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GUANIDINE GROUP SPECIFIC ADP-RIBOSYLTRANSFERASE IN MURINE CELLS

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SUMMARY: We have identified a guanidine group specific ADP-ribosyltransferase activity, capable of transferring an ADP-ribose group from NAD to a low molecular weight guanidine compound [p-(nitrobenzylidine)amino]guanidine and proteins such as histone and poly-L-arginine, in a variety of murine cell lines. The enzyme activity appears to be associated with an integral membrane protein of apparent molecular weight 30-33 kDa. Incubation of the viable cells in isotonic phosphate buffered saline with [^{32}P]NAD results in the incorporation of label into cellular proteins. Dimethyl sulfoxide treatment of the cells downregulates the transferase activity as well as the ADP-ribosylation of cell proteins with extracellular NAD. © 1991 Academic Press, Inc.

Guanidine group specific ADP-ribosyltransferase activity has been described in a large number of eukaryotic cells and tissues (1-11). In eukaryotes the activity is described in cytosolic (1), mitochondrial (12), microsomal (4,13) and nuclear fractions (3,14). The widespread occurrence of transferase activity and a hydrolytic activity that cleaves (ADP-ribosyl)protein linkage (9,15,16) together with the observation that mono(ADP-ribosyl)ation greatly exceeds the nuclear poly(ADP-ribosyl)ation in the extranuclear compartments (17,18), are strong indications that mono(ADP-ribosyl)ation reactions may be a physiologically important cellular regulatory mechanism. Recently, we have reported that guanidine group specific ADP-ribosyltransferases utilize low molecular weight guanylhydrazones as artificial substrates (5,19). Spectrophotometric and high performance liquid chromatography (HPLC) assay methods have been reported using guanylhydrazones as chromophoric substrates (5,19). To better understand this reaction at the cellular level, we have investigated several cell lines for direct demonstration of the activity in cell lysates and subcellular fractions using synthetic guanylhydrazones as artificial acceptors. In this paper we report the presence of guanidine group specific ADP-ribosyltransferase activity in a variety of murine cells including lymphocytes, T cell lymphomas, T cell hybridomas and myoblasts.

Abbreviations used: HPLC, high performance liquid chromatography; NBAG, [p-(nitrobenzylidine)amino]guanidine; PBS, phosphate buffered saline (10 mM phosphate-0.154 M NaCl, pH 7.4); TLC, thin layer chromatography; DMSO, dimethyl sulfoxide.

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MATERIALS AND METHODS

Chemicals and reagents. Snake venom phosphodiesterase 1, Histone type VII S and other chemicals were obtained from Sigma Chemical Company, St.Louis, MO. [α-^{32}P]adenylate labeled NAD (800 Ci/mmol= 29.6 TBq/mmol) was obtained from New England Nuclear, MA. [p-(Nitrobenzylidine)amino]guanidine (NBAG) was synthesized as described earlier (12). The culture media used were DMEM (Flow laboratories, Inc., McLean, VA) supplemented with 1 mM glutamine, 0.1 mM sodium pyruvate (Gibco, Grand Island, NY) or RPMI 1640. Cells were grown in the media supplemented with 10% heat inactivated (56°C, 30 minutes) bovine calf serum (BCS) (Cell culture laboratories, Cleveland OH) 100 U/ml penicillin and 100 µg/ml streptomycin.

Cell lines. The cell lines used in this study are from ATCC. The T cell hybridomas used were constructed in our laboratory by using standard fusion techniques using BW5147 line as the tumor partner (20). The T cell partner for the herpes simplex virus specific hybridoma G was obtained from Balb/c mice immunized with herpes simplex virus type 1 (mP strain) (21). Similarly the T cell partner for the vesicular stomatitis virus specific T cell hybridoma E15 was obtained from Balb/c mice immunized with vesicular stomatitis virus (Indiana strain) (22). The murine skeletal muscle derived mononuclear cell or myoblast line SP25 was derived in our laboratory from adult C57BL/6 mice by a modification of the method described by Koningsberg (23). T lymphocytes were purified from 5-8 weeks old Balb/c mouse splenocytes by passing through Nylon wool column. The nonadherent T cells were eluted using warm medium (37^{0}) and the adherent cells (B cells and macrophages) were eluted by pressing the column with ice cold PBS. The red cells were removed by centrifugation over Ficoll-hypaque gradient followed by ammonium chloride lysis.

Preparation of cell membranes and cell lysates. Membranes were prepared from cells in the exponential growth phase, by a procedure similar to that reported by Koski and Klee (24). Briefly, cells were collected by centrifugation at 900 x *g*, washed three times with PBS, resuspended in 10 mM Tris-HCl, 0.1mM EDTA (pH 7.4) buffer, and disrupted by one cycle of quick freezing and thawing, and then homogenized in glass homogenizer by using 25 strokes. The homogenate was centrifuged at 900 x *g* to remove cell nuclei and debris. The supernatant was centrifuged at 100,000 x *g* in a Beckman 70.1 Ti rotor in Beckman model L8-M ultracentrifuge. The residue was suspended in 10 mM Tris-0.1 mM EDTA (pH 7.4) buffer and centrifuged at 100,000 x *g* for one more time. The resulting pellet was suspended in 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.4), 10% sucrose at a concentration of 4-5 mg protein/ ml and quick frozen in liquid nitrogen and stored at -70°C.

Cell lysates for transferase assay were prepared from cells in the exponential growth phase. Cells were collected by centrifugation at 900 x *g* for 10 min, washed three times in PBS and lysed by incubating in 1 ml of 2% polyoxyethylene-9-lauryl ether in 10 mM Tris-HCl, 0.1mM EDTA (pH 7.4) per $2x10^{7}$ cells for 1 h at 4 ^{o}C. The lysate is then centrifuged at 900 x *g* for 10 min and the supernatant was used for transferase assay.

ADP-ribosyltransferase assay. Guanidine group specific mono ADP-ribosyl transferase activity was measured using the synthetic guanylhydrazone, NBAG, as the substrate by the spectrophotometric method (19). Briefly, reaction systems were set up with 1 mM NBAG, 10 mM dithiothreitol, 5 mM NAD, 50 mM Tris-HCl (pH 7.4) and 50 µl of the cell lysate or 200 µg/ml of membrane proteins in a total volume of 100 µl. Control samples lacking either NAD or NBAG or enzyme source were also set up simultaneously. After 1-2 h incubation at 37°C, the reaction was terminated by adding 400 µl of a 1:1 slurry of Dowex 50W-X4 resin suspended in 10 mM phosphate (pH 7.4) and 1.6 ml of 10 mM phosphate (pH 7.4). Samples were mixed well, the resin was allowed to settle and 900 µl of supernatant mixed with 100 µl of 1 N NaOH, and the optical density at 375 nm was recorded. The optical density was converted to amount of product formed by determining the molar absorption coefficient of NBAG under identical conditions. The identity of the ADP-ribosylated product was confirmed by HPLC analysis of the reaction system as described by Soman *et al* (5) followed by measuring absorption characteristics of the product peaks. A Waters model 600E pump with a Waters model 464 detector and microsorb C18 column (4 mm x 15 cm) from Rainin Instrument Co., MA, were used for analysis.

Endogenous ADP-ribosylation of membrane proteins. Membranes (100-150 µg/ml) were incubated with 10 µM [^{32}P]NAD (4-5 Ci/mole) in the presence of the indicated ligands for 30 min at 37°C . At the end of reaction the proteins were precipitated with 10% ice cold trichloroacetic acid. Precipitated proteins were collected by centrifugation, washed once with 5% trichloroacetic acid followed by a wash in water saturated ice cold ether. The pellet was dissolved in SDS- PAGE sample buffer. SDS -PAGE was performed by the procedure of Laemmli (25). Gels were stained in Coomassie blue, destained, dried and exposed to Kodak XAR-5 film.

Triton X114 phase separation was performed as described by Bordier (26). The detergent phase was reconstituted with 10 mM Tris-HCl, 0.1 mM EDTA (pH 7.4) to the original volume of the detergent lysate used for separation of phase. Activity staining of SDS-PAGE separated membranes was performed as described by Godeau *et al.*(6). Protein concentrations in membrane preparations were determined using Lowry reagent (27). Protein concentration in detergent solubilized cell lysate was determined using a modified Biuret reaction (28).

RESULTS AND DISCUSSION

In order to have a better understanding of the distribution of guanidine group specific ADP-ribosyltransferase activity in eukaryotic cells, we screened a variety of cell lines for the detection of this activity. The spectrophotometric method (19) using NBAG as artificial substrate was used for screening cell lysates and subcellular fractions. The results in Table 1A suggest that the activity is present in some activated murine cells, like myoblast line SP25, thymoma BW5147.G.1.4, lymphoma Yac-1, and T cell hybridomas E15 and G. A number of other murine T cell lymphomas (EL4, S49.1, R1.1, RAW 8.1, and BW 5147.3), two murine B cell tumor lines A20, and NS1, and more than 15 human cell lines tested (including T cell lymphomas, B cell lymphomas, erythrolekemic cells and Hela cells) did not show any detectable activity. The ADP-ribosylation of NBAG by cell lysates and membrane preparation was confirmed by HPLC analysis of the reaction system as described earlier (12) (data not shown). In all cases where the activity was detected, it was primarily localized in membrane preparations (Table 1 B) with very little (5-10%) or no activity in the cytosolic and nuclear fractions (data not shown). Incubation of NBAG with ADP-ribose (5 mM) alone or with the membranes under reaction conditions identical to that used for ADP-ribosyltransferase assay did not show any positive readings indicating that nonenzymatic ADP-ribosylation of NBAG does not occur under these conditions. The time and protein concentration dependency was evaluated using lysates and membranes from the myoblast (SP25) and T cell hybridoma (G). The activity showed time linearity for 2 h at 37^{0}C with 1 mg/ml lysate protein concentration or 100 µg/ml membrane protein concentration and linear correlation with protein concentration in the range (0.5-2.5 mg/ml) for cell lysate and (80-800 µg/ml) for

TABLE 1A

ADP-RIBOSYLTRANSFERASE ACTIVITY IN MURINE CELLS

CELL LINE	ACTIVITY [µmoles/h/mg protein+/-(SE)]
BW5147	0.035 (0.016)
E15	0.047 (0.01)
G	0.106 (0.019)
Yac-1	0..11 (0.03)
SP25	0.082 (0.013)
A20	nd*
NS1	nd
EL4	nd

ADP-ribosyltransferase activity in detergent solubilized cell lysate is measured using the synthetic guanylhydrazone NBAG as the acceptor as described in Materials and Methods.

*no activity detected.

TABLE 1B

ADP-RIBOSYLTRANSFERASE ACTIVITY IN MEMBRANE PREPARATIONS FROM DIFFERENT MURINE CELLS

CELL LINE	ACTIVITY [μmoles/h/mg membrane protein +/-(SE)]	
	+Dithiothreitol (10 mM)[a]	- Dithiothreitol[b]
BW5147.G.1.4	0.12 (0.012)	0.056 (0.005)
E15	0.37 (0.018)	0.230 (0.002)
Yac-1	0.6 (0.048)	0.2500 (0.005)
G	1.0 (0.035)	0.280 (0.005)
SP25	0.56 (0.010)	0.250 (0.012)
A20	nd*	nd
EL4	nd	nd

Membranes were prepared from different cells and ADP-ribosyltransferase activity toward the model substrate NBAG measured as described in Materials and Methods.

[a]Activity measured in the presence of 10 mM dithiothreitol
[b]Activity measured in the absence of dithiothreitol
*no activity detected

membrane in a 60 minute assay. The membrane preparations were capable of ADP-ribosylating histone and poly-L-arginine as determined by their ability to incorporate radioactivity from [^{32}P]NAD to these proteins as measured by a filter paper assay (13)(data not shown).

The activity in the membrane preparations was not extracted with high salt low detergent buffers (0.6M NaCl, 0.02% Triton X100) but was solubilized in 1-2% nonionic detergents. Upon solubilization in 2% Triton X114 followed by phase separation according to the procedure of Bordier (26); 60-70% of the activity was found to be associated with the detergent phase while more than 90% of the total proteins (including most of the major protein bands observed on SDS-PAGE) separated in the aqueous phase (data not shown). This result suggest that the transferase activity is associated with an integral membrane protein.

We analyzed the membrane preparations from different cells for ADP-ribosyltransferase activity using the zymographic *in situ* gel staining method described by Godeau *et al.* (6). Intense staining was observed in the 30-33 kDa range with the membranes from all the positive cell lines and no staining was observed with membranes from negative cell lines (Figure 1). The results confirm the presence of an ADP-ribosyltransferase activity in the positive cells and also suggest that the activity may be associated with a 33 kDa protein .

Because our results suggested that the transferase activity was associated with an integral membrane protein, it was of interest to see the effect of exogenous NAD added to viable cells under isotonic conditions. Viable cells (obtained by fractionation over Ficoll-hypaque gradient) were suspended in PBS and incubated with 10 μM α-[^{32}P]adenylate labeled NAD and 1 mM dithiothreitol for 30 min at 37°C. During this period there was extensive labeling of a number of proteins in the transferase positive cell lines and no labeling in the transferase negative cell lines (Figure 2). There was no difference in the viability of cells, as determined by trypan blue exclusion, before and after the treatment. A protein of 80-90 kDa was extensively labeled. Other major labeled proteins had apparent molecular weights of 200, 165, 145, 110, 54, 48, 38, 34, 27

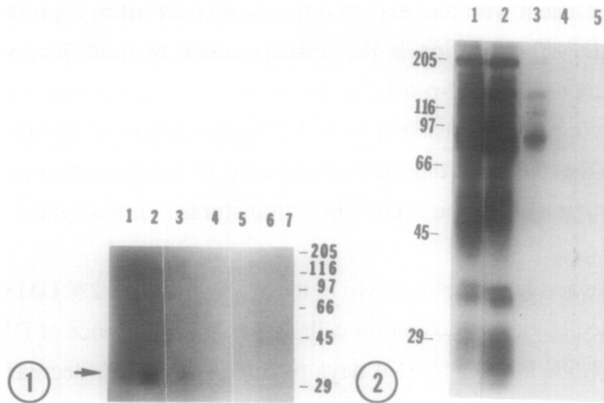

Figure 1. ***In situ* zymographic detection of ADP-ribosyltransferase activity.** Membranes were solubilized in 62.5 mM Tris (pH 6.8), 10 % glycerol, 1% SDS, 5 mM dithiothreitol. Samples were incubated for 5 min at room temperature and run on 10% SDS-PAGE. The proteins were renatured by incubating the gel in 2% Triton X100 for 1 h at room temperature followed by another 15 min wash in 2% Triton X100 and two 15 min washes in distilled water. The renatured gel was incubated with 100 mM phosphate (pH 7.4) 1 mM DTT, 1 mg/ml Histone type V11 S and 100 μM [^{32}P]NAD (100-200 cpm/pi mole) for 1 h. The medium is removed and gel is fixed in 50% trichloroacetic acid, 0.25% Coomassie brilliant blue for 30 min. The fixed gel is destained and dried and exposed to Kodak XAR film as described by Godeau *et al.* (6). SP25 (lane 1), G (lane 2), E15 (lane 3), BW5147.G.1.4 (lane 4), A20 (lane 5), two variants of K562 (lane 6 &7).

Figure 2. **Labeling of cell proteins with exogenous [^{32}P]NAD.** Cells were washed in isotonic PBS and incubated in PBS containing 1 mM dithiothreitol and 10 mM [^{32}P]NAD [2.5 Ci/ m mole (2 million cells/200 μl)] for 30 min at 37°C and reaction terminated by adding 10 ml of ice cold PBS containing 1 mM unlabeled NAD. Cells were collected by centrifugation at 900 x *g* for 10 min at 4°C and washed four times in 10 ml portions of ice cold PBS. The cells were then lysed in 500 μl of buffer A (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X100, 1% deoxycholate, 0.1%SDS, 100 units/ml aprotinin, 1 mM phenylmethylsulphonyl fluoride, 5 mM EDTA , 20 μM vanadate and 10 μg/ml trypsin inhibitor, pH 7.4). The nuclei and debris were removed by centrifugation at 900 x *g* for 10 min and supernatant analyzed by SDS-PAGE and autoradiography under reducing conditions. SP25 (lane 1), G (lane 2), E15 (lane 3), A20 (lane 4) and K562 (lane 5).

and 24 kDa. Labeling was also observed in the absence of dithiothreitol, but to a much lesser extent (data not shown). The membrane association of the activity and the labelling of cell proteins with extracellular NAD suggest that the transferase activity may be associated with a cell surface protein. Further studies are required to see whether the transferase is truly an ectoprotein.

Incubation of isolated membranes with [^{32}P] adenylate labeled NAD resulted in the labeling of a number of proteins. Dithiothreitol (10 mM), polylysine (25 μg/ml) spermine and spermidine (10 mM) stimulated the labeling of proteins with NAD and L-arginine (50 mM), ADP-ribose, ATP, ADP, and AMP (10 mM) significantly reduced the labeling (data not shown).

In order to understand the nature of the label incorporated into the cell, the labeled cells were suspended in isotonic PBS with 10 mM Mg^{++} and snake venom phosphodiesterase 1 for 1 h at 37°C. More than 70% of the incorporated label was released into the medium under these conditions. The supernatant was mixed with an equal volume of chilled ethanol (-70° for 1 h) and the precipitated proteins were removed by centrifugation. Analysis of the supernatant by TLC as described by Edmonds *et al.*(29) revealed that more than 70% of the label comigrated with 5'-AMP. Histones labeled with membrane and [^{32}P]NAD were also digested with snake venom phosphodiesterase and Mg^{++} and TLC analysis revealed 5'- AMP as the major product (data not

shown). This was consistent with that expected for mono(ADP-ribosyl)protein adduct and hence prove that mono(ADP-ribosyl)ation is the primary mode of modification. Analysis of the hydroxylamine stability of the ADP-ribosylated proteins under conditions similar to that reported by Hsia *et al.*(30) gave results consistent with ADP-ribosylation of arginine residues (data not shown). This result, together with the ADP-ribosylation of the guanidino compound NBAG and of poly-L-arginine suggest that the ADP-ribosyltransferase investigated is guanidine group specific.

The transferase activity in cells grown in the presence of 1.3-2% DMSO was considerably lower compared to the activity in the control cells grown in the absence of DMSO. Culturing the cells in presence of 1.5% DMSO for 3-4 days resulted in 50-80% decrease of the transferase activity (data not shown). Consistent with this decrease in activity, the labeling of cell proteins with exogenously added [^{32}P] NAD was also reduced (not shown). The effect of DMSO on the transferase activity and ADP-ribosylation of cell proteins was studied in more detail in the myoblast line SP25 and T cell hybridoma G. These cells were grown and maintained for one month in presence of 1.5% DMSO. During this period there was no significant difference in cell growth (determined by hematocytometer counting) or in cell viability (determined by trypan blue dye exclusion) of the cells grown in presence of DMSO compared to that grown in the absence of DMSO (not shown). After 3-4 days in DMSO the transferase activity as measured using NBAG was reduced to 20-30% of the activity in the control cells maintained in the absence of DMSO. Prolonged culture in presence of DMSO did not further lower the transferase activity significantly. Even after continous growth in DMSO for one month period there was 10-15% of activity compared to that of the control cells (not shown). The transferase activity in membranes prepared from DMSO treated and control cells showed similar pattern. Consistent with this the *in situ* zymographic detection of tranferase activity showed intense staining of a 33 kDa band in the control membrane and little or no staining in the membranes from DMSO treated cells (Figure 3A) Similarly there was a corresponding reduction in the labeling of cell proteins using exogenously added NAD (Figure 3B) or in the labeling of proteins in isolated membrane (Figure 3C) While the possibility of the occurrence of more than one ADP-ribosylating activity in these cell lines can not be ruled out the apparent parallelism in the effect of DMSO on the transferase activity measured using the synthetic compound, the *in situ* zymographic activity staining and the *in vitro* labeling of cell proteins and labeling of proteins in isolated membranes with [^{32}P] NAD suggest to us that these processes are likely to be due to the same activity. DMSO has been reported to be a potential differentiation inducer for several cell lines (30,31) and has been reported to affect a variety of biochemical functions in HL60 cells (32-34). Further detailed studies are required to find the effect of DMSO on these cell lines and to establish the role of the DMSO induced downregulation of the transferase activity on the cell growth and differentiation.

Because the transferase activity was detected in T cell hybridomas and T lymphomas, we looked for similar activity in murine splenocytes and purified T lymphocytes. Using the spectrophotometric assay with NBAG as substrate, transferase activity was detected in Balb/c mouse splenocytes (0.025 μmoles/h/mg), in purified splenic T cells (0.032 μmoles/h/mg) and nylon wool adherent cells (0.048 μmoles/h/mg). However, more than 50% of the transferase activity in purified T cells was associated with the nuclear pellet, 30-35% in the cytosol and less

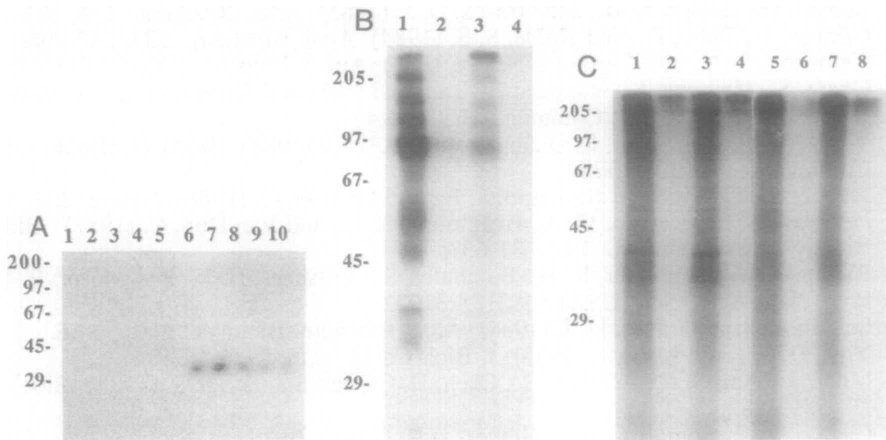

Figure 3. **Effect of DMSO on the ADP-ribosylation process.** Cells were grown in DMEM complete medium containing 10% BCS in the presence or absence of 1.5% DMSO. After specified period of culture cells were collected by centrifugation, washed in PBS and incubated with [^{32}P] NAD as described in figure 2 or processed for membrane preparation as described in Materials and Methods.

A. ***In situ* zymographic detection of ADP-ribosyltransferase activity** (for details see legends to figure 1). Lanes (1-5) membranes prepared from cells grown in the presence of 1.5 % DMSO for the indicated periods and (6-10) control cells grown in the absence of DMSO. Lanes (1-3 and 8-10) SP25 cells, (4-7) G cells). Lanes (1 & 10) 20 days in culture, (2 & 9) 12 days in culture, (3 & 8) 5 days in culture, (4 & 7) 5 days in culture and (5 & 6) 10 days in culture.

B. **Labelling of cell proteins with extracellular [^{32}P]NAD** (for details see legends to figure 2) Lanes (1 & 2), G cells and (3 & 4) , SP25 cells. Lanes (1 & 3) are control cells grown in the absence of DMSO and lanes (2 & 4) cells grown in the presence of 1.5% DMSO for 10 days.

C. **Labeling of proteins in isolated membranes.** Membrane (150 µg/ml) was incubated with 10 mM thymidine, and 10 µM [^{32}P] NAD for 30 min at 37^{0}C and subjected to SDS-PAGE and autoradiography as described in Materials and Methods. Lanes (1-4) G cells and (5-8) SP25. Lanes (1,3, 5 & 7) Membranes prepared from cells grown in the absence of DMSO and (2,4,6 &8) membranes prepared from cells grown in presence of 1.5% DMSO. Lanes (1,2, 5 & 6) cells grown for 5 days in culture and (3,4, 7 & 8) cells grown for 10 days in culture.

than 15% in the membrane pellet. Consistent with this finding, incubation of the purified lymhocytes with exogenous [^{32}P]NAD did not label the cell proteins. Whether the tranferase activity in the splenic lymphocytes and the T cell hybridomas and lymphomas are due to the same protein and the different subcelluar localization is a consequence of transformation remains to be worked out.

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