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Redox-linked ligand-independent cell surface triggering for extensive protein tyrosine phosphorylation

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Exposure of lymphocytes to 0.2–2 mM $HgCl_2$, a thiol-reactive heavy metal, induced extensive tyrosine phosphorylation of multiple cellular proteins. The phosphorylation started as quickly as 5 s after exposure to $HgCl_2$, and was irreversible. Another 3 thiol-reactive chemicals also displayed similar, though less marked, actions, whereas dithiothreitol, a reducing agent, antagonized the $HgCl_2$ action. The demonstrated new action of $HgCl_2$ indispensably required membrane-intact cells as a target. Whereas exposure of lymphocytes to >0.2 mM $HgCl_2$ caused rapid cell death, 0.01–0.1 mM $HgCl_2$ affected the cells so as to accelerate their c-*fos* transcription. These results suggest a novel redox-linked mechanism of cell surface triggering of intracellular protein kinase activity, which is independent of receptor–ligand interactions.

Tyrosine phosphorylation; Redox potential; Thiol-reactive chemical; $HgCl_2$; Signal transduction

1. INTRODUCTION

A number of cellular proteins of lymphocytes are quickly phosphorylated at the tyrosine residues following stimulation of the cell surface receptor with ligand, mitogen or anti-receptor antibody, and such phosphorylation is, in many cases, the earliest event of the receptor-mediated signal transduction into lymphocytes [1,2]. This event is normally controlled strictly by the receptor-specific ligand (antigen) action. This brief communication reports an unexpected observation that extensive tyrosine phosphorylation is induced on multiple proteins of lymphocytes through a ligand-independent redox-linked mechanism when membrane-intact lymphocytes are exposed to thiol-reactive chemicals [3] such as $HgCl_2$, *p*-chloromercuryl phenylsulfonic acid (CMPSA), $HAuCL_4$ and *N*-ethylmaleimide (NEM).

2. MATERIALS AND METHODS

2.1. *Animals and cells*

Single cell suspensions of thymocytes and spleen cells in Eagle's MEM were prepared from C57BL/6 mice as described [4]. A thymoma cell line, BW5147, originating from a AKR mouse, and a pre-B lymphoma cell line, ret01, from a Eu/ret transgenic mouse [5], were also used.

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Abbreviations: PTYR, phosphotyrosine; CMPSA, *p*-chloromercuryl phenylsulfonic acid; NEM, *N*-ethylmaleimide.

2.2. *Reagents and cell treatment*

Suspensions of cells (5–10×10^6 cells/100 μl) in MEM were incubated in the presence or absence of 0.01–10 mM of $HgCl_2$, CMPSA (Sigma, St. Louis, MO), $HAuCl_4$ (Sigma), NEM (Sigma), dithiothreitol (Sigma), LiCl, $MgCl_2$ or $MnCl_2$, at 37°C for 5 s–30 min, and lysed for assay of phosphotyrosine (PTYR)-containing proteins. Before incubation with $HgCl_2$ some cells were pretreated with staurosporin (Kyowa Hakko, Tokyo, Japan) (2 μg/ml; this concentration was not directly cytotoxic to lymphocytes by dye exclusion test) as a protein kinase inhibitor [6], 0.1–1% digitonin (Wako, Osaka, Japan) or 0.01% saikosaponin d (Wako), sonicated (Tomy Seiko, Tokyo, Japan) for 5 s or irradiated with UV light (15 W; Toshiba, Tokyo, Japan) at a distance of 10 cm for 10 min.

2.3 *Assay of PTYR-containing proteins*

SDS-PAGE and immunoblot were performed as described [4,7]. Briefly, cells were lysed by adding an equal volume of sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-ME, 10% glycerol) and heated in boiled water for 3 min. The cell lysates, containing 50–100 μg of proteins per lane, were applied on SDS-7.5% polyacrylamide gels. After electrophoresis, proteins were transferred electrophoretically to a nitrocellulose filter and stained with affinity-purified anti-PTYR rabbit antibody followed by ^{125}I-labeled protein A (ICN, Irvine, CA). Autoradiography was performed on X-ray film for 15–48 h. Specificity of the anti-PTYR antibody has been extensively studied and reported [4,7].

2.4. *Northern blotting*

This was done according to the method described [5]. Briefly, cells were lysed with solution D (4 M guanidinium thiocyanate chloride, 100 mM 2-ME). Total RNA was extracted and assayed for mRNA by Northern blot using a 2.4 kb *Bam*HI–*Sal*I fragment of mouse *fos* DNA [8] (donated by T. Tokuhisa, Kobe University) as a probe.

3. RESULTS AND DISCUSSION

Only a few proteins from normal thymocytes and spleen cells were stained faintly with the anti-PTYR

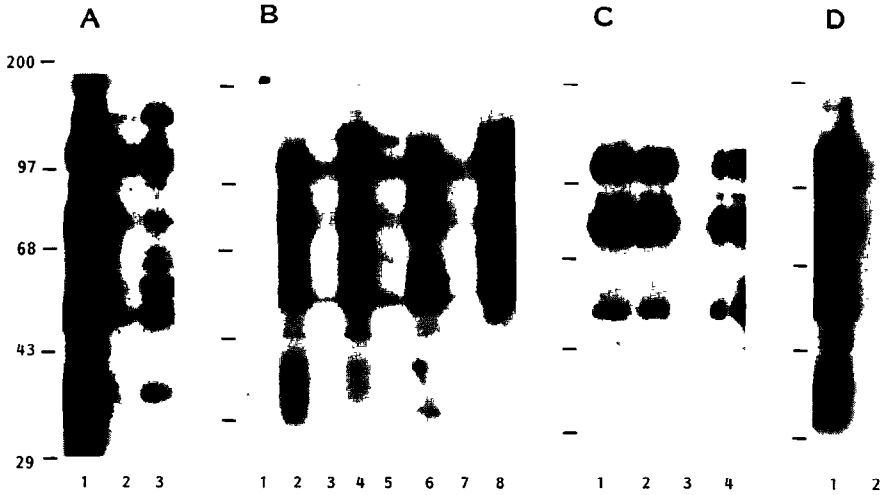

Fig. 1. Demonstration of the unique action of $HgCl_2$ provoking cell-type linked extensive tyrosine phosphorylation of multiple proteins in lymphocytes. Suspensions of lymphocytes in MEM were incubated in the presence or absence of 1 mM $HgCl_2$, and then lysed for the immunoblot assay of PTYR-containing proteins. (A) Lane 1, spleen cells treated with $HgCl_2$; lane 2, no treatment control; lane 3, cells treated with 10 μg/ml of concavalin A. (B) Odd numbered lanes, no $HgCl_2$ controls; even numbered lanes, cells treated with $HgCl_2$; lanes 1 and 2, thymocytes; lanes 3 and 4, spleen cells; lanes 5 and 6, thymoma cells; lanes 7 and 8, pre-B lymphoma cells. (C) Lanes 1–3, added with free phosphoserine (lane 1), phosphothreonine (lane 2) or PTYR (lane 3) at a concentration of 1 mM before staining of proteins from $HgCl_2$-treated thymocytes with anti-PTYR antibodies for inhibition; lane 4, no inhibitor positive control. (D) Lanes 1 and 2, thymocytes treated with $HgCl_2$ in the presence (lane 2) or absence (lane 1) of staurosporin. Molecular weights (kDa) of standard proteins are shown on the left.

antibody and radiolabeled protein A (Fig. 1A, lane 2; 1B, lanes 1 and 3). To our surprise, by the same procedure, a number of proteins from those cells previously exposed to 1 mM $HgCl_2$ for 2 min were heavily stained (Fig. 1A, lane 1; 1B, lanes 2 and 4). This heavy staining was confirmed to be PTYR-specific through specific inhibition by PTYR (Fig. 1C), and was shown to be linked to staurosporin-sensitive kinase activity (Fig. 1D). The molecular sizes of the major protein bands developed for the $HgCl_2$-treated thymocytes (Fig. 1B, lane 2) and thymoma cells (lane 6) were around 120, 80 and 56 kDa, whereas those for spleen cells (lane 4) and pre-B lymphoma cells (lane 8) were around 145, 120, 80, 65 and 56 kDa. This showed that the pattern of distribution of the major protein bands of phosphorylation developed by exposure to $HgCl_2$ was cell-type linked. Furthermore, the pattern resembled that of bands developed by stimulation with concanavalin A (Fig. 1A, lane 3) or anti-CD3 mAb (not shown), which included proteins of 145, 120, 80, 65 and 56 kDa. This suggested a close relationship in the mechanism of the two ways to induce protein tyrosine phosphorylation.

Heavy tyrosine phosphorylation occurred on proteins around 56, 80 and 120 kDa as rapidly as 5 s after exposure of thymocytes to 0.5 mM $HgCl_2$, followed soon after by further phosphorylation of proteins around 200, 180, 145, 90, 65, 43, 40, 30 kDa, which did not fade by 30 min (Fig. 2A). The action of $HgCl_2$ was dependent on the concentration of $HgCl_2$, and required >0.2 mM for heavy phosphorylation (Fig. 2B). Lower concentrations (0.01–0.1 mM) of $HgCl_2$ weakly accelerated the phosphorylation of the 56 kDa protein.

Definite protein tyrosine phosphorylation was also induced by other thiol-reactive chemicals, namely CMPSA (Fig. 3A, lane 3), $HAuCl_4$ (lane 4) and NEM (lane 5), optimal concentrations of which were 10, 2 and 10 mM, respectively (data not shown). However, $HgCl_2$ was not equalled in the strength of action by any of these chemicals at either 1 mM (Fig. 3A) or their optimal concentration (not shown), and $HgCl_2$ and NEM provoked phosphorylation of different proteins. No definite action was observed with dithiothreitol (lane 6), a reducing reagent, or with LiCl, a phosphatase inhibitor (lane 7), suggesting that the action of $HgCl_2$ was definitely more than that of the phosphatase inhibitor. $MnCl_2$ (lane 8) and $MgCl_2$ (lane 9), known co-factors of tyrosine kinases, were also not active. In addition, mixing 10 mM (Fig. 3B, lane 4) or 1 mM (not shown) dithiothreitol with 1 mM $HgCl_2$ completely (in the case of 10 mM) or partially (1 mM) ablated the action of the latter. This proved that the action was redox linked.

Interestingly, $HgCl_2$ was not active on membrane-disrupted cells; treatment of the cells with digitonin (Fig. 4, lanes 3 and 4) or saikosaponin (lane 7), or sonication (lane 5), but not UV irradiation (lane 6) prior to $HgCl_2$, prevented the induction of tyrosine phosphorylation. By the dye exclusion test, the majority (>80%) of the cells exposed to 1 mM $HgCl_2$ were alive within 2 min after the exposure, whereas most of them (>90%) had died by 20 min (the concentration of $HgCl_2$ which induced death of 50% cell population by 20 min was 0.13 mM). Therefore, $HgCl_2$ should affect live cells to signal for heavy protein tyrosine phosphorylation and cell death.

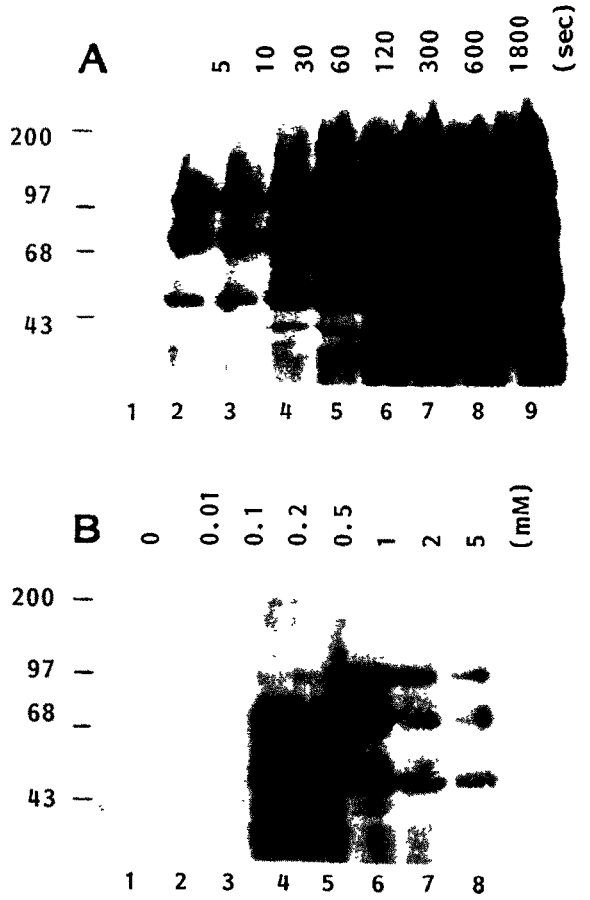

Fig. 2. Characterization of the $HgCl_2$-provoked protein tyrosine phosphorylation. Suspensions of thymocytes in MEM were incubated in the presence or absence of $HgCl_2$, and then lysed for the immunoblot assay of PTYR-containing proteins. (A) Lane 1, no $HgCl_2$ control; lanes 2–9, thymocytes incubated in the presence of 0.5 mM $HgCl_2$ for the indicated time (s). (B) Lane 1, no $HgCl_2$ control; lanes 2–8, thymocytes incubated in the presence of $HgCl_2$ at the indicated concentration (mM) for 2 min.

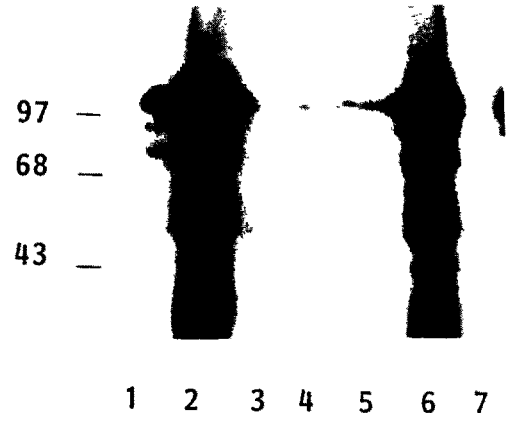

Fig. 4. The unique action of $HgCl_2$ indispensably requires membrane-intact cells as a target. Lane 1, no $HgCl_2$ control; lane 2, no pretreatment control of cells incubated in the presence of 1 mM $HgCl_2$; lanes 3–7, cells first treated with 1% (lane 3) or 0.1% (lane 4) digitonin or saikosaponin d (lane 7) for 20 min, sonicated (lane 5) or irradiated with UV light (lane 6) before incubation in the presence of $HgCl_2$.

A study was made to determine if the $HgCl_2$-provoked early signal would be transmitted into the nucleus for regulating the transcription of genes. As shown in Fig. 5, exposure of thymocytes to 1.0 mM of $HgCl_2$ for 30 min, which caused cell death, decreased the recovery of total RNA (lane 4). On the other hand, exposure to 0.01–0.1 mM, which marginally promoted protein tyrosine phosphorylation (Fig. 2B), accelerated the transcription of c-*fos* (lanes 2 and 3) as an immediate early gene.

The recent report by Bauskin et al. [9] is partially comparable with ours. They showed that alkylating diamide, which penetrated the cell within seconds, selectively activated endoplasmic reticulum-localized tyrosine kinases. Whereas both this report and ours relate

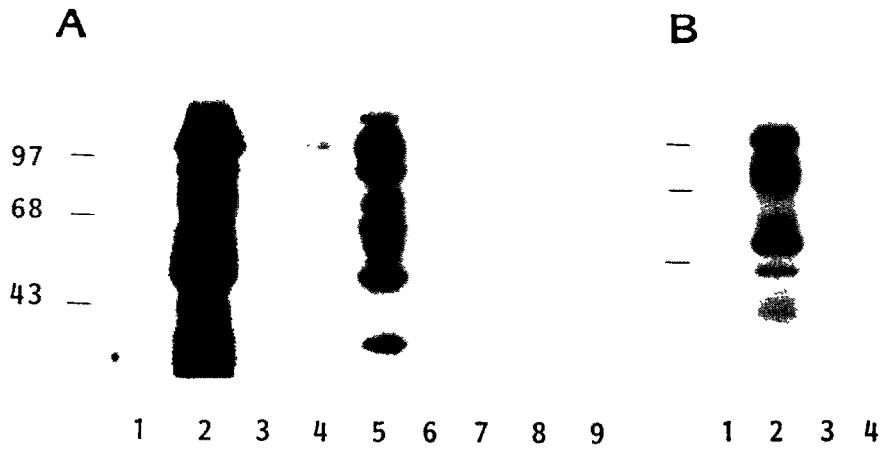

Fig. 3. A number of thiol-reactive chemicals share the unique activity for provoking protein tyrosine phosphorylation through a redox-linked mechanism. Suspensions of thymocytes in MEM were incubated in the presence or absence of one or two of various chemicals, and then lysed for the immunoblot assay of PTYR-containing proteins. (A) Lane 1, no chemicals control; lanes 2–9, cells incubated in the presence of 1 mm of, respectively, $HgCl_2$, CMPSA, $HAuCl_4$, NEM, dithiothreitol, LiCl, $MnCl_2$ or $MgCl_2$. (B) Lane 1, no $HgCl_2$ control; lanes 2–4, cells incubated in the presence of, respectively, 1 mM $HgCl_2$, 10 mM dithiothreitol, or 1 mM $HgCl_2$ plus 10 mM dithiothreitol.

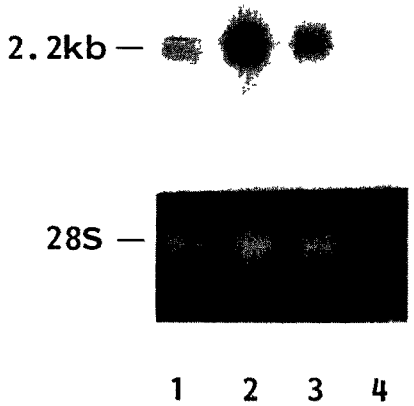

Fig. 5. The $HgCl_2$-provoked early signal modulates the transcription of c-*fos*. Suspensions of thymocytes (3×10^7 cells/100 μl) in MEM were incubated for 30 min in the presence or absence of $HgCl_2$, and lysed for assay of c-*fos* mRNA. Lane 1, no $HgCl_2$ control; lanes 2–4, cells incubated in the presence of 0.01 (lane 2), 0.1 (lane 3) or 1 (lane 4) mM $HgCl_2$. The size of the transcript is shown on the left. Ethidium bromide staining of the gel containing 28 S RNA is shown below to indicate equal loading for lanes 1–3 as compared with poor recovery of total RNA due to cell death for lane 4.

to the previously recognized phenomenon of the regulation of protein activity in response to environmental cues through a redox mechanism [10,11], our study has discovered a novel redox-linked cell triggering mechanism that works selectively on the surface of membrane-intact cells. The exact molecular site of the action of thiol-reactive chemicals remains to be clarified, but our preliminary study has suggested that the action of $HgCl_2$ to dimerize thiol group-bearing proteins is included (Nakashima, I., unpublished). What kind of tyrosine kinase or kinases would be activated by the cell surface-acting redox mechanism is currently being investigated. However, in a preliminary observation, we have found that at least one tyrosine kinase, namely $p56^{lck}$, is involved (Pu, M. and Nakashima, I., unpublished).

Our result that relatively low concentrations (10^{-5}–10^{-4} M) of $HgCl_2$ promoted the transcription of c-*fog* might relate to another observation, by Jung and Endo, that very low concentrations of $HgCl_2$ or $HAuCl_4$ into mice and rats induces immune disorders accompanying autoimmunity [13–15]. It is possible that disregulation through the redox mechanism of the receptor-mediated specific signal delivery for lymphocyte activation underlies the mechanism of the reported mercury/gold-induced autoimmune diseases.

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