JUN* transformed fibroblasts before and after treatment with the MEK1 inhibitor PD98059. Two proteins identified appeared to be truncated. It has been reported that cytokeratins accumulate as processed forms of lower molecular weight in tumor cells [14, 15] and that the increased degradation rate is caused by increased expression of proteasome subunits and of various ubiquitinylation components [16]. We found high levels of a C-terminal fragment of EF-2 in c-*JUN* transformed cells, and the amount of this fragment decreased after inhibition of MEK1 with PD98059. Similar data were found for an N-terminal segment of hnRNP A3. These findings suggest that a consequence of activation of the Raf-MEK-ERK pathway in transformed cells is increased protein degradation.

Pirin was recently described as an evolutionarily conserved nuclear protein which interacts with NF-1 [12]. Pirin was found to be weakly expressed in normal human tissues. However, strong pirin expression was detected in c-*JUN* and *RAS* transformed rat fibroblasts, although decreased pirin expression was observed in c-*JUN* transformed cells after inhibition of MEK1 by PD98059. Low levels of a larger polypeptide (41 kDa) were also detected in Western blot experiments. In human cells, in addition to the 33-kDa form of pirin, a 58-kDa protein also bound anti-pirin antibody [12]. The observed upregulation of pirin in transformed cells is interesting, since it can potentially lead to an altered NF-1 function.

In conclusion, using 2D gel electrophoresis and mass spectrometry, we have identified changes in protein expression after inhibition of MEK1 in c-*JUN* transformed cells, an important first step in determining potential target genes downstream of MEK1 in the signal transduction pathway.

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