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Evidence of Redox-Linked Signaling for Producing a Giant Signal Complex

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Abstract Previously we showed that a thiol-reactive heavy metal, $HgCl_2$, crosslinked multiple cell surface receptors through a ligand-independent pathway, which produced massive aggregates of phosphotyrosine (PTYR)-containing proteins beneath plasma membrane [Nakashima et al. (1994): J Immunol 152:1064–1071]. In this study we characterized these unique aggregates at the molecular level. The lysates in Brij 96 of thymocytes treated with $HgCl_2$ were separated into the supernatant and pellet fractions by simple centrifugation. Selected PTYR-containing proteins and $p56^{lck}$ appeared in the pellet fraction as quickly as 5 s after exposure to $HgCl_2$, and were further increased in amount by 5 min. Although the mechanism of triggering these events was redox-linked, the majority of proteins in the Brij 96-insoluble aggregates were dissociated in SDS-PAGE under nonreducing condition. This suggested that PTYR-containing proteins and $p56^{lck}$ themselves do not form dimer or polymer directly by thiol-mediated bond. The pellet fraction was further found to include some other signal delivery elements, such as GTPase activating protein, phosphatidylinositol 3 kinase, and mitogen-activated protein kinase. Finally, all of these signal elements and selected PTYR-containing proteins were collected in the same fraction by the sucrose density gradient centrifugation. These results suggest a unique redox-linked pathway of formation of a giant signal complex.

Key words: redox, $HgCl_2$, tyrosine phosphorylation, $p56^{lck}$, signal complex

The function of lymphocytes are basically controlled through the signal triggered by crosslinkage of cell surface receptors with specific ligands, which activates a number of cellular elements in a chain reaction [Perlmutter et al., 1993; Klausner and Samelson, 1991; Weiss, 1993; Janeway, 1992]. These elements include nonreceptor tyrosine kinases and their substrates, such as phospholipase C(PLC)-γ 1, GTPase activating protein (GAP), and phosphatidylinositol 3 kinase (PI3-K), which may form a signal complex after the receptor crosslinkage to be coimmunoprecipitated by antireceptor antibody from the detergent-lysed cells. However, knowledge of the dynamics of formation of signal complex after receptor crosslinkage in lymphocytes is still limited.

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It is known that chronic injection of thiol-reactive heavy metals, such as $HgCl_2$ and $HAuCl_4$, into rodents initiates lymphoproliferative disorders with autoimmunity manifestations [Goldman et al., 1991; Aten et al., 1991; Schumann et al., 1990]. In light of this phenomenon, we recently analyzed the molecular basis of the action of these heavy metals to modulate the lymphocyte dynamics, and have found that the heavy metals crosslink lymphocyte receptors by a ligand-independent redox-linked mechanism [Rahman et al., 1993; Nakashima et al., 1994]. Studies using a fluorescent antibody technique revealed that multiple cell surface antigens on T-lymphocytes, such as CD3, CD4, CD45, and Thy-1, were cocrosslinked by cell surface-acting $HgCl_2$, which accompanied linked accumulation of $p56^{lck}$ and tyrosine phosphorylated proteins and activation of $p56^{lck}$ [Nakashima et al., 1994]. This result suggested formation of a giant signal complex as a result of signal delivery through an alternate pathway. We have here analyzed the molecular dynamics

of formation and structural properties of this potentially unique complex of cell signaling elements. The results show that the cell surface-acting mercury does induce a giant signal complex which is sedimentable from the detergent-lysed cells at low speed centrifugation without addition of antireceptor antibody, and includes $p56^{lck}$, GAP, PI3-K, mitogen-activated protein kinase (MAP-K), and selected phosphotyrosine (PTYR)-containing proteins. They further have defined the mechanism of formation of the giant complex as involving both a redox-linked mechanism for triggering and a non-redox-linked one for secondary association of individual proteins in the complex.

MATERIALS AND METHODS

Animals and Cell Suspensions

C57BL/6 strain mice were bred in the Institute for Laboratory Animal Research, Nagoya University School of Medicine. Mice aged 6 to 8 weeks were used as the source of thymocytes. Single cell suspensions of thymocytes in Eagle's minimum essential medium (MEM) were prepared as described [Nakashima et al., 1982].

Antibodies

Monoclonal antibody (mAb) specific to murine $p56^{lck}$ (MOL171, purified mAb) [Moroi et al., 1991], affinity-purified rabbit antibody (Ab) specific to PTYR [Hamaguchi et al., 1988], rabbit Ab specific to GAP (donated by Dr. S. Hattori, National Center of Neurology and Psychiatry), rabbit Ab specific to PI3-K (donated by Dr. Y. Fukui, University of Tokyo), rabbit Ab specific to MAP-K (donated by Dr. E. Nishida, University of Kyoto) [Gotoh et al., 1991], and anti-mouse IgG rabbit Ab (MBL, Nagoya, Japan) were used.

Confocus Laser Microscope

Cells were fixed by treating with 4% paraformaldehyde at room temperature for 30 min. For staining submembranous PTYR-containing proteins, the fixed cells were further treated with 0.2% of Triton X-100 at 4°C for 3 min. Cells were stained in two steps—with anti-PTYR affinity-purified rabbit Ab for the first step, and FITC-labeled anti-rabbit Ig (Cappel, Cochranville, PA) for the second. The stained cells were suspended in 80% glycerol in Tris-HCl buffer, pH 8.0, added with p-phenylenediamine (1 mg/ml) and mounted on slides. The stained cells were observed under a confocus laser microscope.

Reagents and Cell Treatment

Suspensions of cells ($1–10 \times 10^7$ cells/100 μl) in MEM were incubated in the presence or absence of 1 mM of $HgCl_2$ at 37°C for 5–300 s. They were then washed twice with MEM and solubilized in 200 μl of Brij 96 (Sigma, St. Louis, MO) lysis buffer (10 mM Tris HCl, pH 7.4/150 mM NaCl/1 mM EDTA/1% Brij 96) for 30 min on ice [Beyers et al., 1992]. Lysates were centrifuged at 15,000*g* for 10 min at 4°C and separated into supernatant and pellet fractions, although many of the $HgCl_2$-induced aggregates were big enough for being sediment at lower speed centrifugation. Pellets were added with the same volume of distilled water as supernatants. For other experiments, however, 1,000 μl of cell lysates were fractionated into the 12 subfractions from the top layer by sucrose density gradient (10–50% sucrose in Brij 96 lysis buffer) centrifugation.

Immunoblot

Samples were mixed with the same volume of a twofold concentrated sample buffer (62.5 mM Tris HCl, pH 6.8/2% SDS/5% 2ME/10% glycerol), and were heated in boiling water for 3 min. Portions of these solutions for individual samples were applied on SDS-12.5% polyacrylamide gels. After electrophoresis, proteins were transferred electrophoretically to a polyvinylidene difluoride membrane and stained with antibody followed by ^{125}I-labeled protein A (ICN, Irvine, CA). The molecular size of the stained protein was estimated by comparison with protein m.w. standards (Bethesda Research Laboratories, Gaithersburg, MD). Autoradiography was performed on X-ray film for 5–120 h.

RESULTS

Demonstration of Submembranous Accumulation of PTYR-Containing Proteins in $HgCl_2$-Treated Cells

Previously we showed that exposure of thymocytes to $HgCl_2$, which is a thiol-reactive heavy metal, induced heavy tyrosine phosphorylation of multiple cellular proteins [Rahman et al., 1993; Nakashima et al., 1994]. Study by use of fluorescent antibody technique revealed that $HgCl_2$ induced crosslinkage of multiple cell sur-

face antigens, followed by linked development of PTYR-containing proteins [Nakashima et al., 1994]. Observation of slices of a single cell by confocus microscopy confirmed the submembranous massive localization of the PTYR-containing proteins (Fig. 1).

Development of Brij-Insoluble Aggregates of PTYR-Containing Proteins in $HgCl_2$-Treated Cells

The lysates in Brij 96 of thymocytes treated with $HgCl_2$ were separated into the supernatant and pellet fractions by centrifugation, and were analyzed for distribution of PTYR-containing proteins in the two fractions (Fig. 2). Both number and amount of PTYR-containing proteins in normal thymocytes were low, and the majority of those proteins were found in the supernatant fraction of the cell lysate. When thymocytes were incubated with 1 mM $HgCl_2$, extensive tyrosine phosphorylation occurred on proteins around 28, 40/45, 56, 70, 80/85, 110/120, and 180 kDa at 5–300 s after exposure of thymocytes to $HgCl_2$. As time passed after incubation with $HgCl_2$, each of these PTYR-containing proteins was distributed into the supernatant fraction and pellet fraction at the individually distinct ratio. The major parts of proteins around 56, 120, and 180 kDa moved from the supernatant fraction to the pellet fraction in 5 min. On the other hand, proteins around 28 and 80 kDa preferentially remained in the supernatant fraction. This result suggests that after the exposure of thymocytes to $HgCl_2$ a number of cellular proteins were quickly phosphorylated on tyrosine residues, some of which were selectively incorporated into Brij 96-insoluble aggregates with time.

Mechanism of Development of Aggregates of PTYR-Containing Proteins

All the actions of $HgCl_2$ to induce tyrosine phosphorylation and aggregates of cellular proteins were neutralized by thiol-donating reagent dithiothreitol [Rahman et al., 1993; Nakashima et al., 1994; and data not shown], which confirmed the involvement of a redox-linked mechanism. However, it was indefinite whether the PTYR-containing proteins themselves were polymerized directly by thiol-linked bond. When Brij 96-insoluble aggregates in the pellet fraction were incubated in the Brij 96 lysis buffer containing reducing reagent 2ME, a part of them were released from the aggregates (Fig. 3A). This also supported the view of involvement of the thiol-mediated linkage in this mechanism. However, the linkage of the majority of the PTYR-containing proteins in the $HgCl_2$-induced aggregates were dissociated in SDS-PAGE under nonreducing condition. As shown in Figure 3B, the pattern of PTYR-containing protein bands in SDS-PAGE under nonreducing condition did not much differ from that in SDS-PAGE under reducing condition. These results reveal that PTYR-containing proteins in the $HgCl_2$-induced aggregates barely form dimer or polymer directly by thiol-linked bonds.

$p56^{lck}$ Is Involved in the $HgCl_2$-Induced Aggregate of Cellular Proteins

Intracellular $p56^{lck}$ of thymocytes treated with $HgCl_2$ was analyzed similarly with PTYR-containing proteins. As shown in Figure 4A, the majority of $p56^{lck}$ protein in normal thymocyte lysates resided in the supernatant fraction and very little in the pellet fraction. A considerable amount of $p56^{lck}$ was, however, found in the

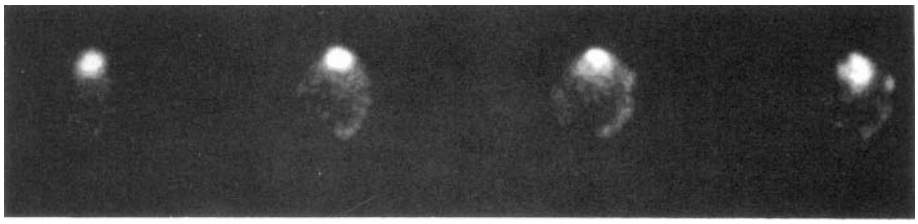

Fig. 1. Demonstration by confocus microscopy of $HgCl_2$-induced PTYR-containing proteins localized beneath cell membrane. Suspensions of thymocytes in MEM were incubated at 37°C for 2 min in the presence of 1 mM $HgCl_2$. The cells were stained with anti-PTYR followed by FITC-labeled anti-Ig. Fluorescence was examined under a confocus laser microscope. Photographs were taken at every 0.6 μm from the bottom of the cell and arranged in order from the left to the right. Although not shown in the figure, no significant fluorescence was detected on unstimulated control thymocytes.

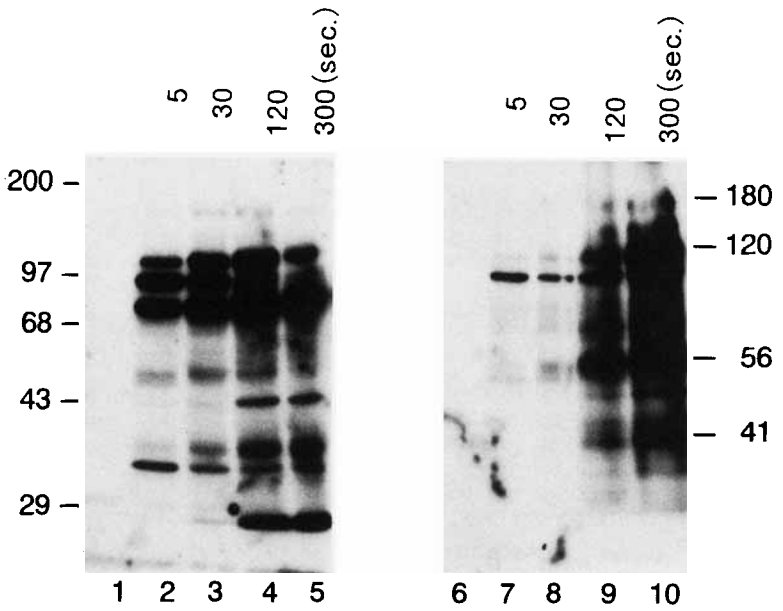

Fig. 2. Time course of development of Brij 96-insoluble aggregates of PTYR-containing proteins after exposure of cells to $HgCl_2$. Suspensions of thymocytes in MEM were incubated in the presence of $HgCl_2$, lysed in Brij 96 lysis buffer, and then separated into the supernatant (lanes 1 to 5) and pellet (lanes 6 to 10) fractions by centrifugation. PTYR-containing proteins in lysates were analyzed by immunoblot. Lanes 1 and 6: No $HgCl_2$ control. Lanes 2 to 5 and 7 to 10: Cells treated with $HgCl_2$ for the indicated time (s). Molecular weights (kDa) of standard proteins (**left**) and major stained bands (**right**) are shown.

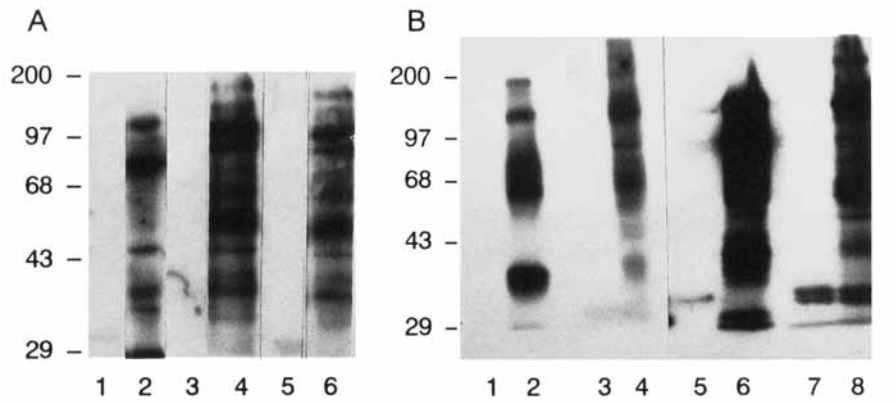

Fig. 3. Both redox-linked and unlinked mechanisms operate in $HgCl_2$-induced formation of aggregates of PTYR-containing proteins. The supernatants and the pellets of cell lysates were prepared from the thymocytes treated with $HgCl_2$ for 5 min as in Figure 2. **A:** The pellets were incubated in Brij 96 lysis buffer containing 2ME for 30 min, and then divided into the soluble fraction and the insoluble fraction. Lanes 1 and 2, supernatant; lanes 3 and 4, 2ME soluble fraction; lanes 5 and 6, 2ME insoluble fraction; lanes 1, 3, and 5, no $HgCl_2$ control; lanes 2, 4, and 6, cells treated with $HgCl_2$. **B:** The supernatants and the pellets were mixed with sample buffer containing (reducing) or not containing (nonreducing) 2ME for SDS-PAGE and were analyzed for PTYR by immunoblot. Lanes 1, 2, 5, and 6, supernatant; lanes 3, 4, 7, and 8, pellet; lanes 1 to 4, SDS-PAGE under nonreducing condition; lanes 5 to 8, under reducing condition; lanes 1, 3, 5, and 7, no $HgCl_2$ control; lanes 2, 4, 6, and 8, cells treated with $HgCl_2$. Molecular weights (kDa) of standard proteins are shown on the left.

pellet fraction as quickly as 5 s after exposure of thymocytes to $HgCl_2$. Interestingly, the amount of $p56^{lck}$ in the pellet fraction was increased with the passage of incubation time with $HgCl_2$, and during 5 min incubation, the majority of $p56^{lck}$ protein moved to the pellet fraction from the supernatant fraction. These results showed that $p56^{lck}$ was aggregated by the action of $HgCl_2$. However, as shown in Figure 4B and C, only a small portion of $p56^{lck}$ was released from the $HgCl_2$-induced aggregates by the treatment with 2ME-containing buffer, and the majority of $p56^{lck}$ in the aggregates were dissociated into monomers in SDS-PAGE under nonreducing condi-

tion. This suggested that $p56^{lck}$ itself does not form dimer or polymer directly by thiol-mediated bond, even though the mechanism of aggregation needed the triggering by thiol-linked mechanism.

Involvement of Known Signal Delivery Elements in the $HgCl_2$-Induced Aggregates of Cellular Proteins

A study was carried out to examine whether some known elements of signal transduction following tyrosine kinase activation were involved in the $HgCl_2$-induced aggregates of cellular proteins. As shown in Figure 5, the majority of GAP, PI3-K, and MAP-K proteins, which resided in the supernatant fraction of normal cell lysates, moved to the pellet fraction after the short time incubation with $HgCl_2$.

Isolation of $HgCl_2$-Induced Aggregates of Cellular Proteins by Sucrose Density Gradient Centrifugation

We next tried to fractionate the cell lysates by sucrose density gradient in order to isolate the Brij 96-insoluble aggregates. As shown in Figure 6A, the PTYR-containing proteins in $HgCl_2$-treated cell lysates were well separated in the gradient centrifugation, and the majority of selected PTYR-containing proteins in the aggregates was collected in the 7th fraction from the top. The same fraction included $p56^{lck}$ (Fig. 6B). Furthermore, GAP (Fig. 6C), PI3-K (Fig. 6D), and MAP-K (Fig. 6E), which were found in the 2nd fraction of normal cell lysates, were demonstrated mostly in the 7th fractions in the $HgCl_2$-treated cell lysates.

DISCUSSION

This study showed that cell surface-acting $HgCl_2$ delivered a signal intracellularly to produce a giant complex of PTYR-containing proteins which is insoluble in Brij 96 and sedimentable at low speed centrifugation. The process started at as early as 5 s after the exposure of lymphocytes to $HgCl_2$. This result corresponded to the observation by fluorescent antibody technique that PTYR-containing proteins developed at well-localized area beneath cell membrane immediately after exposure to $HgCl_2$. Interestingly, selected PTYR-containing proteins (molecular sizes 56, 120, and 180 kDa) were preferentially incorporated in the complex, whereas some other proteins (28 and 80 kDa) largely

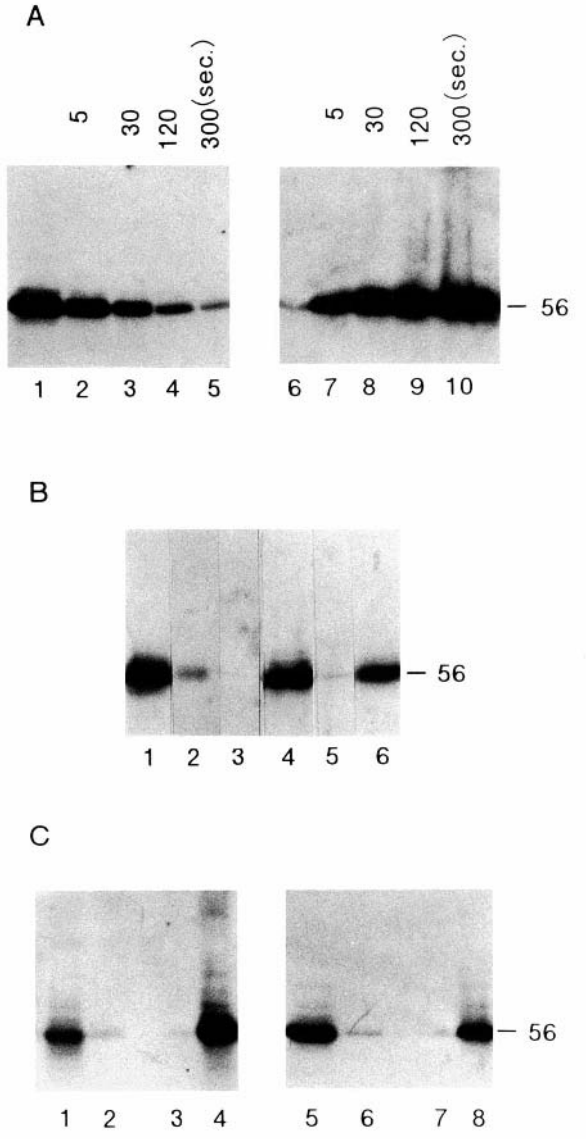

Fig. 4. Evidence of involvement of $p56^{lck}$ in the $HgCl_2$-induced aggregate. Cell lysates were prepared as in Figures 2 and 3, and then analyzed for $p56^{lck}$ by immunoblot. **A:** Lanes 1 to 5, supernatant; lanes 6 to 10, pellet; lanes 1 and 6, no $HgCl_2$ control; lanes 2 to 5 and 7 to 10, cells were treated with $HgCl_2$ for the indicated time (s) before preparation of cell lysates. **B:** Lanes 1 and 2, supernatant; lanes 3 and 4, 2ME soluble fraction of pellet; lanes 5 and 6, 2ME insoluble fraction of pellet; lanes 1, 3, and 5, no $HgCl_2$; lanes 2, 4, and 6, cells treated with $HgCl_2$. **C:** Lanes 1, 2, 5, and 6, supernatant; lanes 3, 4, 7, and 8, pellet; lanes 1 to 4, SDS-PAGE under nonreducing condition; lanes 5 to 8, under reducing condition; lanes 1, 3, 5, and 7, no $HgCl_2$; lanes 2, 4, 6, and 8, cells treated with $HgCl_2$. The position of $p56^{lck}$ is shown on the right.

remain outside the complex. Indeed, there was much variance in the extent of time-dependent incorporation into the complex among different PTYR-containing proteins: some molecules were increasingly incorporated into the complex with

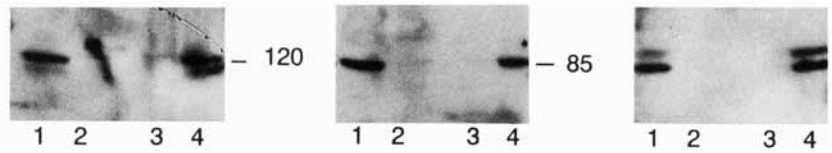

Fig. 5. Evidence of involvement of GAP, PI3-K, and MAP-K in the $HgCl_2$-induced aggregate. The supernatants (lanes 1 and 2) and the pellets (lanes 3 and 4) of cell lysates were prepared from the thymocytes treated with $HgCl_2$ for 5 min as in Figure 2, and analyzed for GAP **(A)**, PI3-K **(B)**, and MAP-K **(C)** by immunoblot. Each position of GAP (120 kDa), PI3-K (85 kDa), and MAP-K (43/41 kDa) is shown on the right.

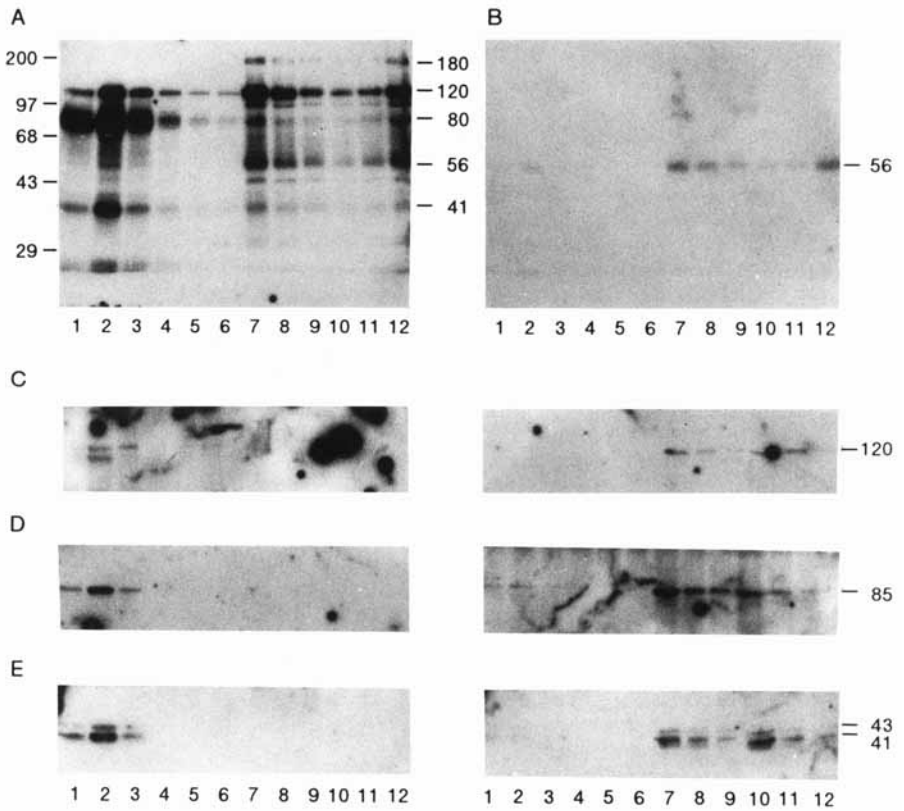

Fig. 6. Partial isolation of the $HgCl_2$-induced aggregate of cellular proteins by sucrose density gradient centrifugation. Suspensions of thymocytes in MEM were incubated in the presence of $HgCl_2$ for 5 min, and lysed in Brij 96 lysis buffer. The lysates were centrifuged with sucrose density gradient (10–50%) at 500*g* for 10 min, and fractionated by 1 ml from the top to the bottom. Each fraction was assayed for PTYR-containing proteins **(A)**, $p56^{lck}$ **(B)**, GAP **(C)**, PI3-K **(D)**, and MAP-K **(E)** by immunoblot. Lane number is arranged in order from left to right. Lane 1 is the top layer and lane 12 is the bottom layer. For panels C–D: left pictures, no $HgCl_2$ control; right pictures, cells treated with $HgCl_2$. Molecular weights (kDa) of standard proteins are shown on the left. The positions of GAP, PI-3K, and MAP-K are shown on the right.

time, whereas others preferentially remain outside the complex from early to late stages even though they are phosphorylated from the early stage. This indicated that the formation of complex is regulated by some specific properties of the individual PTYR-containing proteins.

The action of $HgCl_2$ to induce heavy tyrosine phosphorylation and aggregation of cellular proteins are inhibited by the thiol-donating chemicals such as dithiothreitol [Rahman et al., 1993; Nakashima et al., 1994]. Moreover, the complex was partially dissociated by treatment with 2ME. These results showed that the event was initially triggered by a redox-linked mechanism. However, not all aggregates were dissociated by the treatment with 2ME, and most of proteins

in the aggregate were dissociated into molecular components in SDS-PAGE under either reducing or nonreducing condition. This suggested that other mechanisms than redox-linked mechanism worked in producing the complex. It is known that many tyrosine kinases and their substrates bear two characteristic structures termed Src homology 2 (SH2) and SH3: the former reacts with specific sequences containing phosphorylated tyrosine, and the latter with proline-rich sequences [Pawson and Gish, 1992]. A likely explanation is that crosslinkage of cell surface receptors induces aggregation of intracellularly associating nonreceptor tyrosine kinases and their substrates, which causes tyrosine phosphorylation of these proteins through kinase activation and therefore promotes association between SH2 and the PTYR-containing sequence and between SH3 and the proline-rich sequence. If this is the case, proteins bearing SH2 and SH3 should be selectively included in the complex.

We next showed that aggregation of $p56^{lck}$ quickly occurred preceding phosphorylation and aggregation of other proteins. This suggested that the initial intracellular event following receptor crosslinkage, starting as quickly as 5 s after exposure of thymocytes to $HgCl_2$ [Nakashima et al., 1994], is aggregation and activation of nonreceptor tyrosine kinase $p56^{lck}$, which subsequently phosphorylate substrate proteins for complex formation. The aggregate of $p56^{lck}$ was partially dissociated by 2ME, confirming the involvement of a redox mechanism in forming the aggregate. However, not all $p56^{lck}$ was dissociated by 2ME, and almost all $p56^{lck}$ molecules in the aggregate were dissociated in SDS-PAGE under nonreducing condition. This once again supported the view that $p56^{lck}$ molecules are aggregated by indirect mechanisms, possibly including crosslinkage of $p56^{lck}$-associating cell surface proteins and/or SH2/SH3-mediated secondary protein interaction. Further study proved that the large portion of cellular GAP (120 kDa) and PI3-K (85 kDa) which bear SH2/SH3 in the molecules are incorporated in the $HgCl_2$-induced complex together with $p56^{lck}$. This supports the above-mentioned view that proteins bearing SH2/SH3 are involved in the complex. Both 43 kDa and 41 kDa MAP-K as the late signal elements just before gene transcription regulation was also included. These results support that the cell surface acting $HgCl_2$-induced giant signal complex consists of various signal elements in the cascade of tyrosine kinase-mediated signal transduction.

We lastly tried to partially isolate the $HgCl_2$-induced giant signal complex from other proteins by density gradient centrifugation. In the isolated fraction, several PTYR-containing proteins and $p56^{lck}$, GAP, PI3-K, and MAP-K were enriched separately from other PTYR-containing proteins. This lastly confirmed that the giant complex consists of selected PTYR-containing proteins, including common signal delivery elements, and supported the view that these signal elements were accumulated together through a unique pathway alternative to conventional ligand-mediated one. These results may also provide a new tool analyzing properties and molecular dynamics of signal elements in T-lymphocytes.

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