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# The Molecular Basis of the Differing Kinetic Behavior of the Two Low Molecular Mass Phosphotyrosine Protein Phosphatase Isoforms*

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**The low molecular mass phosphotyrosine protein phosphatase is a cytosolic enzyme of 18 kDa. Mammalian species contain a single gene that codifies for two distinct isoenzymes; they are produced through alternative splicing and thus differ only in the sequence from residue 40 to residue 73. Isoenzymes differ also in substrate specificity and in the sensitivity to activity modulators. In our study, we mutated a number of residues included in the alternative 40–73 sequence by substituting the residues present in the type 2 isoenzyme with those present in type 1 and subsequently examined the kinetic properties of the purified mutated proteins. The results enabled us to identify the molecular site that determines the kinetic characteristics of each isoform; the residue in position 50 plays the main role in the determination of substrate specificity, while the residues in both positions 49 and 50 are involved in the strong activation of the type 2 low $M_r$ phosphotyrosine protein phosphatase isoenzyme by purine compounds such as guanosine and cGMP. The sequence 49–50 is included in a loop whose N terminus is linked to the $\beta$2-strand and whose C terminus is linked to the $\alpha$2-helix; this loop is very near the active site pocket. Our findings suggest that this loop is involved both in the regulation of the enzyme activity and in the determination of the substrate specificity of the two low $M_r$ phosphotyrosine protein phosphatase isoenzymes.**

Protein tyrosine phosphorylation is involved in the regulation of several biological processes that include cell growth and transformation (1–3). In fact, several protein-tyrosine kinases are components of growth-signaling pathways, and a number of viral oncogene products have unregulated protein-tyrosine kinase activities that cause cell transformation (4, 5). Extracellular signals, such as those elicited by growth factors and hormones, cause the enhancement of the protein-tyrosine kinase activities of the receptor proteins, and this leads to increased phosphorylation levels in the proteins involved in cell activation pathways.

The role of protein-tyrosine phosphatases (PTPs)[1] is currently under discussion; most of them have the potential to return activated systems to the ground state, since their activities greatly exceed those of protein-tyrosine kinases. Furthermore, a number of PTPs are regulated enzymes. In fact, some PTPs (such as the leukocyte common antigen CD45) share membrane receptor-like structures, while others (such as the SH-PTP2), which contain SH2-domains, are recruited by activated systems, thus causing a change in their cellular localization (6).

The low $M_r$ phosphotyrosine protein phosphatase (LMW-PTP) is a cytosolic enzyme that is expressed in all eukaryotic organisms. Recently, Mondesert *et al*. (7) have identified a gene coding for this enzyme in *Schizosaccharomyces pombe* (7), which is able to rescue cdc25-deleted cells. The crystal structure of bovine liver LMW-PTP has been recently determined (8), and several residues involved in the catalytic mechanism have been identified (9–11). The three-dimensional structure of the LMW-PTP active site is very close to those of both human placenta PTP1B (8) and *Yersinia* PTP (12); it consists of a loop characterized by the C*XXXXX*R motif, conserved in all PTPs (11). Since there are no other sequence homologies between LMW-PTP and other PTPs, the active site structure common to all PTPs is originated through convergent evolution of different genes (12). Cysteine and arginine are included in the conserved loop, and both are essential for the catalysis (12). Arginine is involved in the binding of the substrate phosphate group, whereas cysteine thiol causes the nucleophilic attack on the substrate phosphorus, producing a thiol-phosphate covalent enzyme intermediate. The hydrolysis of this covalent intermediate is the limiting step of the catalytic process as shown in Scheme I:

$$E + RO-P \underset{k_1}{\overset{k_{-1}}{\rightleftarrows}} E \cdot RO\text{-}P \xrightarrow{k_2} \begin{matrix} E\text{-}P \\ + \\ ROH \end{matrix} \xrightarrow[H_2O]{k_3} E + P_i$$

SCHEME I

where RO-P is protein phosphotyrosine and E-P is the covalent intermediate (11).

The LMW-PTP is expressed in two molecular forms that differ only in the sequence from residue 40 to residue 73 (13, 14). These forms are produced from a single gene through an alternative splicing mechanism.

Fig. 1 shows the alignment of some LMW-PTP sequences. They are divided into two types according to the degree of homology in the 40–73 sequence. Type 1 (IF1) includes human erythrocyte PTP-fast and rat liver AcP1, whereas type 2 (IF2) includes human erythrocyte PTP-slow, rat liver AcP2, and bovine liver LMW-PTP. All members of the IF2 group are strongly activated by purine compounds, such as guanosine and cGMP, while those included in IF1 are weakly activated by

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[1] The abbreviations used are: PTP, protein-tyrosine phosphatase; LMW-PTP, low molecular weight phosphotyrosine protein phosphatase; IF1, isoform 1; IF2, isoform 2; pNPP, *p*-nitrophenyl phosphate.

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                        10        20        30        40        50        60        70        80
IF1   ACP1    Ac-AEVGSKSVLFVCLGNICRSPIAEAVFRKLVTDENVSDNWRIDSAATSTYEVGNPPDYRGQNCMKKHGIHMQHIARQITRED
      Bfast   Ac---QAT--------------------------Q-I-E---V------G--I---------S---R---P-S-V-----K--

IF2   ACP2    Ac----------------------------------------A---S-V-DWN--R---P-AV--LRN---STA-K--------
      Bslow   Ac---QAT--------------------------Q-I-E--V---G-V-DWN--RS--P-AVS-LRN----TA-K-----K--
      LMW-PTP Ac---QVT--------------------------Q-I----V---G-V-DWN--RS--P-AVS-LRN---NTA-K---V-K--

                      90        100       110       120       130       140       150
IF1   ACP1    FATFDYILCMDESNLRDLNRKSNQVKNCKAKIELLGSYDPQKQLIIEDPYYGNDSDFEVVYQQCLRCCKAFLEKTH
      Bfast   --------------------------T-------------------------------T-----V---R-----A-

IF2   ACP2    ------------------------------------------------------------------------------
      Bslow   --------------------------T-------------------------------T-----V---R-----A-
      LMW-PTP -V------------------------R-----------------------A---T-----V---R-----VR
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FIG. 1. **Sequence alignment of the two isoforms of LMW-PTP.** *AcP1* and *AcP2* are from rat liver (13), *Bslow* and *Bfast* are from human erythrocytes (14, 30), and *LMW-PTP* is from bovine liver (31).

these substances. Other purine compounds such as GMP, GDP, GTP, adenosine, cAMP, and ATP and pyrimidine derivatives show no or weak activation abilities on IF1 and IF2 (15).

This paper deals with the identification of the IF2 molecular site involved in the strong cGMP activation. IF1 does not contain this site, and thus it is not able to bind cGMP as we have previously demonstrated (15). We used site-directed mutagenesis of a number of residues included in the isoenzyme alternative sequence (residues 40–73) to reveal the molecular basis of the kinetic differences between the two LMW-PTP isoenzymes. Our findings demonstrate that the molecular site which determines purine compound activation is also involved in the determination of the different $k_{cat}$ and substrate specificity of IF1 and IF2. In fact, the mutation of Asn-50 (contained in IF2) into Glu (contained in IF1) is sufficient to convert the kinetic constants of IF2 to those of IF1.

## EXPERIMENTAL PROCEDURES

*Materials*—The pGEX-KT vector was a gift from J. E. Dixon. *Escherichia coli* DH5 alpha strain (code 18265–017, from Life Technologies, Inc.) was used for mutagenesis, and *E. coli* TB1 strain (New England Biolabs) was used for the expression of recombinant fusion proteins. Restriction enzymes, T4 DNA ligase, and *Taq* DNA polymerase were obtained from Promega. Sequenase was obtained from the U. S. Biochemical Co. [$\alpha$-$^{32}$P]ATP (3000 Ci/mmol) were obtained from DuPont NEN.

Oligonucleotides and the unique restriction site elimination mutagenesis kit were obtained from Pharmacia Biotech Inc. The BCA protein assay reagent was from Pierce. Glutathione, glutathione-linked agarose beads, L-phosphotyrosine, pNPP, cGMP, and guanosine were from Sigma. All other reagents were of analytical grade or the best commercially available.

*Enzyme Assay and Determination of the Kinetic Parameters*—The activities on pNPP and L-phosphotyrosine were determined as follows. The substrates were dissolved in 1 ml of 0.1 M acetate buffer, pH 5.5, containing 1 mM EDTA, and the reaction (performed at 37 °C) was started by adding an appropriate volume of enzyme solution. The reaction was stopped by adding 1 ml of 1 M NaOH. The produced phenols were measured spectrophotometrically using the extinction coefficients $\epsilon_{400} = 18{,}000\ M^{-1}\ cm^{-1}$ for *p*-nitrophenol and $\epsilon_{293.5} = 2{,}330\ M^{-1}\ cm^{-1}$ for tyrosine in alkaline solution. $K_m$ and $V_{max}$ were determined by measuring the initial substrate hydrolysis rate in duplicate and then by plotting it *versus* the initial substrate concentration using a non-linear fitting of the Michaelis equation (Fig.P program from Biosoft). The activation of the enzyme at saturating cGMP concentration was determined according to Ref. 15.

*Oligonucleotide-directed Mutagenesis*—The unique restriction site elimination method for site-directed mutagenesis (16) was done with a unique restriction site kit following the manufacturer's instructions with minor modifications and was performed directly on the pGEX-KTPTP construct. For this purpose, bovine liver LMW-PTP (an IF2-isoenzyme) cDNA was cloned in pGEX-KT (17) expression vector as described previously (18). The pGEX-KT-PTP vector was used as a template for the unique restriction site elimination oligonucleotide-directed mutagenesis reaction (16).

We substituted the amino acids in positions 48, 49, 50, 53, 64, and 73 of the IF2 family members using six different 25 base-long synthetic oligonucleotides for introducing single mutations, together with the *Apa*I restriction site-eliminating oligonucleotide. The changed codons were GAC to AAC (D48T), TGG to TAT (W49Y), AAC to GAG (N50E), CGC to AAC (R53N), CGC to AAG (R64K), and AAG to ATC (K73I). The mutated genes were completely resequenced using the Sanger method to make sure that the desired single mutation occurred.

*Expression and Purification of Recombinant and Mutated Enzymes*—10-ml overnight cultures were added to 1 liter of LB broth containing 100 mg/liter ampicillin. The culture was incubated at 37 °C under continuous shaking until it reached an optical density of 0.4 at 600 nm. Isopropyl-1-thio-$\beta$-D-galactopyranoside was added to a final concentration of 0.3 mM, and the culture was grown for 2 h. The fusion proteins, whose expression levels and solubility were not essentially different from those of non-mutated fusion enzyme, were purified by a single step affinity chromatography using a glutathione-linked agarose resin, which was equilibrated with a 20 mM 3,3-dimethylglutarate buffer, pH 7.0, containing 200 mM NaCl, 10 mM 2-mercaptoethanol, and 1 mM EDTA. After application of the cell extract to the column, the resin was washed with the equilibrating buffer and then with a 50 mM Tris/HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol and 1 mM EDTA to remove unbound protein. The elution was performed using the latter buffer containing 5 mM reduced glutathione. A single protein peak that contained the fusion enzyme was obtained. The purified non-mutated and mutated fusion proteins were analyzed by SDS-polyacrylamide gel electrophoresis according to Laemmli (19) to check their purity. Each sample gave a single 44-kDa band, as expected (Fig. 2).

Protein concentration was determined by the Pierce BCA assay method.

## RESULTS

We have previously demonstrated that the kinetic properties of the LMW-PTP IF1 differ from those of LMW-PTP IF2. In fact, they have different $k_{cat}$ and $K_m$ values relative to a number of substrates, which include pNPP, L-phosphotyrosine, and synthetic Tyr-phosphorylated peptides. The sequences of these synthetic peptides were identical to those present in the phosphorylation sites of a number of natural proteins, such as c-Src, epidermal growth factor receptor, insulin receptor, $\beta$-platelet-derived growth factor receptor, and human erythrocyte membrane band 3 (20). Furthermore, IF2 is strongly activated by purine derivatives, such as guanosine and cGMP, while the activity of IF1 is only slightly changed. We have previously studied the mechanism of cGMP activation and found that it causes the increase of the $k_3$ kinetic constant value (see Scheme I), which is the rate-limiting step constant in the catalytic process (15). In the present study, we determined the main kinetic constants ($k_{cat}$ and $K_m$) of non-mutated and mutated fusion enzymes using two different substrates, pNPP and L-phosphotyrosine (Table I). Since we found that glutathione *S*-transferase, which is linked to the N terminus of LMW-PTP

through a short peptide linker, does not modify the kinetic properties of the natural enzyme, we used the fusion enzymes directly. Previously, we have used the same strategy to obtain very important information about the residues that are directly involved in the catalytic mechanism of LMW-PTP (9, 11).

Our findings indicate appreciable differences in the $K_m$ and $k_{cat}$ values for both substrates; in fact, IF1 shows an affinity for L-phosphotyrosine almost one order of magnitude higher than that of IF2. The IF2 $k_{cat}$ value relative to pNPP is two times higher than that of IF1, whereas the IF2 $k_{cat}$ value relative to L-phosphotyrosine differs very little from that of IF1. The $k_{cat}/K_m$ specificity constant value of IF1 acting on L-phosphotyrosine is also 1 order of magnitude higher than that of IF2, while in the case of pNPP the $k_{cat}/K_m$ value of IF1 is only 40% higher than that of IF2.

Furthermore, IF1 differs greatly from IF2 in the extent of activation by cGMP. In fact, IF2 shows a 9-fold increase of $V_{max}$ at cGMP saturating concentration, while IF1 is only weakly activated.

The mutation of IF2 in two different residues (K73I and D48T) hardly affected the kinetic characteristics of this isoform. In contrast, the IF2-R53N and IF2-R64K mutants present greater variations in $k_{cat}$, $K_m$, and $k_{cat}/K_m$ values with respect to both substrates. However, the observed kinetic constant variations of these mutated IF2 family members do not bring them closer to those of IF1, suggesting that these residues are not involved in the determination of different isoenzyme kinetic behavior. On the other hand, the IF2-R53N and IF2-R64K mutants maintain cGMP activations similar to that of IF2.

The IF2-W49Y mutant displays kinetic constant values close to those of the original isoform, whereas it shows decreased activation by cGMP with respect to IF2. The IF2-N50E mutant shows kinetic constants relative to pNPP and L-phosphotyrosine comparable to those of IF1; in addition, this mutant loses its sensitivity to cGMP activation. Thus, the replacement of Asn-50 (conserved in all IF2 members) with Glu (conserved in all IF1 members) transforms all the kinetic properties of IF2 to those of IF1. This residue seems to be the main molecular site determining the kinetic differences between the two LMW-PTP isoenzymes. Furthermore, our study has found that the residue in position 49 is also partially involved in IF2 activation by purine compounds. To better clarify the role of the residue 49 of LMW-PTP, we determined the cGMP/guanosine activation ratio, since guanosine is also able to activate IF2 (15) but differs from cGMP in its phosphate moiety, which is linked to the 3′ and 5′ positions of the nucleoside ribose. The results are shown in Table II. It can be seen that non-mutated IF2 as well as all IF2 mutants except N50E have very similar cGMP/guanosine ratios, while IF2-W49Y, although it has the same ratio, has reduced cGMP activation power. The cGMP/guanosine activation ratio was considered as a parameter to evaluate the type of interaction a residue has with the activator molecule. cGMP activates IF2 by interacting with both the guanosine ring and the cyclic phosphate groups, as indicated by cGMP and guanosine activation values. The results shown in Table II indicate that the residue in position 49 is involved in the interaction with the guanosine ring and not with the cyclic phosphate moiety of the molecule.

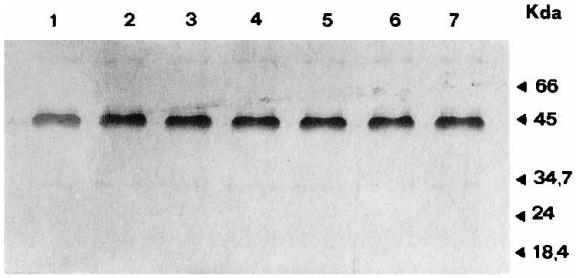


FIG. 2. **SDS-polyacrylamide gel electrophoresis analyses of purified non-mutated and mutated LMW-PTP fusion proteins.** *Lane 1*, non-mutated enzyme. The mutated fusion proteins are as follows: *lane 2*, IF2-D48T; *lane 3*, IF2-W49Y; *lane 4*, IF2-N50E; *lane 5*, IF2-R53N; *lane 6*, IF2-R64K; *lane 7*, IF2-R73I.

## DISCUSSION

LMW-PTP isoenzymes are expressed at the same time and at comparable levels in several mammalian tissues (21). This suggests that each isoenzyme serves a different function or that they are differently regulated.

Gene duplication and genetic drift (22) are known to be molecular mechanisms leading to the formation of several isoenzymatic forms. This phenomenon can involve the whole gene copy (23–25) or only a part of it, leading, in the latter case, to the formation of alternative exons (26–29); in both cases, two copies of the same protein are produced. Initially, these two different exons may have the same function, but later genetic events, such as point mutations and deletion or insertion of bases, may lead to a differentiating process. For example, two isoenzymes that have the same enzymatic activity may assume different kinetic characteristics as well as a different substrate specificity. The selective advantage of gene duplication consists essentially in the release of the additional copy from selective pressure, which continues to act on the functional copy. This event can lead to the evolution of functionally different enzymes (22). Gene duplication can have as consequences: 1) enhanced level of the gene product if the copies of the gene do not diverge; 2) the production of different isoenzymatic forms if divergency is limited, so that the same substrate specificity is maintained; and 3) the creation of a new protein function if divergency is very high.

Previously, we have demonstrated that the two isoforms of LMW-PTP differ mainly in their catalytic properties acting on

TABLE I

*Kinetic constant and cGMP activation of non-mutated and mutated LMW-PTP isoforms*

Values are means of at least three independent determinations. Standard errors are indicated. cGMP activation is expressed as ratios between the apparent $V_{max}$ values determined in the presence and in the absence of the activator.

| | *p*-Nitrophenyl phosphate | | | L-Phosphotyrosine | | | Maximum cGMP activation |
|---|---|---|---|---|---|---|---|
| | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | |
| | $s^{-1}$ | mM | $s^{-1}$ mM$^{-1}$ | $s^{-1}$ | mM | $s^{-1}$ mM$^{-1}$ | |
| IF1 | 15.0 ± 0.5 | 0.07 ± 0.01 | 214 | 14.6 ± 0.6 | 0.61 ± 0.04 | 24.3 | 1.3 |
| IF2 | 29.2 ± 1.1 | 0.19 ± 0.01 | 154 | 11.9 ± 0.5 | 5.82 ± 0.10 | 2.0 | 9.0 |
| IF2-D48T | 26.1 ± 2.0 | 0.21 ± 0.01 | 124 | 13.8 ± 0.4 | 3.95 ± 0.20 | 3.4 | 9.4 |
| IF2-W49Y | 25.0 ± 1.5 | 0.22 ± 0.02 | 114 | 14.1 ± 0.3 | 3.91 ± 0.25 | 3.6 | 5.6 |
| IF2-N50E | 20.4 ± 1.1 | 0.10 ± 0.01 | 204 | 15.1 ± 0.5 | 0.71 ± 0.06 | 21.6 | 1.0 |
| IF2-R53N | 11.5 ± 0.9 | 1.10 ± 0.09 | 10.4 | 6.1 ± 0.2 | 22.51 ± 0.92 | 0.3 | 8.9 |
| IF2-R64K | 19.8 ± 0.7 | 0.54 ± 0.04 | 36.7 | 7.0 ± 0.2 | 9.12 ± 0.41 | 0.8 | 9.4 |
| IF2-K73I | 28.3 ± 0.9 | 0.23 ± 0.02 | 123 | 13.6 ± 0.6 | 5.21 ± 0.22 | 2.6 | 9.4 |

TABLE II
*Relative activation values of LMW-PTP isoforms and IF2-mutants by cGMP and guanosine*

| | cGMP 0.8 mM | Guanosine 0.8 mM | cGMP/guanosine |
|---|---|---|---|
| IF1 | 1.0 | 1.0 | 1.0 |
| IF2 | 3.30 | 2.40 | 1.37 |
| IF2-D48T | 2.95 | 2.20 | 1.34 |
| IF2-W49Y | 1.80 | 1.33 | 1.35 |
| IF2-N50E | 1.0 | 1.0 | 1.0 |
| IF2-R53N | 3.20 | 2.42 | 1.32 |
| IF2-R64K | 3.72 | 2.90 | 1.28 |
| IF2-R73I | 3.10 | 2.30 | 1.35 |

Tyr-phosphorylated peptides (20) and on other synthetic substrates. Furthermore, IF2 shows a great increase in $k_{cat}$ in the presence of cGMP as compared to IF1. The structural basis of these differences is certainly due to the 40–73 sequence, since the isoenzymes differ only in this part of the enzyme molecule.

The three-dimensional structure of bovine LMW-PTP, which is included in the IF2 group, was recently determined by x-ray crystallography (8). It consists of mixed $\alpha$-$\beta$ secondary structures that are folded in a compact globular structure through a number of loops. One of these loops (residues 13–17) links the $\beta$1-strand to the $\alpha$1-helix, creating a surface cavity that, together with Cys-12 and Arg-18, forms the phosphate binding cradle. Furthermore, if we consider the alternative sequences of LMW-PTP isoenzymes, we see that the 46–55 polypeptide stretch forms a loop near the phosphate binding cradle; the same loop, which links the $\beta$2-strand with the $\alpha$2-helix, brings the Trp-49 and Asn-50 side chains very near the active site residues (8). We also see that the 40–45 sequence is included in the $\beta$2-strand structure, the 56–66 sequence is structured in the $\alpha$2-helix, and the 67–73 sequence belongs to another loop that links the $\alpha$2-helix to the $\alpha$3-helix (8).

We hypothesized that site-directed mutagenesis of the 40–73 sequence in the sites where the different isoforms have different residues could help in understanding the molecular basis of their different kinetic behavior. Particularly, we noted that residues 49 and 50, which are the nearest to the active site pocket, are isoenzyme specific. Thus, we planned to perform a series of single mutations of a gene coding for an IF2 isoenzyme (the bovine liver LMW-PTP) to replace some residues (49 and 50, but also others included in the 40–73 alternative sequence) with those present in the IF1. Our aim was to find the residues that determine the different kinetic properties of the two LMW-PTP isoenzymes. The findings reported in Table I clearly demonstrate that we achieved our objective, since we found that a single residue, *i.e.* the one in position 50, plays a crucial regulatory role. In this sequence position, a conserved Glu is present in the known members of IF1, while Asn is conserved in those of IF2 isoenzymes. It appears that the residue in position 49 also has some influence in cGMP activation. Nevertheless, it can be noted that the mutation of IF2-Asn-50 to Glu is sufficient to give this mutant all the kinetic properties peculiar to IF1. Both residues are located very near the surface cavity where the substrate is linked and then hydrolyzed.

The kinetic analysis of the additional enzymes, mutated in positions where isoenzyme-specific conserved residues are present, reveals that they have no roles in the determination of the kinetic properties that are characteristic of each isoenzyme. In fact, we found that mutant IF2-K73I and IF2-D48T have almost the same kinetic properties as non-mutated IF2. Furthermore, the R53N and R64K IF2 mutants show kinetic constants greatly different from both those of IF2 and IF1. However, these mutants are activated by cGMP to the same extent as IF2; thus, they seem to be a third phenotype that is completely different from both LMW-PTP isoenzymes. The properties of these four mutants can be explained considering that residues 73 and 64 are not included in the loop that is close to the active site; the former is located in a loop far from the active site pocket, while the latter is included in an $\alpha$-helix. On the other hand, the residues in positions 48 and 53 are included in the loop that is close to the active site pocket, but their positions are more distant from the active site than the position of residues 49 and 50.

In conclusion, this study demonstrates that the loop linking the $\beta$2-strand to the $\alpha$2-helix and localized near the active site pocket is involved in the regulation of the enzyme activity and in the determination of the substrate specificity of the two low $M_r$ PTPase isoenzymes.

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