Calcium-dependent ADP-ribosylation of high-mobility-group I (HMGI) proteins

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Micrococcal nuclease digestion of nuclei from mouse Lewis lung carcinoma cells releases a protein mixture into the supernatant that lacks histone H1 and contains a full complement of high-mobility-group I (HMGI) proteins (i.e. I, Y and I-C). This implies that all three HMGI proteins are localized at the nuclease-sensitive regions of active chromatin. It is also shown that if Ca^{2+}

ions are present in the nuclear incubation buffer (with or without exogenous nuclease), all three HMGI proteins become ADP-ribosylated. We propose that this modification of HMGI family proteins is part of the general poly(ADP-ribosyl)ation that accompanies DNA damage in apoptosis and other processes.

INTRODUCTION

High-mobility-group I (HMGI) proteins are a family of three nuclear proteins named HMGI, HMGY and HMGI-C, the first of which gives the name to the entire group. They consist of about 100 amino acids and have a high content of both basic and acidic residues (about 20 % each). HMGI and HMGY derive from alternative splicing of the same gene and differ by an 11-amino-acid segment present in HMGI that is absent in HMGY. These two proteins will be referred to collectively as HMGI/Y. HMGI-C is expressed from a different gene but is closely related to the other two proteins, with about 50 % sequence identity [1–6]. Elevated levels of the HMGI proteins have been correlated with neoplastic phenotypes since normal adult cells or tissues show very low or undetectable levels [1,3,4,7–13]. It has recently been shown that HMGI proteins are widely expressed in developing mouse embryos up to day 14, after which they become difficult to detect [14]. We have demonstrated a role for HMGI-C expression in cell transformation by showing that transforming viruses are no longer able to induce neoplastic transformation of rat thyroid cells when these cells also transcribe an HMGI-C antisense message [15].

HMGI/Y proteins have been proposed as structural components of combinatorial promoter complexes together with other transcription factors [16]. In particular it has been demonstrated that complexes of HMGI/Y proteins with NF- κ B and ATF-2/c-Jun heterodimers stimulate transcription of the human interferon β gene [17,18]. Furthermore, HMGI/Y proteins are implicated in the regulation of both the tumour necrosis factor (TNF) β gene in murine leukaemia virus-transformed cells and the interleukin-2 receptor α -chain gene [19,20].

HMGI/Y proteins have been shown to bind AT-rich DNA sequences within the minor groove of certain promoters [17,21–25] and are released from active chromatin when isolated nuclei are treated with micrococcal nuclease [26–30]. The protein mixture released by nuclease digestion does not contain histone H1, a repressor of transcription, and is enriched in HMGI/Y proteins [26]. Corresponding data for HMGI-C protein have not been reported.

To determine whether HMGI-C is positioned at micrococcal nuclease-sensitive sites like HMGI/Y, we used micrococcal nuclease digestion of nuclei isolated from Lewis lung carcinoma (LLC) cells, which are known to contain the complete set of three HMGI proteins [8]. We report that all three HMGI proteins are present on micrococcal nuclease-sensitive chromatin and can be ADP-ribosylated by a calcium-dependent process.

EXPERIMENTAL

Materials

Chemicals and reagents were obtained from the following sources: [γ - ^{32}P]ATP and [α - ^{32}P]NAD $^{+}$, DuPont NEN; benzamide, PMSF and phosphodiesterase I (P 6761, Type VII) from *Crotalus atrox* venom, Sigma; goat anti-(rabbit IgG) conjugated with horseradish peroxidase, Pierce; ECL Western blotting detection reagent (RPN 2209), Amersham; 3,3',5,5'-tetramethylbenzidine-stabilized substrate, Promega; phenyl boronate-agarose gel (PBA-30), Amicon; leupeptin, aprotinin and micrococcal nuclease (*Staphylococcus aureus*), Boehringer. Polyclonal antibodies against recombinant HMGI-C protein and against the synthetic peptides WPQQVVQKKPAQ and SSSKSSQPL-ASKQ, specific to HMGI-C protein and HMGI/Y proteins respectively, were prepared as previously described and then affinity-purified [4].

Isolation of nuclei from LLC cells

Samples (1.0–1.5 g) of tissue were blended three times for 2 min on ice in 6 ml of isolation buffer (3.75 mM Tris/HCl, pH 7.5, 50 μM spermine, 125 μM spermidine, 500 μM EDTA, 500 μM dithiothreitol, 20 mM KCl) containing 1 μM of the protease inhibitors PMSF, aprotinin and leupeptin. The pellet recovered by centrifugation (1200 g) was washed three times with isolation buffer, resuspended in 4 ml of isolation buffer containing 0.1 % digitonin and stroked 15 times in a Dounce homogenizer (pestle S). Nuclei were collected by repeated washing with the same buffer, the last washing lacking EDTA. Isolated nuclei were

stored at -20°C in isolation buffer lacking EDTA/glycerol (1:1, v/v).

Treatment of LLC nuclei with micrococcal nuclease and protein extraction

For chromatin fractionation, 200 A_{260} units of nuclei were washed twice in MB buffer (20 mM Tris/HCl, pH 7.4, 70 mM NaCl, 10 mM MgCl_2 , 50 μM spermine, 125 μM spermidine, 0.1 % digitonin) containing protease inhibitors, resuspended in 1 ml of MB buffer containing 2 mM CaCl_2 and divided into two aliquots of 500 μl each. One aliquot was then treated with 5 units of micrococcal nuclease and the two aliquots were incubated together for 10–20 min at 37°C . These conditions were similar to those used by other authors for the same type of experiments [26]. Digestion was stopped by addition of EGTA up to 3 mM and cooling on ice. After centrifugation for 10 min at 13000 g , two fractions were obtained: S, the supernatant and P, the pellet. For protein extraction, the S supernatant was treated with perchloric acid (PCA) up to 5 % (w/v) and HCl up to 0.3 M and the protein mixture precipitated overnight at -20°C by the addition of 8 vol. of acetone. Pellet P was directly extracted with 5 % PCA and protein recovered as above.

Incubation of LLC nuclei in different media and protein extraction

About 100 A_{260} units of LLC nuclei were resuspended in the presence of protease inhibitors in one of the following media up to a final volume of 250 μl : medium M1 (10 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, pH 7.4), medium M2 (10 mM Tris-acetate, 10 mM sodium acetate, 50 mM potassium acetate, pH 7.4) or medium M3 (10 mM Tris-acetate, 10 mM calcium acetate, 50 mM potassium acetate, pH 7.4).

A set of experiments in medium M3 containing Ca^{2+} in the range 1 μM –10 mM was also carried out.

For phosphorylation studies, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (20 $\mu\text{Ci}/\text{sample}$) was used; for observation of ADP-ribosylation, $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ (10 $\mu\text{Ci}/\text{sample}$) was used. A set of experiments in medium M3 (10 mM Ca^{2+}) was carried out using benzamide in the range 3 μM –3 mM as inhibitor of poly(ADP-ribose) polymerase. Nuclei were incubated at 37°C for 30 min and protein was extracted from the pellet with 5 % PCA and recovered as described above.

Following PCA extraction, some pellets of $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ -treated nuclei were subsequently extracted with 0.2 M sulphuric acid and proteins (core histones) recovered by acetone precipitation as described above.

Treatment of radiolabelled proteins

Phosphodiesterase treatment

Radioactive samples (about 50 μg of total protein) obtained by PCA extraction from nuclei treated with 10 mM Ca^{2+} and $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ were incubated with 0.2 unit of snake venom phosphodiesterase I for 3 h at 37°C in 200 mM Tris/HCl, 20 mM MgCl_2 , pH 8.8, according to the method of Chen et al. [31]. Samples before and after treatment with phosphodiesterase were then electrophoresed in acetic acid/urea gels and radioactivity evaluated by autoradiography.

Alkaline hydrolysis

First-dimension electrophoretic radioactive bands were excised and incubated for 3 h at 60°C in 10 mM Tris base, 1 mM EDTA, 8 mM NaOH, pH 11.4 [32]. Radioactivity in the bands both before and after incubation was measured with a scintillation β -counter, Kontron Betamatic IV.

Immobilization on phenyl boronate-agarose

About 50 μg of radioactive protein was dissolved in 1 ml of 50 mM Mops, pH 8.2, and added to 300 μl of phenyl boronate-agarose gel equilibrated in the same buffer [33]. The mixture was incubated for 30 min by shaking at room temperature. The radioactivity of the starting protein solution (before gel treatment) was compared with that of the supernatant after centrifugation of the gel suspension.

Electrophoresis and protein detection

First-dimension acetic acid/urea and second-dimension SDS electrophoretic analyses were carried out on polyacrylamide gels as described elsewhere [1,34] and protein detected by Coomassie Blue staining and autoradiography (Kodak X-OMAT AR film). For immunostaining, proteins were transferred to a membrane [polyvinylidene difluoride (PVDF), Immobilon P] as reported previously [4,15] and detected using the polyclonal antibodies specified above. Two different substrates for peroxidase were used (see under 'Materials' above). A sample of total protein obtained by PCA extraction of whole LLC tissue was used as reference [8].

RESULTS

Analysis of the HMGI proteins obtained from LLC nuclei treated with micrococcal nuclease

Purified nuclei from LLC cells were treated with micrococcal nuclease in MB buffer with 2 mM CaCl_2 added. S is the protein mixture from chromatin that diffuses from the nuclei into the medium following nuclease incubation, while P is that remaining in the nuclei and recovered by PCA extraction. Figure 1 shows an electrophoretic analysis (acetic acid/urea gels) of these fractions, two different identification methods being used: in panel A, proteins were Coomassie Blue-stained; in panels B and C a Western transfer was immunostained using antibodies raised against recombinant HMGI-C protein (panel B), while panel C shows the same membrane further stained with antibody against a peptide of the HMGI/Y sequence not present in HMGI-C.

Figure 1 Acetic acid/urea electrophoretic gel and Western blot of PCA-soluble proteins from LLC nuclei following incubation with micrococcal nuclease

Purified nuclei from LLC cells were incubated with micrococcal nuclease in MB buffer containing 2 mM CaCl_2 . S is the PCA-soluble protein which diffuses through the nuclear pores (lanes 3); P is the PCA-soluble protein which remains in the nucleus (lanes 1). Lanes 2 and 4 are as lanes 1 and 3 respectively, but incubation was without nuclease. Total protein (about 40 μg) directly extracted with PCA from whole LLC tissue was used as reference (lanes 5). (A) shows Coomassie Blue staining of the gel; (B) shows a Western blot of a gel similar to (A) immunostained with anti-(recombinant-HMGI-C) antibodies; (C) shows the same membrane as in (B) but subsequently also immunostained with antibodies raised against a peptide specific to HMGI/Y proteins. In the immunostaining, tetramethylbenzidine was used as peroxidase substrate. The arrowhead in (A), lane 3, indicates micrococcal nuclease.

Figure 2 Immunostaining of Western blots of HMGI proteins electrophoresed in acetic acid/urea gels

(A) (lanes 2–6) is a Western blot stained with anti-(recombinant HMGI-C) antibodies and (B) is the same membrane subsequently also stained with antibodies against a peptide specific to HMGI/Y proteins. In the immunostaining, tetramethylbenzidine was used as peroxidase substrate. In lanes 1–4 total protein directly extracted with PCA from whole LLC tissue was electrophoresed following incubation in different media: lane 1 (reference), protein sample used without any further treatment and stained with Amido Black; lanes 2, as lane 1, but immunostained; lanes 3, protein sample incubated in MB buffer containing 2 mM CaCl_2 as used in the micrococcal nuclease experiments; lanes 4, protein sample incubated in a medium containing 10 mM EGTA. Lanes 5 and 6 show S and P fractions respectively, obtained by incubation of purified LLC nuclei with micrococcal nuclease in MB buffer containing 2 mM CaCl_2 .

Since protein HMGI-C shows nearly 50% sequence identity with HMGI/Y proteins [3], anti-(recombinant HMGI-C) shows some recognition of proteins HMGI/Y, but with a lower sensitivity [4,13,15]. Figure 1(A) shows that histone H1 is not released (compare lanes 1 and 2 with lane 3). The arrowhead in lane 3 indicates the micrococcal nuclease used in the experiments, as demonstrated by recovering and sequencing the protein from a Western blot (results not shown). This result is consistent with that obtained by Zhao et al. [26] in that the protein mixture which diffuses through the nuclear pores following nuclease incubation does not contain histone H1. Antibody staining was used to identify HMGI proteins. Figure 1(B) shows that protein diffusing through the nuclear pores contains HMGI-C (lane 3), but some also remains in the nucleus (lane 1). Subsequent staining with antibodies specific for HMGI/Y (Figure 1C) demonstrated the presence of these proteins in lanes 1 and 5, but the level of HMGI/Y in lanes 2 and 3 was too low to allow their recognition (see below).

The migration of HMGI proteins in Figures 1(B) and 1(C) shows a striking feature: in lanes 1–3 the proteins run slower than the reference proteins of lane 5. This is true both for nuclease-treated nuclei (lanes 1 and 3) and nuclei incubated in the same medium but without nuclease (lane 2). To demonstrate that S (lanes 3) contains the complete set of three HMGI proteins, overloaded lanes were run and antibodies raised against a peptide specific to HMGI-C protein used in addition to those of Figure 1. The result showed that S contains all three HMGI proteins and these have a mobility much lower than those contained in a total HMGI sample directly extracted from LLC tissue, e.g. lanes 5 of Figure 1 (results not shown). To check that the lower mobility is not due to an artifact, we incubated the total protein sample obtained by direct extraction of whole tumour tissue with PCA [8] using several buffer media before electrophoresis. Some of these experiments are shown in Figure 2 (lanes 3 and 4). The result demonstrates that there is no direct effect of the incubation medium on protein mobility. On the other hand, a clear effect of the buffer medium on protein migration is observable (Figure 2, lanes 5 and 6) when the proteins are

Figure 3 Acetic acid/urea electrophoretic gel and Western blot of PCA-soluble proteins from isolated nuclei of LLC cells

Purified nuclei from LLC cells were incubated in three different media, M1, M2 and M3, then protein extracted with PCA and electrophoresed. The media were Tris-acetate buffers, pH 7.4, containing 50 mM K^+ plus 10 mM Mg^{2+} (M1), 10 mM Na^+ (M2) or 10 mM Ca^{2+} (M3). (A) Gel stained with Coomassie Blue. (B) Immunostaining of Western blot of same samples as in (A) using anti-(recombinant-HMGI-C) antibodies. In the immunostaining, tetramethylbenzidine was used as peroxidase substrate. Lanes 1, total protein PCA-extracted from whole LLC tissue; lanes 2, nuclei incubated in M1 buffer (containing Mg^{2+}); lanes 3, nuclei incubated in M2 buffer (no Mg^{2+} or Ca^{2+}); lanes 4, nuclei incubated in M3 buffer (containing Ca^{2+} but not Mg^{2+}).

extracted from nuclei incubated with nuclease in the same buffer used for Figure 1, lane 3 (i.e. MB buffer + 2 mM CaCl_2). The experiments of Figures 1 and 2 show that the treatment of LLC nuclei with exogenous micrococcal nuclease releases the set of three HMGI proteins, a fraction of which remains in the nucleus. Those released into S exhibit a markedly reduced electrophoretic mobility (lane 5), while those remaining in the nucleus (P) also show reduced mobility (lane 6) but to a lesser extent. Lane 2 of Figure 1 suggests that this reduced mobility is not necessarily related to the use of the micrococcal nuclease.

Analysis of HMGI proteins extracted from LLC nuclei without micrococcal nuclease treatment

To understand the role of the buffer medium, nuclei were incubated in media deprived of individual components and proteins extracted with PCA (see Figure 3). Figure 3(A) shows Coomassie Blue staining and Figure 3(B) is a Western blot of the same samples immunostained with antibodies to recombinant HMGI-C, which recognizes at low dilution all three HMGI proteins. Media M1, M2 and M3 (lanes 2, 3 and 4) are Tris-acetate buffers, pH 7.4, containing 50 mM K^+ plus additionally 10 mM Mg^{2+} in M1, 10 mM Na^+ in M2 and 10 mM Ca^{2+} in M3. Relative to lane 1 (total PCA-extracted protein), no effect on protein migration was seen for the medium containing Na^+ and K^+ (Figure 3B, lane 3), whereas a small retardation is present when the medium contains Mg^{2+} (lane 2). A large retardation and a multiplicity of bands are seen with the Ca^{2+} -containing medium (Figure 3B, lane 4) and the result is such that the well-defined pattern of bands in lane 1 cannot be used to identify the HMGI proteins in lane 4. We conclude that the effect observed in the nuclease digestion experiments was due to the Ca^{2+} ion present in the incubation medium. In order to understand the effect of varying Ca^{2+} concentration, a set of experiments was carried out over the range of Ca^{2+} concentration 1 μM –10 mM. The results showed that the effect of calcium is observable at concentrations higher than 0.1 mM (results not shown). The retarded migration

Figure 4 Acetic acid/urea electrophoretic gel of PCA-soluble proteins from isolated nuclei of LLC cells

Purified nuclei from LLC cells were incubated in the following media, then proteins extracted with PCA and electrophoresed. **(A)** Gel stained with Coomassie Blue; **(B)** autoradiography of gel **(A)**. Lanes 2, buffer M2 (no Mg^{2+} or Ca^{2+}); lanes 3, buffer M3 (containing 10 mM Ca^{2+}); lanes 4, buffer M2 (containing $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$); lanes 5, buffer M2 (containing 80 μM benzamide); lanes 6, buffer M3 (containing 10 mM Ca^{2+} and 80 μM benzamide); lanes 7, buffer M3 (containing 10 mM Ca^{2+} and $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$); lanes 8, buffer M3 (containing 10 mM Ca^{2+} , 80 μM benzamide and $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$). Lane 1 shows total protein extracted with PCA from whole LLC tissue and used without any further treatment.

of the HMGI proteins in this electrophoretic system (acetic acid/urea) could be due to post-translational modification of the proteins, e.g. phosphorylation, acetylation or ADP-ribosylation. To test for phosphorylation, we carried out experiments in which $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was added to the media M1, M2 and M3. The retarded bands of HMGI proteins were not labelled with ^{32}P , implying that the post-translational modification is not phosphorylation (results not shown).

To investigate whether ADP-ribosylation could be the origin of the electrophoretic retardation of HMGI proteins, we used $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ as precursor [35] and benzamide as inhibitor of ADP-ribosylation [36–38]. Autoradiography (Figure 4B) of a Coomassie Blue-stained gel (Figure 4A) shows that strong incorporation of label takes place in nuclei incubated in Ca^{2+} (Figure 4B, lane 7) and this is reduced by benzamide (lane 8). Moreover, a slight incorporation is also seen in the region of the HMGI proteins in the sample incubated in a medium containing neither Ca^{2+} nor Mg^{2+} (Figure 4B, lane 4). This last result could correspond to a basal modification of the HMGI proteins as previously observed by Elton and Reeves [39]. In order to quantitatively evaluate the inhibitory effect of benzamide, we carried out experiments in the concentration range 3 μM –3 mM. The IC_{50} was about 40 μM , whereas 2–3 mM benzamide gave 100 % inhibition (results not shown).

The one-dimensional electrophoresis of Figure 4 allows the undoubted identification of histone H1 as a target for ADP-ribosylation. However, the identification of the three HMGI proteins is not clear cut. A second-dimension SDS analysis of a pattern similar to that of Figure 4 (lane 7) was therefore carried out to distinguish the three proteins HMGY, HMGI and HMGI-C [7]. Panels A and A' of Figure 5 (Coomassie Blue staining and autoradiography, respectively) show a second-dimension analysis of the sample of Figure 4 (lane 4), obtained from nuclei incubated with $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ but no Mg^{2+} or Ca^{2+} . Spots of radioactivity corresponding to HMGY and I are seen to be retarded (Figure 5A') relative to bulk (Figure 5A). This effect is much more pronounced for the sample of lane 7, Figure 4, obtained when Ca^{2+} was included with $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ (Figures 5B and 5B'). To be

Figure 5 Second-dimension SDS electrophoretic analysis

First-dimension radiolabelled patterns as in lanes 4 and 7 of Figure 4 were electrophoresed in a second SDS dimension. **(A)** and **(A')**: sample 4 of Figure 4. **(B)** and **(B')**: sample 7 of Figure 4. **(A)** and **(B)**: Coomassie Blue staining. **(A')** and **(B')**: autoradiography of **(A)** and **(B)** respectively. The inset to **(B')** shows a Western blot from an acetic acid/urea gel of HMGI proteins (similar to sample 4 of Figure 3) immunostained first with antibodies specific to HMGI/Y proteins (track 1), then with antibodies raised against recombinant HMGI-C protein (track 2); at the dilution used in the staining, HMGI and Y are only weakly visible. In the immunostaining, ECL Western blotting detection reagent (RPN 2209) was used as peroxidase substrate. Dotted circles in **(A')** and **(B')** show the positions of HMGY and HMGI spots detected with Coomassie Blue in **(A)** and **(B)**.

certain that the radioactivity-labelled bands are indeed due to the HMGI family proteins, a Western blot of PCA-extracted proteins from LLC nuclei incubated in Ca^{2+} (as in lane 4, Figure 3) was immunostained first with antibodies specific to HMGI/Y proteins (Figure 5B', inset, track 1) and subsequently with antibodies to recombinant HMGI-C protein (Figure 5B', inset, track 2). The results demonstrate that the region of acetic acid/urea gels between histone H1 and HMGI17 contains multiple bands of modified HMGI family proteins.

Since the modified bands are labelled by radioactive NAD^+ , it is likely that the modification is ADP-ribosylation. To be certain that radioactivity detected in Figures 4 and 5 is due to ADP-ribosylated groups, we carried out three procedures that have been reported in the literature as identifying NAD^+ -dependent modification [31–33] (results not shown). In the first experiment (see the Experimental section), radioactive protein extracted with PCA from nuclei incubated in Ca^{2+} and $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ was treated with snake venom phosphodiesterase, an enzyme which hydrolyses ADP-ribosylated groups bound to protein [31]. By comparative electrophoresis and autoradiography we observed that all of the radioactivity was removed from all three HMGI proteins (and also histone H1) after 3 h incubation at 37 °C. In the second experiment, radioactive bands similar to those of Figure 4, lane 7, were excised from the gel and incubated in Tris/EDTA/NaOH buffer according to the method in [32] to hydrolyse ADP-ribosyl groups from proteins (see the Experimental section). Radioactive electrophoretic bands lost 91 % of their radioactivity following incubation for 3 h at 60 °C. In the third experiment, aminophenyl boronic acid-agarose was used

to specifically bind ADP-ribosylated proteins [33]. In a batch experiment, aminophenyl boronic acid-agarose retained 80 % of radioactive proteins following incubation for 30 min at room temperature.

Finally, since ADP-ribosylation of core histones is a well studied process [33,35,38,40–44], we extracted core histones from nuclei treated with 10 mM Ca^{2+} and $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$, subsequent to PCA extraction of histone H1 and HMG proteins. Two-dimensional acetic acid/urea/SDS gel electrophoresis and autoradiography (results not shown) demonstrated considerable ADP-ribosylation of core histones, with histone H2B being the major acceptor. This accords with gels presented in the literature [33] and we conclude that modification of the HMGI family takes place concurrently of the core histones.

DISCUSSION

The HMGI proteins are of particular current interest due to their involvement in processes such as neoplastic transformation and gene activation/regulation [13,15–20]. A role in adipogenesis, mesenchyme differentiation and the mouse pygmy phenotype have recently been demonstrated for HMGI-C [14,43]. Following the method of Zhao et al. [26], we carried out digestion of LLC nuclei using micrococcal nuclease to define the chromatin localization of protein HMGI-C, since those authors demonstrated that the other two proteins of the group (HMGI and HMGY) could be found in the S1 fraction from HeLa nuclei (S fraction in the present paper). Zhao et al. also reported that the proteins of the S1 fraction do not contain histone H1 and are enriched in HMGI/Y proteins. Our data of Figures 1 and 2 show that HMGI-C does not obviously differ in its localization from HMGI/Y since it is present in the H1-depleted S fraction that emanates from nuclei.

The present data also show that HMGI-C, like HMGI/Y, can be ADP-ribosylated. Acetic acid/urea gels show ladder patterns of many closely spaced bands (at least ten), characteristic of this modification [31–33,35–38,40–42,44,45]. The nature of the modification is shown by incorporation of $[\alpha\text{-}^{32}\text{P}]\text{NAD}^+$ into the proteins (see Figures 4 and 5), a process reduced by benzamide, a classic inhibitor of ADP-ribosylation. Our data do not allow us to discriminate between mono(ADP-ribosylation) and poly(ADP-ribosylation); however, the level of electrophoretic retardation is consistent with the presence of several modifying groups on each of the HMGI proteins.

Experiments carried out both with and without exogenous nuclease demonstrate that the ADP-ribosylation is induced by incubation with Ca^{2+} , while Mg^{2+} shows only a slight effect. It is unlikely that Ca^{2+} is the direct cause of the ADP-ribosylation of HMGI proteins since Ca^{2+} and Mg^{2+} have similar effects on the enzyme that catalyses the ADP-ribosylation reaction [46]. However, it is known that endonuclease activity in isolated nuclei requires Ca^{2+} for DNA cleavage [47,48]. The endonuclease activity induced by Ca^{2+} is a part of a spectrum of activities that Ca^{2+} induces in the nucleus: this includes protease, protein kinase and phosphatase activation, gene regulation (such as c-fos, c-jun, interleukin-2, krox 20, and ERG 10A), DNA repair and apoptosis [42–51]. It is therefore possible that ADP-ribosylation of HMGI proteins is the result of the activation of endogenous endonucleases which catalyse DNA fragmentation [45,52–57]. Poly(ADP-ribose) polymerase could be involved in this process since it has been reported [58–61] that this enzyme, which is inactive in unperturbed tissue culture cells, is activated following DNA single-strand and double-strand breaks, to which the polymerase binds and then automodifies. The incubation of isolated nuclei in the Ca^{2+} -containing buffers used here probably

mimics the consequences of extracellular signals, such as $\text{TNF-}\alpha$ [62–65], that trigger release of Ca^{2+} from the endoplasmic reticulum. This cytosolic Ca^{2+} propagates into the nucleus [66,67] and initiates DNA fragmentation through activation of endogenous nucleases. The present work suggests that the ADP-ribosylation of HMGI/Y/I-C proteins seen here, as a consequence of incubating nuclei in Ca^{2+} -containing buffers, is part of the general post-translational modification process that introduces the same group(s) into several nuclear protein families, such as histones, RNA/DNA polymerases and topoisomerases [68] following DNA damage in apoptosis and other processes. Apoptosis is a complex process comprising many cytoplasmic and nuclear biochemical events and interference with Ca^{2+} homeostasis is one of the mechanisms which could trigger it [69]. Ca^{2+} -induced ADP-ribosylation of HMGI proteins at nuclease-sensitive sites could be related to the morphological changes of chromatin which take place during collapse of nuclear structure in apoptosis [69].

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