Stability of centrosome numbers in poly(ADP-ribose) polymerase-1- and poly(ADP-ribose) glycohydrolase-deficient mouse ES cells

By Hideki OGINO,* Akemi GUNJI,* Nobuo KAMADA,** Hitoshi NAKAGAMA,* Takashi SUGIMURA, M. J. A.,* and Mitsuko MASUTANI*),†
(Contributed by Takashi SUGIMURA, M. J. A.)

Abstract: Poly(ADP-ribose) polymerase-1 (Parp-1) localizes mainly in the nucleus and functions in DNA repair, genome stability and cell death regulation. Meanwhile, it also localizes in centrosomes and is involved in the regulation of centrosome duplication. An abnormal increase in centrosome numbers is frequently observed in Parp-1-deficient (Parp-1−/−) mouse embryonic fibroblasts (MEFs) (Kanai et al. (2003) Mol. Cell. Biol. 23, 2451-2462). However, there are no studies on whether the centrosome abnormality occurs also in other cell types under Parp-1 deficiency. In this study, we report that Parp-1−/− mouse embryonic stem (ES) cell lines did not show an abnormally increased number of centrosomes compared to wild-type ES cells. Recently, poly(ADP-ribose) glycohydrolase (Parg) has also been shown to localize in centrosomes (Ohashi et al. (2003) Biochem. Biophys. Res. Commun. 307, 915-921). The number of centrosomes of Parg-deficient (Parg−/−) ES cells was also analyzed in this study and was found to be stable under Parg deficiency. We also examined centrosome numbers in wild-type, Parp-1−/− and Parg−/− ES cell lines after treatment with methylmethanesulfonate (MMS) or γ-irradiation. Although a slight increase in the number of centrosomes is observed in each genotype twenty-four hours after treatment with MMS at 50 µM or with γ-irradiation at 1.4 Gy, there was no difference among the genotypes. These results suggest that loss of Parp-1 and Parg is insufficient to induce abnormality in centrosome numbers in ES cells and that ES cells possibly possess a strict mechanism for the maintenance of a normal number of centrosomes.

Key words: Poly(ADP-ribose) polymerase-1; poly(ADP-ribose) glycohydrolase; centrosome; embryonic stem cell; DNA damage.

Introduction. Poly(ADP-ribose) polymerase-1 (Parp-1) is an enzyme catalyzing polyADP-ribosylation, a post-translational modification.1,2,3 Parp-1 has a dominant role in polyADP-ribosylation in cells, modifying Parp-1 itself and various other proteins associated with chromatin after being activated by DNA strand breaks. Poly(ADP-ribose) is degraded into ADP-ribose by poly(ADP-ribose) glycohydrolase (Parg) by splitting α(1″→2′) glycosidic linkages.3,4 Poly(ADP-ribose) metabolism plays a role in many biological functions including DNA repair,6 cell cycle regulation,7 chromatin remodeling,8 transcriptional activation and repression,9 and protein degradation.10

Recent studies showed that Parp-1 localizes both in nuclei and centrosomes throughout cell cycles.11,12 An abnormal increase in the number of centrosomes is frequently observed in 3-aminobezamide-treated mouse embryonic fibroblasts (MEFs) and Parp-1−/− MEFs,12 and it is shown that replication of chromosomes and duplication of centrosomes are uncoupled in Parp-1−/− MEFs.12 Parp-1 also polyADP-ribosylates centrosomal p5312 and may thus modulate its function in centrosomal regulation.

We previously reported that immortalized Parp-1−/− MEFs show enhanced ploidy increase compared to wild-type (Parp-1+/+) MEFs.12 It could be speculated that an increase in centrosome numbers and ploidy coordinately occurred under Parp-1 deficiency. On the other hand, we reported that Parp-1−/− ES cells did not manifest ploidy increase.14 To clarify the relationship of ploidy increase and abnormality in centrosome numbers and to understand the impact of Parp-1 deficiency on centrosome numbers in cells other than MEFs, we ana-
lyzed the number of centrosomes in Parp-1\(^{-}\) and Parg\(^{-}\) ES cells. Since Parg was recently reported to be localized also in centrosomes,\(^{15}\) we analyzed the number of centrosomes in Parg\(^{-}\) ES cells as well.

**Materials and methods.** **Cell culture.** ES cell lines, J1 (Parp-1\(^{+/+}\), Parg\(^{+/+}\)), 210-58 (Parp-1\(^{-}\)), 226-47 (Parp-1\(^{-}\)),\(^{14}\) B609 (Parg\(^{++}\)), D79 (Parg\(^{++}\)), and D122 (Parg\(^{++}\)) were maintained in the absence of a STO cell feeder layer in Dulbecco’s modified Eagle’s medium (Gibco) containing 20% fetal calf serum supplemented with non-essential amino acids and leukemia inhibitory factor (ESGRO, Chemicon) as described elsewhere.\(^{14}\) For measurement of centrosomes by indirect immunofluorescence, 10\(^{6}\) cells were plated onto 100-mm diameter gelatin-coated dishes (Iwaki). The next day, cells were mock-treated, treated with methylmethanesulphonate (MMS, Sigma) at 50 µM or \(\gamma\)-irradiated at 1.4 Gy using \(^{60}\)Co. Twenty-four hours after the treatment, cells were fixed with 10% formalin and 10% methanol for 20 min at room temperature. All of the subsequent processes were carried out at room temperature. After washing with phosphate-buffered saline (PBS), cells were permeabilized with 1% NP-40 in PBS for 5 min, incubated with blocking solution (2% bovine serum albumin in PBS) for 1 hour and probed with anti-\(\gamma\)-tubulin polyclonal antibody (T3559, Sigma) for 1 hour. The antibody-antigen complexes were visualized with Alexa Fluor\(^{\text{®}}\) 594-conjugated goat anti-rabbit IgG antibody (Molecular Probes) by incubation for one hour. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) and were analyzed with a fluorescence microscope (Axiovert 200, Zeiss).

**Statistical test.** The significance of differences was analyzed by the Mann-Whitney \(U\) test and the Kruskal-Wallis test using SPSS version 6.1 software (SPSS Inc.).

**Results.** The number of centrosomes was counted in an asynchronous culture of Parp-1\(^{+/+}\) and Parp-1\(^{-}\) ES cells after staining with centrosomal \(\gamma\)-tubulin. The result is shown in Fig. 1A. In every genotype, one or two \(\gamma\)-tubulin signals were observed in more than 98% of total cells, and there was no difference in the percentage of the cells containing three or more centrosomes between Parp-1\(^{+/+}\) and Parp-1\(^{-}\) ES cells.

Next, to elucidate whether degradation of poly(ADP-ribose) by Parg is important for centrosome
function, we examined centrosome numbers in Parp−/− ES cell lines, in which total poly(ADP-ribose) degradation activity was reduced approximately by half. We observed a 3-fold accumulation of poly(ADP-ribose) in these Parp−/− ES cells without any treatment compared to Parp+/+ ES cells (Fujihara et al., submitted). An asynchronous culture of Parp−/− ES cells was used for the measurement of centrosome numbers (Fig. 1B). Most of Parp+/+, Parp−/− and Parp−/− ES cells contained either one or two centrosomes per cell and there was no significant difference among Parp genotypes, although D122 cells showed a slightly higher frequency of three or more centrosomes per cell than in Parp−/− ES cells (p = 0.0495 in Mann-Whitney U test). These results indicate that loss of Parp-1 and Parp is insufficient to induce abnormality in the number of centrosomes in ES cells in normal culture conditions.

It is reported that various genotoxic stresses, including treatment with hydroxyurea, aphidicolin and γ-irradiation, induce an abnormal increase of centrosome numbers in various cell lines. Therefore, we speculated that an abnormal increase in centrosome numbers might be induced in ES cells after treatment with genotoxic stresses. Wild-type, Parp-1+/− and Parp−/− ES cell lines were treated with methylmethanesulphonate (MMS) or γ-irradiation. MMS treatment was carried out at a concentration of 50 µM, which causes an approximately 2-fold elevation in the frequency of sister-chromatid exchanges and γ-irradiation was performed at 1.4 Gy. Both treatments did not affect the survival of Parp-1+/−, Parp−/− and wild-type ES cells (data not shown). The results are shown in Table I. After treatment with MMS at 50 µM or γ-irradiation at 1.4 Gy, the percentages of cells containing abnormal numbers of centrosomes, namely 3 or more, showed a tendency to slight increase in every genotype. However, there was no significant difference in the increase of centrosome numbers in Parp-1−/− and Parp−/− ES cells compared to wild-type ES cells.

**Discussion.** In this study, we demonstrated that Parp-1 and Parp deficiency did not induce an abnormal increase in centrosome numbers in non-treated conditions and after treatment with DNA damaging agents. The results with Parp-1−/− ES cells obtained in this study are in clear contrast with those obtained with Parp-1−/− MEFs, with which the number of centrosomes significantly increased compared to wild-type MEFs. Since Parp-1−/− MEFs showed enhanced ploidy increase whereas Parp-1−/− ES cells did not show it, one possible explanation could be that the ploidy increase may be closely related to the abnormal elevation of the number of centrosomes.

It is reported that percentage of the cells containing three or more centrosomes per cell elevated from 2% to

| ES cell line | Treatment | Centrosome number per cell (%) |
|-------------|-----------|--------------------------------|
| J1 (Wild-type) |            |                                |
| Mock-treated | 0.5 ± 0.3  | 0                              |
| MMS | 0.9 ± 0.3  | 0.8 ± 0.1 | 0 | 0 | 0 | 0 |
| IR | 1.9 ± 0.9  | 0.5 ± 0.2 | 0 | 0 | 0 | 0.1 ± 0.1 |
| 210-58 (Parp-1−/−) | |                                |
| Mock-treated | 0.6 ± 0.3  | 0                              |
| MMS | 1.2 ± 0.5  | 0.5 ± 0.1 | 0.3 ± 0.1 | 0 | 0 | 0 |
| IR | 0.8 ± 0.3  | 0.7 ± 0.4 | 0 | 0 | 0 | 0 |
| 226-47 (Parp−/−) | |                                |
| Mock-treated | 0.9 ± 0.5  | 0.1 ± 0.1 | 0 | 0 | 0 | 0 |
| MMS | 0.9 ± 0.6  | 1.1 ± 0.6 | 0 | 0 | 0 | 0 |
| IR | 1.1 ± 0.4  | 0.7 ± 0.1 | 0.1 ± 0.1 | 0 | 0 | 0 |
| D79 (Parp−/−) | |                                |
| Mock-treated | 0.6 ± 0.3  | 0.2 ± 0.2 | 0 | 0 | 0 | 0 |
| MMS | 0.3 ± 0.2  | 0 | 0 | 0 | 0 | 0 |
| IR | 0.1 ± 0.1  | 0.1 ± 0.1 | 0 | 0 | 0 | 0 |
| D122 (Parp−/−) | |                                |
| Mock-treated | 1.5 ± 0.2  | 0.1 ± 0.1 | 0 | 0 | 0 | 0 |
| MMS | 2.3 ± 0.3  | 0.4 ± 0.4 | 0 | 0 | 0 | 0 |
| IR | 1.2 ± 0.2  | 0.1 ± 0.1 | 0 | 0 | 0 | 0 |

a Measurements were done in triplicate. In each measurement, 200-300 cells were counted for centrosome numbers. Cells which contained no centrosome were 0.2 ± 0.2, 0.3 ± 0.1, 0.3 ± 0.1%, respectively, whereas other samples contained at least one centrosome. Values (%) represent mean ± S.E.

b MMS treatment at 50 µM and γ-irradiation (IR) at 1.4 Gy were performed as described in Materials and methods and cells were fixed 24 hrs after each treatment.
30% by Parp-1 deficiency in primary cultures of MEFs.\textsuperscript{12} We observed that in wild-type, Parp-1\textsuperscript{-/-} and Parg\textsuperscript{-/-} ES cells, the percentage of cells containing three or more centrosomes was maintained at the level of less than approximately 2%, even after treatment with DNA damaging agents (Fig. 1 and Table I). ES cells may thus possess a strict mechanism to maintain a normal number of centrosomes. Since Parp-3\textsuperscript{20}\textsuperscript{-} and tankyrase\textsuperscript{21}\textsuperscript{ also localize in centrosomes, these Parp family proteins or other proteins may compensate the Parp-1 function in ES cells. It is also possible that ES cells may not be able to survive under centrosome abnormality, and cells with an abnormal number of centrosomes may be immediately excluded by cell death. In Drosophila embryos, DNA replication defects and DNA damage trigger the inactivation and disruption of centrosomes depending on the function of checkpoint kinase 2 and this appears to maintain genome stability by blocking chromosome segregation and elimination of defective nuclei.\textsuperscript{22}\textsuperscript{-}29\textsuperscript{ We did not observe the elevation of the frequency of cells lacking centrosomes in every genotype, even after treatment with DNA damaging agents (Table I), suggesting the possibility that ES cells may not have the pathway of centrosome inactivation and disruption. A detailed time course analysis of centrosome numbers in ES cells after treatment with DNA damaging agents may further elucidate the maintenance mechanism of centrosome numbers in ES cells.

The present results indicate that the impact of Parp-1 deficiency is substantially different depending on the cell types and Parp-1 deficiency did not induce the abnormal centrosome numbers in ES cells. This could support the evidence that Parp-1\textsuperscript{-/-} mice develop and grow normally without showing extensive genomic instability.

The function of Parg in centrosomes has not been clarified yet, but the presence of polyADP-ribosylated proteins including p53 and Parp-1 itself suggests the requirement of Parg activity for poly(ADP-ribose) degradation in centrosomes. As we presented in this study, Parg deficiency did not induce the abnormal increase of centrosome numbers in ES cells. This may be due to the residual activity of Parg present in Parg\textsuperscript{-/-} ES cells (Fujihara et al., submitted) used in this study. Further study using potent Parg inhibitors or knocking down Parg activity completely with other gene-targeting constructs or with small interference RNA techniques may be helpful for understanding the function of Parg in centrosome regulation.

Acknowledgements. This work was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan and by a Grant-in-Aid for the Second Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare of Japan.

References

1) Sugimura, T. (1973) Prog. Nucleic Acid Res. Mol. Biol. 13, 127-151.
2) Althaus, F. R., and Richter, C. (1987) Mol. Biol. Biochem. Biophys. 37, 1-237.
3) Miwa, M., and Sugimura, T. (1971) J. Biol. Chem. 246, 6362-6364.
4) Lin, W., Ame, J. C., Aboul-Ela, N., Jacobson, E. L., and Jacobson, M. K. (1997) J. Biol. Chem. 272, 11895-11901.
5) Shimokawa, T., Masutani, M., Nagasawa, S., Nozaki, T., Ikota, N., Aoki, Y., Nakagama, H., and Sugimura, T. (1999) J. Biochem. (Tokyo) 126, 748-755.
6) Dantzer, F., Schreiber, V., Niedergang, C., Trucco, C., Flatter, E., de La Rubia, G., Oliver, J., Rolli, V., Ménissier-de Murcia, J., and de Murcia, G. (1999) Biochimie 81, 69-75.
7) Watanabe, F., Masutani, M., Kamada, N., Suzuki, H., Nakagama, H., Sugimura, T., and Teraoka, H. (2003) Proc. Jpn. Acad., Ser. B 79, 248-251.
8) Talin, A., and Spradling, A. (2003) Science 299, 560-562.
9) Simbulan-Rosenthal, C. M., Iy, D. H., Rosenthal, D. S., Konopka, G., Luo, R., Wang, Z. Q., Schultz, P. G., and Smulson, M. E. (2000) Proc. Natl. Acad. Sci. USA 97, 11274-11279.
10) Ullrich, O., Ciftci, O., and Hass, R. (2000) Free Radic. Biol. Med. 29, 995-1004.
11) Kanai, M., Uchida, M., Hanai, S., Uematsu, N., Uchida, K., and Miwa, M. (2000) Biochem. Biophys. Res. Commun. 278, 385-389.
12) Kanai, M., Tong, W. M., Sugihara, E., Wang, Z. Q., Fukasawa, K., and Miwa, M. (2003) Mol. Cell. Biol. 23, 2451-2462.
13) Nozaki, T., Fujihara, H., Kamada, N., Ueda, O., Takato, T., Nakagama, H., Sugimura, T., Suzuki, H., and Masutani, M. (2001) Proc. Jpn. Acad., Ser. B 77, 121-124.
14) Masutani, M., Nozaki, T., Nishiyama, E., Ochiya, T., Nakagama, H., Wakahaysa, K., Suzuki, H., and Sugimura, T. (1998) Proc. Jpn. Acad., Ser. B 74, 233-236.
15) Ohashi, S., Kanai, M., Hanai, S., Uchiimi, F., Maruta, H., Tanuma, S., and Miwa, M. (2003) Biochem. Biophys. Res. Commun. 307, 915-921.
16) Gunji, A., Fujihara, H., Kamada, N., Omura, K., Jishage, K., Nakagama, H., Sugimura, T., and Masutani, M. (2003) Proc. Jpn. Acad., Ser. B 79, 305-307.
17) Wong, C., and Stearns, T. (2003) Nat. Cell Biol. 5, 539-544.
18) D’Assoro, A. B., Busby, R., Suiño, K., Delva, E., Almodovar-Mercado, G. J., Johnson, H., Folk, C., Furrugia, D. J., Vasilé, V., Stivala, F., and Salisbury, J. L. (2004) Oncogene 23, 4068-4075.
19) Sato, N., Mizumoto, K., Nakamura, M., and Tanaka, M. (2000) Exp. Cell Res. 255, 321-326.
20) Augustin, A., Spenlehauer, C., Dumond, H., Ménissier-de Murcia, J., Piel, M., Schmit, A. C., Apiou, F., Vonesch, J. L., Kock, M., Bornens, M., and de Murcia, G. (2003) J. Cell Sci. 116, 1551-1562.
21) Smith, S., and de Lange, T. (1999) J. Cell Sci. 112, 3649-3656.
22) Sibon, O. C., Kelkar, A., Lenstra, W., and Theurkauf, W. E. (2000) Nat. Cell Biol. 2, 90-95.
23) Takada, S., Kelkar, A., and Theurkauf, W. E. (2003) Cell 113, 87-99.

(Received May 25, 2004; accepted June 15, 2004)