Protein that induces cell differentiation causes nicks in double-stranded DNA

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The growth and differentiation of myeloid hematopoietic cells are regulated by different macrophage and granulocyte inducing proteins, those that induce growth and others that induce differentiation. The proteins that induce differentiation but not those that induce growth bind to double-stranded DNA. We now report that purified myeloid cell differentiation-inducing protein causes single strand breaks (nicks) in double-stranded DNA. This DNA nicking may initiate the changes in gene expression that are required for differentiation.

DNA-binding protein *DNA nicking* *Differentiation-inducing protein* *(Myeloid hematopoietic cell)*

1. INTRODUCTION

A normal developmental program requires growth factors to induce cell multiplication and differentiation factors to induce differentiation to mature cells with specific functions. The identification of these factors makes it possible to analyze the mechanism that controls growth and differentiation in cells of a particular lineage. Both these types of factors have been identified for myeloid hematopoietic cells. The myeloid growth factors are proteins called macrophage and granulocyte inducers – type 1 (MGI-1) or colony stimulating factors (CSFs) [1–4]. The myeloid cell differentiation factors are other proteins called MGI – type 2 (MGI-2) or differentiation factors (DFs) [1–3,5,6], and they induce differentiation of myeloid precursors to macrophages or granulocytes. DNA-binding studies have shown that differentiation-inducing protein MGI-2 can bind to double-stranded but not to single-stranded DNA, whereas there was no DNA binding by the growth-inducing proteins MGI-1 [7,8]. We have now found that purified DF MGI-2 can cause single strand breaks (nicks) in double-stranded DNA.

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Abbreviation: MGI-2, macrophage and granulocyte differentiation-inducing protein

2. MATERIALS AND METHODS

2.1. *Differentiation-inducing protein MGI-2*

MGI-2 was obtained from serum-free conditioned medium from a clone of myeloid cells that can be induced to secrete this DF [9]. The protein was purified by procedures that included electrophoresis on a polyacrylamide gel as described [7,8]. The gels were cut into slices and the peak of differentiation-inducing activity eluted from these gels showed one band on silver staining [10] on an SDS gradient polyacrylamide gel [11,12]. Recovery of the loaded MGI-2 from the gels was 60%. Differentiation-inducing activity was assayed by induction of differentiation in a differentiation competent (D^+) clone of myeloid leukemic cells [13] using lysozyme synthesis and secretion as a function that is induced during differentiation [14]. One unit of MGI-2 is the amount that induced the secretion of 1 μg lysozyme equivalent per 5×10^6 cells.

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2.2. *DNA nicking activity*

DNA nicking activity in vitro was measured by incubating 0.5 μg form I SV40 DNA with about 10 ng protein for 1 h at 37°C in buffer A containing 10 mM Tris-HCl, pH 7.4, 25 mM NaCl, 5 mM Mg acetate, 5 mM β-mercaptoethanol, 0.1 mM EDTA and 10% glycerol. The reactions were stopped by an equal volume extraction with phenol/chloroform/isomylalcohol (25:24:1, v/v) followed by 2 equal volume extractions with chloroform/isomylalcohol (14:1, v/v) [15]. The samples with added glycerol and bromophenol blue were then electrophoresed in 1% agarose in TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA) [15] and visualised by staining with ethidium bromide [15,16]. For electron microscopy, 1 μg form I SV40 DNA was incubated in buffer A with 10 ng purified MGI-2. Aliquots were placed on grids, stained with uranyl acetate, prepared for dark-field electron microscopy as described [8] and examined with an EM 400T electron microscope. DNA nicking in vivo was followed by measuring changes in nucleoid sedimentation rate [17,18] in a differentiation competent (D^+) and a differentiation defective (D^-) clone of mouse myeloid leukemic cells incubated with purified MGI-2. The D^+ clone (no.11) can be induced to differentiate by MGI-2 to mature cells, and the D^- clone (no.1) is not induced to differentiate by MGI-2 [1,2,13]. The cells were washed with ice-cold phosphate-buffered saline, resuspended at 5×10^5 cells in 100 μl deposited inside a layer of 400 μl of 0.5% Triton X-100, 10 mM Tris, 10 mM EDTA, 2 M NaCl, pH 8.0, lying on top of a 10.0 ml gradient of 5–20% (w/v) sucrose in 10 mM Tris, 10 mM EDTA, 2 M NaCl, pH 8.0, in a Beckman SW 41 centrifuge tube [18]. After incubation for 15 min at 20°C the gradients were centrifuged at 25000 rpm (100000 $\times g_{max}$) [18] for 40 min at 20°C and collected from the bottom of the tube with the aid of a peristaltic pump. The absorbance of each 0.5 ml fraction was measured at 260 nm.

3. RESULTS AND DISCUSSION

The differentiation-inducing protein MGI-2 was purified by procedures that included electrophoresis on a polyacrylamide gel as described [7,8], the gels were cut into slices and the peak of differentiation-inducing activity eluted from these gels (fig.1A) showed one band when silver stained [10] on an SDS gradient polyacrylamide gel [11,12] (fig.1B). There are enzymes that induce single strand nicks and other structural changes in supercoiled DNA [19–22]. The activity of these enzymes can be detected by agarose gel electrophoresis [23]. Supercoiled SV40 (form I) DNA, nicked circular (form II) DNA, and linear (form III) DNA migrate in agarose gels at different rates. We have used this

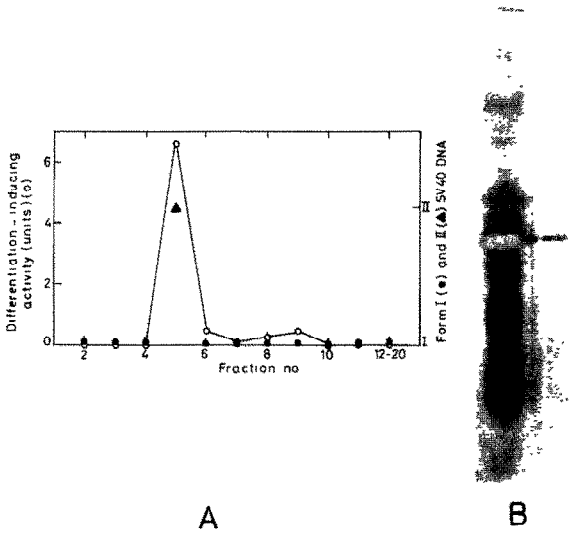

Fig.1. Differentiation-inducing activity and nicking activity on supercoiled SV40 DNA in fractions from a 7% polyacrylamide gel (A). Electrophoresis of the unfractionated and purified MGI-2 on a 7–17% SDS gradient polyacrylamide gel (B). In (A) the gel was cut into slices and the MGI-2 activity eluted as described [7]. Differentiation-inducing activity was assayed by induction of differentiation in a differentiation competent (D^+) clone of myeloid leukemic cells [13]. One unit of differentiation-inducing activity is the amount that induced the secretion of 1 μg lysozyme equivalent per 5×10^6 cells [14]. The SV40 form I nicking activity was measured by incubating 0.5 μg form I SV40 DNA with about 10 ng protein at 37°C. The samples were then electrophoresed in 1% agarose and visualised by staining with ethidium bromide as described in [15,16] and section 2. In (B) the unfractionated conditioned medium containing MGI-2 (lane 1) and fraction 5 from the 7% polyacrylamide gel (lane 2) were electrophoresed on an SDS gradient polyacrylamide gel [11,12] and the proteins visualised by silver staining [10].

assay (fig.2A,B) to detect such enzyme activity in samples eluted from the polyacrylamide gels.

The only sample that contained any activity that changed form I SV40 DNA was the sample with the differentiation factor MGI-2 which changed form I into form II DNA (fig.1A). Unlike some of the other enzymes that produce nicks [19,21,24], this nicking activity did not require ATP. The cells used for the production of MGI-2 also secrete growth-inducing protein MGI-1. In the graph shown in fig.1A, this growth factor was found in fractions after number 8. None of the fractions with growth factor showed any nicking activity (fig.1A).

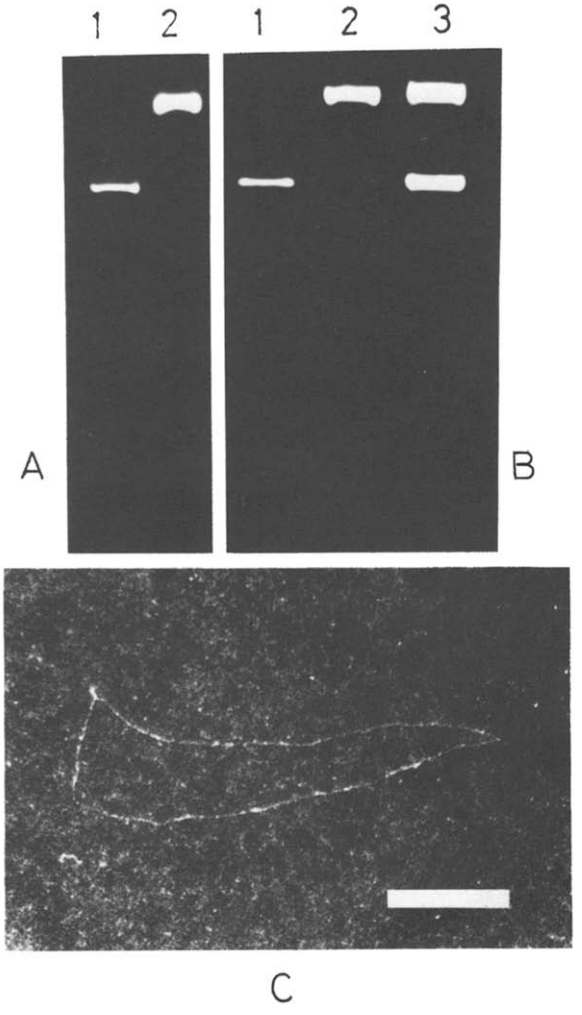

Fig.2. Agarose gel electrophoresis of SV40 DNA after incubation with or without purified MGI-2. 10 ng purified MGI-2 was incubated with 0.5 μg form I SV40 DNA and electrophoresed on a 1% agarose gel [23] in (A) without and in (B) with saturating levels (5 μg/ml) of ethidium bromide [23]. SV40 DNA incubated without (lane 1) or with MGI-2 (lane 2). Standards of form I and II SV40 DNA (B, lane 3). Electron microscope picture of form II SV40 DNA after incubation of form I SV40 DNA with 10 ng purified MGI-2 (C). Enlarged 65660 diameters. The scale bar represents 0.25 μm.

To determine further whether the form II DNA induced by MGI-2 contained single strand breaks, or was a relaxed covalently closed circular DNA which is produced by topoisomerases, the DNA was electrophoresed in agarose gels with ethidium bromide [23,25]. In the presence of saturating levels of ethidium bromide the migration of relaxed covalently closed DNA produced by topoisomerases will be significantly faster than nicked DNA due to induced positive supercoiling. However, a nicked DNA will migrate like the standard of form II nicked DNA. The results indicate (fig.2B, lane 2) that the form II DNA induced by purified MGI-2 migrates like a nicked DNA. The change of form I to form II DNA after incubation with MGI-2 was also seen by electron microscopy (fig.2C). These results indicate that purified MGI-2 causes DNA nicking in vitro. Nicking of DNA in vivo has been found in differentiating muscle cells [17]. Differentiation competent (D^+) and differentiation defective (D^-) mouse myeloid leukemic cells [1,2] were therefore incubated with purified MGI-2 and analyzed for single strand DNA breaks in vivo by measuring changes in their nucleoid sedimentation rate [17,18]. DNA nicking leads to a slower rate of sedimentation [17,18]. After 4 h incubation of the myeloid leukemic cells with purified MGI-2 the nucleoid sedimentation rate had decreased by 14.6 ± 2.1% in the differentiation competent but not in the differentiation defective leukemic cells. Nucleoid sedimentation in the presence of ethidium bromide [17] has indicated that the change in sedimentation rate in the D^+ leukemic cells incubated with MGI-2 was due to DNA nicking. This suggests that induction of myeloid cell differentiation by MGI-2 is associated with in vivo nicking of DNA. DNA nicking in vivo has also been reported after induction of differentiation by dimethylsulphoxide in a line of human myeloid leukemic cells [26].

Our results indicate that purified myeloid cell DF MGI-2 can cause nicks in double-stranded

DNA. The purified protein with both nicking and differentiation-inducing activity showed a single band following silver staining of an SDS-polyacrylamide gel. This indicates that the MGI-2 protein has nicking activity, or that the nicking activity was very tightly bound to MGI-2 and was not separated in the procedures used. Either of these alternatives is compatible with the suggestion that the DNA nicking activity may initiate the changes in gene expression that are required for differentiation. Other proteins that regulate differentiation of other cell types include the homoeotic gene proteins [27] which show structural homology to DNA-binding proteins [28]. It will be interesting to determine whether the homoeotic proteins and others like the DNA-binding *myc* protein [29], the nuclear *fos* protein [30] and the *Drosophila* proteins that bind to Z-DNA [31], also cause nicking. The sequence specificity of nicking or related enzyme activities on DNA by different differentiation regulating proteins may determine which genes are activated in differentiation of specific cell types.

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