Volume 222, number 2, 261–265 FEB 05163 October 1987

Epidermal and transforming growth factors modulate secretion of a 69 kDa phosphoprotein in normal rat kidney fibroblasts

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Received 1 July 1987; revised version received 14 August 1987

Our study shows that the secretion of a major non-glycosylated, phosphoprotein of 69 kDa (pp69) is a specific marker for non-transformed NRK-49F cells. Treatment of NRK-49F cells with EGF alone or with different combinations of EGF plus TGF-β modulates the secretion of pp69, suggesting its relationship with cellular proliferation. Antibody raised against pp69 recognizes, in addition to pp69, another major phosphoprotein of 62 kDa (pp62) secreted by RR1022 and spontaneously transformed NRK-49F cells. Immunoprecipitation of total cell lysates from both NRK-49F and RR1022 cells with anti-pp69 antibody detected only pp69. These observations suggest a precursor-product relationship between pp69 secreted by non-transformed NRK-49F cells and pp62 secreted by transformed cells.

Phosphoprotein pp69; Epidermal growth factor; Transforming growth factor β; Phosphoprotein; Protein secretion; (NRK-49F cell)

1. INTRODUCTION

Mammalian fibroblast cells in culture secrete into their extracellular environment a wide variety of proteins [1], including a few major phosphoproteins [2–4]. One of these phosphoproteins is a polypeptide of approx. 62 kDa, which has been shown to be associated with cell transformation [5]. We have previously reported that non-transformed normal rat kidney (NRK-49F) fibroblasts, as well as retinoic acid-treated RR1022 and SR-1T cells which reversibly acquire non-transformed phenotype, predominantly secrete a non-glycosylated 69 kDa phosphoprotein (pp69) [6]. Here we show that pp69 secretion by NRK-49F cells is modulated upon treatment with EGF and TGF-β. In addition, our data show that pp69 is immunologically related to a 62 kDa phosphoprotein secreted by many transformed cells.

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Abbreviations: NRK-49F, normal rat kidney fibroblasts; KNRK, Kirsten murine sarcoma virus transformed NRK; RR1022, Rous sarcoma virus (strain Schmidt-Ruppin) transformed rat cells; SR-1T, avian sarcoma virus transformed field vole cells; EGF, epidermal growth factor; TGF-β, transforming growth factor-β; DMEM, Dulbecco's modification of Eagle's medium; NCS, normal calf serum

2. MATERIALS AND METHODS

NRK-49F and RR1022 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). All cells were grown in DMEM (Gibco) supplemented with 10% NCS. Cells were re-fed daily and passaged 24–48 h prior to labeling. Monolayers were incubated with either phosphate- or methionine-free medium with or without growth factors for 1 h before incubation for 4 h in 0.75 or 1 ml growth medium containing 0.5–1.0% serum and 0.13 mCi/ml of

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00145793/87/$3.50

[^{35}S]methionine or 0.5 mCi/ml of [^{32}P]orthophosphate (Amersham). EGF (Sigma) and/or TGF-β (a gift from Dr Anita Roberts, NIH, Bethesda) were used at a final concentration of 5 or 50 ng/ml and 0.1 or 1 ng/ml, respectively.

For the analysis of secreted proteins, conditioned medium was removed and centrifuged in an Eppendorf microfuge for 3 min. 100-μl aliquots were taken and precipitated with 4 vols cold acetone and pelleted by centrifugation. The pellet was washed with a small volume of cold acetone, dried, solubilized in 50 μl sample buffer (0.07 M Tris-HCl, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 3% SDS, 0.01% bromophenol), and samples containing equal cpm were applied to 7.5 or 10% SDS-PAGE gels and analyzed [7]. Gels containing [^{35}S]methionine-labeled samples were soaked for 30 min in 1 M sodium salicylate [8], rinsed, dried and exposed to Fuji-RX X-ray film at -70°C. Two-dimensional analyses of secreted proteins were carried out according to O'Farrell [9].

Phosphoamino acid analysis was done either by using ^{32}P-labeled proteins from gel slices, or from acetone-precipitated conditioned media. Proteins were hydrolyzed in 6 N HCl at 110°C for 1 h and the hydrolysates were resuspended in small volumes of distilled water, mixed with unlabeled phosphoamino acids, and then separated either by ascending thin-layer chromatography using 0.05 M NH_4OH:isobutyric acid (5:3, v/v) or by high-voltage electrophoresis in pyridine, acetic acid and water 1:10:189 [9].

Antibody directed against pp69 was prepared using concentrated conditioned medium or pp69 eluted from gel slices; samples were mixed with Freund's adjuvant and injected into rabbits as described [5].

3. RESULTS AND DISCUSSION

Non-transformed NRK-49F cells, characterized by their inability to grow in soft agar [11], and the lack of phosphorylation of a group of low-molecular-mass membrane proteins [12], secrete a major phosphoprotein of 69 kDa (pp69) (fig.1A,B, lanes 1). Densitometric tracings of the audioradiograms revealed that treatment of NRK-49F cells with 5 ng/ml of EGF, which was found to be mitogenic for these cells [13], caused an approx. 3-fold increase in the levels of ^{32}P-labeled pp69 (fig.1A, lane 4), as compared to un-

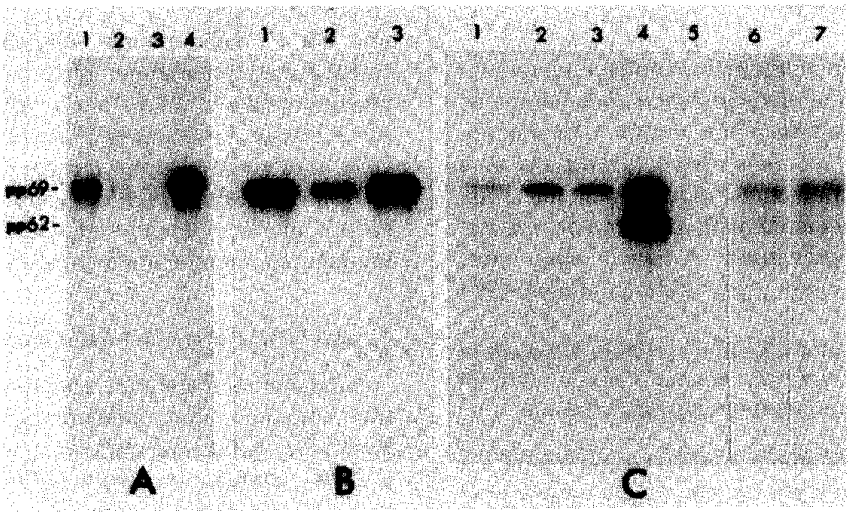

Fig.1. Analysis of ^{32}P-labeled secreted pp69 by SDS-PAGE. (A,B) Acetone-precipitated secreted proteins; (C) immunoprecipitated secreted proteins and total cell lysates. All treatments were for 96 h. (A) Lanes: (1) NRK-49F untreated, (2) 1 ng/ml TGF-β, (3) 1 ng/ml TGF-β plus 5 ng/ml EGF, (4) 5 ng/ml EGF. (B) Lanes: (1) NRK-49F untreated, (2) 0.1 ng/ml TGF-β, (3) 0.1 ng/ml TGF-β plus 5 ng/ml EGF. (C) Lanes: (1) NRK-49F untreated, (2) 0.1 ng/ml of TGF-β plus 5 ng/ml EGF, (3) 0.1 ng/ml TGF-β plus 50 ng/ml EGF, (4) RR1022 cells, (5) normal rabbit serum, (6) total NRK-49F cell lysate, (7) total RR1022 cell lysate.

treated cells. On the other hand, TGF-β, a known inhibitor of cell proliferation [14], at 0.1 and 1 ng/ml caused a 2- and 4-fold decrease respectively, of ^{32}P-labeled pp69 (fig.1A,B, lanes 2) relative to levels observed for untreated cells.

This putative growth-related modulation of the levels of ^{32}P-labeled pp69 was further tested by treating NRK-49F cells with both TGF-β and EGF. Simultaneous treatment with 0.1 ng/ml of TGF-β plus 5.0 ng/ml of EGF, which induced colony formation of NRK-49F cells in soft agar (not shown), caused a 3-fold increase in the levels of ^{32}P-labeled pp69 (fig.1B, lane 3) as compared to untreated cells. When the concentration of TGF-β was increased 10-fold (1.0 ng/ml) but the EGF concentration remained the same (5.0 ng/ml), an 18-fold decrease in ^{32}P-labeled pp69 was observed (fig.1A, lane 3). These observations were further confirmed by immunoprecipitation of ^{32}P-labeled secreted proteins with a polyclonal anti-pp69 rabbit antibody (fig.1C). Phosphoamino acid analysis of pp69 secreted by both untreated and EGF plus TGF-β-treated NRK-49F cells as well as of pp62 secreted by RR1022 and KNRK cells revealed that only serine residues are phosphorylated (not shown).

Immunoprecipitation of secreted phosphoprotein from RR1022 cells with anti-pp69 antibody showed two major phosphoprotein bands, one of 69 kDa (pp69) and the other of 62 kDa (pp62) (fig.1C, lane 4). This lower molecular mass phosphoprotein has been shown to be a specific marker for most transformed cells [5,6,15]. It is apparent, therefore, that the 62 kDa transformation-associated phosphoprotein is immunologically related to pp69. Peptide mapping of partially digested pp69 and pp62 with V8 protease, carried out in our previous study, showed considerable fragment homology [6]. Immunoprecipitation of ^{32}P-labeled total cell lysates from both non-transformed NRK-49F and transformed RR1022 cells showed only pp69 (fig.1C, lanes 6 and 7, respectively).

To determine whether the increased levels of ^{32}P-labeled pp69 found in the conditioned media of EGF and TGF-β treated cells were due to increased secretion of phosphorylation of this protein, secreted proteins were labeled with [^{35}S]methionine and analyzed (fig.2A). It can be seen that treatments of NRK-49F cells with

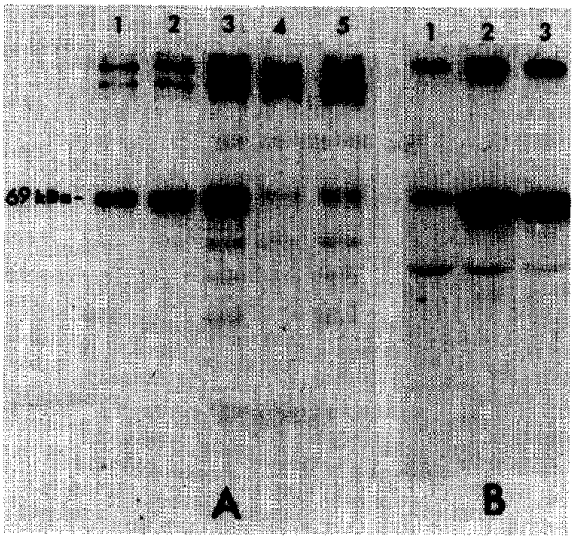

Fig.2. Analysis of [^{35}S]methionine-labeled secreted pp69 by SDS-PAGE. (A) Acetone-precipitated secreted proteins; (B) immunoprecipitated secreted proteins. All treatments were for 96 h. (A) Lanes: (1) 50 ng/ml EGF, (2) 0.1 ng/ml TGF-β plus 50 ng/ml EGF, (3) 0.1 ng/ml TGF-β plus 5 ng/ml EGF, (4) 0.1 ng/ml TGF-β, (5) NRK-49F untreated. (B) Lanes: (1) NRK-49F untreated, (2) 0.1 ng/ml TGF-β plus 5 ng/ml EGF, (3) 0.1 ng/ml TGF-β plus 50 ng/ml EGF.

50 ng/ml of EGF, 0.1 ng/ml TGF-β plus 50 ng/ml of EGF, and 0.1 ng/ml of TGF-β plus 5 ng/ml of EGF, showed significant increase in the amount of labeled pp69 (fig.2A, lanes 1–3, respectively). Treatment of NRK-49F cells with 0.1 ng/ml of TGF-β alone caused a slight decrease in radioactivity associated with pp69 (fig.2A, lane 4) as compared to untreated cells (lane 5). Immunoprecipitation of [^{35}S]methionine-labeled pp69 with an anti-pp69 antibody confirmed that treatment of NRK-49F cells with EGF and TGF-β significantly increases the secretion of pp69 (fig.2B). These observations suggest that EGF and TGF-treated cells secrete more pp69 than their untreated counterparts.

Since EGF treatment alone caused increased secretion of pp69 by NRK-49F cells (fig.2A, lane 1), we determined the length of EGF treatment needed for such an increase. Initially, long- and short-term treatments were carried out. Treatment of NRK-49F cells with EGF for 48, 96 and 120 h significantly increased the secretion of pp69 (fig.3A, lanes 3–5). A 4 h EGF treatment did not

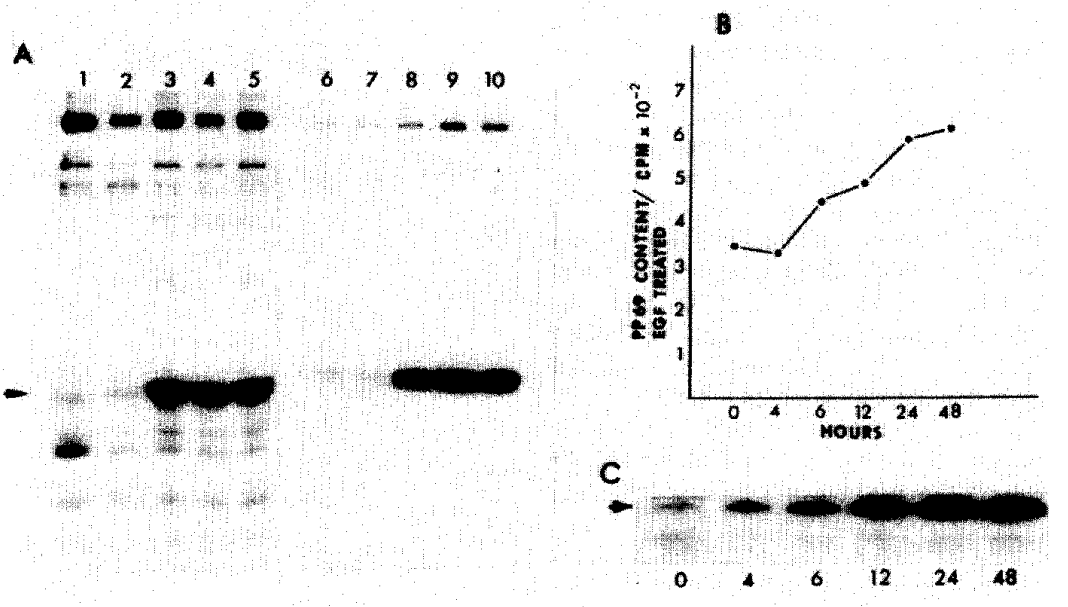

Fig.3. Time-course study of the effects of EGF treatment (50 ng/ml) on the secretion of pp69 by NRK-49F cells. (A) Lanes 1–5, acetone-precipitated secreted proteins; 6–10, immunoprecipitated secreted proteins. Lanes: (1) 0 h, (2) 4 h, (3) 48 h, (4) 96 h, (5) 120 h, (M) markers, (6) 0 h, (7) 4 h, (8) 48 h, (9) 96 h, (10) 120 h. (B) Radioactivity (cpm) present in pp69 band from samples taken at various time points after EGF treatment. (C) Typical SDS-PAGE pattern of immunoprecipitated pp69 taken at various time points after EGF treatment. The numbers under each band represent hours of EGF treatment. Arrows indicate the position of pp69.

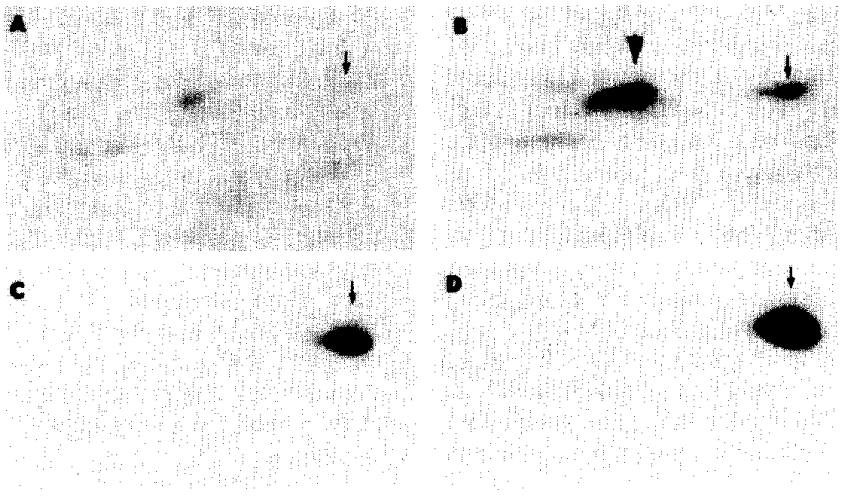

Fig.4. Two-dimensional gel analysis of [^{35}S]methionine (A,B) and [^{32}P]orthophosphate (C,D) secreted proteins; (A,C) NRK-49F untreated, (B,D) 0.1 ng/ml TGF-β plus 5 ng/ml EGF. Small arrow indicates pp69 and large arrowhead denotes the non-phosphorylated 69 kDa protein.

increase section of pp69 (fig.3A, lane 2). Analysis of immunoprecipitated pp69 confirmed the above results (lanes 6–10). In order to determine the exact length of EGF treatment needed for the increased secretion of pp69, the time course study was extended to include time points between 4 and

48 h. As can be seen in fig.3B and C, a significant increase in levels of pp69 could be detected between 4 and 6 h of EGF treatment, and a maximal level was reached at approx. 48 h. Estimation of radioactivity in pp69 bands taken from four gels resulting from two separate experiments confirmed the above observation (fig.3B).

Two-dimensional electrophoretic analysis of [^{35}S]methionine-labeled secreted proteins demonstrated that two distinct 69 kDa proteins are excreted by NRK-49F cells; the phosphorylated form (pp69) which has a p*I* of approx. 6.0, and a more acidic non-phosphorylated protein (np69) (fig.4). Secretion of both of these proteins was increased upon treatment with 5 ng/ml of EGF plus 0.1 ng/ml of TGF-β.

Following prolonged culture of NRK-49F cells, a proportion of the cell population undergoes spontaneous transformation. As a result, significant levels of pp62 could be detected in the conditioned media of these cultures (fig.5, lane 1). It is clear, therefore, that the secretion of pp69 can be used as a specific marker for non-transformed NRK-49F cells. Treatment of NRK-49F cultures containing spontaneously transformed cells with 5 ng/ml EGF plus 0.1 ng/ml of TGF-β caused an increase in the amount of both ^{32}P-labeled pp69 and pp62 (lane 2). When these cultures were treated with 1 ng/ml of TGF-β plus 5 ng/ml EGF, the level of radioactivity in the pp69 band significantly decreased, and pp62 was undetectable (lane 3). These results show that EGF and TGF-β modulate the secretion of both pp69 and pp62.

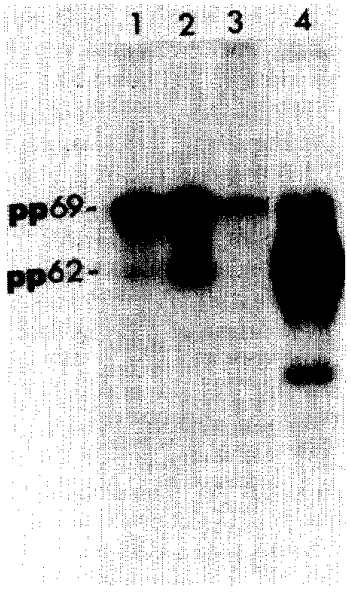

Fig.5. Analysis of immunoprecipitated ^{32}P-labeled secreted proteins from partially transformed NRK-49F cultures and RR1022 cells. Lanes: (1) NRK-49F untreated, (2) 0.1 ng/ml TGF-β plus 5 ng/ml EGF, (3) 1 ng/ml TGF-β plus 5 ng/ml EGF, (4) RR1022, acetone-precipitated control.

ACKNOWLEDGEMENTS

We thank Robert Pentney for membrane phosphorylation analysis of NRK-49F cells, Michael DeVouge for critical evaluation of the manuscript, Cathy Hughes for typing the manuscript, and Robert Lamarche and Guy L'Heureux for photographic assistance. This work was supported by a grant (MT-2169) from the Medical Research Council of Canada.

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