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Phosphorylation affects the DNA affinity of the nuclear protein 24/7 from human tumor cells

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In the present study we investigated the binding behavior of nuclear proteins from human tumor cells to human placental DNA coupled on CNBr-activated Sepharose. When nuclear proteins soluble in 5 M urea prepared from serum-stimulated cells and containing the majority of the nonhistone proteins were applied onto a dsDNA column, next to several other proteins one prominent group consisting of at least 2 distinct proteins with a p*I* at 7 and a molecular mass near 24 kDa bound to DNA. The DNA-binding ability of one of them is lost on phosphorylation and is recovered after dephosphorylation using alkaline phosphatase. Additionally, normal human fibroblasts taken as controls exhibit comparatively low levels of these 24/7 proteins, indicating a particular function in tumor cells.

DNA binding; Protein phosphorylation; Affinity chromatography; Nonhistone protein

1. INTRODUCTION

Nuclear proteins in the eukaryotic genome play a crucial role in controlling gene activity. Their reversible binding to specific DNA sequences after covalent modification such as phosphorylation appears to be an essential step in replication and transcription as has been shown for SV40 T-antigen [1]. Even more phosphoproteins which bind to DNA in a non-sequence-specific manner like the HMG-proteins [2] may be involved in gene structure and function. On the other hand, proteins coded by some oncogenes exhibit kinase activity with specificity for nuclear proteins. Hence, they might act as modulators of gene activity. To look for tumor-specific nonhistone phosphoproteins with high affinity for dsDNA we used nuclear proteins from the human fibrosarcoma cell line HT 1080 [3], normal diploid fibroblasts and human glioblastoma cell line HeRoSV [4]. Proteins were fractionated according to their DNA affinity by chromatography on placental dsDNA. Treatment of protein samples with protein kinase in the presence of [γ-^{32}P]ATP and dephosphorylation with alkaline phosphatase additionally allow one to investigate the influence of modifications on the DNA-binding ability of those proteins.

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; 2D, two-dimensional; PAGE, polyacrylamide gel electrophoresis; dsDNA, double-stranded DNA; HMG, high-mobility-group proteins; NEPHGE, nonequilibrium pH gradient gel electrophoresis

2. MATERIALS AND METHODS

Human placental DNA was prepared [5], sheared with a sonifier to an average length of 0.5–2.5 kb and coupled (0.5 mg/ml) to CNBr-Sepharose 4B (Pharmacia) as recommended by the manufacturer. Normal diploid human fibroblasts derived from foreskin biopsies, human glioblastoma cell line HeRoSV described previously [4] and the fibrosarcoma cell line HT 1080 [3] (Flow Labs) were cultured as monolayers in minimal essential medium (MEM) containing 10% fetal calf serum and antibiotics. To focus on those proteins characteristic as markers for the onset of cell division, cells were synchronized in G_1-phase by 24 h serum starvation followed by a stimulation period of 1.5 h prior to protein isolation. Nuclear proteins were obtained from the different cell cultures using our standard procedure [6]. Briefly, nuclei obviously free of cytoplasmic contamination were stirred on ice for 1 h in 5 M urea, 50 mM Tris-HCl (pH 8.0), 1 mM PMSF and dialyzed vs 50 mM Tris-HCl (pH 8), 0.1 mM PMSF. Phosphorylation was carried out

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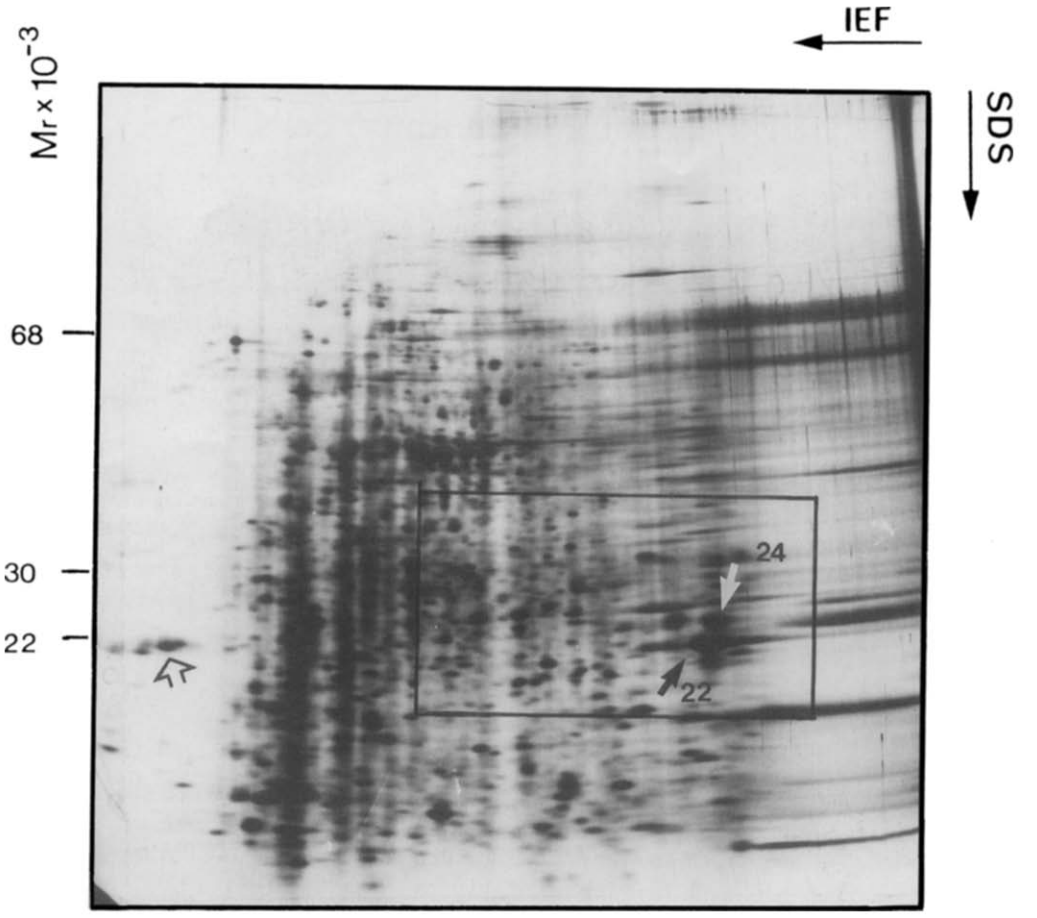

Fig.1. Nuclear protein pattern from the fibrosarcoma cell line HT 1080 analysed by 2D PAGE and silver staining; 180 μg protein were applied. The white arrow indicates the 24/7 (molecular mass/p*I*) protein, whose DNA-binding ability – depending on the phosphorylation state – is shown in figs 2,3. Similarly, the black arrow indicates the lower molecular mass protein of this group (22/7). The area enclosed by a rectangle is shown as an enlargement in figs 2,3.

as in [7] but with the addition of exogenous kinase (500 U, catalytic subunit, bovine heart, Sigma) for 30 min at 37°C. Hydrolysis of phosphate moieties was performed with alkaline phosphatase (CI, Sigma, 15–12 U/mg protein). Untreated and treated proteins, respectively, were loaded on the DNA-Sepharose column and eluted by a linear gradient of 0.1–2.0 NaCl, 50 mM Tris-HCl (pH 8). Radioactivity was determined via trichloroacetic acid precipitation and scintillation counting. At least two fractions corresponding to the flowthrough and bound material were collected, dialyzed vs 20 mM NH_4HCO_3, 0.1 mM PMSF, lyophilized and subsequently separated on standard 2D gels [6] followed by silver staining and autoradiography, respectively.

3. RESULTS AND DISCUSSION

High-resolution 2D gel electrophoresis of the nuclear protein fraction extractable with 5 M urea from fibrosarcoma cell line HT 1080 in connection with sensitive silver staining revealed more than 500 polypeptides, demonstrating the extent of chromosomal protein heterogeneity (fig.1). Comparison of the electrophoretic patterns of proteins from normal human fibroblasts and human fibrosarcoma cell line HT 1080 revealed several differences [9]. The most striking finding was the occurrence of two prominent proteins with a p*I* of 7 and a molecular mass near 24 kDa in the tumor cells. Consequently, they may be regarded as tumor-specific at least for the cell lines analyzed. Previously, we have shown that phosphorylation of a set of acidic proteins (23/4, molecular

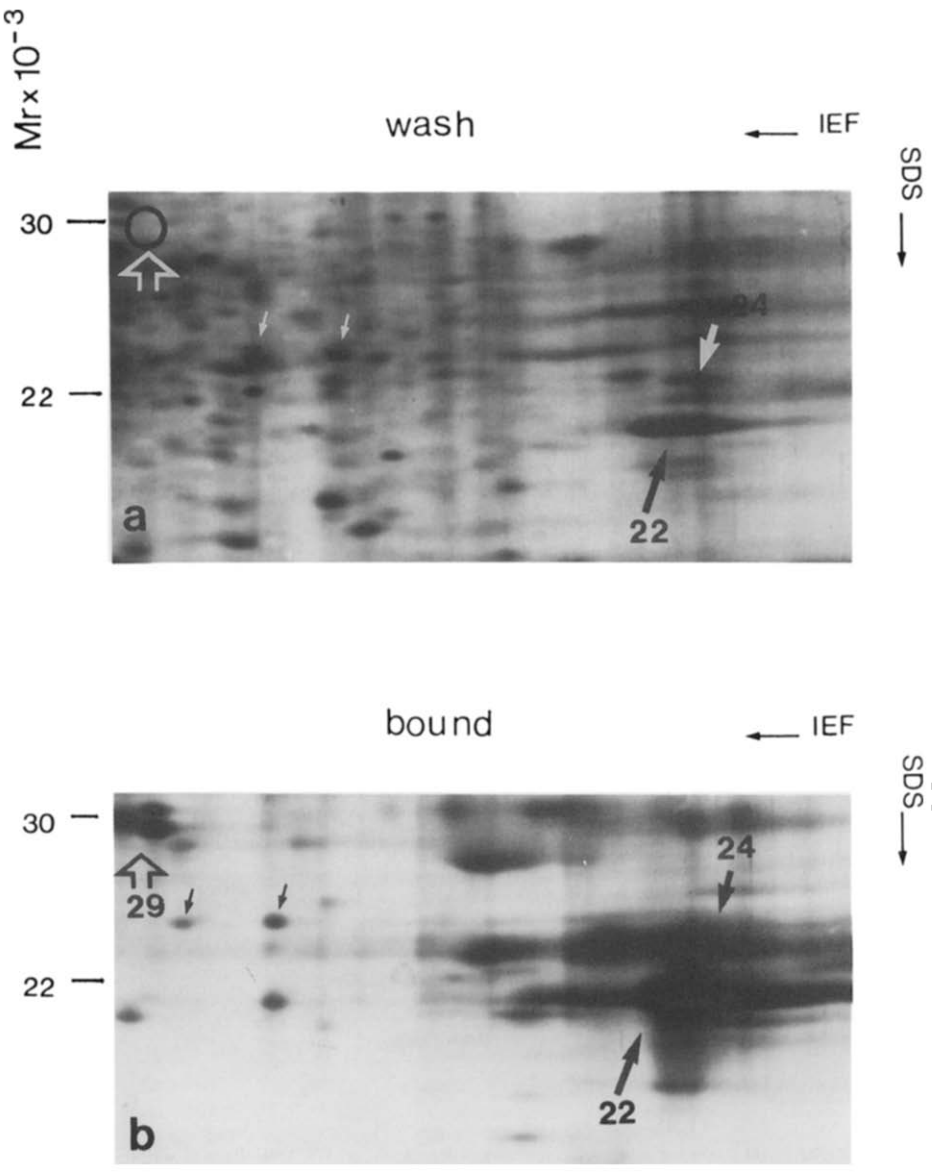

Fig.2. 2D PAGE (close-up from the lower basic region) of nuclear proteins fractionated on placental DNA-Sepharose as described visualized by silver staining. (a) Flowthrough and (b) bound material. Open arrow and large arrows (a,b) indicate proteins (in kDa) highly enriched in the DNA-bound material. Small arrows indicate some proteins occurring at comparable levels in both fractions.

mass/p*I*) constitutes one of the early events in the prereplicative phase of the cell cycle in different human tumor cells [9]. Here, using DNA-affinity chromatography, we examined the influence of phosphorylation on the DNA-binding ability of nuclear proteins derived from two different human tumor cell lines. A number of reports have appeared dealing with DNA-affinity chromatography of nuclear proteins (review [10]). Several approaches were tackled including single/double-stranded DNA from different sources (homologous/heterologous) and various matrices. To perform DNA chromatography under conditions as close to physiological as possible, we used human placental dsDNA with an average length of 0.5–1.5 kb coupled on CNBr-Sepharose. When

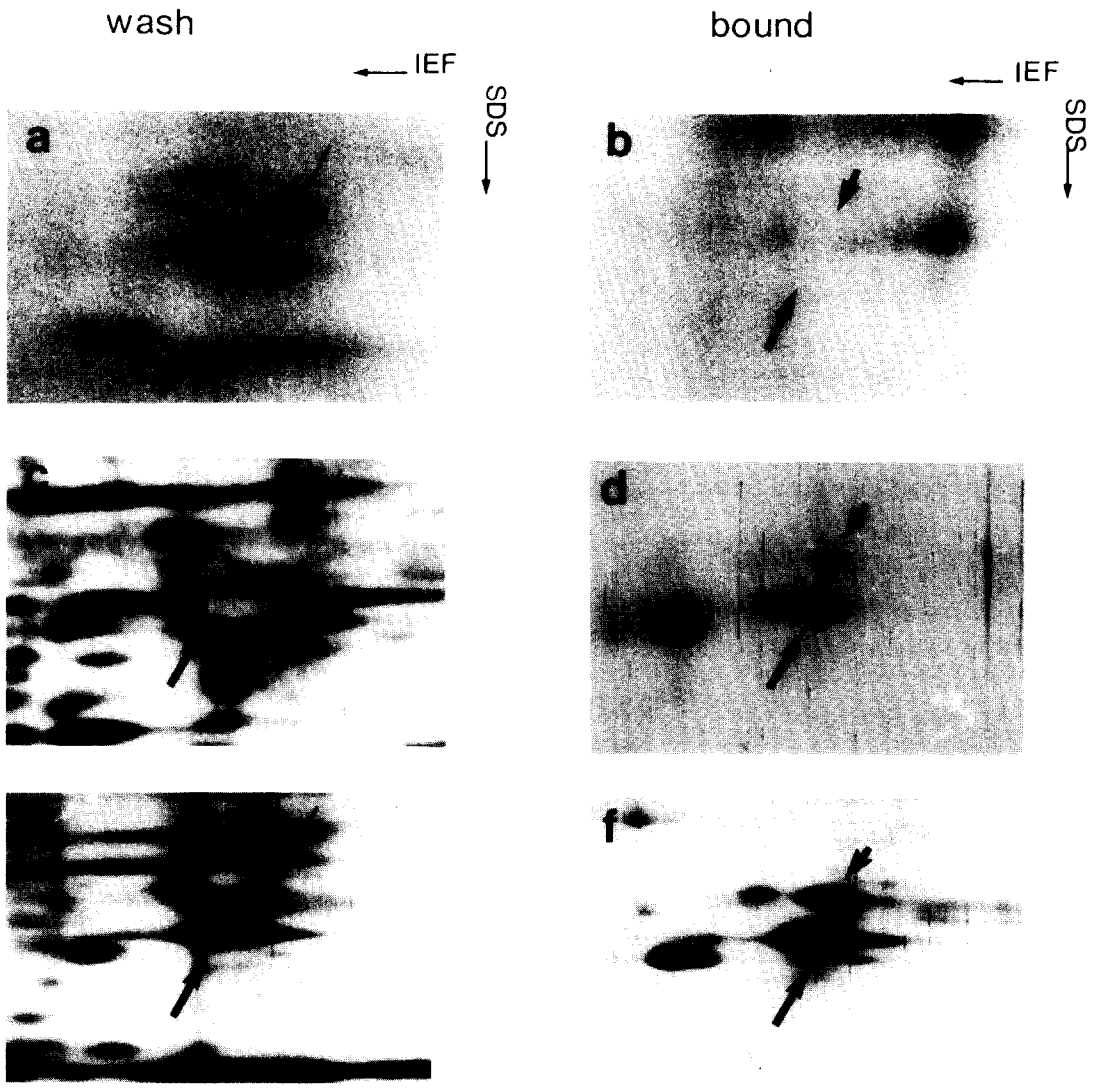

Fig.3. 2D PAGE of nuclear proteins (HT 1080) after chromatography on a placental DNA-Sepharose column. Only the relevant region of the gel containing the basic protein group is shown. (a,c,e) Eluted wash and (b,d,f) bound fraction. (a,b) Sample treated with exogenous kinase in the presence of [γ-^{32}P]ATP prior to chromatography. Phosphoproteins were detected by autoradiography. (c,d) Silver-stained gels corresponding to a,b. (e,f) Proteins fractionated on dsDNA-Sepharose after treatment with alkaline phosphatase (silver staining). Large arrows indicate the 24/7 and 22/7 proteins whereas a small arrow in a,c points to an additional phosphoprotein occurring in the wash.

the 5 M urea extract from tumor cell nuclei (HT 1080 and HeRoSV) was applied onto the column, the majority of the proteins occurred in the flowthrough. A close-up of the corresponding gels (HT 1080) is shown in fig.2. One prominent protein group retained on the DNA column corresponds to the 24/7 proteins, probably characteristic of at least the tumor cell lines we analysed in the present study. This protein group is detectable only at low levels in human fibroblasts (not shown). Since both tumor cell lines behave similarly with respect to these particular proteins, the patterns shown here are restricted to cell line HT 1080. The exact composition of this

group remains to be determined using extended pH-gradient gels and NEPHGE which permits more detailed analysis in the basic region of a 2D gel. Previously, we studied the extent of modification of nuclear proteins derived from human tumor cells. Through in vitro phosphorylation of nuclear proteins (5 M urea extract) more than 50 polypeptides were phosphorylated [7]. DNA-affinity chromatography of such treated proteins revealed a dramatic change in binding behavior as shown in fig.3. One of the most important observations made was that the 24/7 proteins lost their binding ability following phosphorylation. Treatment of the phosphoproteins with alkaline phosphatase resulted in the restoration of DNA binding of this specific group. This observation is in agreement with the results on HMG-17 reported by Palvimo et al. [11] who demonstrated a change in DNA binding of this particular nuclear protein after phosphorylation. It is unlikely that the proteins 24/7 in the present investigation correspond to the HMG proteins since they are not soluble in 2% trichloroacetic acid as is characteristic for the HMGs (not shown) [2]. Additionally, that these proteins are most abundant in the fibrosarcoma cell line HT 1080 and glioblastoma cell line HeRoSV points to their specific function in these tumor cells. We are currently investigating whether these proteins occur in other human tumor cell lines and trying to determine the time of their synthesis during the G_1-phase.

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