Increased expression of α-enolase in c-*jun* transformed rat fibroblasts without increased activation of plasminogen

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Abstract Two-dimensional gel electrophoresis was used to identify polypeptides differentially expressed between normal and c-*jun* transformed rat fibroblasts. The level of a 49 kDa polypeptide was 3-fold elevated in c-*jun* transformed cells. Sequence analysis by ion trap mass spectrometry identified the polypeptide as rat α-enolase. Enolase functions as a cell surface receptor for plasminogen, suggesting that upregulation may increase plasminogen activation and cell surface proteolysis important for tumor growth. However, no difference was observed between normal and transformed cells in formation of plasmin, suggesting that upregulation of α-enolase may contribute to an increased metabolic capacity, but not to increased plasminogen activation.

Key words: Two-dimensional gel electrophoresis; Mass spectrometry; Cell transformation; Plasminogen activation

1. Introduction

c-Jun is the major component of the AP-1 transcription factor. The activity of c-Jun is regulated by serine/threonine phosphorylations, triggered by activation of receptor Tyr kinases, Src-family kinases and H-Ras [1,2]. Overexpression of c-Jun induces transformation of rat fibroblasts [3].

Little is known about c-Jun target genes and the outcome of signal transduction pathways acting via c-Jun. Decreased expression of cellular matrix proteins has been reported in c-*jun* transformed cells and in *ras*-oncogene transformed cells [4]. *Ras* transformed cells show invasive characteristics [5,6], and have been shown to overexpress metalloproteases and urokinase plasminogen activator [7,8].

For some cells, binding of plasminogen is rate-limiting for cell surface mediated activation of plasminogen by urokinase [9]. Plasminogen receptors are present in large numbers and show relatively low affinity for plasminogen. The receptors recognize the lysine binding sites associated with the kringle domains of plasminogen [9]. Proteins with carboxyl-terminal lysine residues such as α-enolase and annexin II show plasminogen binding activities [10]. Plasminogen binding to cells enhances plasmin activity by augmenting plasminogen activation, and protecting plasmin from inactivation by inhibitors [10].

In this study, we have used two-dimensional gel electrophoresis to screen for genes differentially expressed between normal and c-*jun* transformed fibroblasts. A 49 kDa protein overexpressed in transformed cells was identified by electrospray collision induced dissociation ion trap mass spectrometry of its tryptic peptides. The functional significance of this protein was examined with regard to reports showing that it is a cell surface plasminogen receptor.

2. Materials and methods

2.1. Cell lines and reagents

FR3T3 is an immortalized rat fibroblast cell line [11]. Jun1 and Jun3 were derived from FR3T3 by transfection with a human c-*jun* cDNA expression vector [4]. FR3T3-RAS was derived from FR3T3 by transfection with T24-H-*ras*.

Human Glu-plasminogen was prepared by affinity chromatography of plasma on lysine-Sepharose [12]. Human one-chain tissue plasminogen activator was purified from melanoma cell cultures [13]. Flavigen-Pli (2AcOH. H-D-But-CHT-lys-PNA) was obtained from Biopool (Umeå, Sweden). Resolyte (pH 4–8) was from BDH, Poole, UK.

2.2. Peptide synthesis

Peptides were synthesized with Fmoc chemistry using an Applied Biosystems 430A peptide synthesizer. Cleavage of the peptide from the resin and removal of the side chain protecting groups were carried out using reagent K. The peptides were purified by HPLC on Vydac C-8 with a gradient of acetonitrile in 0.05% aqueous TFA, and their masses were confirmed by electrospray ionization mass spectrometry on a Finnigan-MAT TSQ 7000.

2.3. Plasminogen activation assay

Cells were non-enzymatically detached in Cell Dissociation Solution (Gibco, Paisley, UK) for 15 min at 37°C. Cells were counted in a hemocytometer and resuspended in 0.1 M Na-phosphate, pH 7.3. The cell suspension was added to quartz or plastic microtiter plates and reagents were added to final concentrations of 0.3 μM plasminogen, 0.4 nM tPA and 400 μM Flavigen-Pli in a volume of 250 μl. 6-Aminohexanoic acid (ε-aminocaproic acid; EACA) was added, where indicated, to a final concentration of 40 mM. All samples were run in duplicate. Plates were incubated at 30°C, and the absorbance at 450 nm was monitored every 5 min in a 2001 Anthos (Salzberg, Austria) microplate reader.

2.4. Two-dimensional electrophoresis and image analysis

Cells were labelled with [^{35}S]methionine for 16 h in methionine-free medium containing 5% fetal calf serum. Cell extracts were prepared as described [14]. Two-dimensional gel electrophoresis [15,16] utilized Resolyte (2%, pH 4–8) for isoelectric focusing, and 10–13% gradient SDS polyacrylamide gels for the second dimension. Gels were dried and exposed to X-ray film, or stained with Coomassie brilliant blue for subsequent in-gel digestion of protein spots.

Autoradiography films were scanned at 100 μm resolution using a

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Molecular Dynamics laser densitometer. Data were analyzed using the PDQUEST software [17] (Pharmacia Biotech AB). Background was subtracted, peaks located and the individual polypeptide quantities were expressed as ppm of the total integrated optical density.

2.5. In-gel digestion and mass spectrometric sequence analysis

Gel pieces were excised and destained by end-over-end mixing in 12 ml 30% methanol for 48 h (room temperature), washed twice for 30 min with 150 ml of 1:1 acetonitrile/0.1 M ammonium bicarbonate, pH 8. The washed gel pieces were dried in Eppendorf tubes in a Savant Speed Vac.

Rehydration of gel pieces, with 0.1 M ammonium bicarbonate, pH 8, containing 0.2 μg/μl (8 pmol/μl) trypsin (modified trypsin from Boehringer Mannheim, dissolved in 1 mM HCl and stored at −20°C in aliquots until use), was achieved in a volume just enough to cover the pieces. Additional buffer without trypsin was added if necessary. After incubation at 37°C for 20 h, the condensate was collected by spinning the tubes briefly and excess liquid was removed into a new Eppendorf tube. Peptides remaining in the gel matrix were extracted with 150 μl 60% aqueous acetonitrile, 0.1% formic acid at 30°C for 30 min. The combined extracts were dried under a stream of nitrogen.

All mass spectrometric data regarding tryptic peptides from in-gel digestions were obtained from collision induced dissociation (CID) spectra with a Finnigan-MAT LCQ ion trap mass spectrometer after introduction via a polyamide coated fused silica microcapillary HPLC [18].

3. Results

3.1. Overexpression of a 49 kDa protein in c-jun transformed FR3T3 cells

Two-dimensional gel electrophoresis was used to screen for differences in protein expression between FR3T3 cells (an untransformed rat fibroblast cell line) and two c-*jun* transformed derivatives of FR3T3 (Jun1 and Jun3). Autoradiographs of [^{35}S]methionine labelled polypeptides were scanned, and the images analyzed with the PDQUEST software [17].

Increased expression of two polypeptides with molecular weights of 46 kDa but with different p*I*s was observed in Jun1 (Fig. 1) and Jun3 cells (not shown). Quantitation by PDQUEST showed an approximately 3-fold increase of the major (basic) 49 kDa protein and an approximately 6-fold increase of the acidic protein. A number of other differences were also observed. The cytoskeletal proteins tropomyosin-2 and -3 were down-regulated in c-*jun* transformed cells, whereas proliferating cell nuclear antigen (PCNA) was upregulated (Fig. 1).

The 49 kDa proteins were more prominently expressed in FR3T3-RAS cells, a derivative of FR3T3 cells which expresses 12V-H-Ras (data not shown).

3.2. Identification of the basic 49 kDa protein as α-enolase

The major form of the 49 kDa protein was digested in the gel with trypsin and fragments were analyzed by electrospray CID ion trap mass spectrometry. The masses of the tryptic fragments (Table 1) showed 13 perfect matches to the published sequence of rat α-enolase. All except peptide fragments 8 and 11 were also verified by sequence interpretation from the CID spectra.

3.3. Plasminogen activation by c-jun transformed and non-transformed cells

Cells were incubated in the presence of plasminogen, tPA and a plasmin chromogenic substrate. The interactions of plasminogen and tPA with FR3T3 cells induced a cell concentration dependent increase in the rate of plasmin generation (Fig. 2A). Activation was inhibited by the lysine analogue EACA, and did not occur in the absence of tPA or plasminogen.

Comparison of plasminogen activation by c-*jun* transformed and parental FR3T3 cells (Fig. 2B) showed no difference in the efficiency of plasminogen activation in the presence of tPA. Jun1 cells, but not FR3T3 cells, showed weak but reproducible plasminogen activation in the absence of tPA.

3.4. An α-enolase peptide does not inhibit plasminogen activation

A peptide (C13) corresponding to a 13 residue C-terminal portion of α-enolase was added to Jun1 cells and the corresponding 12 residue peptide lacking the C-terminal lysine residue, desLys-C13, was also studied as a control (Fig. 2C). No effect on plasminogen activation was observed using either peptide. Note that a plastic microtiter plate was used for

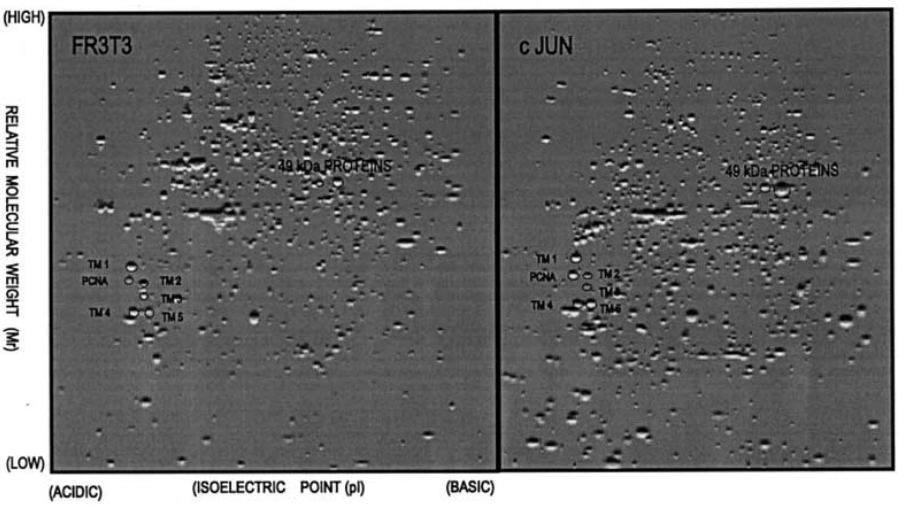

Fig. 1. Autoradiographs of two-dimensional gel separations of [^{35}S]methionine labelled polypeptides from FR3T3 and Jun1 cells. The two 49 kDa polypeptides found to be increased in c-*jun* transformed cells are encircled. TM, tropomyosin; PCNA, proliferating cell nuclear antigen.

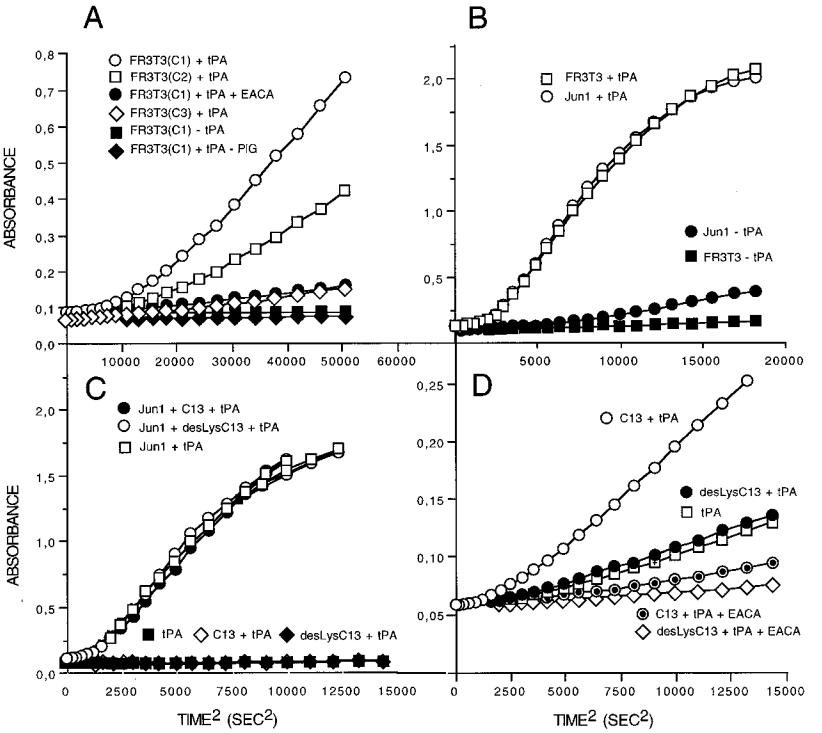

Fig. 2. Cell dependent activation of plasminogen. Cells were incubated with tPA, plasminogen (PlG) and EACA as indicated. Plasmin activity was detected by following the cleavage of a chromogenic substrate (Flavigen Pli). The absorbance values were plotted as a function of time squared, and the slopes of the curves represent estimates of plasminogen activation rates. A: Various numbers of FR3T3 cells were added to a quartz microtiter plate (C1: 5×10^4 cells; C2: 2×10^4 cells; C3: 5×10^3 cells) in a 250 μl reaction. B: FR3T3 and Jun1 cells (3×10^5 cells per well) were incubated in the presence or absence of tPA. A quartz microtiter plate was used. C: 2.5×10^5 Jun1 cells were added to each well of a plastic microtiter plate, and incubated in the presence or absence of 30 μM peptide. Peptide C13 corresponds to the 13 C-terminal amino acids of rat α-enolase, peptide desLys-C13 was identical to peptide C13 except that it lacked the C-terminal lysine. D: Peptide C13 and desLys-C13 were added to glass wells and incubated overnight. After washing away excess peptide, plasminogen, tPA and Flavigen Pli were added. Note the activation of plasminogen by peptide C13 (13 C-terminal amino acids of rat α-enolase) and the inhibition by 40 mM EACA.

this experiment, since the peptides adhered to the glass substrate (see Fig. 2D).

To examine whether the α-enolase C-terminus stimulates plasminogen activation, peptide 13C was immobilized on a glass surface. Peptide C13 stimulated plasminogen activation by tPA, and this stimulation was sensitive to EACA (Fig. 2D). DesLys-C13 did not stimulate plasminogen activation.

4. Discussion

Using two-dimensional gel electrophoresis and ion trap mass spectrometry, we found that α-enolase expression was increased in c-*jun* and *ras* transformed fibroblasts. Recombinant α-enolase binds plasminogen [19] and α-enolase is known to be a cell surface plasminogen receptor on some cell types [20,21]. The finding that α-enolase is upregulated in transformed cells raised the question whether cell-associated plasminogen activation is increased. Increased formation of plasmin at the cell surface would lead to increased proteolysis, important for tumor cell invasion and metastasis.

Table 1
Mass analysis of tryptic fragments

Peptide	MS found	MS calculated[a]	Residues[a]
1	1648.0	1647.8	343–357
2	1406.6	1406.7	15–27
3	2738.1	2736.3	80–102
4	2610.0	2608.2	81–102
5	1961.1	1960.9	202–220
6	2047.2	2047.0	306–325
7	1805.1	1804.9	32–49
8	1439.5	1439.7	269–280
9	2209.2	2208.0	233–252
10	1929.0	1929.0	162–178
11	3024.4	3023.5	372–399
12	2368.5	2367.2	372–393
13	3046.2	3043.4	281–305

[a]Based on rat α-enolase (SwissProt.r34 accession number: P04764).

FR3T3 and Jun1 cells enhanced the activation of plasminogen to plasmin in the presence of tPA. This effect was inhibited by EACA, suggesting that binding of plasminogen to lysine residues on cell surfaces was necessary for enhancement. However, no difference in stimulation of plasminogen activation by tPA was observed between FR3T3 cells and their c-*jun* transformed derivatives, and further experiments showed that a peptide corresponding to the C-terminal portion of α-enolase also did not affect plasminogen activation by c-*jun* transformed cells. Together, these data suggest that α-enolase is not an important plasminogen cell surface receptor on rat fibroblasts. Plasminogen receptors are characterized by their relatively low affinity and their exceptionally high density on many cells. It is possible, therefore, that changes in the levels of a single type of plasminogen receptor do not lead to detectable differences in cell-associated plasminogen activation.

As expected [20,21], control experiments showed that immobilized α-enolase C-terminal peptide stimulates plasminogen activation by tPA.

Jun1 cells, but not non-transformed FR3T3 cells, activated plasminogen in the absence of tPA. This result is consistent with reports showing that transformed fibroblasts express plasminogen activators [22].

Two-dimensional gel electrophoresis of proteins is a valuable approach to studying changes in gene expression in biological systems. One disadvantage of this technique was difficulties in monitoring changes in the expression of multiple polypeptides. This problem has been overcome by the availability of software such as PDQUEST [17] and Melanie II [23] which have made computer analysis of gel patterns possible. Another disadvantage was the difficulty of obtaining sequence information from the limited amounts of protein on the gels, a problem solved by the increased sensitivity of mass spectrometry. We conclude from this study identifying α-enolase that two-dimensional gel electrophoresis in combination with mass spectrometry can be used to rapidly identify changes at the translational and posttranslational levels.

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References

[1] Binétruy, B., Smeal, T. and Karin, M. (1991) Nature 351, 122–127.
[2] Smeal, T., Binétruy, B., Mercola, D., Grover-Bardwick, A., Heidecker, G., Rapp, U.R. and Karin, M. (1992) Mol. Cell. Biol. 12, 3507–3515.
[3] Schütte, J., Minna, J.D. and Birrer, M.J. (1989) Proc. Natl. Acad. Sci. USA 86, 2257–2261.
[4] Mettouchi, A., Cabon, F., Montreau, N., Vernier, P., Mercier, G., Blangy, D., Tricoire, H., Vigier, P. and Binetruy, B. (1994) EMBO J. 13, 5668–5678.
[5] Thorgeirsson, U.P., Turpeenniemi-Humanen, T., Williams, J.E., Westin, E.H., Heilman, C.A., Talmadge, J.E. and Liotta, L.A. (1985) Mol. Cell. Biol. 5, 259–262.
[6] Bondy, G.P., Wilson, S. and Chambers, A.F. (1985) Cancer Res. 45, 6005–6009.
[7] Bortner, D.M., Langer, S.J. and Ostrowski, M.C. (1993) Crit. Rev. Oncogen. 4, 137–160.
[8] Lindblom, A. and Linder, S. (1996) Crit. Rev. Oncol./Hematol. 24, 71–96.
[9] Namiranian, S., Naito, Y., Kakkar, V.V. and Scully, M.F. (1995) Biochem. J. 309, 977–982.
[10] Redlitz, A. and Plow, E.F. (1995) Baillière's Clin. Haematol. 8, 313–327.
[11] Seif, R. and Cuzin, F. (1977) J. Virol. 24, 721–728.
[12] Wiman, B. and Rånby, M. (1986) Thromb. Haemost. 55, 189–193.
[13] Wallén, P., Pohl, G., Bergsdorf, N., Rånby, M. and Jörnvall, H. (1983) Eur. J. Biochem. 132, 681–686.
[14] Hunt, D.F., Michel, H., Dickinson, T.A., Shabanowitz, J., Cox, A.L., Sakaguchi, K., Appella, E., Grey, H.M. and Sette, A. (1992) Science 255, 1261–1263.
[15] Franzén, B., Okuzawa, K., Linder, S., Kato, H. and Auer, G. (1993) Electrophoresis 14, 382–390.
[16] O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[17] Anderson, N.L. (1991), pp. 17–97, Large Scale Biology Press, Rockville, MD.
[18] Garrels, J.I. (1989) J. Biol. Chem. 264, 5269–5282.
[19] Andronicos, N.M., Ranson, M., Bognacki, J. and Baker, M.S. (1997) Biochim. Biophys. Acta 1337, 27–39.
[20] Miles, L.A., Dahlberg, C.M., Plescia, J., Felez, J., Kato, K. and Plow, E.F. (1991) Biochemistry 30, 1682–1691.
[21] Nakajima, K., Hamanoue, M., Takemoto, N., Hattori, T., Kato, K. and Kohsaka, S. (1994) J. Neurochem. 63, 2048–2057.
[22] Duffy, M.J. and O'Grady, P. (1984) Eur. J. Cancer Clin. Oncol. 20, 577–582.
[23] Wilkins, M.R., Hochstrasser, D.F., Sanchez, J.C., Bairoch, A. and Appel, R.D. (1996) Trends Biochem. Sci. 21, 496–497.