Differential Regulation of the Human Tyrosine Hydroxylase Isoforms via Hierarchical Phosphorylation*

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Tyrosine hydroxylase (TH) \([\text{EC}1.14.16.2]\) is the rate-limiting enzyme in the biosynthesis of the catecholamines dopamine, noradrenaline, and adrenaline. In response to short term stimuli TH activity is primarily controlled by phosphorylation of serine 40. We have previously shown that phosphorylation of serine 19 in TH can indirectly activate TH via a hierarchical mechanism by increasing the rate of phosphorylation of serine 40. Here we show that phosphorylation of serine 31 in rat TH increases the rate of serine 40 phosphorylation 9-fold in vitro. Phosphorylation of serine 31 in intact bovine chromaffin cells potentiated the forskolin-induced increase in serine 40 phosphorylation and TH activity more than 2-fold. Humans are unique in that they contain four TH isoforms but to date no significant differences have been shown in the regulation of these isoforms. Phosphorylation of the human TH isoform 1 at serine 31 by extracellular signal-regulated protein kinase (ERK) also produced a 9-fold increase in the rate of phosphorylation of serine 40, whereas little effect was seen in the TH isoforms 3 and 4. ERK did not phosphorylate human TH isoform 2. The effect of serine 19 phosphorylation on serine 40 (44 in TH2) phosphorylation is stronger in TH2 than in TH1. Thus hierarchical phosphorylation provides a mechanism whereby the two major human TH isoforms (1 and 2) can be differentially regulated with only isoform 1 responding to the ERK pathway, whereas isoform 2 is more sensitive to calcium-mediated events.

Tyrosine hydroxylase (TH) is the rate-limiting enzyme in the biosynthesis of the catecholamines dopamine, noradrenaline, and adrenaline. Short term regulation of TH is accomplished by dynamic changes in the phosphorylation state of the enzyme (2, 3). Although four serine residues have been shown to be phosphorylated in TH, only three of these serine residues (Ser19, Ser31, and Ser40) are regulated in vivo (4). The most important mechanism of TH activation is phosphorylation of Ser40, which decreases the feedback inhibition by the catecholamines (5–7). Phosphorylation of dopamine bound TH at Ser40 by protein kinase A (PKA) can activate TH by up to 20-fold (5). The direct effect of Ser19 and Ser31 phosphorylation on TH activation is much more modest. Phosphorylation of Ser19 by calcium/calmodulin-dependent protein kinase (CaMKII) will only increase TH activity in the presence of the 14-3-3 protein (8–10), and this results only in a 2-fold increase in the activity. The phosphorylation of Ser31 by extracellular signal-regulated protein kinase (ERK) produces less than a 2-fold increase in TH activity, primarily by decreasing the affinity of the cofactor tetrahydrobipterin (BH4) (11–13). Phosphorylation of Ser31 in TH has also been shown to increase the stability of TH (14).

Humans have four TH protein isoforms, whereas anthropoids have two, and other mammalian species only have one (15). TH is encoded by a single gene, and the multiple isoforms are because of multiple mRNAs generated by alternative splicing of the single gene (16–18). The human TH1 (hTH1) variant is like the subunits in all other species. The other human TH isoforms hTH2, hTH3, and hTH4 have inserts that lead to the expression of proteins containing 4, 27, and 31 (4 + 27) amino acids inserted immediately N-terminal to Ser31 in the hTH1 isoform. The hTH1 and hTH2 isoforms are the most prominent forms in human tissue samples and human cell lines (15, 19, 20). Analysis of recombinant forms of the four human isoforms indicate that their steady state kinetic parameters are comparable among the non-phosphorylated forms and among the phosphorylated forms (12, 21–23). All four human TH isoforms showed the same dopamine binding characteristics as rat TH (rTH) (23).

As there is little direct effect of Ser19 and Ser31 phosphorylation on TH activity we have been exploring the possibility that phosphorylation of these sites may indirectly effect TH activation by hierarchical phosphorylation (3). That is, phosphorylation of Ser19 and Ser31 could alter the rate of Ser40 phosphorylation and therefore TH activation. We have shown that phosphorylation of Ser19 increases the rate of phosphorylation of Ser40 in TH 3-fold, whereas Ser40 phosphorylation has no effect on the rate of Ser19 phosphorylation (24). Therefore the phosphorylation of Ser19 increases the rate of phosphorylation of Ser40 in a hierarchical manner. This result has been confirmed both qualitatively and quantitatively by others using a different methodology (10). We have further shown that Ser19 phosphorylation can potentiate Ser40 phosphorylation and TH activation in intact cells (25).

In this report we have investigated whether Ser31 phosphorylation could also alter the rate of Ser40 phosphorylation. The results in this report show that Ser31 phosphorylation has a major effect on the rate of Ser40 phosphorylation and TH activation in intact cells and in vitro. These results also provide, for the first time, a mechanism by which the four human TH isoforms can be differentially regulated by hierarchical phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—Protease Inhibitor Mixture was from Roche Applied Science. Angiotensin II (AngII), HEPES, phenol red, EGTA, EDTA, and PKA were obtained from Sigma. BH4 was obtained from Dr. B. Schirck’s laboratories, Jona, Switzerland. Forskolin was obtained from Biomol. SDS-PAGE reagents were from Bio-Rad Laboratories. Molecular
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Measurement of Site-specific TH Phosphorylation, TH Activity, Ser19 Kinase Activity and the Stoichiometry of Ser31 Phosphorylation in Bovine Adrenal Chromaffin Cell Extracts—Bovine adrenal chromaffin cells (BACCs) were prepared as described (31) with modifications (32). BACCs were plated and treated as described (25) except that cells were initially treated with 100 nM AngII for 30 min rather than anisomycin. Site-specific TH phosphorylation, TH activity, and Ser19 kinase activity in BACC extracts were measured as described (25). For measurement of the stoichiometry of Ser31 phosphorylation in BACCs, recombinant rat TH was maximally phosphorylated at Ser31 by incubation with ERK and was used as a calibration standard. A stoichiometry of 0.45 was assumed for the ERK-phosphorylated TH, as this was the average stoichiometry determined in a number of experiments. The stoichiometry of Ser31 phosphorylation in untreated BACCs was determined as described for Ser19 and Ser40 phosphorylation (25).

Statistical Analysis—For multiple comparisons statistical significance was assessed by the Tukey’s test for multiple comparisons, protected by a one-way analysis of variance. Student’s t test was used for pairwise comparisons.

RESULTS

The Effect of Ser19 Phosphorylation on the Forskolin-induced Increase in Ser40 Phosphorylation and TH Activity in Intact Cells—To examine the effect of Ser31 phosphorylation on Ser40 phosphorylation in intact cells we needed to establish conditions in which BACCs could be stimulated independently to increase the level of Ser31 or Ser40 phosphorylation without increasing Ser19 phosphorylation. The data in Fig. 1A show that when BACCs were incubated with AngII for 30 min, the level of Ser31 phosphorylation increased 3–4-fold with respect to control, whereas Ser40 phosphorylation and Ser19 phosphorylation did not change compared with the control (p > 0.05). An incubation of BACCs with forskolin for 4 min increased only Ser40 phosphorylation. The stoichiometry of phosphorylation of Ser19 was measured as described under “Experimental Procedures.” A value for Ser31 stoichiometry in untreated chromaffin cells of 0.04 ± 0.003 (mean ± S.E., n = 8) was obtained. This indicates that the stoichiometry of Ser31 phosphorylation after AngII treatment would be between 0.12 and 0.16.

When the BACCs were treated first with AngII and then forskolin, the change in the levels of phosphorylation of Ser19 and Ser31 were the same as when the cells were individually treated with AngII or forskolin. This suggests that the effects of AngII and forskolin on BACCs are independent of each other. In contrast to this result, the increase in the level of Ser40 phosphorylation in cells treated with AngII plus forskolin was almost 2-fold greater than the increase in Ser31 phosphorylation in cells treated with forskolin alone. This difference was significant (p < 0.001). The effect of AngII and forskolin on TH activity is also shown in Fig. 1A. The treatment of cells with AngII produced no significant increase in TH activity when compared with the activity in untreated cells (p > 0.05). The addition of forskolin increased TH activity in BACCs 2–3-fold. In cells treated with forskolin plus AngII, the increase of TH activity over control was 2.2-fold greater than that in cells treated with forskolin alone. This difference was statistically significant (p < 0.001).

The data shown above suggest that phosphorylation of Ser31 mediated by AngII can potentiate the forskolin-induced increase in Ser40 phosphorylation. If this were true then AngII should not increase the Ser31 phosphorylation in forskolin-treated cells. The level of TH Ser31 kinase activity in extracts of cells was determined as described under “Experimental Procedures” using exogenous TH as a substrate. The cells were treated with AngII, forskolin, or both forskolin and
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AngII. The data in Fig. 1B show the results from a phosphopeptide analysis of TH under the conditions used for the Ser40 kinase assay after treatment with AngII and forskolin. The results show a single radioactive peak with an elution position similar to that obtained for the Ser(P)8 peptide in our system (33). This indicates that essentially only the Ser40 is phosphorylated. The very low stoichiometry of Ser19 and Ser31 under these conditions would mean that we would not expect any potentiation of exogenous TH Ser40 phosphorylation because of Ser19 or Ser31 phosphorylation in the Ser40 kinase assay and so would be measuring the true Ser40 kinase activity. The results in Fig. 1B show that treatment of cells with forskolin produced ~17-fold increase in Ser40 kinase activity in the cell extracts. When extracts of cells treated with AngII and forskolin were analyzed for Ser40 kinase activity, the activity was not significantly different from that in extracts of cells treated with forskolin alone (p > 0.05). Therefore the phosphorylation of Ser31 mediated by AngII must be potentiating the forskolin-induced increase in TH Ser40 phosphorylation.

Stoichiometry and Site Analysis of the Phosphorylation of rTH by ERK and PKA—The data shown above indicated that phosphorylation of Ser31 could increase the rate of phosphorylation of Ser40 and activation of TH in intact cells. To understand the mechanism of this we examined the effect of Ser31 phosphorylation on Ser40 phosphorylation and TH activation in vitro. In these experiments ERK was used to phosphorylate Ser31. To phosphorylate Ser31 in rTH, recombinant ERK was expressed in E. coli and purified as described under “Experimental Procedures.” The specificity of ERK phosphorylation of rTH was determined. rTH was maximally phosphorylated with ERK. The ERK-phosphorylated rTH was then subjected to trypsic digestion, and the resulting labeled phosphopeptides were analyzed using HPLC as described under “Experimental Procedures.” The results in Fig. 2, panel E show a major peak eluting at ~20 min and three minor peaks. Taking into account the elution position of the Ser(P)40 peptide (PKA-phosphorylated rTH; see Fig. 2, panel P) the elution position of the major peak was consistent with that previously determined for the Ser(P)31 peptide from rat TH (11). Essentially identical results were obtained with the ERK phosphorylation of dopamine-bound rTH (Fig. 2, panel DE). Previous results have shown that ERK could also phosphorylate Ser31 in rTH but only at a low level (11). More recent results suggested that ERK could phosphorylate Ser31 at a rate ~9-fold less than Ser31 (34), so it was possible that under the conditions used to maximally phosphorylate Ser31 there may have been significant phosphorylation of Ser31. The elution position of the smallest of the three minor peaks was consistent with that previously defined to be the elution position of the Ser(P)31 peptide in rTH (11). To confirm this, ERK was used to phosphorylate a S8A mutant of rTH. As can be seen in Fig. 2, panel E (S8A), the middle peak of the three minor peaks disappears in the S8A mutant, confirming that this peak is in fact the Ser(P)8 peptide. The very low level of Ser31 phosphorylation suggested that it was unlikely to have any significant impact on Ser40 phosphorylation and so wild-type rTH was used in all subsequent experiments. The nature of the two other minor peaks is unclear. Once rTH was maximally phosphorylated by ERK, the subsequent addition of PKA only resulted in phosphorylation of Ser40 in dopamine-free (Fig. 2, panel EP) or dopamine-bound (Fig. 2, panel DE) rTH.

Analysis of the Effect of Ser31 Phosphorylation on Ser40 Phosphorylation and TH Activity in rTH—We first determined whether the effect of Ser31 phosphorylation on TH in intact cells was because of Ser31 phosphorylation increasing the ability of PKA to phosphorylate Ser40 and activate dopamine-bound rTH. The results in Fig. 3A show that addition of dopamine inhibited rTH activity ~40-fold (D). Phosphorylation of Ser31 by ERK did not significantly activate dopamine-bound rTH (Fig. 3A, DE). The addition of very high levels of PKA was able to fully reactivate the dopamine-bound rTH (DPISO). The addition of a lower con-
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FIGURE 2. Site analysis of ERK and PKA phosphorylation of dopamine-free rTH and dopamine-bound rTH. TH and Ser31 rTH were phosphorylated by ERK and/or PKA and subject to site analysis as described under “Experimental Procedures.” The elution profiles of the [γ-32P]-labeled peptides are shown. E, rTH (6 μM) was phosphorylated in a buffer containing [γ-32P]ATP by ERK (0.8 μM) for 20 min at 30 °C. P, rTH (2 μM) was phosphorylated in a buffer containing [γ-32P]ATP by PKA (0.8 μM) for 20 min at 30 °C. DE, dopamine-bound rTH (4 μM) was phosphorylated in a buffer containing [γ-32P]ATP by ERK (0.8 μM) for 20 min at 30 °C. DP, dopamine-bound rTH (4 μM) was phosphorylated in a buffer containing [γ-32P]ATP by 5 units PKA for 10 min at 30 °C. EP, rTH (4 μM) was phosphorylated in a buffer containing unlabeled ATP and [γ-32P]ATP by ERK (0.8 μM) for 20 min at 30 °C. EP was then added to the reaction mix followed by 10 units PKA and the reaction incubated for a further 5 min. DEP, dopamine-bound rTH (4 μM) was phosphorylated in a buffer containing unlabeled ATP by PKA (0.8 μM) for 20 min at 30 °C. [γ-32P]ATP was then added to the reaction mixture followed by 400 units of PKA, and the reaction mixture was incubated for a further 10 min.

Prior phosphorylation of Ser31 by ERK had no effect on the ability of the lower PKA concentration to reactivate rTH (DEP is not significantly different from DP). The effect of Ser31 phosphorylation on Ser40 phosphorylation in dopamine-bound TH was examined directly (Fig. 2B). Again, prior phosphorylation of Ser31 did not alter the PKA-mediated increase in the phosphorylation of Ser40 in dopamine-bound TH (DEP is not significantly different from DP). The results show that phosphorylation of Ser31 could not increase the rate of phosphorylation of Ser40 nor increase the activation of dopamine-bound rTH by PKA.

We therefore determined whether the potentiation of TH activation by Ser31 phosphorylation in intact cells could be because of Ser31 phosphorylation increasing the ability of PKA to phosphorylate Ser40 in dopamine-free TH. As there is only a very small effect on rTH activity by Ser40 phosphorylation in dopamine-free TH and as Ser31 phosphorylation produces only a small change in rTH activity, we examined Ser40 phosphorylation directly. The effect of Ser31 phosphorylation on the initial rate of Ser40 phosphorylation by PKA in dopamine-free rTH was determined, and the results are presented in Fig. 3C. The data show that the rate of Ser40 phosphorylation by PKA for Ser31 phosphorylated rTH (Fig. 3C, EP) is 9-fold greater than that for rTH not phosphorylated at Ser31 (P). In contrast, prior phosphorylation of Ser40 did not alter the rate of phosphorylation of Ser31 by ERK (not shown), indicating that the effect of the phosphorylation of Ser31 is hierarchical.

Stoichiometry and Site Analysis of the Phosphorylation of the Human TH Isoforms by ERK and CaMKII—The sequence comparison of the human TH isoforms is shown in Fig. 4A. It can be seen that the human TH isoforms differ in sequence only around the Ser31 site in hTH1. This suggested that the human TH isoforms may differ with respect to the effect of hierarchical phosphorylation via Ser31 (or its equivalent) phosphorylation. The four human isoforms were expressed and purified as described under “Experimental Procedures.” The phosphorylation of the isoforms by ERK was examined. hTH1 was phosphorylated to a maximum stoichiometry of 0.5, whereas hTH3 and hTH4 could be phosphorylated to a maximum stoichiometry of 1.0, but there was very little phosphorylation of hTH2 by ERK (not shown). These results are consistent with that determined previously (12).

The site analysis of hTH1 phosphorylated by ERK and CaMKII is shown in Fig. 4B. The relative positions of the peaks found for the Ser31, Ser19, and Ser35 tryptic phosphopeptides are consistent with those determined previously (12). When hTH1 was phosphorylated by ERK, there was also a small peak that eluted at 20 min. This peak was also found when hTH2 was phosphorylated by ERK, which would suggest that this peak is because of the phosphorylation of a protein in the ERK preparation rather than hTH1. hTH2 contains an additional four amino acids inserted N-terminal to Ser31 in hTH1. There is no information in the literature on the elution position of the hTH2 tryptic Ser35 phosphopeptide (GQS5PR). The hTH2 tryptic Ser35 phosphopeptide was therefore synthesized, and as can be seen in Fig. 4B this peptide elutes very early under the conditions used. As expected, when hTH2 was phosphorylated by ERK the major

FIGURE 3. Analysis of the effect of Ser31 phosphorylation on Ser40 phosphorylation and TH activity in rTH. A, rTH activity was determined in vitro as described under “Experimental Procedures” after various treatments. TH, not treated; DP150, dopamine-bound rTH phosphorylated with 150 units of PKA for 20 min; DP, dopamine-bound rTH phosphorylated with 20 units of PKA for 20 min; DE, dopamine-bound rTH maximally phosphorylated with ERK; DEP, dopamine-bound rTH maximally phosphorylated with ERK and then phosphorylated with 20 units of PKA for 20 min. rTH activity is shown relative to untreated rTH, which was defined as 100%. For each condition the number of individual experiments was three. Statistical analysis, DEP was not significantly different from DP (p > 0.05). B, Ser40 phosphorylation of rTH was determined using Western blotting as described under “Experimental Procedures” after various treatments. P, dopamine-free rTH phosphorylated with 20 units of PKA for 20 min; DP, dopamine-bound rTH phosphorylated with 20 units of PKA for 20 min; DEP, dopamine-bound rTH maximally phosphorylated with ERK and then phosphorylated with 20 units of PKA for 20 min. Ser40 phosphorylation is shown relative to dopamine-free rTH phosphorylated with PKA, which was defined as 100%. For each condition the number of individual experiments was six. Statistical analysis, DEP was not significantly different from DP (p > 0.05). C, dopamine-free rTH was incubated with ERK (P) or without ERK (P) in the presence of unlabeled ATP for 25 min. [γ-32P]ATP was then added, and the incorporation of radiolabel into rTH was measured as described under “Experimental Procedures” for 3.5 min after addition of PKA. Initial rates were determined by linear regression analysis. For each condition three rate experiments were performed. The results are presented relative to the mean rate of phosphorylation of rTH by PKA (P), which was assigned a value of one. Statistical analysis, EP was significantly different from P (p < 0.001).
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FIGURE 4. Site analysis of hTH1 and hTH2 after phosphorylation by ERK or CaMKII. A, sequence comparison of human TH isoforms. Sequences of the human TH isoforms are identical except for sequences inserted between amino acid 30 and 31 in hTH1. The sequence of hTH1 around that region is shown together with the inserted sequences in hTH2, hTH3, and hTH4. Figure is adapted from Ref. 1. B, hTH1 and hTH2 were phosphorylated by ERK or CaMKII in the presence of \([\gamma-32P]ATP\) and subject to site analysis as described under “Experimental Procedures.” The elution profiles of the 32P-labeled peptides are shown. hTH2 Ser\(^{35}\) (pSer\(^{35}\)) peptide, the synthesized GOQ\(^{35}\)PR peptide run on the same HPLC column.

![Image](https://example.com/image.png)

peak found in hTH1 was no longer found (Fig. 4B, hTH2 ERK). Rather, in addition to the same small peak found in hTH1 that eluted at around 20 min, there was an additional minor peak that eluted very early. This early eluting peak was not found when hTH1 was phosphorylated by ERK, which suggests that it is derived from the phosphorylation of hTH2. This “peak” appeared to be two to three unresolved peaks. These peaks consistently eluted at least 2 min later than the synthesized hTH2 tryptic Ser\(^{35}\) phosphopeptide and so this suggests that ERK cannot phosphorylate hTH2 at Ser\(^{35}\) but may be phosphorylating another site(s) at very low levels. When hTH2 was phosphorylated by CaMKII (Fig. 4B, hTH2 CaMKII), the result was essentially the same as that for hTH1 phosphorylated by CaMKII (Fig. 4B, hTH1 CaMKII). This was surprising as it has been claimed that the Ser\(^{35}\) site in hTH2 could be phosphorylated by CaMKII (22). We could find no evidence for the phosphorylation of an hTH2 tryptic Ser\(^{35}\) phosphopeptide by CaMKII with mobility similar to that of the synthesized Ser\(^{35}\) phosphopeptide. This result is consistent with previous work where attempts to demonstrate the phosphorylation of hTH2 Ser\(^{35}\) in intact human neuroblastoma cells (prelabeled with \(^{32}P\), treated with veratridine, forskolin, or phorbol ester, immunoprecipitated with isoform-specific antibodies, trypsinized, and analyzed by HPLC with radiochemical detection) indicated, rather, that hTH2 Ser\(^{35}\) was essentially not phosphorylated under any of the conditions.3

Effect of ERK Phosphorylation on the Human TH Isoforms—We examined the effect of ERK phosphorylation on the rate of activation of the dopamine-bound human TH isoforms by PKA. The data is shown in Fig. 5A. All four isoforms showed similar levels of inhibition by dopamine. Addition of low concentrations of PKA produced partial reactivation of each isoform (Fig. 5, DP). In all four isoforms, prior phosphorylation by ERK (Fig. 5, DEP) had no effect on the PKA-mediated activation as, in each case, DEP was not significantly different from DP.

We next investigated whether Ser\(^{31}\) phosphorylation could potentiate Ser\(^{40}\) phosphorylation in dopamine-free human TH isoforms. The results in Fig. 5B show that the effect seen in hTH1 was essentially the same as that for rTH in that Ser\(^{31}\) phosphorylation by ERK increased the rate of Ser\(^{40}\) phosphorylation by PKA ~9-fold. In contrast, phosphorylation of Ser\(^{31}\) in hTH3 by ERK increased the rate of Ser\(^{40}\) phosphorylation by PKA only ~1.7-fold. In the hTH4 isoform, Ser\(^{32}\) phosphorylation by ERK did not alter the rate of Ser\(^{40}\) phosphorylation by PKA. The Ser\(^{35}\) residue in hTH2 could not be significantly phosphorylated by ERK or CaMKII and so for this isoform of TH the equivalent potentiation could not occur.

Determination of the Role of Ser\(^{40}\) Phosphorylation in the Phosphorylation of Ser\(^{40}\) in hTH1 and Ser\(^{44}\) in hTH2—When the phosphorylation of hTH1 and hTH2 was examined by site analysis we con-

3 J. W. Haycock, unpublished observations.
sistentley found that when hTH1 and hTH2 were phosphorylated by CaMKII, the ratio of Ser44 to Ser19 phosphorylation in hTH2 was higher than the ratio of Ser40 to Ser19 phosphorylation in hTH1 (see Fig. 4B). This raised the possibility that the 4-amino-acid insert in hTH2 was either directly altering the rate of phosphorylation of Ser44 or altering the effect of Ser19 phosphorylation on the rate of Ser44 phosphorylation. We therefore examined the rate of phosphorylation of Ser19, Ser40, and Ser44 in the two isoforms by CaMKII. This was done by quantitating the radioactivity incorporated into Ser19, Ser40, and Ser44 by site-analysis as described in methods. The results in Fig. 6A show that when hTH1 and hTH2 are maximally phosphorylated by the 4-amino-acid insert in hTH2, the 4-amino-acid insert in hTH2 directly effecting CaMKII phosphorylation of Ser44, we prepared mutants of hTH1 and hTH2 where Ser19 was converted to alanine so Ser19 could not be phosphorylated. We compared the results of phosphorylation of Ser44 in hTH2 was increased by 150% when compared with the rate of phosphorylation of Ser19 in hTH1. To examine whether this was because of the effect of Ser19 phosphorylation or because of the 4-amino-acid insert in hTH2 directly effecting CaMKII phosphorylation of Ser44, we prepared mutants of hTH1 and hTH2 where Ser19 was converted to alanine so Ser19 could not be phosphorylated. We compared the rates of phosphorylation by CaMKII of Ser40 in S19A hTH1 with that of Ser40 in S19A hTH2. The results in Fig. 6B show that CaMKII could phosphorylate Ser40 in hTH1 faster than Ser40 in hTH2 in the absence of Ser19 phosphorylation, but the effect was small, with there being only a 30% increase. This direct effect (30% increase) can only contribute in a minor way to the effect seen in the presence of Ser19 phosphorylation (150% increase). We therefore believe that it is the phosphorylation of Ser19 that is primarily responsible for the increased rate of Ser44 phosphorylation in hTH2. The rate of phosphorylation of Ser40 in hTH1 by PKA (which does not phosphorylate Ser19) was the same as the rate of phosphorylation of Ser40 in hTH2 by PKA (Fig. 6C). Thus the presence of the 4-amino-acid insert in hTH2 does not affect the rate at which PKA phosphorylates Ser44 compared with the rate at which PKA phosphorylates Ser40 in hTH1.

DISCUSSION

Previously we have shown that phosphorylation of Ser19 in TH can potentiate the phosphorylation of Ser40 in vitro (24) and potentiate the phosphorylation of Ser40 and lead to TH activation in situ (25). The data reported here extends this work by showing that the phosphorylation of Ser31 can also increase the rate of Ser40 phosphorylation and TH activation both in vitro and in situ. The phosphorylation of Ser40 had no effect on the rate of phosphorylation of either Ser19 or Ser31. Therefore the effect of phosphorylation of Ser19 and Ser31 is hierarchical in nature. In light of the very modest effect that phosphorylation of Ser19 or Ser31 have on TH activity we would suggest that the main role of the phosphorylation of these sites is to potentiate the rate of phosphorylation of Ser40.

Phosphorylation of Ser31 increased the rate of phosphorylation of Ser40 in dopamine-free TH but did not have any effect on the PKA activation of dopamine-bound TH. This is the same as the situation with Ser19 where Ser19 phosphorylation increased the rate of Ser40 phosphorylation in dopamine-free TH (24) but had no effect on the PKA activation of dopamine-bound TH.4 The phosphorylation of Ser40 in dopamine-free TH has very little effect on TH activity (5) and so the question arises as to how Ser31 (or Ser19) phosphorylation produces the potentiation of TH activation that we have found in intact cells. In response to a stimulus Ser40 phosphorylation in TH increases in the first 2–3 min. Clearly, phosphorylation of Ser19 or Ser31 does not effect the initial activation of catecholamine-bound TH. If there is continued stimulation the level of Ser40 phosphorylation remains relatively stable (35). This means that after the initial activation phase there is an equilibrium phase where the rate of phosphorylation of Ser40 must equal the rate of dephosphorylation of Ser40 in order that the level of phosphorylation of Ser40 in the cell remains constant. This equilibrium phase is shown diagrammatically in Fig. 7. In the absence of Ser31 phosphorylation, the level of Ser40 phosphorylation will reach a stable point when the rate of phosphorylation equals the rate of dephosphorylation (Fig. 7, dotted line). Phosphorylation of Ser31 will increase the rate of phosphorylation of

4 J. Daniel, P. Dunkley, and P. Dickson, unpublished observations.
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Ser^{31} in catecholamine-free TH. This will have the effect of altering the equilibrium to favor the phosphorylated form and so the level of Ser^{31} phosphorylation will increase. Ultimately a new equilibrium will be established but it will be at higher level of Ser^{31} phosphorylation than in the absence of Ser^{31} phosphorylation (Fig. 7, solid line). Therefore we believe that the role of Ser^{31} (and Ser^{31}) phosphorylation in catecholamine-free TH is to increase the rate of rephosphorylation of Ser^{31} in TH after it has been dephosphorylated (but before dopamine is rebound). This will have the effect of increasing Ser^{31} phosphorylation in intact cells and, in particular, reducing the possibility of the catecholamine binding to the dephosphorylated TH and reinitiating TH.

This work also provides a major insight into the way the human TH isoforms are regulated. Previous work has failed to identify any significant differences in the activity or regulation of the four human TH isoforms (12, 21–23). The fact that the only sequence difference among the four isoforms is around Ser^{31} in hTH1 suggests that the effect of Ser^{31} (or its equivalent) phosphorylation in increasing Ser^{31} (or its equivalent) phosphorylation may be different in different isoforms. This was in fact the case with the strong potentiation via Ser^{31} phosphorylation being only found in the hTH1 isoform. The other major human TH isoform hTH2 showed stronger potentiation via Ser^{19} phosphorylation than hTH1. How would these differences between the two major TH isoforms impact on the regulation of TH in cells? When cells are stimulated either via depolarization or receptor-mediated mechanisms the level of phosphorylation of Ser^{19} increases rapidly and thereafter starts to decrease (3). In contrast, the level of Ser^{31} phosphorylation only increases after extended stimulation of the cell (3). This means that in response to short term stimuli, hTH2 will be activated to a greater extent than hTH1 because of the stronger potentiation via Ser^{19} phosphorylation. When cells are stimulated for a longer period of time, the ERK pathway is activated. Activation of the ERK pathway will strongly potentiate the activation of hTH1 but will have no effect on hTH2. Therefore hTH1 and hTH2 can be differentially activated depending on the length of the stimulus involved.

Hierarchical phosphorylation via Ser^{31} may also provide a mechanism by which hTH1, or the TH in other mammalian species that are homologous to hTH1, may be differentially activated in different tissues. We have shown here that in unstimulated adrenal chromaffin cells the stoichiometry of Ser^{31} phosphorylation is very low with only 4% of TH phosphorylated at Ser^{31}. This is similar to the results from unstimulated adrenal-derived PC12 cells where only 7% of TH molecules are phosphorylated at Ser^{31} (36). In contrast, the basal level of Ser^{31} phosphorylation was much higher in the brain with as much as 32% of TH phosphorylated at Ser^{31} in the striatum (36). This would mean that in response to short term stimuli the high stoichiometry of Ser^{31} phosphorylation the striatum would lead to strong potentiation of hTH1 activation, whereas this would not occur in adrenal cells where the stoichiometry of Ser^{31} phosphorylation is very low. For many years it was thought that ERK was the only kinase that could phosphorylate Ser^{31} in TH. The recent discovery that Ser^{31} can also be phosphorylated by CDK5 (14, 37) means that this potentiation of activation of hTH1 can be modulated by at least two different pathways and so could provide the basis of quite complex regulation of hTH1.

In summary we have shown that the Ser^{31} phosphorylation can strongly potentiate Ser^{40} phosphorylation and TH activation. This has also provided for the first time a mechanism by which the different human TH isoforms are differentially regulated.

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