

# Direct evidence of involvement of glycosylphosphatidylinositol-anchored proteins in the heavy metal-mediated signal delivery into T lymphocytes

Meiyi Pu<sup>a</sup>, Li Ma<sup>a</sup>, Kozo Ohkusu<sup>a</sup>, Ken-Ichi Isobe<sup>a</sup>, Ryo Taguchi<sup>b</sup>, Hiroh Ikezawa<sup>b</sup>,  
Michinari Hamaguchi<sup>c</sup>, Izumi Nakashima<sup>a,\*</sup>

<sup>a</sup>Department of Immunology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466, Japan

<sup>b</sup>Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya 467, Japan

<sup>c</sup>Institute of Disease Mechanism and Control, Nagoya University School of Medicine, Nagoya 466, Japan

Received 17 January 1995; revised version received 14 February 1995

**Abstract** The biological significance of the action of glycosylphosphatidylinositol (GPI)-anchored proteins in cell physiology and pathology when stimulated with their natural agonists is not known. Here we provide evidence that GPI-anchored proteins play a crucial role in the recently defined heavy metal (HgCl<sub>2</sub>)-triggered signal delivery to T lymphocytes. Thiol-reactive HgCl<sub>2</sub>, a multi-potent crosslinker of cell membrane proteins, induced heavy aggregation of Thy-1, a representative GPI-anchored protein, on murine thymocytes, and delivered a signal to induce heavy tyrosine phosphorylation of cellular proteins. This rather unusual signal delivery by HgCl<sub>2</sub> is diminished by the pre-treatment of cells with phosphatidylinositol-specific phospholipase C, which partially cleaved GPI-anchored proteins from the cell surface. Direct evidence for the involvement of GPI or GPI-anchored proteins in the HgCl<sub>2</sub>-mediated signaling is provided by the loss of signaling in a mutant thymoma cell line defective in the phosphatidylinositol glycan-class A gene (PIG-A), and its restoration in a transfectant with PIG-A.

**Key words:** Glycosylphosphatidylinositol (GPI)-anchored protein; Mercuric chloride; Signal transduction; Phosphatidylinositol-specific phospholipase C (PIPLC); Phosphatidylinositol glycan-class A gene (PIG-A)

## 1. Introduction

It is known that a number of cell surface antigens, including Thy-1, Qa-2, and TAP, are anchored to cell membranes with glycosylphosphatidylinositol (GPI) [1–3]. These GPI-anchored proteins have been shown to associate with non-receptor tyrosine kinases such as p56<sup>lck</sup> and p60<sup>src</sup> across the plasma membrane [4–6], and to transduce or modulate signals for promotion of protein tyrosine phosphorylation, increase the intracellular Ca<sup>2+</sup> level, and accelerate cell growth or cell death when crosslinked with specific antibodies [7–12]. However, natural ligands of these GPI-anchored proteins for crosslinking largely remain unknown, and this has made it difficult to elucidate the biological significance of the signal delivery through these GPI-anchored proteins in the physiology and pathology of T lymphocytes.

Injection of heavy metal salts such as HgCl<sub>2</sub> and HAuCl<sub>4</sub> into

rodents induces immunological disorders with autoantibody production [13–15]. Therapeutic use of HAuCl<sub>4</sub> [15] and exposure to HgCl<sub>2</sub> from the polluted environment [16] in man also causes related disorders. Namely, these chemicals could incidentally affect cells in man and animals for their dysfunctions. Correspondingly, HgCl<sub>2</sub> has recently been found to work as a multi-potent crosslinker of cell membrane proteins on T lymphocytes through reaction with cysteine thiol groups on the proteins [17], and to deliver a signal for inducing heavy tyrosine phosphorylation of cellular proteins [17–19], which was linked to activation of non-receptor tyrosine kinase p56<sup>lck</sup> [17]. This signal was most remarkably provoked by relatively high concentrations (0.5 mM or more) of HgCl<sub>2</sub>, which caused quick (within 5 min) cell death, IL-2 production and cell proliferation [17], however, were accelerated by the signal triggered by lower concentrations (0.001–0.01 mM) of HgCl<sub>2</sub>, which only slightly promoted protein tyrosine phosphorylation. The present study aimed to determine the potential role of GPI-anchored proteins in the mechanism of the HgCl<sub>2</sub>-mediated intracellular signal transduction. The high concentration of HgCl<sub>2</sub>-mediated signal has been monitored in this study by measuring the level of protein tyrosine phosphorylation as its initial event. The results provide direct evidence for the first time of a crucial role for GPI-anchored proteins in intracellular signal delivery by a multi-potent receptor crosslinker from a natural source.

## 2. Materials and methods

### 2.1. Cells, cell lines and reagents

Single cell suspensions of thymocytes in MEM were prepared from C57BL/6 mice as described previously [20]. Murine thymoma cell line BW5147 of AKR mouse origin and its Thy-1<sup>−</sup> mutant class A (Thy-1<sup>−</sup>a) defective in PIG-A [21,22], were donated by Dr. R. Hyman, Salk Institute, through Dr. T. Kinoshita, Osaka University. PIG-A-transfected BW5147 Thy-1<sup>−</sup>a was a kind gift of Drs. T. Kinoshita and N. Inoue, Osaka University. This transfectant was prepared by transfecting BW5147 Thy-1<sup>−</sup>a with PIG-A cDNA plasmid into which a neomycin resistance gene had been introduced, followed by selection in medium with G418 [23]. PIPLC was obtained from the culture supernatant of *Bacillus thuringiensis*, and was purified to give a single band on SDS-PAGE [24]. This PIPLC was protease-free and was not toxic to cells by the dye exclusion test at the concentration (500 mU/ml) used. HgCl<sub>2</sub> was from Sigma (St. Louis, MO).

### 2.2. Confocal laser microscopy

Thymocytes (10<sup>7</sup>/100 μl), which had been treated or untreated with 0.5 mM HgCl<sub>2</sub> at 37°C for 2 min, were fixed with 4% paraformaldehyde at room temperature for 30 min, and then stained with FITC-labelled anti-Thy-1.2 mAb (Becton Dickinson, Mountain View, CA). They were mounted on a glass slide in the presence of *p*-phenylenediamine (1 mg/ml), and were observed under a confocal laser microscope as serial 0.6 μm sections.

\*Corresponding author. Fax: (81) (52) 741 2295.

**Abbreviations:** GPI, glycosylphosphatidylinositol; PIPLC, phosphatidylinositol-specific phospholipase C; PIG-A, phosphatidylinositol glycan-class A gene; PTYR, phosphotyrosine.

### 2.3. Laser flow cytometry

Thymocytes from C57BL/6 mice (Thy-1.2) and thymoma cell lines of AKR mouse origin (Thy-1.1) ( $5 \times 10^6/100 \mu\text{l}$ ) were stained with FITC-labelled anti-Thy-1.2 mAb and a two step combination of anti-Thy-1.1 mAb (OX7; donated by Dr. A. Williams, University of Oxford) and FITC-labelled anti-mouse IgG (Tago, Burlingame, CA), respectively. They were analyzed on a laser flow cytometer (EPICS Profile; Coulter, Hialeah, FL). Measurement of the level of fluorescence (log) was done on 5000 cells per sample.

### 2.4. Immunoblot analysis

Immunoblot assays were carried out as described [17,18,25]. Briefly, thymocytes ( $10^7/100 \mu\text{l}$ ) and thymoma cell lines ( $2 \times 10^6/100 \mu\text{l}$ ) were lysed in an equal volume of  $2 \times$  sample buffer and boiled for 3 min. The cell lysates were passed through by syringe with a 26G needle before being applied on 10% SDS-PAGE gels. The protein-transferred membrane was incubated with anti-phosphotyrosine (PTYR) rabbit antibody [25] followed by  $^{125}\text{I}$ -labeled protein A (ICN, Irvine, CA). The autoradiograph on X-ray film was analyzed by comparing with protein molecular weight (MW) standards (Gibco, Gaithersburg, MD).

## 3. Results and discussion

Fig. 1 compares localization of Thy-1, as a representative of GPI-anchored proteins, on a normal murine thymocyte and thymocytes treated with 0.5 mM  $\text{HgCl}_2$  for 2 min, in sections of single cells by confocus microscopy. Whereas Thy-1 was distributed diffusely on the surface of the normal control cell, it was aggregated to form a cap on the cell previously exposed to  $\text{HgCl}_2$ . Heavy aggregation of Thy-1 on one pole of the latter cell was noted in the serial sections. This action of  $\text{HgCl}_2$ , however, was neutralized by thiol-donating dithiothreitol (data not shown, see also [17]), suggesting that the event was redox-linked. Moreover, bivalent  $\text{HgCl}_2$  but not monovalent  $\text{Hg}_2\text{Cl}_2$  was active in inducing aggregation of Thy-1 (not shown, see also [17]). These results basically confirm our earlier conclusion that the thiol-reactive bivalent  $\text{HgCl}_2$  works as a multi-potent crosslinker of cell membrane molecules, including GPI-anchored proteins [17].

Previously, we showed that treatment of murine thymocytes with 30–500 mU/ml of PIPLC from *B. thuringiensis* for 30 min

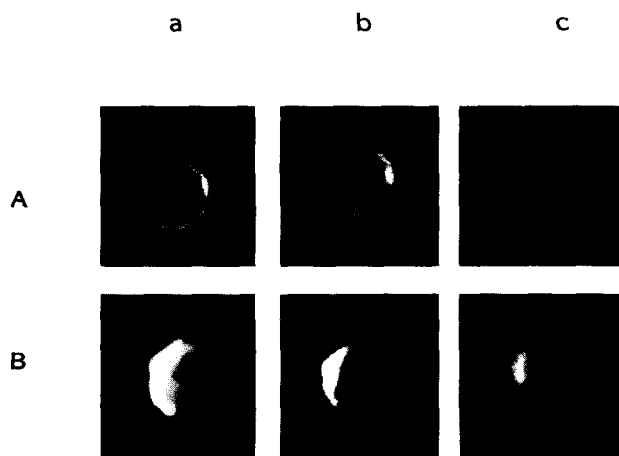


Fig. 1.  $\text{HgCl}_2$  induces aggregation of GPI-anchored Thy-1 on thymocytes. Thymocytes were stained with FITC-labeled anti-Thy-1 mAb and observed under confocal laser microscope. Compare the  $\text{HgCl}_2$ -treated cell (lower panel, B) with the untreated normal cell (upper panel, A). Photograph was taken for every  $0.6 \mu\text{m}$  thick (a–c) of a single cell from the center (a) to one edge (c) as a representative of over 100 cells observed.

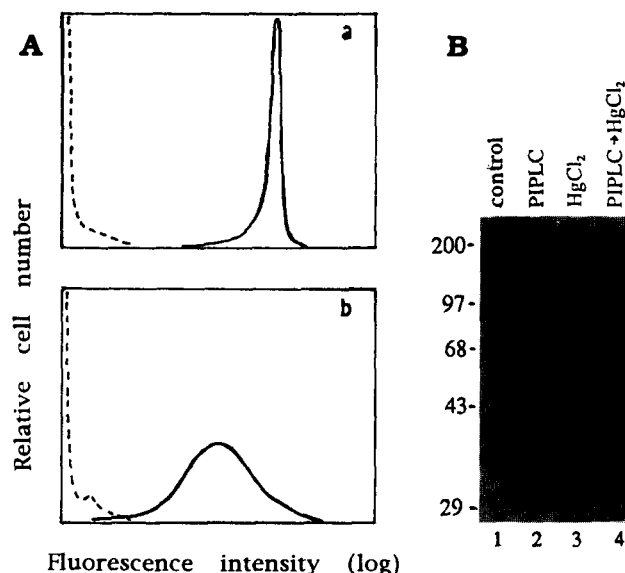


Fig. 2. Partial elimination of GPI-anchored proteins with PIPLC diminishes the cellular response to  $\text{HgCl}_2$  for protein tyrosine phosphorylation. (A) Flow cytometric analysis. Thymocytes were treated (b) or untreated (a) with 500 mU/ml PIPLC at  $37^\circ\text{C}$  for 30 min and then stained with FITC-labelled anti-Thy-1.2 mAb. Broken line, unstained control. (B) Immunoblot analysis. Thymocytes were treated with 500 mU/ml PIPLC at  $37^\circ\text{C}$  for 30 min. They were then stimulated with 0.5 mM  $\text{HgCl}_2$  for 2 min and lysed for immunoblotting against PTYR. Lane 1, untreated control; lane 2, PIPLC-treated only; lane 3,  $\text{HgCl}_2$ -stimulated; lane 4, PIPLC-treated and  $\text{HgCl}_2$ -stimulated. Positions of MW marker proteins (kDa) are indicated. Shown is a representative of three experiments.

cleaves large percentages of Thy-1 from their surface, although the remaining was resistant to any higher concentration of PIPLC because of minor structural heterogeneity of the GPI anchors with different susceptibilities to PIPLC [26]. Correspondingly, treatment of thymocytes from C57BL/6 mice with 500 mU/ml of PIPLC for 30 min partially but extensively cleaved Thy-1 as a representative of GPI-anchored proteins on the cells (Fig. 2A). Expression of transmembrane proteins such as CD4, however, was not affected by the same treatment (not shown). We then tested whether the partial elimination of Thy-1, and potentially other GPI-anchored proteins from the surface of the cells, would affect the signaling triggered by  $\text{HgCl}_2$  for heavy protein tyrosine phosphorylation. Stimulation of normal thymocytes with 0.5 mM  $\text{HgCl}_2$  for 30 s induced extensive heavy tyrosine phosphorylation of multiple cellular proteins (Fig. 2B, compare lane 3 with lane 1 as unstimulated control). This  $\text{HgCl}_2$ -mediated induction of protein tyrosine phosphorylation was partially but definitely inhibited by pre-treatment of the cells with PIPLC before  $\text{HgCl}_2$  (lane 4), which barely modulated the background phosphorylation level of control (lane 2). This suggested that GPI-anchored proteins played a definite role in the  $\text{HgCl}_2$ -mediated signal transduction.

Next, we asked whether a mutant of murine thymoma defective in PIG-A, that codes for the enzyme catalysing the early step of GPI anchor biosynthesis and is functionally defective in the blood of paroxysmal nocturnal hemoglobinuria patients [23,27,28], would respond to  $\text{HgCl}_2$  for protein tyrosine phosphorylation. As shown in Fig. 3A, wild-type BW5147, but not the PIG-A-defective mutant, Thy-1<sup>-</sup> expressed Thy-1 as a

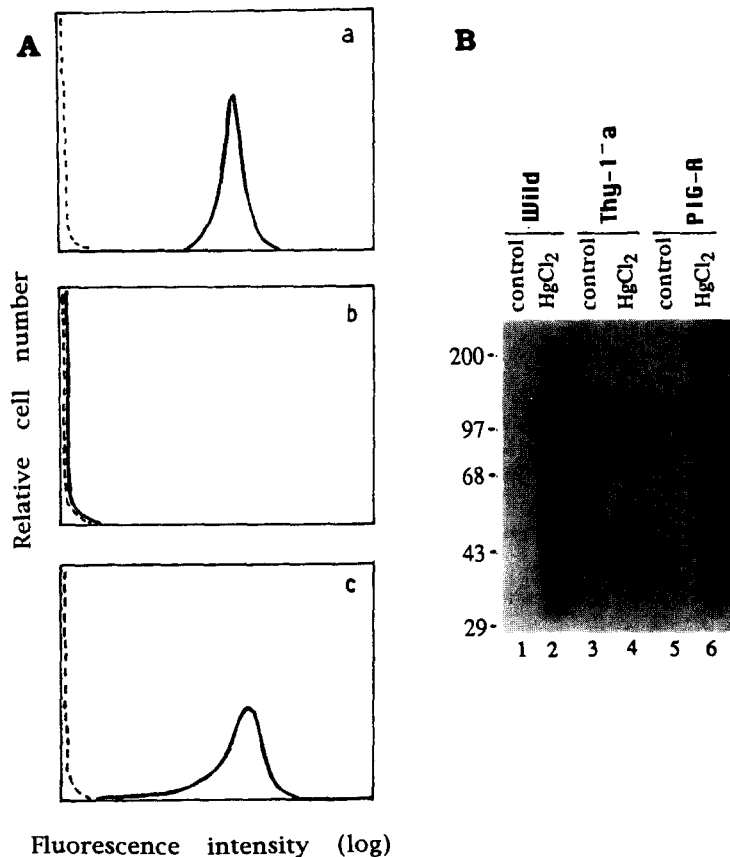


Fig. 3. PIG-A controls the HgCl<sub>2</sub>-mediated signal transduction. (A) Flow cytometric analysis of Thy-1 expression on wild-type BW5147 (a); PIG-A-defective Thy-1<sup>-a</sup> (b); and PIG-A-transfected Thy-1<sup>-a</sup> (c). Broken line, unstained control. (B) Western blot analysis. Wild-type BW5147 (lane 2), PIG-A-defective Thy-1<sup>-a</sup> (lane 4) and PIG-A-transfected Thy-1<sup>-a</sup> (lane 6) were stimulated with 0.5 mM HgCl<sub>2</sub> for 2 min and then lysed for immunoblotting against anti-PTYR. Lanes 1, 3 and 5 are untreated controls of BW5147, Thy-1<sup>-a</sup> and PIG-A-transfected Thy-1<sup>-a</sup>, respectively. Positions of MW marker proteins (kDa) are indicated. Shown is a representative of three experiments.

representative of GPI-anchored proteins on the surface of the cell. The poor Thy-1 expression on Thy-1<sup>-a</sup>, however, was recovered on the surface of PIG-A-transfected Thy-1<sup>-a</sup>. Wild-type BW5147 cells showed high response to HgCl<sub>2</sub> for tyrosine phosphorylation of multiple proteins (Fig. 3B, lane 2). In contrast, PIG-A-defective Thy-1<sup>-a</sup> failed to respond definitely to HgCl<sub>2</sub> for protein tyrosine phosphorylation (lane 4), the level of which was only slightly more than that in unstimulated Thy-1<sup>-a</sup> (lane 3). This poor response of Thy-1<sup>-a</sup> to HgCl<sub>2</sub>, however, was restored in PIG-A-transfected Thy-1<sup>-a</sup> (lane 6). These results proved that PIG-A-controlled GPI-anchors are indispensable for HgCl<sub>2</sub>-mediated signal delivery.

Our results, however, do not necessarily indicate that GPI anchored proteins alone worked for all the HgCl<sub>2</sub>-mediated signaling. This is because HgCl<sub>2</sub>, a multi-potent receptor crosslinker, was previously shown to aggregate a number of transmembrane receptor proteins such as CD3, CD4 and CD45, together with GPI-anchored Thy-1 on T lymphocytes [17], and GPI-anchored proteins and transmembrane receptors could cooperate to generate extensive signals when crosslinked together [11,12]. Physiologically, cooperation between two different types of receptors might occur through simultaneous binding of each receptor with its corresponding ligand. Our current study has demonstrated an alternative mechanism of co-operation among different types of receptors, which is

through redox-linked binding between sulfhydryl groups on receptors and sulfhydryl-reactive chemicals [17,18]. The present study has for the first time provided evidence that GPI-anchored proteins are indispensable for generating signals into T lymphocytes through the redox-linked crosslinkage with a natural chemical from the environment. However, we should evaluate this conclusion with some reservation since it was based entirely on results for a rather unusual signal provoked by high concentrations of HgCl<sub>2</sub>.

**Acknowledgements:** We thank Dr. A. Williams for donating mAb OX7, and Dr. R. Hyman and Dr. T. Kinoshita for providing cell lines BW5147 and Thy-1<sup>-a</sup>. We would like to extend our great thanks to Dr. T. Kinoshita and Dr. N. Inoue for donating PIG-A-transfected Thy-1<sup>-a</sup>, which was essential for this study.

## References

- [1] Low, M.G. and Saltiel, A.R. (1988) *Science* 239, 268–275.
- [2] Fergusen, M.A.J. (1988) *Annu. Rev. Biochem.* 57, 285–320.
- [3] Low, M.G. (1989) *Biochim. Biophys. Acta* 988, 427–454.
- [4] Stefanova, I., Horejsi, V., Anstotegui, I.J., Knapp, W., and Stockinger, H. (1991) *Science* 254, 1016–1021.
- [5] Thomas, P.M. and Samelson, L.E. (1992) *J. Biol. Chem.* 267, 12317–12322.
- [6] Shenoy-Scaria, A.M., Kwong, J., Fujita, T., Olszowy, M.W., Shaw, A.S. and Lublin, D.M. (1992) *J. Immunol.* 149, 3535–3541.

- [7] Kroccek, R.A., Gunter, K.C., Germain, R.N. and Shevach, E.M. (1986) *Nature* 322, 181–184.
- [8] Rock, K., Yeh, E.T.H., Gramm, C.F., Haber, S.I., Reiser, H. and Benacerraf, B. (1986) *J. Exp. Med.* 163, 315–333.
- [9] Robinson, P.J., Millrain, M., Antoniou, J., Simpson, E. and Mellor, A.L. (1989) *Nature* 342, 85–87.
- [10] Massaia, M., Perrin, L., Ianchi, A., Ruedi, J., Attisano, C., Altieri, D., Rijkers, G.T. and Thompson, L.F. (1990) *J. Immunol.* 145, 1664–1674.
- [11] Nakashima, I., Zhang, Y.-H., Rahman, S.M.J., Yoshida, T., Isobe, K., Ding, L., Iwamoto, T., Hamaguchi, M., Ikezawa, H. and Taguchi, R. (1991) *J. Immunol.* 147, 1153–1162.
- [12] Nakashima, I., Pu, M.-Y., Hamaguchi, M., Iwamoto, T., Rahman, S.M.J., Zhang, Y.-H., Kato, M., Ohkusu, K., Katano, Y., Yoshida, T., Koga, Y., Isobe, K. and Nagase, F. (1993) *J. Immunol.* 151, 3511–3520.
- [13] Goldman, M., Druet, P. and Gleichmann, E. (1991) *Immunol. Today* 12, 223–227.
- [14] Aten, J., Veninga, A., Heer, E.D., Rozing, J., Nieuwenhuis, P., Hoedemaeker, P.J. and Weenig, J.J. (1991) *Eur. J. Immunol.* 21, 611–616.
- [15] Shuhmann, D., Kubicka-Muranyi, M., Mirtschewa, J., Gunther, J., Kind, P. and Gleichmann, E. (1990) *J. Immunol.* 145, 2132–2139.
- [16] Takafuji, S., Suzuki, S., Muranaka, M. and Miyamoto, T. (1989) *Ciba Foundation Symposium* 147, 188–204.
- [17] Nakashima, I., Pu, M.-Y., Nishizaki, A., Rosila, I., Ma, L., Katano, Y., Ohkusu, K., Rahman, S.M.J., Isobe, K., Hamaguchi, M. and Saga, K. (1994) *J. Immunol.* 152, 1064–1071.
- [18] Rahman, S.M.J., Pu, M.-Y., Hamaguchi, M., Iwamoto, T., Isobe, K. and Nakashima, I. (1993) *FEBS Lett.* 317, 35–38.
- [19] Lander, H.M., Levine, D.M. and Novogrodsky, A. (1992) *Cell. Immunol.* 145, 146–155.
- [20] Nakashima, I., Mizoguchi, K., Nagase, F., Isobe, K., Saito, M. and Suzuki, K. (1982) *Eur. J. Immunol.* 12, 713–719.
- [21] Hyman, R. and Starlingo, V. (1974) *J. Natl. Cancer Inst.* 53, 429–436.
- [22] Conzelmann, A., Spiassi, A., Bron, C. and Hyman, R. (1988) *Mol. Cell. Biol.* 8, 674–678.
- [23] Miyata, T., Takeda, J., Iida, Y., Yamada, N., Inoue, N., Takahashi, M., Maeda, K., Kitani, T. and Kinoshita, T. (1993) *Science* 259, 1318–1320.
- [24] Ikezawa, H., Nakabayashi, T., Suzuki, K., Nakajima, M., Taguchi, T. and Taguchi, R. (1983) *J. Biochem.* 93, 1717–1719.
- [25] Hamaguchi, M., Grandori, C. and Hanafusa, H. (1988) *Mol. Cell. Biol.* 8, 3035–3042.
- [26] Nakashima, I., Yoshida, T., Zhang, Y.-H., Pu, M.-Y., Taguchi, R., Ikezawa, H., Isobe, K., Iwamoto, T., Kato, M. and Parvin, M., (1992) *Immunobiology* 185, 466–474.
- [27] Takeda, J., Miyata, T., Kawagoe, K., Iika, Y., Endo, Y., Fujita, T., Takahashi, M., Kitani, T. and Kinoshita, T. (1993) *Cell* 73, 703–711.
- [28] Bessler, M., Mason, P., J., Hillmen, P., Miyata, T., Yamada, N., Takeda, J., Luzzatto, L. and Kinoshita, T. (1994) *EMBO J.* 13, 110–117.