Suppression of a High-Affinity Transport System for Manganese in Cadmium-Resistant Metallothionein-Null Cells

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ABSTRACT

Cadmium is a hazardous heavy metal existing ubiquitously in the environment, but the mechanism of cadmium transport into mammalian cells has been poorly understood. Recently, we have established a cadmium-resistant cell line (Cd-rB5) from immortalized metallothionein-null mouse cells, and found that Cd-rB5 cells exhibited a marked decrease in cadmium uptake. To investigate the mechanism of altered uptake of cadmium in Cd-rB5 cells, incorporation of various metals was determined simultaneously using a multitracer technique. Cd-rB5 cells exhibited a marked decrease in manganese incorporation as well as that of cadmium. However, the reduced uptake of manganese was observed only at low concentrations, suggesting that a high-affinity component of the Mn\(^{2+}\) transport system was suppressed in Cd-rB5 cells. Competition experiments and kinetic analyses revealed that low concentrations of Cd\(^{2+}\) and Mn\(^{2+}\) share the same high-affinity pathway for their entry into cells. The mutual competition of Cd\(^{2+}\) and Mn\(^{2+}\) uptake was also observed in HeLa, PC12, and Caco-2 cells. The highest uptake of Cd\(^{2+}\) and Mn\(^{2+}\) by parental cells occurred at neutral pH, suggesting that this pathway is different from a divalent metal transporter 1 that can transport various divalent metals including Cd\(^{2+}\) and Mn\(^{2+}\) under acidic conditions. These results suggest that a high-affinity Mn\(^{2+}\) transport system is used for mammalian cellular cadmium uptake, and that the suppression of this pathway caused a marked decrease in cadmium accumulation in cadmium-resistant metallothionein-null cells.

Cadmium is an environmental pollutant that causes adverse effects in various organs. Chronic exposure to cadmium in animals and humans results in preferential renal cadmium accumulation, thereby leading to nephrotoxicity (Guerer et al., 1995). Although detrimental effects of cadmium have been well documented, the mechanism of cadmium transport, especially in mammalian cells, remains poorly understood. Several animal studies have shown that cadmium intestinal absorption can be affected by dietary components such as iron, zinc, calcium, vitamin D, and phytic acid (Valberg et al., 1976; Omori and Muto, 1977; Waskho and Cousins, 1977; Rose and Quartenman, 1984; Foulkes, 1985; Moon, 1994). In in vitro studies, Cd\(^{2+}\) has been extensively used as a potent calcium channel blocker (Jacobson and Turner, 1980; Tsien et al., 1987; Lopez et al., 1989) because the ionic radius of Cd\(^{2+}\) is close to that of Ca\(^{2+}\). However, cellular uptake of Cd\(^{2+}\) can be inhibited by calcium channel blockers (Hinkle et al., 1987), suggesting that Cd\(^{2+}\) is incorporated into cells at least partly via calcium channels. Other trace elements, such as zinc and copper, also have been reported to inhibit cadmium uptake in hepatocytes (Blazka and Shaikh, 1992) and intestinal cells (Jumarie et al., 1997). Because cadmium is not an essential trace element, transporters for metals such as calcium, zinc, copper, or iron may also be used for cadmium incorporation. However, the process of transport of each metal into mammalian cells has not yet been fully elucidated (Nelson, 1999).

Recently, Gunshin et al. (1997) isolated a divalent metal transporter 1 (DMT1) from rat intestine as a transporter responsible for transferrin-independent iron uptake in the intestine. DMT1 exhibited a broad substrate specificity for divalent metals such as Zn\(^{2+}\), Mn\(^{2+}\), Co\(^{2+}\), Cu\(^{2+}\), Ni\(^{2+}\), Pb\(^{2+}\), and Cd\(^{2+}\). Thus, DMT1 is the first and only characterized mammalian metal transporter that can facilitate the cellular uptake of cadmium. However, the actual contribution of DMT1 to cadmium uptake remains unclear.

The most important cellular factor responsible for cadmium resistance in animals is known to be metallothionein (MT). MT is a low-molecular-weight cysteine-rich protein that can be induced by metals, including cadmium, and can attenuate the toxicity of metals by sequestering them (Kagi, 1993). Previously, to investigate the contribution of non-MT factors for cadmium resistance, we established cadmium-resistant cell lines (Yanagiya et al., 1999) from immortalized MT-null embryonic fibroblasts (Kondo et al., 1999) derived from transgenic mice deficient in MT-I and -II, and MT-null embryonic fibroblasts (Kondo et al., 1999) derived from transgenic mice deficient in MT-I and -II, the major

ABBREVIATIONS: DMT1, divalent metal transporter 1; MT, metallothionein; DMEM, Dulbecco’s modified Eagle’s medium.
isoforms of MT (Michalska and Choo, 1993). The cadmium-resistant MT-null cells exhibited a marked decrease in cadmium uptake and an increase in cadmium release compared with the parental MT-null cells, suggesting that these changes in cadmium transport have conferred resistance to cadmium (Yanagiya et al., 1999). Thus, the characterization of cadmium accumulation in the cadmium-resistant MT-null cells can lead to an improved understanding of mammalian cellular cadmium transport mechanism.

In this study, the changes in the incorporation of various elements into cadmium-resistant cells were determined using a multitracer technique, which permitted measurement of the incorporation of 20 radioactive tracers simultaneously. Interestingly, the incorporation of Mn$^{2+}$ in cadmium-resistant MT-null cells was approximately 10% of that in parental cells. Therefore, we further examined time- and dose-dependent incorporation of Mn$^{2+}$ and competition of Cd$^{2+}$ and Mn$^{2+}$ uptake by each other in these cell lines. The results indicate that the high-affinity transport system for Mn$^{2+}$ was suppressed in cadmium-resistant cells, and that this pathway may be used for the entry of Cd$^{2+}$ into mammalian cells.

**Materials and Methods**

**Cell Culture.** A clone of cadmium-resistant MT-null cells, Cd-rB5, established from the SV40-transformed embryonic cells of MT-null mice was used (Yanagiya et al., 1999). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Grand Island, NY) with 10% fetal bovine serum under 5% CO$_2$ at 37°C. Cd-rB5 and parental cells were maintained in the medium containing 10$^{-5}$ M CdCl$_2$, and were cultured in Cd$^{2+}$-free medium for 10 days before the following assays were performed.

**Preparation of a Radioactive Multitracer Solution.** The detailed procedure for preparation of the multitracer solution was described previously (Ambe et al., 1995; Enomoto et al., 1996; Hirunuma et al., 1997). Briefly, a plate of silver was irradiated with the 135 MeV/nucleon$^{14}$N beam from the RIKEN Ring Cyclotron (Saitama, Japan). After irradiation, the silver target, which contained incorporation was expressed as a percentage of the total amount of each sample were determined by Lowry’s method (Lowry et al., 1951).

**Inhibition of Mn$^{2+}$ and Cd$^{2+}$ Uptake.** Cells (2 $\times$ 10$^5$ cells/6-well dish) were preincubated in serum-free media for 30 min and were treated with 0.03 $\mu$M [109$^{109}$Cd]CdCl$_2$ (Amersham Pharmacia Biotech, Tokyo, Japan) in the presence of 0, 0.03, 0.06, 0.1, and 0.3 $\mu$M MnCl$_2$. After a 15-min incubation, cells were washed three times with 2 ml of PBS containing 0.05% EDTA and were harvested with 1 ml of PBS containing 2% SDS and were transferred to a test tube. $^{54}$Mn concentrations remaining in the cells were determined by the radioactivity of $^{54}$Mn as mentioned above. Protein concentrations in each sample were determined by Lowry’s method (Lowry et al., 1951).

**Measurement of Uptake and Release of Mn$^{2+}$.** Cd-rB5 and parental cells (2 $\times$ 10$^5$ cells/6-well dish) were preincubated in serum-free medium for 30 min and then exposed to 0.03 $\mu$M [54$^{54}$Mn]MnCl$_2$ (DuPont-NEN, Boston, MA) for 0, 15, 30, 60, and 120 min. After three washings with 2 ml of PBS containing 0.05% EDTA, cells were harvested with 1 ml of PBS containing 2% SDS and were transferred to a test tube. The radioactivity of $^{54}$Mn was measured with an auto well gamma counter (ALOKA, Tokyo, Japan). Similarly, dose-dependent uptake of manganese was determined at 15 min after the addition of 0.01, 0.1, 0.3, 1.0, 3.0, and 10.0 $\mu$M [54$^{54}$Mn]MnCl$_2$. For the measurement of manganese efflux, Cd-rB5 and parental cells were treated with 0.03 or 0.3 $\mu$M [54$^{54}$Mn]MnCl$_2$ in serum-free medium for 120 min, washed three times with 2 ml of PBS containing 0.05% EDTA, and incubated for 0, 5, 15, 30, 60, and 120 min in a freshly supplemented $^{54}$Mn-free medium. After rinsing three times with 2 ml of PBS containing 0.05% EDTA, the cells were harvested with 1 ml of PBS containing 2% SDS and were transferred to a test tube. $^{54}$Mn concentrations remaining in the cells were determined by the radioactivity of $^{54}$Mn as mentioned above. Protein concentrations in each sample were determined by Lowry’s method (Lowry et al., 1951).

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**pH-Dependent Uptake of Cd$^{2+}$ and Mn$^{2+}$.** Cd-rB5 and parental cells (2 $\times$ 10$^5$ cells/6-well dish) were preincubated in serum-free media for 30 min and were exposed to 0.03 $\mu$M [109$^{109}$Cd]CdCl$_2$ or [54$^{54}$Mn]MnCl$_2$ for 15 min in the pH-adjusted medium. The pH of the medium was adjusted by the addition of 1 N HCl (pH 5.5–6.5) or 7.5% NaHCO$_3$ solution (pH 6.5–8.0) to DMEM. After washing three times with 2 ml of PBS containing 0.05% EDTA, the cells were harvested with 1 ml of PBS containing 2% SDS and were transferred to a test tube. The radioactivity of $^{109}$Cd or $^{54}$Mn was measured by an ALOKA auto well gamma counter.

**Data Analyses.** All experiments were performed in triplicate. Data were expressed as mean ± S.D., and statistical significance was determined by Student’s t test. Lineweaver-Burk plots were used to determine values of $K_M$ and $V_{max}$ of metal uptake. $K_M$ values were derived from Dixon plots.

**Results**

**Altered Metal Accumulation in Cd-rB5 Cells.** A multitracer technique was used to identify metal(s) that exhibit altered accumulation in cadmium-resistant MT-null cells in which cadmium accumulation was markedly repressed. Figure 1 shows the incorporation of metals in Cd-rB5 and parental cells that were exposed to multitracer solutions for 120 min. Radioactivity from 11 of 20 elements in the multitracer solution (Fig. 1) was detected. Other elements could not be measured due either to their rapid half-lives or to extremely low cellular incorporation. Incorporations of Be$^{2+}$, Sc$^{3+}$,
Cr$^{3+}$, Fe$^{3+}$, Zn$^{2+}$, SeO$_4^{2-}$, Rb$^+$, Y$^{3+}$, and ZrO$_2^{2+}$ were similar between Cd-rB5 and parental cells. However, the incorporation of Mn$^{2+}$ in Cd-rB5 cells was approximately 10% of that in parental cells. A reduced accumulation of Mn$^{2+}$ in Cd-rB5 cells was also observed when the multitracer solution was dissolved in DMEM with 10% fetal bovine serum (data not shown). Furthermore, the incorporation of Co$^{2+}$ into Cd-rB5 cells was approximately half of that in parental cells (Fig. 1).

**Alteration in Mn$^{2+}$ Uptake in Cd-rB5 Cells.** Because screening with multitracer solutions suggested a suppressed transport of Mn$^{2+}$ in cadmium-resistant MT-null cells, time- and dose-dependent uptake of Mn$^{2+}$ was determined in Cd-rB5 and parental cells using commercially available $^{54}$Mn$^{2+}$-loaded MnCl$_2$. As shown in Fig. 2A, the incorporation of Mn$^{2+}$ (0.03 μM) into Cd-rB5 cells was markedly suppressed, whereas the parental cells exhibited a time-dependent accumulation of Mn$^{2+}$. At 120 min after the addition of Mn$^{2+}$ in the medium, Mn$^{2+}$ incorporation into Cd-rB5 cells was approximately 10% of that in parental cells.

In a previous study (Yanagiya et al., 1999) reported from this laboratory, it was demonstrated that the reduced accumulation of Cd$^{2+}$ in Cd-rB5 cells was caused not only by a reduction in Cd$^{2+}$ uptake but also by an enhancement of Cd$^{2+}$ release from the cells. To compare the rate of Mn$^{2+}$ release in Cd-rB5 and parental cells, both cell lines were exposed to MnCl$_2$ for 120 min and were incubated in Mn$^{2+}$-free medium. However, both Cd-rB5 and parental cells released 20 to 40% of the loaded Mn$^{2+}$ 120 min after the medium change, and no increase in the rate of Mn$^{2+}$ release was observed in Cd-rB5 cells (Fig. 2B). Thus, the reduced accumulation of Mn$^{2+}$ in Cd-rB5 cells may be attributable primarily to a reduced rate of Mn$^{2+}$ uptake.

Figure 2C shows dose-dependent uptake of Mn$^{2+}$ in Cd-rB5 and parental cells. When cells were exposed to Mn$^{2+}$ at the concentrations less than 1.0 μM, the incorporation of Mn$^{2+}$ into Cd-rB5 cells was approximately 10% of that in parental cells. However, Mn$^{2+}$ incorporation into Cd-rB5 cells gradually increased with Mn$^{2+}$ concentration over 1.0 μM, and reached the same level as that in parental cells at 10.0 μM. These data suggest that there are two or more pathways of Mn$^{2+}$ entry into cells, and that only the pathway for low concentration of Mn$^{2+}$ is suppressed in Cd-rB5 cells.

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**Fig. 1.** Incorporation of various elements into Cd-rB5 and parental cells. Cd-rB5 (solid column) and parental (open column) cells, which were cultured in serum-free media, were exposed to radioactive multitracers for 120 min. After cells were washed with PBS containing 0.05% EDTA, the radioactivities of the elements incorporated into the cells were determined simultaneously by a Ge-detector. The quantification of radioactivity of each element was performed as described in Materials and Methods. The incorporation of 11 of 20 radioactive elements was successfully determined and expressed as a percentage of the amount added in the medium. Mean ± S.D. (n = 3). Asterisks indicate significant differences from parental cells by t test ($^*P < .05$; $^{**}P < .01$).

**Fig. 2.** Time- and dose-dependent uptake and release of manganese in Cd-rB5 and parental cells. A, Cd-rB5 (closed circles) and parental (open circles) cells were preincubated in serum-free media and exposed to 0.03 μM $^{54}$Mn$^{2+}$MnCl$_2$ for 0, 15, 30, 60, or 120 min. After washing the cells with PBS containing 0.05% EDTA, $^{54}$Mn radioactivity in each sample was measured by gamma counter. B, Cd-rB5 (closed circles and triangles) and parental (open circles and triangles) cells were exposed to 0.03 μM (triangles) or 0.3 μM (triangles) $^{54}$Mn$^{2+}$MnCl$_2$ for 120 min, washed with PBS containing 0.05% EDTA, and then supplemented freshly with $^{54}$Mn-free media. The radioactivity of $^{54}$Mn remaining in the cells was measured at 0, 15, 30, and 60 min after the medium change. C, Cd-rB5 (closed circles) and parental (open circles) cells were exposed to 0.01, 0.1, 0.3, 1.0, 3.0, and 10.0 μM $^{54}$Mn$^{2+}$MnCl$_2$ for 15 min. $^{54}$Mn radioactivity in each sample was measured as described above. Mean ± S.D. (n = 3). Asterisks indicate significant differences from parental cells by t test ($^{**}P < .01$).
Mutual Inhibition of Cd$^{2+}$ and Mn$^{2+}$ Uptake in Parental Cells. To test whether the transport of low concentrations of Mn$^{2+}$ and Cd$^{2+}$ into cells is mediated via the same pathway, we determined whether the uptake of Cd$^{2+}$ and Mn$^{2+}$ is mutually competitively inhibited. As shown in Fig. 3A, Cd$^{2+}$ uptake by parental cells was inhibited by Mn$^{2+}$ in a dose-dependent manner. Similarly, the uptake of Mn$^{2+}$ was also inhibited by Cd$^{2+}$ in a dose-dependent manner (Fig. 3B). However, when Cd-rB5 cells were used, Mn$^{2+}$ did not inhibit Cd$^{2+}$ uptake (Fig. 3A), nor did Cd$^{2+}$ inhibit Mn$^{2+}$ uptake (Fig. 3B). These results suggest that Mn$^{2+}$ and Cd$^{2+}$ share the same process of transport into cells at low concentrations, and that this process is not functioning in Cd-rB5 cells.

To examine further the mode of inhibition in parental cells, the uptake rates of Cd$^{2+}$ at various concentrations in the presence (0.3 μM) or absence of Mn$^{2+}$ were determined and subsequently analyzed by Lineweaver-Burk plot. Figure 4A shows that Mn$^{2+}$ competitively inhibited the uptake of Cd$^{2+}$. The inhibition constant ($K_i$) of Mn$^{2+}$ for the uptake of Cd$^{2+}$ as determined by Dixon plot (Fig. 4B) was 0.14 μM. However, a meaningful $K_i$ value of Cd$^{2+}$ for Mn$^{2+}$ uptake could not be obtained by Dixon plot, probably due to an overlapping of both high- and low-affinity transport systems within a narrow range of Mn$^{2+}$ concentrations used in this experiment.

The uptake of Cd$^{2+}$ and Mn$^{2+}$ by parental cells in a low concentration range (0.01–0.1 μM) was determined in a separate experiment using more points of metal concentration and analyzed by Lineweaver-Burk plot. The apparent $K_m$ values were 40 and 36 nM, and $V_{max}$ values were 1.21 and 1.18 pmol/min/mg protein for Cd$^{2+}$ and Mn$^{2+}$, respectively. These kinetic data and the results of competition experiments suggest that mouse fibroblast cells have a high-affinity transport system for Mn$^{2+}$ uptake, and that this pathway is also used for Cd$^{2+}$ uptake.

Inhibition of Cd$^{2+}$ and Mn$^{2+}$ Uptake by Other Metals. To investigate the effects of other metal ions on the uptake of Cd$^{2+}$ and Mn$^{2+}$ at low concentrations, the uptake of Cd$^{2+}$ or Mn$^{2+}$ was determined in the presence of a 5-fold excess amount of Zn$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Fe$^{2+}$, and Cu$^{2+}$. As shown in Fig. 5A, Cd$^{2+}$ uptake was inhibited by Zn$^{2+}$ as well as by Mn$^{2+}$, but the other metals did not exhibit inhibitory effects. Similarly, Mn$^{2+}$ uptake was also inhibited by both Cd$^{2+}$ and Zn$^{2+}$, but inhibition by other metals was not observed (Fig. 5B). In Cd-rB5 cells, however, Zn$^{2+}$ did not inhibit either Cd$^{2+}$ or Mn$^{2+}$ uptake (data not shown). These results suggest that Zn$^{2+}$ may also have an affinity for the high-affinity Mn$^{2+}$ and Cd$^{2+}$ transport system. However, Zn$^{2+}$ incorporation was not reduced in Cd-rB5 cells compared with parental cells when Zn$^{2+}$ was added to the medium as a component of multitracer solution (Fig. 1). Thus, Zn$^{2+}$ may not be incorporated into cells solely via the high-affinity Mn$^{2+}$ and Cd$^{2+}$ uptake system.

The results obtained in the multitracer experiment (Fig. 1)
Mutual Inhibition of Cd$^{2+}$ and Mn$^{2+}$ Uptake in Other Mammalian Cells. To test whether the high-affinity transport system for Mn$^{2+}$ and Cd$^{2+}$ is present in other mammalian cells, the influence of low concentrations of Cd$^{2+}$ and Mn$^{2+}$ on uptake of each metal was determined in HeLa, PC12, and Caco-2 cells. As shown in Table 1, the presence of 5-fold excess amounts of Cd$^{2+}$ or Mn$^{2+}$ efficiently inhibited the uptake of 0.03 μM Mn$^{2+}$ or Cd$^{2+}$, respectively, in HeLa, PC12, and Caco-2 cells, although the extents of inhibition differed among cell lines. Thus, the high-affinity Mn$^{2+}$ and Cd$^{2+}$ transport system may exist ubiquitously in mammalian cells.

pH-Dependent Uptake of Cd$^{2+}$ and Mn$^{2+}$. The influence of medium pH on the uptake of Cd$^{2+}$ and Mn$^{2+}$ was determined because the optimal pH of rat DMT1, which can transport both Cd$^{2+}$ and Mn$^{2+}$ as well as Fe$^{2+}$, was reported to be 5.5 (Gunshin et al., 1997). Figure 6 demonstrated that the highest uptake of both Cd$^{2+}$ and Mn$^{2+}$ by parental cells was observed at pH 7.4 to 7.6. These peaks of metal uptake at neutral pH were diminished in Cd-rB5 cells, suggesting that the high-affinity Cd$^{2+}$ and Mn$^{2+}$ transport system functions effectively at physiological pH, and that the transporter that was suppressed in Cd-rB5 cells is not DMT1.

Discussion

In this study, we demonstrate that the high-affinity component of the manganese transport system in mammalian cells is also used for cadmium uptake. Previously, we have established cadmium-resistant cell lines from MT-null mouse cells that exhibited a marked decrease in the uptake of cadmium (Yanagiya et al., 1999). The application of multitracer technique in this study revealed that the uptake of ultratrace amount of manganese in Cd-rB5 cells was reduced to approximately 10% of that in parental cells, whereas no change in the incorporation of zinc, copper, or iron was observed. Because cadmium accumulation in the Cd-rB5 cells was also reduced to 10% of that in parental cells, the same mechanism may be responsible for the reduction of the incorporation of both cadmium and manganese into Cd-rB5 cells.

The results of time- and dose-dependent uptake of Mn$^{2+}$ in Cd-rB5 and parental cells (Fig. 2 A and C) suggest that there are at least two components, having high and low affinity to Mn$^{2+}$, for Mn$^{2+}$ uptake into these cells, and that only the high-affinity component is suppressed in Cd-rB5 cells. However, the low-affinity system(s) for Mn$^{2+}$ transport may not be altered in Cd-rB5 cells nor involved in Cd$^{2+}$ transport. In support of this notion, Cd-rB5 cells did not exhibit cross-

TABLE 1

Inhibition of cadmium or manganese uptake in mammalian cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>109Cd Uptake</th>
<th>54Mn Uptake</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>−Mn</td>
<td>+Mn</td>
</tr>
<tr>
<td>HeLa</td>
<td>30.0 ± 0.4</td>
<td>16.5 ± 1.5</td>
</tr>
<tr>
<td>PC12</td>
<td>15.7 ± 4.0</td>
<td>4.9 ± 0.8</td>
</tr>
<tr>
<td>Caco-2</td>
<td>9.1 ± 1.6</td>
<td>3.3 ± 0.3</td>
</tr>
</tbody>
</table>

HeLa, PC12, and Caco-2 cells were preincubated in serum-free medium, and were treated with 0.03 μM [109Cd] CdCl$_2$ or 0.03 μM [54Mn] MnCl$_2$ for 15 min in the presence of a 5-fold excess amount of Mn$^{2+}$ or Cd$^{2+}$, respectively. Asterisks indicate significant differences from the value obtained in the absence of metal inhibitors by $t$ test ($*P < .05$; **$P < .01$).
resistance to MnCl₂ as determined by cell survival assay (data not shown).

In accordance with our results, several studies have suggested the existence of high- and low-affinity pathways of Mn²⁺ incorporation into cells. In rabbit reticulocytes, a high-affinity (Kₘ = 0.4 μM) and a low-affinity (Kₘ = 48 μM) manganese transport system were reported (Chua et al., 1996). Using rat liver slices, Galeotti et al. (1995) reported that there are three saturable systems for Mn²⁺ uptake with different Kₘ values of 0.075, 2, and 100 μM. Two components of Mn²⁺ uptake were also observed in hepatoma cells (Galeotti et al., 1995). Thus, it appears reasonable that the mouse fibroblast cells used in this study also have both high- and low-affinity pathways of manganese incorporation. To date, however, no specific manganese transporter has been identified.

The apparent Kₘ values for the uptake of Cd²⁺ and Mn²⁺ at low concentrations were 40 and 36 nM, respectively. The competition study shown in Fig. 3 demonstrated that uptake of low concentrations of Cd²⁺ and Mn²⁺ (0.03 μM) was inhibited by the counterpart metal in parental cells. Kinetic analysis (Fig. 4) revealed that Mn²⁺ inhibited the uptake of Cd²⁺ competitively with a Kᵢ value of 0.14 μM. On the other hand, no inhibition by either metal was observed in Cd-rB5 cells. These data strongly suggest that low concentrations of Cd²⁺ and Mn²⁺ use the same high-affinity pathway for their entry into cells. Furthermore, the absence of the inhibition of Cd²⁺ and Mn²⁺ uptake by one another in Cd-rB5 cells indicates that this pathway is suppressed in Cd-rB5 cells, thereby leading to reduced accumulation of cadmium.

A limited numbers of studies have indicated an interaction between Cd²⁺ and Mn²⁺ in mammalian cell transport systems. Frame and Milanick (1991) demonstrated that the Na⁺-Ca²⁺ exchanger in ferret erythrocytes is able to transport both Mn²⁺ and Cd²⁺ as alternative substrates for Ca²⁺ in Na⁺-free solution. However, the Kₘ values for uptake of Cd²⁺ and Mn²⁺ in these cells were in a range of 5 to 20 μM, which is much higher than those obtained in this experiment. It has been demonstrated that Cd²⁺ and Mn²⁺ can enter the plasma membrane as surrogates for Ca²⁺ via calcium channels (Shibuya and Douglas, 1992). However, most of these findings were obtained using relatively high concentrations of Cd²⁺ or Mn²⁺ (1–100 μM). Furthermore, the addition of Ca²⁺ in the medium, even at 5 mM, did not inhibit the uptake of 0.03 μM Cd²⁺ or Mn²⁺ in parental cells (data not shown). Thus, it is unlikely that the high-affinity transport system for Mn²⁺ and Cd²⁺ uptake that was observed in this study is mediated via calcium channels.

Recently, the gene for the transferrin-independent iron transporter, DMT1, has been isolated from rat intestine (Gunshin et al., 1997). DMT1 has a broad substrate range, including Zn²⁺, Mn²⁺, and Cd²⁺. Thus, it can be assumed that the transport of Cd²⁺ and Mn²⁺ observed in this study is attributable to DMT1. However, the analysis of pH dependence of Cd²⁺ and Mn²⁺ uptake in Cd-rB5 and parental cells (Fig. 6) revealed that the highest uptake of both Cd²⁺ and Mn²⁺ by parental cells occurred at neutral pH, and the uptake of Cd²⁺ and Mn²⁺ at this range of pH was suppressed in Cd-rB5 cells. On the contrary, DMT1 was reported to be functioning as a proton symporter under acidic conditions like intestinal lumen, and the optimal pH for metal uptake by DMT1 was 5.5 (Gunshin et al., 1997). Furthermore, in the competition experiment (Fig. 5), the addition of other divalent metals such as Fe²⁺, Co²⁺, Cu²⁺, or Ni²⁺ did not inhibit the uptake of Cd²⁺ or Mn²⁺ in parental cells. Thus, a novel transporter that is distinct from DMT1 may be responsible for the high-affinity transport of Mn²⁺ and Cd²⁺ in mouse fibroblast cells. Because the mutual inhibition of the uptake of Mn²⁺ and Cd²⁺ at low concentrations was also observed in other mammalian cells such as HeLa, PC12, and Caco-2 cells (Table 1), the high-affinity Mn²⁺ and Cd²⁺ transport system observed in this study may be present in various mammalian cells.

Several lines of evidence have indicated that cadmium uptake in mammalian cells is mediated at least partly via calcium channels (Jumari et al., 1997) or the iron transport system (Foulkes, 1985; Moon, 1994). Calcium channels may play a significant role in cadmium transport in excitable cells, and DMT1 can be used for cadmium absorption in the acidic milieu of intestinal lumen. However, little information has been available on the mechanism of cadmium transport in other tissues or cells, and no data have indicated the involvement of manganese in cadmium transport. Due to the existence of both high- and low-affinity Mn²⁺ transport systems, total concentration of manganese in cells or tissues may not exhibit a drastic change when cadmium is applied to cells or administered to animals. This might have led other workers to overlook the involvement of manganese in cad-
mum transport. Only a few studies have reported that manganese can ameliorate cadmium toxicity (Stacy and Klaasen, 1981; Goering and Klaassen, 1985), although the reduction of cadmium accumulation was not observed in these experiments.

Recent studies in yeast have enabled molecular cloning of multiple metal transporters responsible for the influx of zinc, copper, iron, and manganese (Dancis et al., 1994; Dix et al., 1994; Supek et al., 1996; Zhao and Eide, 1996a,b). However, no specific transporter for metal influx has been isolated in mammals except for DMT1. In this study, establishment of a cadmium-resistant MT-null cell line and the application of multitracer technique have permitted the detection of a novel transport system for both Mn\(^{2+}\) and Cd\(^{2+}\).

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