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Phosphotyrosine-Containing Lactate Dehydrogenase Is Restricted to the Nuclei of PC12 Pheochromocytoma Cells

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There are five lactate dehydrogenase (LDH) isoenzymes, composed of various combinations of two types of subunits. LDH-5, which contains only the LDH A subunit, is known to be present in both the cytoplasm and the nucleus, to act as a single-stranded DNA-binding protein possibly functioning in transcription and/or replication, and to undergo phosphorylation of tyrosine 238 in approximately 1% of the enzyme after cell transformation by certain tumor viruses. We have characterized LDH from wild-type PC12 pheochromocytoma cells and from a PC12 variant (MPT1) that exhibits altered lactate metabolism and altered expression of multiple genes. Wild-type and MPT1 cells contain different proportions of LDH isoenzymes, with LDH-5 being more predominant in wild-type cells than in the variant. A small fraction of LDH from PC12 cells contains phosphotyrosine. Approximately 99% of the total LDH activity is located in the cytoplasm, but all of the phosphotyrosine-containing LDH is located in the nucleus. Furthermore, essentially all of the nuclear LDH contains phosphotyrosine. These results suggest that tyrosine phosphorylation can affect its role in the nucleus.

Lactate dehydrogenase (LDH, EC 1.1.1.27) catalyzes the reversible conversion of pyruvate to lactate. Five LDH isoenzymes exist because the enzyme is a tetramer that is composed of various combinations of two kinds of M_r 35,000 subunits named A and B (9). The isoenzymes and their respective structures are LDH-5 (A_4), LDH-4 (A_3B), LDH-3 (A_2B_2), LDH-2 (AB_3), and LDH-1 (B_4). The LDH isoenzyme composition varies from tissue to tissue.

In addition to its role in the metabolism of lactate and pyruvate, one of the LDH isoenzymes, LDH-5, may function in the regulation of gene transcription and/or DNA replication, since LDH-5 is present in the nucleus (5) and it has recently been found to be a previously studied single-stranded DNA-binding protein (14, 25, 31). It had been shown earlier that antibody raised against this DNA-binding protein cross-reacts with single-stranded DNA-binding proteins from heterologous species including *Drosophila* spp. (22). Furthermore, studies of *Drosophila* polytene salivary chromosomes revealed that this protein is associated nonrandomly with the chromosomes and is usually concentrated in certain chromosome puffs that were active sites of transcription, e.g., heat shock puffs after heat shock treatment (22). A possible role in replication is suggested by the finding that LDH-5 stimulates the activity of DNA polymerase α in vitro (14).

Another LDH variation arises from phosphorylation. A small percentage of chicken fibroblast LDH-5 is phosphorylated at serine residues; after transformation of these cells by Rous sarcoma virus, 0.5 to 1% of LDH-5 is also phosphorylated at a single tyrosine residue, tyrosine 238 (6, 7). It is not clear that this tyrosine phosphorylation affects glycolysis, because such a small fraction of the LDH in the transformed cells contains phosphotyrosine. The intracellular location of phosphotyrosine-containing LDH-5 has not previously been studied; therefore, little is known about whether tyrosine phosphorylation occurs on nuclear LDH-5 and hence whether it could alter transcription or replication. Phosphorylation of tyrosine 238 may regulate the DNA-binding activity of LDH-5, since nitration of this residue decreases the ability of LDH-5 to bind to DNA (31).

In the work described in this paper we have characterized LDH from wild-type and variant PC12 cells. PC12 is a clonal line of rat pheochromocytoma cells derived from the adrenal medulla (13). We initiated these studies of LDH because the PC12 variant, which is resistant to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), exhibits three properties that might be related to a difference in LDH. First, the variant exhibits altered lactate metabolism in that it produces abnormally low levels of lactate when oxidative phosphorylation is inhibited (8). Second, the variant exhibits altered the expression of multiple genes, with some mRNA species being markedly decreased (1) and other being markedly increased (our unpublished data). Third, the growth characteristics of the variant in culture are more typical of noncancer cells than of cancer cells; unlike wild-type PC12 cells, variant cells are nonrefractile, adhere tightly to the plate, do not grow in multiple layers, and do not multiply when cultured in 0.3% agar (our unpublished observations). The LDH isoenzyme composition of cancer cells is known to differ from that of noncancer cells (11).

We report that the composition of LDH isoenzymes in wild-type PC12 cells differs from that of isoenzymes in the variant, but each cell type contains LDH-5 that is phosphorylated at tyrosine residues. An unexpected finding is that all of this tyrosine-phosphorylated LDH is located in the nucleus and that essentially all of the nuclear LDH-5 contains phosphotyrosine. These results suggest that tyrosine phosphorylation of nuclear LDH-5 can affect its role in the nucleus.

MATERIALS AND METHODS

Cell culture. PC12 cells were obtained from David Schubert, Salk Institute, and the MPT1 variant was selected as described previously (8). Cells were cultured on plastic dishes (100 by 20 mm) at 37°C under an atmosphere of 10% CO_2–90% air in Dulbecco modified Eagle medium containing 5% fetal calf serum, 10% horse serum, 50 μg of streptomycin per ml, and 50 U of penicillin per ml. The cell density was

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TABLE 1. Protein, DNA, and enzyme levels in extracts from whole cells and isolated nuclei

PC12 cell type	Amt (mg/extract from 10^7 cells) of:		DNA/protein ratio	% Recovery of DNA	Amt of:			
	Protein	DNA			LDH[a]	Galactosyl-transferase[b]	NADPH-cytochrome *c*(P-450) reductase[c]	Succinate dehydrogenase[d]
Wild type								
Whole cells	2.15	0.14	0.07		38.0	248.5	0.353	4.2
Nuclei	0.58	0.12	0.21	86	0.32	4.5	0.031	0.16
MPT1 variant								
Whole cells	1.95	0.13	0.07		6.50	ND[e]	ND	ND
Nuclei	0.53	0.12	0.23	92	0.067	ND	ND	ND

[a] Units of activity in extract from 10^7 cells.
[b] cpm (10^3) in extract from 10^7 cells.
[c] ΔE_{550} per minute in extract from 10^7 cells.
[d] ΔE_{490} per 15 min in extract from 10^7 cells.
[e] ND, Not determined.

approximately 10^7 cells per plate when the cells were harvested.

Isolation and disruption of nuclei. Nuclei were isolated by a slight variation of the procedure used to isolate nuclei for nuclear runoff experiments (12). Cells from 10 to 100 plates were washed in phosphate-buffered saline (10 mM sodium phosphate, 137 mM NaCl [pH 7.4]) and suspended for 10 to 15 min at 4°C in phosphate-buffered saline containing 0.075% Nonidet P-40. The lysed cells were centrifuged for 5 min in a tabletop clinical centrifuge, and the pellet of nuclei was washed three times with phosphate-buffered saline. The nuclei were disrupted by homogenization in a tight fitting Dounce homogenizer or by brief sonication in a bath sonicator. When the LDH phosphotyrosine content was being examined, all solutions contained 100 μM sodium orthovanadate and 0.1% Nonidet P-40 to inhibit phosphotyrosine phosphatase activity (16).

Cellulose acetate strip electrophoresis. LDH was electrophoresed on cellulose acetate strips, which were subsequently stained for LDH activity, by using a Gelman electrophoresis chamber and conditions described in Gelman bulletin 822130. For Fig. 1, 50 to 60 μg of protein was electrophoresed per lane. For Fig. 4 and 5, 50 to 60 μg of nuclear protein or 5 to 6 μg of whole-cell protein was electrophoresed per lane.

Purification of LDH. Whole cells from 10 to 20 plates or nuclei isolated from cells from 10 to 100 plates were homogenized in 10 mM potassium phosphate–1 mM dithiothreitol (pH 6.5) in a tight-fitting Dounce homogenizer and centrifuged at 25,000 × *g* for 20 min at 0°C. The supernatant mixture was applied to a 1-cm-diameter column containing 1 ml of N^6-(6-aminohexyl)-AMP-Sepharose (Pharmacia, Inc.). The column was washed, and the LDH was eluted with NAD-pyruvate adduct as described previously (20). The LDH-containing fractions were pooled, dialyzed against a pH 8.6 buffer, applied to a chromatofocusing column of PBE94 resin (1 by 10 cm; Pharmacia) and eluted first with 100 ml of pH 8.6 to 6.0 gradient and then with 1.0 M NaCl as described previously (30). When the phosphotyrosine content was being examined, all solutions contained 100 μM sodium orthovanadate and 0.1% Nonidet P-40 to inhibit phosphotyrosine phosphatase activity (16).

Measurement of phosphoamino acids. Eleven plates of cells were incubated for 18 h in phosphate-free Dulbecco modified Eagle medium containing dialyzed calf serum, horse serum, and carrier-free $^{32}P_i$ (0.3 mCi/ml; Amersham Corp.). The cells were washed with phosphate-buffered saline containing 100 μM sodium orthovanadate and harvested in 40 ml of the same solution. ^{32}P-labeled whole-cell LDH was prepared by mixing 0.4 ml of this suspension with a suspension of unlabeled cells from 10 plates, homogenizing the mixture, and purifying the LDH by affinity chromatography. Labeled nuclear LDH was prepared from the remaining 39.6 ml, which was centrifuged and suspended in 20 ml of phosphate-buffered saline containing 100 μM sodium orthovanadate. To this suspension was added 20 ml of phosphate-buffered saline containing 100 μM sodium orthovanadate and 0.15% Nonidet P-40. After 10 min, the suspension was centrifuged to obtain a nuclear pellet, which was mixed with unlabeled PC12 cells from 10 plates and homogenized, and LDH was isolated as above. The affinity column eluate containing LDH was precipitated with trichloroacetic acid, and the precipitate was collected by centrifugation, washed three times with acetone, and air dried. LDH was partially hydrolyzed by incubation for 1.5 h at 110°C in 6 M HCl and dried under nitrogen. The hydrolyzate was mixed with marker phosphoserine, phosphothreonine, and phosphotyrosine, and the phosphoamino acids were separated on thin-layer plates by electrophoresis at pH 1.9 and at pH 3.5 as described by Hunter and Sefton (15). The position of the marker phosphoamino acids was determined with ninhydrin.

Determination of enzyme activities, protein, and DNA. The LDH activity was assayed in 0.03 M sodium phosphate (pH 7.4)–1.0 mM sodium pyruvate–0.15 mM NADH. The rate of oxidation of NADH was measured fluorimetrically at an excitation wavelength of 360 nm and an emission wavelength of 457 nm. One unit of enzyme activity was taken as the amount of enzyme that caused an initial rate of oxidation of 1.0 μmol of NADH per min at 25°C (20). The assay was linear with time and protein concentration under the conditions used. Published procedures were used to measure the activities of galactosyltransferase (3), succinate dehydrogenase (23), and NADPH-cytochrome *c*(P-450) reductase (29) and the levels of protein (2) and DNA (4).

RESULTS

Isolation of nuclei. We characterized LDH from wild-type PC12 cells and the MPTP-resistant variant called MPT1. LDH was obtained from extracts of whole cells and from isolated nuclei. The nuclei were prepared after lysis of the plasma membranes by the detergent Nonidet P-40. Table 1 gives the protein and DNA contents of nuclei obtained from the wild-type and variant MPT1 cells. The data show that the isolation procedure used resulted in a 86 to 92% recovery of nuclei. The ratio of DNA to protein in the isolated nuclei was

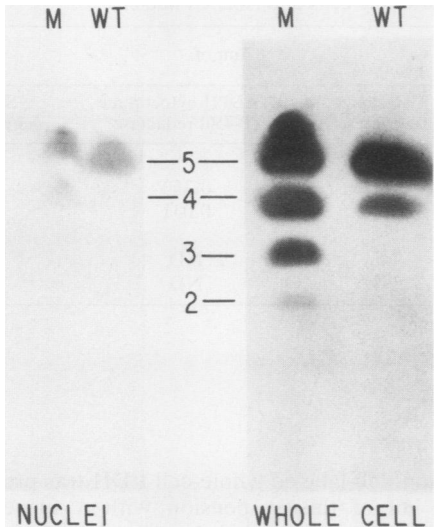

FIG. 1. LDH isoenzymes of wild-type and variant PC12 cells. Proteins were extracted from whole cells or isolated nuclei of wild-type (WT) PC12 or the MPT1 variant (M) and subjected to electrophoresis on cellulose acetate strips, which were then stained for LDH activity. The two lanes on the right were overloaded to show minor isoenzyme forms. The identification of the LDH isoenzymes is indicated in the middle of the figure. The anode is at the bottom.

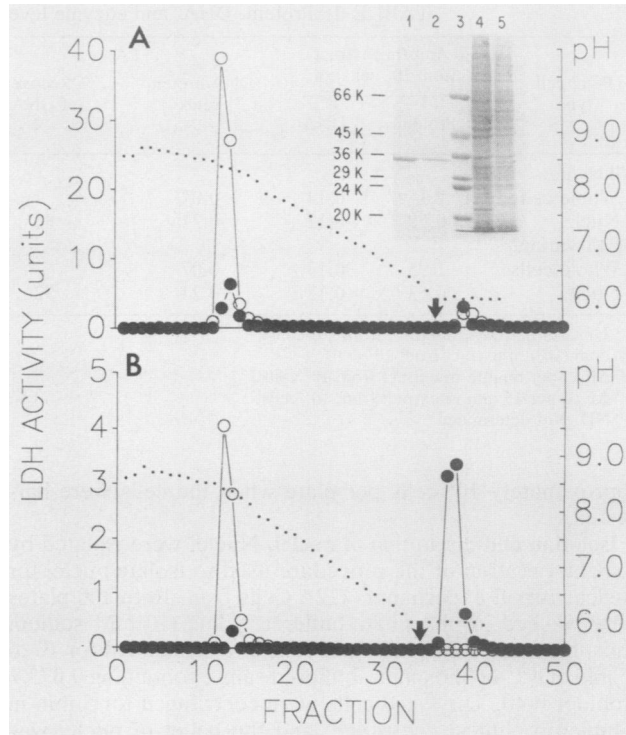

FIG. 2. Chromatofocusing column elution profile of LDH. (A) LDH from wild-type (○) or MPT1 variant (●) whole cells was purified by affinity chromatography as described in Materials and Methods. Approximately 50 μg of protein was applied to a PBE94 column, and the protein was eluted first with a pH gradient (·····) and then with 1.0 M NaCl (▼). Fractions were assayed for LDH activity. Inset: Coomassie blue stain of a sodium dodecyl sulfate slab gel after electrophoresis (19). Lanes: 1 and 2, fraction 13 from column with wild-type and MTP1 cells, respectively; 3, protein standards of indicated molecular weights; 4 and 5, whole-cell proteins from MPT1 and wild-type cells, respectively. (B) Wild-type nuclear LDH was prepared in the presence (●) or absence (○) of inhibitors of phosphotyrosine phosphatase activity. Approximately 5 μg of protein from an affinity column was applied to a PBE94 column and eluted as described for panel A.

0.21 to 0.23, a value that is consistent with a high state of purity of the nuclei (28). We further assessed the purity of the isolated nuclei by assaying the preparation of nuclei for the activities of galactosyltransferase, NADPH-cytochrome *c*(P-450) reductase, and succinate dehydrogenase, which are markers for the Golgi complex, endoplasmic reticulum, and mitochondria, respectively. We found that the isolated nuclei contained less than 2% of the galactosyltransferase activity present in the whole cell, less than 9% of the NADPH-cytochrome *c*(P-450) reductase activity of the whole cell, and less than 4% of the succinate dehydrogenase activity of the whole cell (Table 1).

The MPT1 variant contains only 20% of the LDH activity found in wild-type cells (Table 1). Approximately 1% of the cellular LDH is located in the nucleus. In subsequent sections we refer to LDH isolated from whole cells as cytoplasmic LDH, since such a small fraction of this LDH is from the nucleus.

Isoenzyme composition. Figure 1 shows the isoenzyme composition of LDH in whole-cell extracts (i.e., cytoplasmic LDH) and in nuclei prepared from wild-type or MPT1 cells. Wild-type PC12 cells contain mainly LDH-5 and a small amount of LDH-4. The LDH isoenzyme pattern in MPT1 cells is clearly shifted away from LDH-5 in favor of LDH-4, and the cells also contain LDH-3 and a trace of LDH-2. Wild-type nuclei contain only LDH-5, whereas the nuclei of MPT1 cells contain LDH-5 and a small amount of an isoenzyme that migrates like LDH-4. Northern (RNA) blot analysis indicates that the difference in LDH isoenzyme composition in the variant is due to a decreased level of LDH A mRNA in the variant compared with that in wild-type cells (our unpublished results).

Purification of LDH-5. We next directed our attention specifically to the LDH-5 isoenzyme because of its possible role in the regulation of gene expression and/or replication. In particular, we asked whether it was only a coincidence that 1% of the cellular LDH activity was in the nucleus (Table 1) and that 1% of the LDH in a cell contains phosphotyrosine after viral transformation (7). Therefore, we examined the phosphotyrosine content of LDH-5 from the nucleus and the cytoplasm.

LDH was purified by a two-step procedure. An extract of whole cells or isolated nuclei was passed through an aminohexyl-AMP-Sepharose affinity column, and after being washed, the LDH was eluted with a NAD-pyruvate adduct. This preparation was almost pure but contained a contaminating peptide of lower molecular weight (data not shown). LDH-5 was separated from the contaminant and the other LDH isoenzymes by a chromatofocusing column. Protein was eluted by a pH gradient; cytoplasmic LDH-5 eluted at a buffer pH of 8.3 to 8.5, whereas the remaining LDH eluted subsequently when the buffer contained 1.0 M NaCl (Fig. 2A). The LDH-5 was pure (Fig. 2A, inset). The elution profiles for cytoplasmic LDH from wild-type and MPT1 cells were consistent with the isoenzyme composition shown in Fig. 1 in that a much greater fraction of the wild-type LDH was LDH-5. The LDH that was eluted by 1.0 M NaCl was LDH-4 in the case of wild-type cytoplasm and LDH-4 and LDH-3 in the case of MPT1 cytoplasm (data not shown).

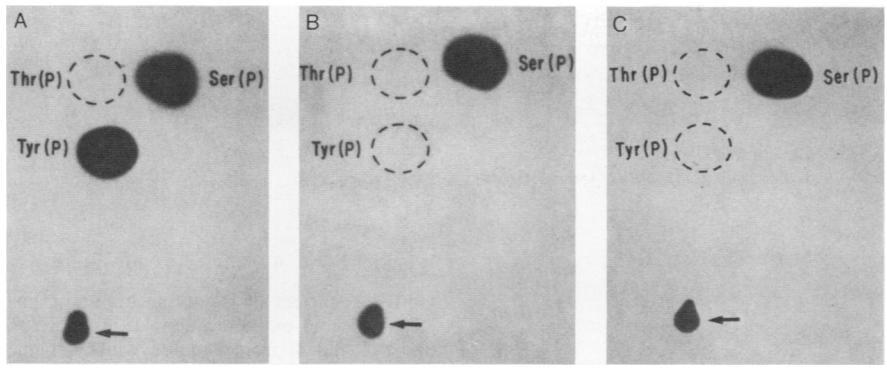

FIG. 3. Autoradiograph of phosphoamino acids of LDH from wild-type whole cells (B) and nuclei (A and C). As described in Materials and Methods, wild-type cell proteins were labeled with $^{32}P_i$, and the LDH was purified by affinity chromatography from whole cells or nuclei, partially hydrolyzed, and separated on thin-layer plates by two-dimensional electrophoresis. A total of 10,000 counts was applied. The film was exposed for 96 h. The origin (←) and the positions of the marker phosphoamino acids are indicated. Sodium orthovanadate (100 μM) was present (panels A and B) or absent (panel C) during the enzyme isolation procedure.

Elution of wild-type nuclear LDH-5 from the chromatofocusing column depended on the composition of the buffer used to extract the enzyme. When the usual extraction buffer (see Materials and Methods) was used, wild-type nuclear LDH-5 eluted at pH 8.3 to 8.5, similarly to cytoplasmic LDH-5. In contrast, when precautions were taken during the enzyme extraction to minimize phosphotyrosine phosphatase activity, e.g., by including sodium orthovanadate in the extraction buffer, nuclear LDH-5 was not eluted by the pH gradient, but only by 1.0 M NaCl (Fig. 2B). The specific LDH activities of the two peaks in Fig. 2B were the same. Wild-type cytoplasmic LDH eluted at pH 8.3 to 8.5 even when sodium orthovanadate was present.

Phosphotyrosine content. The results in Fig. 2B suggested that wild-type nuclear LDH-5 may differ from cytoplasmic LDH-5 in the phosphorylation of tyrosine. Therefore, we tested for the presence of ^{32}P-labeled phosphotyrosine in LDH purified from whole wild-type cells grown with $^{32}P_i$ and in LDH from nuclei obtained from these cells. The purified LDH was hydrolyzed, the amino acids were separated by two-dimensional thin-layer electrophoresis, and the phosphoamino acids were detected by autoradiography. Phosphotyrosine was found only in LDH purified from the nuclei (Fig. 3A and B). Both cytoplasmic and nuclear LDH-5 contained phosphoserine. After autoradiography, the spots on the thin-layer plates where the phosphoamino acids migrated were scraped, and the radioactivity was determined. The amount of radioactivity in the spots was 2,630, 49, and 110 dpm for, respectively, phosphoserine, phosphothreonine, and phosphotyrosine from whole-cell LDH and 1,127, 31, and 1,889 dpm for the same respective phosphoamino acids from nuclear LDH. The values for phosphothreonine correspond to background levels. The results are consistent with the autoradiography shown in Fig. 3A and B.

We were concerned that a finding that phosphotyrosine-containing LDH-5 was confined to the nucleus could be an artifact, since nuclear LDH-5 might be protected from cytoplasmic phosphatases that were removed during the washing of the nuclei. Therefore, it should be noted that in the experiments shown in Fig. 3A, the nuclei isolated from cells grown in $^{32}P_i$ were mixed with unlabeled whole cells before the LDH was extracted and purified.

The detection of ^{32}P-labeled phosphotyrosine in nuclear LDH-5 (Fig. 3A) depended on the use of sodium orthovanadate during the isolation procedure to inhibit phosphotyrosine phosphatase. The autoradiograph of electrophoresed phosphoamino acids from nuclear LDH-5 isolated in the absence of sodium orthovanadate is shown in Fig. 3C; it looks like that of Fig. 3B; i.e., the enzyme contained phosphoserine but not phosphotyrosine.

The finding that phosphotyrosine-containing LDH from the nucleus migrated on a chromatofocusing column differently from phosphotyrosine-free LDH from the cytoplasm (Fig. 2) indicates that most, if not all, of the nuclear LDH contains phosphotyrosine. The chromatofocusing column we used separates proteins on the basis of differences in the isoelectric point, and Cooper et al. (7) have demonstrated that the isoelectric point of phosphotyrosine-containing LDH is much more acidic than that of phosphotyrosine-free LDH. Our conclusion on the stoichiometry of tyrosine phosphorylation of nuclear LDH is further supported by the fact that the migration of nuclear LDH on a chromatofocusing column depended on whether inhibitors of phosphotyrosine phosphatase were used during its isolation (Fig. 2B).

Another indication that essentially all of the wild-type nuclear LDH contains phosphotyrosine comes from the cellulose acetate strip electrophoresis shown in Fig. 4A. Proteins were extracted from whole cells or isolated nuclei in buffer either containing sodium orthovanadate or not. The proteins were electrophoresed on cellulose acetate strips, which were then stained for LDH activity. The charge of wild-type nuclear LDH, but not of cytoplasmic LDH, was more negative when sodium orthovanadate was present to inhibit phosphotyrosine phosphatase activity. Similarly, the charge of MPT1 nuclear LDH was more negative when sodium orthovanadate was present (Fig. 4B), indicating that essentially all of the nuclear LDH of MPT1 cells contains phosphotyrosine. To date, we have not obtained sufficient nuclear LDH from the MPT1 variant to study its phosphorylation directly.

When nuclei were isolated and disrupted in the presence of sodium orthovanadate and 1 mM EDTA, the nuclear LDH had the more negative charge of phosphotyrosine-containing LDH (Fig. 5A). This result indicates that tyrosine phosphorylation of LDH did not occur in vitro after the nuclei were prepared, since the EDTA would inhibit any protein tyrosine kinase activity.

The charge of phosphotyrosine-containing nuclear LDH reverted to that of phosphotyrosine-free LDH after incubation with acid phosphatase (Fig. 5A). Thus, the LDH charge shift observed when sodium orthovanadate is not present during enzyme extraction can be attributed to a dephosphor-

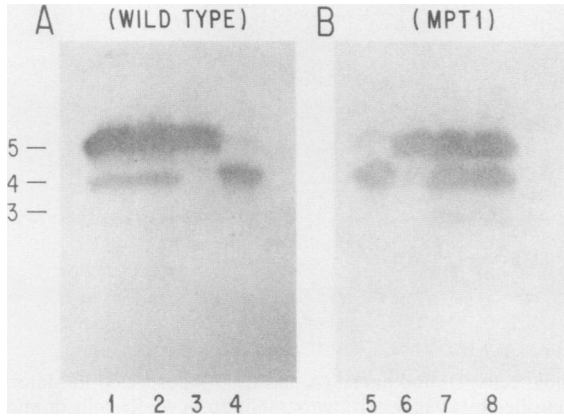

FIG. 4. Effect of orthovanadate on the charge of cytoplasmic and nuclear LDH from wild-type (A) and MPT1 (B) cells. Protein was extracted from whole cells (lanes 1, 2, 7, and 8) or isolated nuclei (lanes 3 to 6) in the presence (lanes 2, 4, 5, and 7) or absence (lanes 1, 3, 6, and 8) of orthovanadate. The protein was subjected to electrophoresis on cellulose acetate strips, which were then stained for LDH activity. The identification of the LDH isoenzymes is indicated to the left. The anode is at the bottom.

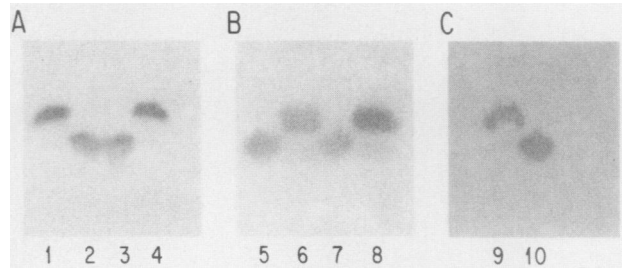

FIG. 5. Effect of isolation conditions on the charge of nuclear LDH. Protein extracts (lanes 1, 2, and 5 to 10) or purified LDH (lanes 3 and 4) from isolated wild-type nuclei were subjected to electrophoresis on cellulose acetate strips, which were then stained for LDH activity. The anode is at the bottom. (A) Lanes: 1 and 2, nuclei were isolated and sonicated in buffer containing 100 μM sodium orthovanadate and 1 mM EDTA (lane 2) or in buffer containing no orthovanadate or EDTA (lane 1); 3 and 4, nuclei were isolated and homogenized in orthovanadate-containing buffer, and then LDH was separated from orthovanadate by passage through and elution from an affinity column as described in Materials and Methods. The purified LDH was incubated for 60 min at room temperature in 50 mM morpholineethanesulfonic acid (MES; pH 6.0)–100 mM KCl–4 μg of potato acid phosphatase per ml (lane 4) or not treated with acid phosphatase (lane 3). (B) Nuclei were isolated and/or homogenized in orthovanadate-containing buffer. Lanes: 5, orthovanadate was absent during isolation of the nuclei but present during homogenization of the nuclei; 6, orthovanadate was present during isolation of the nuclei but was removed by centrifugation before the nuclei were homogenized; 7, orthovanadate was present during both the isolation and homogenization of the nuclei; 8, the nuclei were both isolated and homogenized in orthovanadate-free buffer. (C) PC12 cells from 18 plates were homogenized in 5 ml of 10 mM Tris hydrochloride (pH 7.4)–2 mM $MgCl_2$ in a loose-fitting Dounce homogenizer, and the homogenate was centrifuged for 5 min in a tabletop clinical centrifuge. The pellet was suspended in 11 ml of 2.2 M sucrose and centrifuged in a Beckman SW41 rotor at 60,000 × *g* for 60 min. The pellet was sonicated in buffer containing sodium orthovanadate (lane 10) or no vanadate (lane 9).

ylation reaction. For this experiment we isolated nuclei and extracted the LDH in the presence of orthovanadate, removed the orthovanadate by passage of the enzyme through an affinity column as described in Materials and Methods, and incubated the orthovanadate-free LDH with acid phosphatase. The fact that the mobility of the phosphatase-treated LDH was the same as that of LDH lacking only phosphotyrosine indicates that dephosphorylation of phosphoserine did not contribute to the shift in charge of the treated LDH. Phosphoserine in some proteins is a poor phosphatase substrate (27), so there may have been little dephosphorylation of the LDH phosphoserine. Furthermore, the bulk of the LDH may not even be phosphorylated at serine residues; Cooper et al. (6, 7) found only a low level of phosphoserine in LDH from chicken fibroblasts. Since phosphotyrosine is more acid labile than phosphoserine (24), Fig. 3A may not accurately reflect the relative amounts of phosphoserine and phosphotyrosine in nuclear LDH.

The preparation of nuclear LDH involved two steps: isolation of nuclei followed by their disruption with extraction of nuclear proteins. We examined whether the orthovanadate-inhibitable dephosphorylation of tyrosine (Fig. 2 to 4) occurs in isolated nuclei or subsequent to disruption of the nuclei. The results are shown in Fig. 5B. For this study we made protein extracts from wild-type PC12 nuclei treated in one of four ways. In one case the nuclei were isolated in the absence of orthovanadate but disrupted (homogenized) in orthovanadate-containing buffer (lane 5). In a second case the nuclei were isolated in the presence of orthovanadate but homogenized in orthovanadate-free buffer (lane 6). As controls, nuclei were both isolated and homogenized either in orthovanadate-containing buffer (lane 7) or in orthovanadate-free buffer (lane 8). It was not necessary to have orthovanadate prior to homogenization of the nuclei in order to preserve the more negative charge of nuclear LDH (Fig. 5B). We conclude, therefore, that tyrosine dephosphorylation occurred only after the nuclei were homogenized. A similar result was obtained with nuclei from MPT1 cells (data not shown).

The results of Fig. 5B also indicate that phosphotyrosine-containing LDH is present in a membrane-enclosed compartment, since it was resistant to phosphatase activity until this compartment was disrupted by homogenization. We performed one additional procedure to ensure that this compartment was indeed the nucleus. Nuclei prepared from several animal tissues can be separated from contaminating microsomes by sedimentation of the nuclei through 2.2 M sucrose (27). PC12 nuclei prepared by treatment of PC12 cells with Nonidet P-40, as described in Materials and Methods, did not sediment through 2.2 M sucrose. (None of the material, including the DNA-containing organelles, was more dense than 2.2 M sucrose.) Apparently, the Nonidet P-40 treatment removed some nuclear material that increases the density of the nuclei. However, nuclei prepared by homogenization of PC12 cells in 10 mM Tris (pH 7.4)–2 mM $MgCl_2$ in a loose-fitting Dounce homogenizer did sediment in 2.2 M sucrose. The material in the resultant pellet contained LDH-5, and the specific LDH activity (0.5 U/mg of protein) was comparable to that of the preparation described in Table 1. When the material that sedimented in 2.2 M sucrose was disrupted (sonicated) in orthovanadate-containing buffer, the LDH had the same migration as phosphotyrosine-containing LDH on cellulose acetate strip electrophoresis (Fig. 5C).

It should be noted that the migration pattern of Fig. 1 was obtained with LDH isolated in the absence of orthovanadate, so all differences in mobility were due only to differences in isoenzyme composition. The results shown in Fig. 4

or 5 for nuclear LDH were obtained with the same LDH isoenzyme, LDH-5, but under different conditions of isolation or treatment, so mobility differences can be attributed to differences in phosphotyrosine content.

DISCUSSION

We have found that only 1% of the total LDH activity of PC12 cells is located in nuclei isolated from these cells. This percentage is substantially lower than that reported in early studies of the subcellular location of LDH (17, 18, 21, 26). Perhaps most of the LDH activity associated with nuclei in the early studies resulted form cytoplasmic contamination. It is also possible that the detergent treatment we used resulted in significant loss of LDH from the nuclei, but we doubt that this is the case. Cattaneo et al. (5) used immunofluorescence microscopy to locate LDH-5 in nuclei of PC12 and other cells. Treatment of these cells with 0.1% Nonidet P-40 did not result in significant loss of LDH from the nuclei. Furthermore, these studies also showed that 75% of the nuclear LDH was associated with chromatin and apparently not free to exit isolated nuclei readily (5).

We have shown that the LDH of wild-type PC12 cells, which are cancer cells, contain phosphotyrosine and that the phosphotyrosine-containing LDH is restricted in location to the nucleus, although 99% of the total LDH activity is located in the cytoplasm. Furthermore, under purification conditions that have been shown to inhibit phosphotyrosine phosphatase activity, nuclear LDH had a more acid isoelectric point and a more negative charge than when the phosphatase activity was not inhibited (Fig. 2B and 4). These results indicate that essentially all of the nuclear LDH in PC12 cells has phosphotyrosine. The compartmentalization (in nuclei) of phosphotyrosine-containing LDH suggests the possibility that other proteins phosphorylated at tyrosine residues are also concentrated in some cell compartment. Thus, the finding of a low stoichiometry of tyrosine phosphorylation does not necessarily exclude a physiological role of this phosphorylation in the protein activity. The roles of nuclear LDH and its phosphorylation remain to be determined.

Cooper et al. (7) reported that only 1% of LDH-5 from chicken fibroblasts contained phosphotyrosine after transformation by Rous sarcoma virus. Our results are consistent with their finding if all of the phosphotyrosine-containing LDH were located in the nuclei of the fibroblasts and all of the fibroblast nuclear LDH contained phosphotyrosine. The fibroblasts studied did not distinguish between nuclear and cytoplasmic LDH.

It is of interest that the LDH of the MPT1 variant contains phosphotyrosine, even though this variant in culture appears to have lost some of the transformed phenotype of wild-type PC12 cells and the LDH isoenzyme composition of the variant is more typical of noncancer cells (11). Nuclear LDH from MPT1 cells contains LDH-5 and a minor species that is more negatively charged (Fig. 1). We do not know the basis of this minor species, but its presence in nuclei prepared from MPT1 cells is a repeatable finding. It may result from contamination by cytoplasm or from the effect of some phosphotyrosine-containing LDH that escaped phosphatase activity, even though phosphatase inhibitors were not used in this experiment.

MPT1 cells also differ from wild-type PC12 cells with respect to their lactate metabolism. The MPT1 cells do not exhibit as much of an increase in lactate production as do wild-type cells when oxidative phosphorylation is inhibited (8). This may be attributed at least in part to the different composition of LDH isoenzymes present in MPT1 cells, which have a greater proportion of LDH B-containing isoenzymes than do wild-type cells (Fig. 1). Because of its kinetic properties, LDH B is less efficient than LDH A in catalyzing the reduction of pyruvate to lactate (10).

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