Stimulus-Coupled Interaction of Tyrosine Hydroxylase with 14-3-3 Proteins†

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ABSTRACT: Tyrosine hydroxylase (TH) is phosphorylated by CaM kinase II and is activated in situ in response to a variety of stimuli that increase intracellular Ca^{2+}. We report here, using baculovirus-expressed TH, that the 14-3-3 protein binds and activates the expressed TH when the enzyme is phosphorylated at Ser-19, a site of CaM kinase II-dependent phosphorylation located in the regulatory domain of TH. Site-directed mutagenesis showed that a TH mutant in which Ser-19 was substituted by Ala retained enzymatic activity at the same level as the non-mutated enzyme, but was a poor substrate for CaM kinase II and did not bind the 14-3-3 protein. Likewise, a synthetic phosphopeptide (FRRAV*p*SELDA) corresponding to the part of the TH sequence, including phosphoSer-19, inhibited the interaction between the expressed TH and 14-3-3, while the phosphopeptide (GRRQ*p*SLIED) corresponding to the site of cAMP-dependent phosphorylation (Ser-40) had little effect on complex formation. The complex was very stable with a dissociation constant of 3 nM. Furthermore, analysis of PC12nnr5 cells transfected with myc-tagged 14-3-3 showed that 14-3-3 formed a complex with endogenous TH when the cultured cells were exposed to a high K^+ concentration that increases intracellular Ca^{2+} and phosphorylation of Ser-19 in TH. These findings suggest that the 14-3-3 protein participates in the stimulus-coupled regulation of catecholamine synthesis that occurs in response to depolarization-evoked, Ca^{2+}-dependent phosphorylation of TH.

Tyrosine hydroxylase (TH)[1] [EC1.14.16.2] is a key enzyme in catecholamine biosynthesis and has a role in normal and pathological functions of many endocrine systems. Although the activity of TH is controlled by a variety of cellular mechanisms, such as regulation of enzyme expression and cofactor biosynthesis, the phosphorylation-dependent regulation of enzyme activity is believed to be of functional significance with respect to stimulus-response coupling (for reviews, see refs *1* and *2*). It has previously been shown that TH is phosphorylated at four sites in vivo and in vitro. These are at Ser-8, -19, -31, and 40, all of which are located in the regulatory domain of the enzyme (*1*, *2*). It has also been shown that Cdc/cyclin A is responsible for the phosphorylation at Ser-8, CaM kinase II at Ser-19 and -40, Erk 1 and 2 at Ser-31, and PKC and PKA at Ser-40 (*2*). Phosphorylation of TH by Erk 1 and 2 at Ser-31 and by PKA at Ser40 result in a direct increase in its enzymatic activity. However, CaM kinase II requires an additional protein factor for the activation of TH (*3*, *4*), which is now identified as the 14-3-3 protein (*5*, *6*).

The 14-3-3 protein is a family of acidic, dimeric proteins consisting of at least 8 distinct subunit isoforms, each isoform having a molecular mass of ∼30 kDa (for a review, see ref *7*). This protein family is found in many eukaryotic cells from yeast to mammals, and participates in a variety of cell signaling processes that direct cell proliferation, differentiation, and function. In yeast, this protein family has a role in checkpoint signaling for cell division, and null mutants are lethal (*7*−*9*). Recent genetic studies in *Drosophila* show that mutations in 14-3-3 genes disrupt Ras-mediated differentiation of photoreceptor R7 cells during eye development and decrease the capacity of olfactory learning and memory (for a review, see ref *10*). There are many proteins that have been

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[1] Abbreviations: TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; CaM, calmodulin; CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase type II; PKA, cAMP-dependent protein kinase; Erk: extracellular signal-regulated kinase; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; GST, glutathione *S*-transferase; W13, *N*-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide; KN-62, 1-(*N*,*O*-bis-[5-isoquinoline-sulfonyl]-*N*-methyl-L-tyrosyl)4-phenyl-piperazine: PAGE, polyacrylamide gel electrophoresis.

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identified as targets of the 14-3-3 protein family such as those involved in the Ras-MAPK signaling cascade and in an apoptosis-signaling pathway (*11*−*15*). In many cases, the capacity of the 14-3-3 protein to bind and form a complex with a target protein depends on the latter's state of phosphorylation, particularly at specific Ser residues. On the basis of these findings,it has been proposed that the 14-3-3 family is a type of chaperon or adapter that modulates the activity, conformation, stability, interaction, or intracellular localization of target proteins.

We have previously shown that the 14-3-3 protein activates tyrosine and tryptophan hydroxylases (TH and TPH) when the enzymes are phosphorylated by CaM kinase II (*5*). Subsequent studies of the TPH activation process demonstrate that 14-3-3 binds specifically to the phosphorylated form of TPH to activate the enzyme (*16*, *17*). The site of 14-3-3 involved in this interaction has been defined as the box-1 region of the protein (*16*). On the other hand, results are contradictory regarding the effect of 14-3-3 on the activity of TH, and no direct evidence has been provided for interaction between these two proteins. We show here, using a baculovirus-expressed TH: (i) that the phosphorylation of TH at Ser-19 triggers its formation of a complex with the 14-3-3 protein that leads to activation of the enzyme and (ii) that this complex is formed in PC12nnr5 cells when they are depolarized to induce Ca^{2+} influx. We propose that this is one of the mechanisms by which TH-expressing cells regulate their catecholamine levels in response to a variety of signals that increase intracellular Ca^{2+}.

EXPERIMENTAL PROCEDURES

Materials. GST-14-3-3η was generated by PCR amplification and cDNA cloning into the pGEX-3X vector as described (*16*). The expressed protein was purified with glutathione-agarose beads (*16*). CaM and CaM kinase II were purified from bovine and rat brains, respectively (*16*). Phosphopeptides were synthesized and were purified by C18 reverse-phase high performance liquid chromatography as described previously (*18*).

Plasmid Constructions. The cDNA for histidine-tagged TH was generated by PCR using the oligonucleotides (5′-AGG ATC CTT CAC AGA GCC ATG CCC AC-3′ and KS primer, 5′-TCG AGG TCG ACG GTA TC-3′) and the human TH1 cDNA (*19*). The PCR fragment was digested with *Bam*HI and *Eco*RI and the *Eco*RI site was blunted with Klenow. Then the PCR fragment was inserted into the *Bam*HI and *Hind*III sites (where the *Hind*III site was blunted) of baculovirus expression vector pBlueBacHisC (*20*). Site-directed mutagenesis was performed as described previously (*21*) using the mutagenic primer: 5′-GGG CCG TGG CTG AGC TGG AC-3′. The cDNAs for rat 14-3-3β and bovine 14-3-3η were synthesized by PCR using the oligonucleotides (5′-CCA CGC GTA TGA CCA TGG ACA AAA GT-3′ and 5′-AAG GAT CCT TAG TTC TCT CCC TCT CC-3′, for β; 5′-TCA CGC GTA TGG GGG ACC GCG AGC AG-3′ and 5′-CAG GAT CCT CAG TTG CCT TCT CCG GC-3′ for η), and the PCR fragments were inserted into the *Mlu*I and *Bam*HI sites of myc-tag cloning vector, pMUM1 (*22*). The myc-tagged 14-3-3 cDNAs were then digested with *Hind*III and *Xba*I and the *Xba*I sites were blunted with Klenow. Then the cDNAs were inserted into the *Hind*III and *Xho*I sites (where the *Xho*I site was blunted) of retroviral expression vector pLNCX5 as described (*23*).

Expression and Purification of Histidine-Tagged TH. Expression of histidine-tagged TH was conducted by infecting Sf21 cells according to the procedure described previously (*20*). After 5 days, the cells were spun down and resuspended in buffer A (50 mM Tris-HCl, 300 mM sucrose, 1 mg/mL leupeptin and pepstatin, 100 μg/mL phenylmethylsulfonyl fluoride, pH 7.5). The cells were lysed by sonication and centrifuged for 20 min at 27500*g*. Ammonium sulfate was added to 30% saturation at 4 °C; after stirring for 20 min, the precipitate was removed by centrifugation for 15 min at 12000*g*. Solid ammonium sulfate was added to the supernatant to give 42% saturation; after 20 min at 4 °C, this was centrifuged at 12000*g* and the supernatant was removed. The pellet was suspended in 20 mL of buffer A and incubated with Ni^{2+}-agarose beads (800 μL) previously equilibrated with the same buffer at 4 °C for 120 min on a rotating platform. The beads were then washed extensively with buffer A containing 20 mM imidazole and bound TH was eluted with buffer A containing 500 mM imidazole. The TH eluate was diluted about 8-fold with buffer B (20 mM Tris-HCl, 10% glycerol, 0.5 mM DTT, 0.1 mM EDTA, 1 mg/mL leupeptin and pepstatin, pH 7.5) and loaded onto a heparin-Sepharose column (0.7 × 5 cm) preequilibrated with buffer B containing 100 mM KCl. Bound TH was then eluted with buffer B containing 500 mM KCl. SDS-PAGE showed that the purified his-TH and his-S19A-TH were 83 and 80% pure, respectively.

Phosphorylation and Binding Assays. Phosphorylation of his-TH by CaM kinase II was carried out at 30 °C for 10 min in a reaction mixture (50 mM Hepes, pH 7.6, 5 mM $Mg(CH_3COO)_2$, 0.1 mM $CaCl_2$, 0.5 mM ATP, 1 μg CaM, 0.3 μg CaM kinase II, and various amounts of GST-fused proteins (see figure legends)) in a final volume of 50 μL. Phosphorylation of his-TH by PKA was performed under the above conditions except that 1 μg of the catalytic subunit of PKA and 2 mM DTT were used instead of CaM and CaM kinase II. For the quantification of phosphate content, 1 μg his-TH was phosphorylated under the above conditions in the presence of 5 μCi of [γ-^{32}P]ATP. Proteins were analyzed by SDS-PAGE followed by autoradiography, and ^{32}P incorporation was measured by scintillation counting of excised TH bands. For binding assays, glutathione-agarose beads (~60 μL) were added to the reaction mixture and incubated for 30 min at 4 °C, and the protein complexes bound to the beads were washed five times (with 20 mM Tris-HCl, 150 mM NaCl, 0.1 mM DTT, pH 7.5) and solubilized in SDS sample buffer. The bound his-TH was analyzed by SDS-PAGE followed by Western blotting using a TH polyclonal antibody (*24*). In the experiments shown in Figure 2, the enzymatic activity of bound his-TH was measured using the washed beads directly. The densities of his-TH and GST-fused 14-3-3 bands, shown in Figure 2, lane 4, were quantitated with a Fast Scan Personal Scanning Imager (Molecular Dynamics). The linearity range was established with a calibration curve.

Surface Plasmon Resonance Analysis. All studies were performed on a Biacore 2000 instrument (Biacore). GST or GST-14-3-3η was immobilized on a CM5 sensor chip via an anti-GST monoclonal antibody. For the immobilized protein, the concentration and flow rate were adjusted to yield

500−1000 response units, which were utilized for each experiment. TH binding was assayed at 25 °C at a flow rate of 20 μL/min at concentrations between 5.3 nM and 20 nM. The rates of association (k_{on}) and dissociation (k_{off}) were determined using the BIAevaluation 3.0 software program by nonlinear curve-fitting methods. Phosphopeptide binding was assayed at 25 °C at a flow rate of 5 μL/min at concentrations between 25 μM and 500 μM. The steady-state resonance (R_{eq}) was calculated at each concentration for analytes using BIAevaluation 3.0 software program. The equilibrium dissociation constant (K_D) was calculated by K_D = 1/association constant (K_a) in which K_a was determined from the plot of R_{eq}/[analyte] vs R_{eq} at different analyte concentrations by Scatchard plot analysis (*17*). K_D was also determined from the rate of k_{off}/rate of k_{on}.

Cell Culture and Depolarization Treatment. PC12nnr5 cells were grown on collagen-coated tissue culture plates in RPMI1640 medium supplemented with 10% heat-inactivated donor horse and 5% fetal bovine sera, as described previously (*25*). The PC12nnr5 cells are mutants from PC12 pheochromocytoma cells that possess low- but not high-affinity nerve growth factor-binding sites (*23*). Retroviral infections were carried out as described (*23*) by adding 0.5−1 mL of virus-containing supernatant, recovered from packaging cells (Bosc23), to 50% confluent PC12nnr5 cell cultures. Infected cells were cultured for 1 week and then subjected to selection with G418 for at least 1 month. Surviving cells were polyclonally expanded and cultured (termed PC12-14b and -14e cells). For depolarization treatment, cells were placed on 60-mm collagen-coated dishes ($3-5 \times 10^6$ cells/dish) 3 days before use. The cells were gently washed twice with 5 mL of prewarmed Krebs−Ringer−Hepes (KRH) buffer (125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM Hepes, 5.6 mM glucose, pH 7.4) and preincubated for 15−30 min at 37 °C. The cells were washed once more with prewarmed KRH buffer and then depolarized for 5 min with 5 mL of high (56 mM) KCl in KRH. During this treatment, the NaCl content was decreased to 85 mM to keep the tonicity of KRH buffer constant.

Immunoprecipitation. After stimulation, the medium was removed and the cells were immediately frozen on dry ice and scraped into 200 μL of a buffer containing 25 mM Hepes (pH 7.4), 50 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, and 1mM phenylmethylsulfonyl fluoride. The suspension was homogenized, and the homogenate was centrifuged at 20000*g* for 10 min at 4 °C. The supernatant was mixed with protein G-agarose preconjugated with the monoclonal myc antibody 9E10. Incubations were at 4 °C for 90 min on a rotating platform. Immunoprecipitants were washed six times (with 20 mM Tris-HCl, 150 mM NaCl, 0.1 mM DTT, pH 7.5) and solubilized in SDS sample buffer. Samples were analyzed by SDS-PAGE followed by Western blotting using the indicated primary antibody (see Figure 6).

Others. TH activity was assayed radiometrically by measuring the amount of [^{3}H]H_2O formed from [3,5-^{3}H]-tyrosine. The assay mixture contained 100 mM MES, pH 6.5, 0.1 mM L-[3,5-^{3}H]tyrosine (0.4 μCi), 1 mM D,L-6-methyl-5,6,7,8-tetrahydropterin, 2 mM dithiothretol, 0.5 mM ferrous ammonium sulfate, 0.16 mg catalase, and a suitable amount of the enzyme solution in a final volume of 100 μL. The reaction was carried out at 30 °C for 10 min with shaking. One unit of TH was defined as the amount that catalyzes the formation of 1 nmol of 3,4-dihydroxyphenylalanine (Dopa) per min at 30 °C. SDS-PAGE and Western blotting were carried out as described (*16*). The amounts of recombinant proteins were evaluated from the densitometric quantitation of the protein bands obtained after SDS-PAGE (Coomassie Blue staining).

RESULTS

Characterization of Baculovirus-Expressed TH and Its Activation by CaM Kinase II and 14-3-3. We used the baculovirus expression system that produces proteins in insect cells as His6-tagged fusion proteins, because it permitted affinity purification of the soluble and active TH (*26*, *27*). An His6-tagged human TH (termed his-TH) was expressed in this system, and was purified by Ni^{2+}-agarose followed by heparin-Sepharose chromatography (see Experimental Procedures). The purified his-TH was almost homogeneous on SDS-PAGE with an expected molecular mass of ~60 kDa (Figure 1A, lane 2), cross-reacted with a TH antibody (lane 4), and had a specific activity of 390 nmol/min/mg protein.

The purified his-TH was phosphorylated by CaM kinase II and PKA similarly to the brain TH (ref *28*, 0.8−0.9 mol and 0.4 mol of phosphate/mol of TH monomer, respectively, under conditions described in Experimental Procedures). In addition, the PKA-mediated phosphorylation induced a increase in the activity of this recombinant TH to about 2.5-fold (Figure 1B, left panel). In contrast, the phosphorylation by CaM kinase II was insufficient to cause activation (Figure 1B, left panel). In this case, activation was also observed, as analyzed in the presence of GST-fused 14-3-3 protein (bovine η-isoform, Figure 1C). Other 14-3-3 isoforms such as GST-fused rat β and θ (*20*) also activated this enzyme to the similar extent (data not shown). No activation occurred, however, in the absence of CaM kinase II or in the presence of GST alone (Figure 1C, dotted lines), suggesting that this activation needs both phosphorylation of the hydroxylase and 14-3-3 proteins. Thus, the mode of activation compares with that of TPH (*16*, *17*), a structural homologue that regulates serotonin synthesis.

Interaction of 14-3-3 and TH. We examined whether 14-3-3 binds directly to TH. The purified his-TH was incubated with the GST-fused η-isoform of 14-3-3 in the presence or absence of CaM kinase II, and glutathione-agarose beads were added to recover the fused protein; his-TH bound to the fused 14-3-3 was then assayed by its enzymatic activity and by Westem blot with a TH antibody. As illustrated in Figure 2 (lanes 3 and 4), his-TH bound to the fused 14-3-3 only under conditions in which it was phosphorylated by CaM kinase II. The complex was not detected with a GST moiety alone (lanes 1 and 2) or with his-TH phosphorylated by PKA (lane 5), suggesting that 14-3-3 binds directly to TH only when the enzyme is phosphorylated by CaM kinase II. From the densitometric quantitation of the TH and the fused 14-3-3 bands in lane 4 (see also Experimental Procedures), one mol of phosphorylated TH monomer appeared to bind ~0.7 mol of 14-3-3η dimer.

The direct binding of phosphorylated TH to 14-3-3 was also examined using surface plasmon resonance spectroscopy. This method detects binding in real time and allows the rates of association (k_{on}) and dissociation (k_{off}) to be

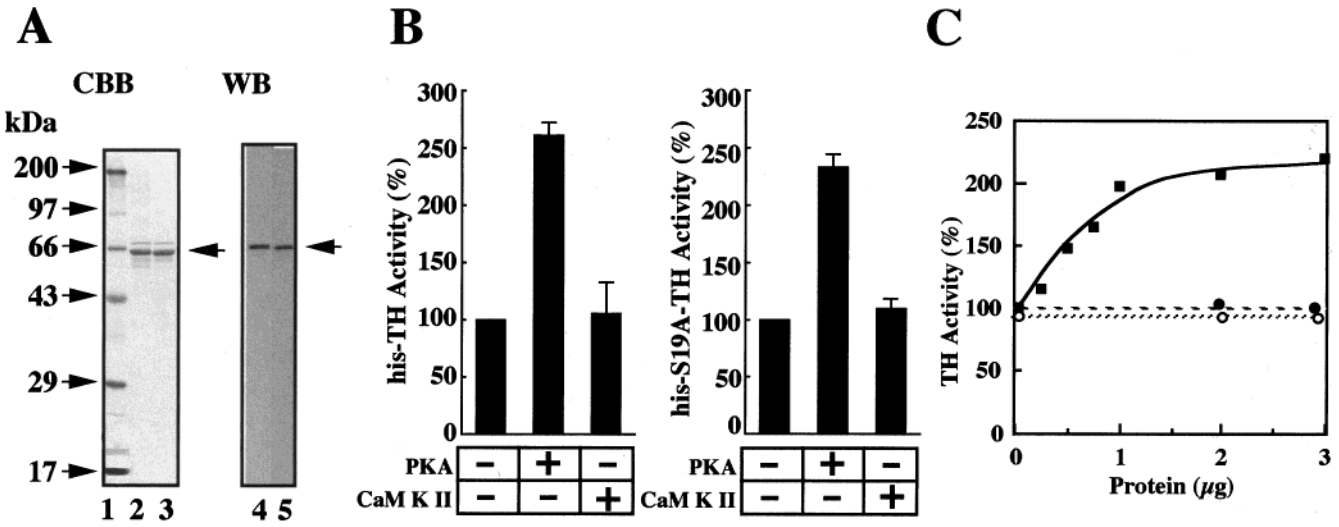

FIGURE 1: Characterization of baculovirus-expressed TH. (A) Purified his-TH and his-S19A-TH (each 1 μg) were analyzed by SDS-PAGE (Coomassie blue staining, CBB, lanes 2 and 3) and Western blotting with a TH antibody (WB, lanes 4 and 5). Molecular mass markers are shown in lane 1. Arrows indicate the position of TH. (B) Activation of TH by PKA. his-TH (left panel) and his-S19A-TH (right panel) (each 1 μg) were phosphorylated by the indicated protein kinase for 10 min and analyzed for their enzymatic activities as described under Experimental Procedures. Results are expressed as a percentage of the activity in the absence of the kinases. Vertical bars represent standard errors calculated on means of three experiments. (C) Activation of TH by 14-3-3. his-TH (1 μg) was incubated with the indicated amounts of GST-fused 14-3-3η isoform in the presence of CaM kinase II and then analyzed for its activity (■) as described under Experimental Procedures. Control experiments were performed under the same conditions without CaM kinase II (○) or with GST alone (●). The results are the means of triplicate experiments and are expressed as a percentage of the activity in the absence of the GST fused 14-3-3η.

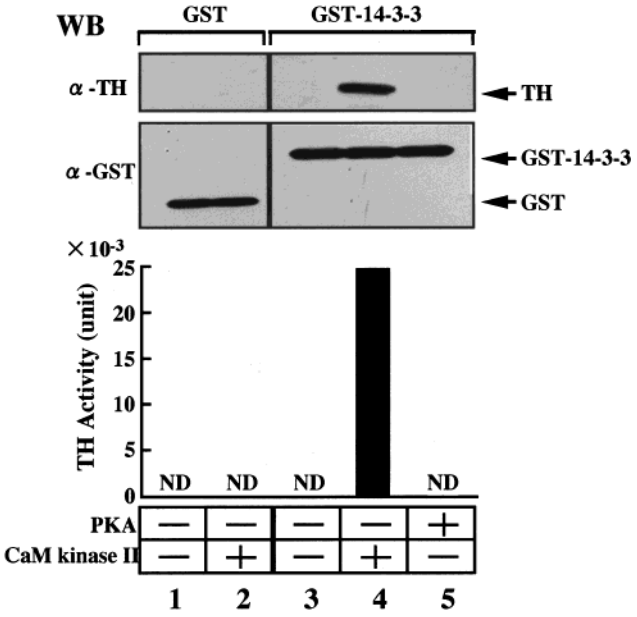

FIGURE 2: Binding of phosphorylated TH with 14-3-3. his-TH (~1 μg) was incubated for 10 min with GST alone (0.2 μg, lanes 1 and 2) or GST-fused-14-3-3η (0.2 μg, lanes 3−5) in the absence (lanes 1 and 3) or presence of CaM kinase II (lanes 2 and 4) or PKA (lane 5), and glutathione-agarose beads were added to the mixture. TH bound to the GST-fused 14-3-3 protein and immobilized on the beads was then assessed by its enzymatic activity (bottom panel) or by Western blot with a TH polyclonal antibody (top panel). The same blot was also stained with a GST monoclonal antibody to see GST and the fused 14-3-3 protein (middle panel). ND, not detected.

determined directly. GST-fused 14-3-3η was attached to the sensor surface through a GST antibody and was tested for its ability to bind his-TH. As expected, the fused 14-3-3 bound specifically the phosphorylated form of his-TH (Figure 3A). The GST-moiety alone had no affinity to the recombinant enzyme regardless of phosphorylation (data not shown). According to a quantitative analysis performed using the Biacore curve-fitting program, the 14-3-3 protein associates with the phosphorylated TH at a fast rate ($k_{on} = 5.4 \times 10^5 \pm 4 \times 10^4$ M^{-1} s^{-1}, mean ± standard error) and dissociates at a very slow rate ($k_{off} = 1.6 \times 10^{-3} \pm 2 \times 10^{-4}$ s^{-1}) at 25 °C. The equilibrium dissociation constant (K_D) calculated from k_{off}/k_{on} was 3.0 ± 0.6 nM. The association and dissociation rate constants were not influenced by the amount of immobilized 14-3-3. Figure 3B shows that the observed binding of phosphorylated TH to the immobilized 14-3-3 is saturable, and the association constant (K_a) calculated from the slope of the straight line of the corresponding Scatchard plot (inset) is 3.0×10^8 M^{-1}. The equilibrium dissociation constant (K_D) calculated from $1/K_a$ was 3.3 nM, in good agreement with the K_D value estimated as described. These results indicate that the 14-3-3/TH complex is very stable and is almost comparable to the 14-3-3/TPH (30 nM, ref *17*) and 14-3-3/ExoS (7 nM, ref *29*) complex.

Involvement of Ser-19 Phosphorylation in the TH/14-3-3 Interaction and TH Activation. Accumulating evidence suggests that 14-3-3 is a phosphoserine-binding protein (*18*, *30*). Because previous studies indicated that CaM kinase II phosphorylates TH predominantly at Ser-19, we anticipated that this might trigger the interaction between 14-3-3 and TH. To test this possibility, a point mutation was introduced in his-TH to replace Ser-19 with Ala, and the mutant TH (termed his-S19A-TH) was expressed in insect cells (Figure 1A, lane 3). The purified his-S19A-TH was stable and shared many properties in common with his-TH; e.g., reactivity to a TH antibody (Figure 1A, lane 5), a specific enzyme activity of 350 nmol/mg protein, and activation by ~2.5-fold by PKA-mediated phosphorylation (Figure 1B, right panel; 0.4 mol of phosphate/mol of S19A-TH monomer). However, unlike his-TH, his-S19A-TH was no longer bound to (Figure 4A) or activated by the 14-3-3 protein (data not shown), even

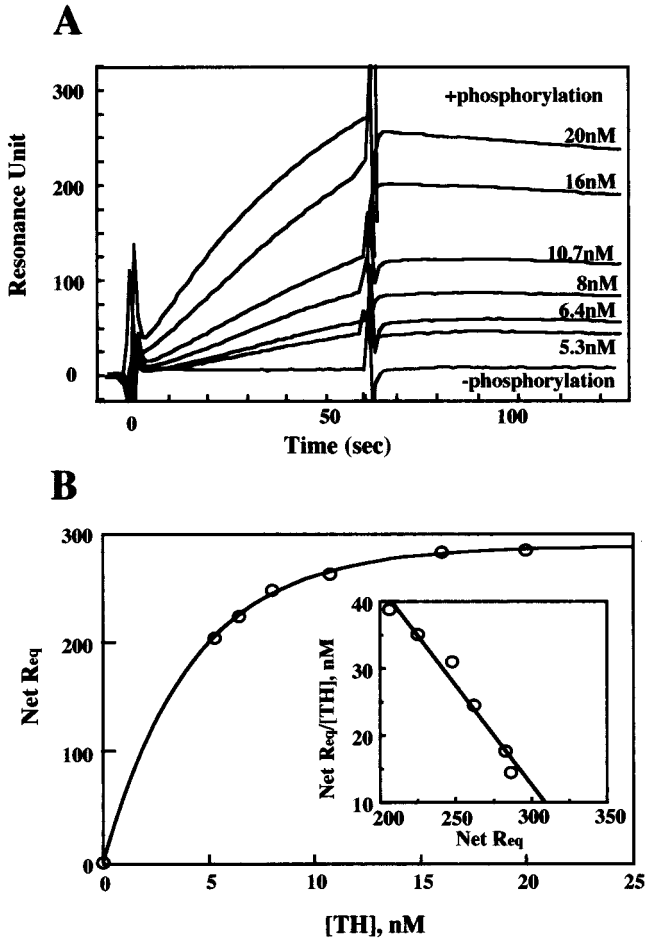

FIGURE 3: Biacore sensorgram of TH binding to 14-3-3. (A) Analysis of 14-3-3 binding to phosphorylated TH by surface plasmon resonance spectrometry. The Biacore 2000 was used to analyze the interaction of 14-3-3 with phosphorylated TH. GST-fused 14-3-3η was immobilized on a CM5 sensor chip and the surface plasmon response was utilized to measure the interaction with phosphorylated (5.3∼20 nM) or nonphosphorylated (20 nM) his-TH (flow rate, 20 μL/min). The response unit, a relative indicator of protein−protein interaction, was plotted as a function of time in seconds. (B) Affinity of the interaction of phosphorylated TH and 14-3-3. Steady-state resonance (R_{eq}) was calculated at each concentration of phosphorylated TH using BIAevaluation 3.0 software from the binding data corrected for nonspecific binding. Inset, Scatchard plot derived from the data in *B* for phosphorylated TH. The straight line was drawn by the method of least squares.

in the presence of CaM kinase II. This suggests that phosphorylation at Ser-19 triggers and is required for the interaction between 14-3-3 and TH.

In an alternative approach, two phosphopeptides were synthesized based on the TH sequence that include Ser-19 (FRRAV*p*SELDA) and Ser-40 (IGRRQ*p*SLIED), and these were examined for their effects on the 14-3-3/his-TH interaction. As shown in Figure 4B, the phosphoSer-19-containing peptide inhibited both formation of the 14-3-3/his-TH complex (upper panel, lanes 3 and 4, compare with lane 2) and activation of phosphorylated his-TH (lower panel, lanes 3 and 4), while the phosphoSer-40-containing peptide had little effect on the interaction (lanes 5 and 6). These results indicate that the Ser-19 phosphorylation is necessary and sufficient to trigger the complex formation. Further studies by surface plasmon spectrometry indicated that 14-3-3 directly binds the phosphoSer-19 peptide (Figure 5).

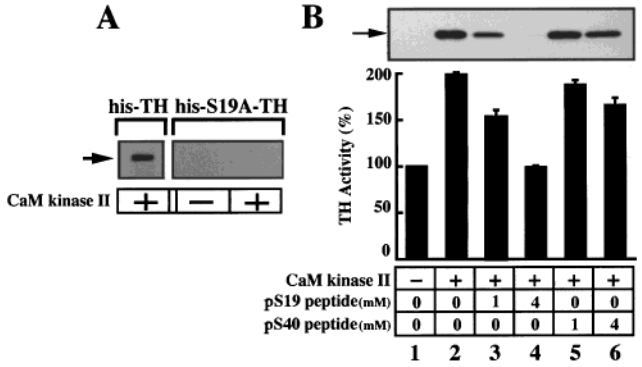

FIGURE 4: Phosphorylation of Ser-19 is essential for the interaction of TH and 14-3-3. (A) 14-3-3 does not interact with his-S19A-TH. Purified his-S19A-TH (∼1 μg) was incubated for 10 min with GST-fused 14-3-3η in the presence or absence of CaM kinase II, and then analyzed for its binding to 14-3-3 as in the experiments shown in Figure 2. (B) Disruption of TH/14-3-3 interaction by a phosphoSer-19-containing peptide. Purified his-TH (∼0.2 μg) was incubated for 10 min at 30 °C in the absence (lane 1) or presence of CaM kinase II (lanes 2−6), and then mixed with GST-fused 14-3-3η (each 0.6 μg) in the presence of the indicated concentrations of the phosphopeptides. The activity of TH was measured using the mixture directly, and the results (lower panel) are expressed as a percentage of the activity in the absence of CaM kinase II (lane 1). Vertical bars represent standard errors calculated on means of three experiments. In parallel experiments, TH bound to the GST-fused 14-3-3 protein was analyzed by Western blotting (upper panel) as in the experiments shown in Figure 2. (pS 19 peptide, FRRAVpSELDA; pS40 peptide, IGRRQpSLIED)

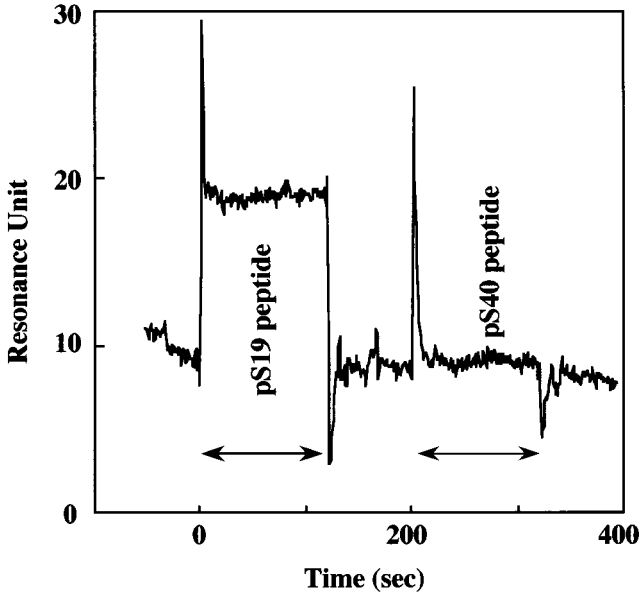

FIGURE 5: Analysis of 14-3-3 binding to phosphopeptides by surface plasmon resonance spectrometry. The phosphoSer19 or phosphoSer40 peptide (500 μM) was passed over a CM5 sensor chip immobilized with GST-fused 14-3-3η (flow rate, 5 μL/min), and the peptide-protein interaction was analyzed as in the legend to Figure 3A. Association constant (K_a) was determined by Scatchard plot analysis (*17*) using peptide concentrations between 25 and 500 μM. The equilibrium dissociation constants for the pS19 and pS40 peptides calculated from $1/K_a$, were 0.081 mM and 9.6 mM, respectively.

However, it is not clear whether the sequence surrounding Ser-19 is the only site of interaction with the 14-3-3 protein.

Depolarization-Evoked Interaction of 14-3-3 and TH. In intact PC12 cells, TH is phosphorylated at Ser-19 in response to membrane depolarization, apparently by the action of

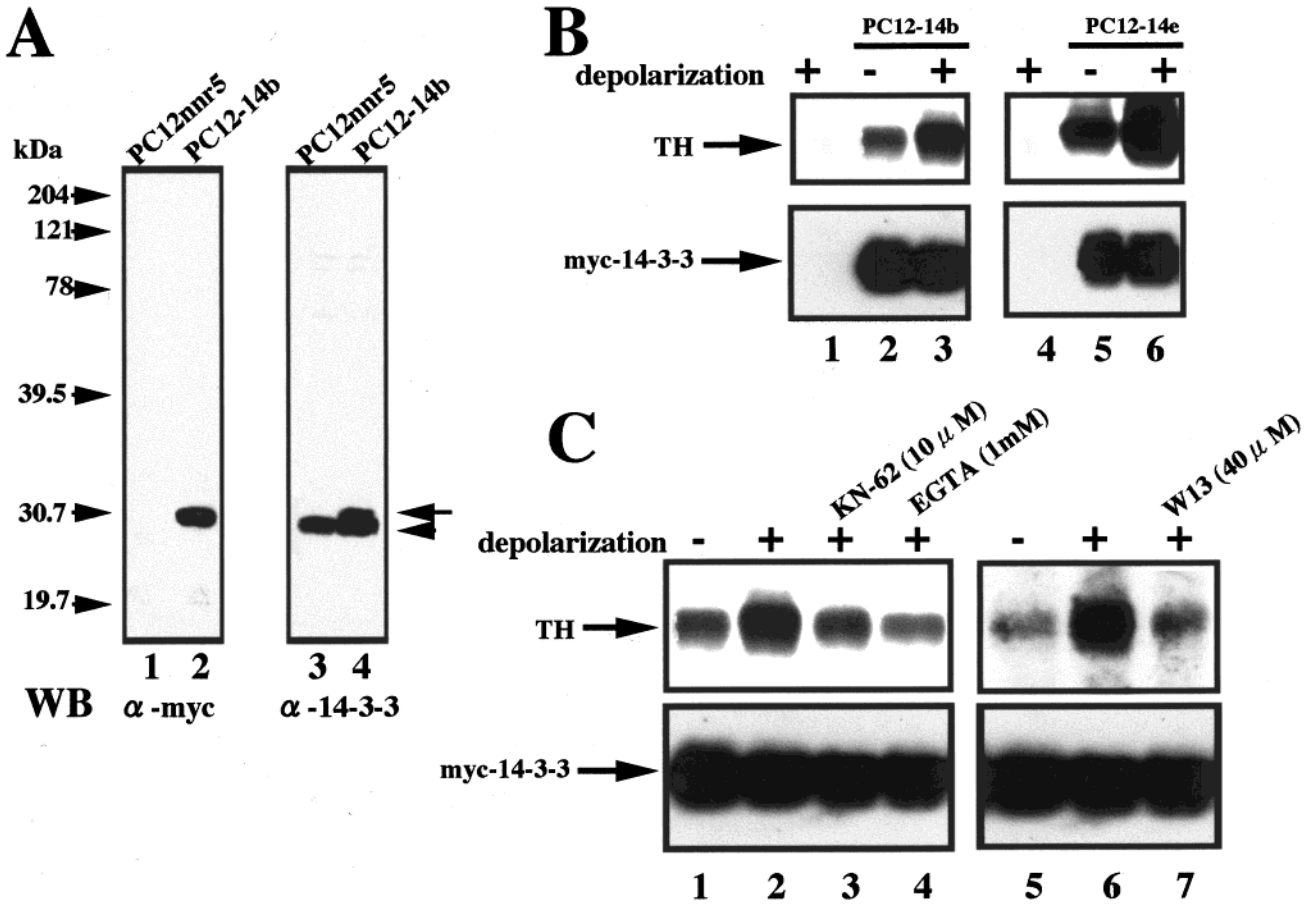

FIGURE 6: Co-immunoprecipitation of TH and 14-3-3 from depolarized PC12nnr5 cells. (A) Expression of myc-tagged 14-3-3β. Proteins (20 μg each) from PC12-14b cells (lanes 2 and 4) or from non-transfected PC12nnr5 cells (lanes 1 and 3) were analyzed by Western blotting with a myc monoclonal antibody (lanes 1 and 2) or with a 14-3-3 polyclonal antibody (Santa Cruz, sc-629, lanes 3 and 4). The positions of molecular mass markers are indicated. The arrow and arrowhead indicates myc-14-3-3β and endogenous 14-3-3, respectively. (B) Depolarization-evoked interaction between TH and myc-14-3-3. The cells were incubated for 15−30 min at 37 °C, washed with prewarmed KRH buffer, and then depolarized for 5 min with high (56 mM) KCl in KRH. Cell extracts (containing 0.5 mg of protein) from PC12-14b cells (lanes 2 and 3) and PC12-14e cells (lanes 5 and 6) before (lanes 2 and 5) and after depolarization (lanes 3 and 6) were subjected to immunoprecipitation with a myc monoclonal antibody as described under Experimental Procedures. Control experiments were performed with nontransfected PC12nnr5 cells (lanes 1 and 4). The immunoprecipitants were subjected to Western blotting with a TH polyclonal antibody (upper panel) and a myc monoclonal antibody (lower panel). The experiment was performed at least 5 times with constant results. (C) Effects of EGTA, W13, and KN-62 on the complex formation of TH and 14-3-3 in PC12-14b cells. The experimental conditions were same as in *B*, except that KRH buffer included the indicated concentrations of agents. Only in the experiment with EGTA, $CaCl_2$ was also omitted from KRH buffer. The concentrations of EGTA and KN-62 were from refs *42* and *33*, respectively, and that of W13 was from our recent study (T. Ichimura, and L.A. Greene, unpublished data). The blots were stained with a TH polyclonal antibody (upper panel) and a myc monoclonal antibody (lower panel).

endogeneous CaM kinase II (*31*−*33*). To assess the formation of TH/14-3-3 complexes in vivo,we first attempted to detect the endogenous complex in stimulated PC12 cells by co-immunoprecipitation with antibodies against 14-3-3 protein (*34*). However, these attempts were unsuccessful, probably due to the low binding affinity of these antibodies. We used, therefore, PC12-14b and -14e cells both of which are PC12nnr5-derived cells and constitutively express myc-tagged 14-3-3β (Figure 6A) and η (data not shown), respectively. In this case, the tagged proteins are strongly recognized and can be precipitated by an antibody against the myc-sequence (see Figure 6B, lower panel, lanes 2 and 3, for β; and lanes 5 and 6, for η). We have mainly used PC12-14b cells for this study, because the expression of this isoform is proven in PC12 (*35*). PC12-14b cells expressing the myc-tagged version of 14-3-3β grew normally and exhibited no apparent phenotypes.

The expressed myc-β isoform was immunoprecipitated with a myc antibody from PC12-14b cells before and after exposure to high (56 mM) K^+ medium, and the precipitates were analyzed by SDS-PAGE and Western blotting to detect myc-β and TH. A significant amount of TH was detected in the immunoprecipitates after the cells were depolarized with high K^+ (Figure 6B, upper panel, lane 3). A much lower, but detectable amount of TH was also found to associate with 14-3-3 before the depolarization (lane 2). In contrast, no TH was detected in the immunoprecipitates obtained from the control cells not expressed myc-β (lane 1). The myc-tagged 14-3-3η also bound TH in PC12-14e cells when the cells were stimulated to induce Ca^{2+} influx (lanes 4−6 in Figure 6B).

The biological significance of the TH/14-3-3 complex was evaluated by removing Ca^{2+} from the incubation media. As shown in Figure 6C, lane 4, this treatment inhibited the depolarization-evoked interaction of TH and 14-3-3 in PC12-14b cells (compare with lanes 1 and 2). Similarly, treatment of cells with KN-62 (a CaM kinase II inhibitor, ref *33*) and W13 (a CaM inhibitor, refs *36*, *37*) abolished the K^+-

stimulated association of TH and 14-3-3 (lanes 3 and 7, respectively). Thus, the complex formation between TH and 14-3-3 appears to be triggered by activation of CaM kinase II. However, since KN-62 and W13 could also block the voltage-dependent Ca^{2+} channels and prevent the Ca^{2+} entry induced by high K^+ (*38*, *39*), further study might be necessary to determine whether CaM kinase II directly participates in the complex formation in vivo.

DISCUSSION

In this study, we present the first experimental evidence that 14-3-3 binds directly to TH and regulates its activity in concert with the phosphorylation of TH at Ser-19, a major phosphorylation site of CaM kinase II in vitro and possibly in vivo (*1*, *31*−*33*). We have also shown that the 14-3-3 protein does not bind to TH when Ser-40 is phosphorylated by PKA (Figure 2). Furthermore, the complex formation between TH and 14-3-3 is not inhibited by the addition of the synthetic peptide including phosphoSer-40 (Figure 4B) or by the addition of PKA.[2] These results are consistent to the previous observation that PKA directly activates TH without 14-3-3 and that the stimulatory effect of PKA is additive to the effect of CaM kinase II plus 14-3-3 (*40*). Thus, the TH activity is regulated independently by CaM kinase II and PKA, where the 14-3-3 protein acts as a CaM kinase II-specific, phosphorylation-dependent activator of TH.

Previous studies suggest that 14-3-3 recognizes a consensus sequence, R(S)X*p*SXP (*18*, *30*). In the crystal structure of the 14-3-3ζ complexed with a phosphopeptide designed for the polyomavirus middle T antigen, 14-3-3 binds the phosphoserine through ionic interaction and via proline at the +2 position. Interestingly, this proline residue is fixed in all cis-configuration in the complex (*30*). This implies that 14-3-3 may exert its biological activity by inducing a conformational change in target proteins through proline isomerization or by stabilizing proline in a cis-configuration against its cis−trans equilibrium. In the TH sequence, the proposed consensus sequence is found at positions 315-320 (RHA*p*SSP), while there are no experimental results published on the phosphorylation of Ser-318 both in vivo and in vitro. On the other hand, the present study has defined a 10-residue fragment in TH, FRRAV*p*SELDA, as a recognition site for the 14-3-3 protein. Although this sequence closely resembles the proposed consensus sequence, leucine is found at the +2 position in place of proline. Thus, 14-3-3 may have broader specificity in target recognition and exert its activity by more diverse mechanism than previously expected. Because the 14-3-3/TH complex is more stable than the 14-3-3/phosphopeptide complex (K_D values, 3 nM vs 81 μM), it is possible that additional residue(s) in TH molecule participates in the interaction, once the initial complex formation is triggered by phosphorylation at Ser-19. The structural requirement and the molecular mechanism by which 14-3-3 recognizes and reacts with its target molecules await more systematic investigation and analysis of quaternary structure.

The TH/14-3-3 complex is detected in PC12-14b/14e cells, particularly when the cells are stimulated to increase intracellular Ca^{2+} (Figure 6B). In addition, the formation of TH/14-3-3 complex is inhibited by the treatment of cells with EGTA, W13, or KN-62 (Figure 6C), suggesting that the observed TH/14-3-3 complex is functionally significant in vivo. On the basis of this finding, we propose the following mechanism for the Ca^{2+}-dependent activation of TH. Depolarization of cells with high K^+ medium leads to activation of voltage-dependent Ca^{2+} channels and elevation of intracellular free Ca^{2+}. Subsequently, CaM binds Ca^{2+} and activates CaM kinase II. Activated CaM kinase II then phosphorylates TH at Ser-19 and to the phosphorylated TH binds the 14-3-3 protein to activate the TH activity (Figure 7). The confirmation of this model should have an important implication towards understanding a role of 14-3-3 in Ca^{2+} signaling, because the 14-3-3 protein also appears to participate in the Ca^{2+}-dependent catecholamine secretion in adrenal chromaffin cells (*41*).

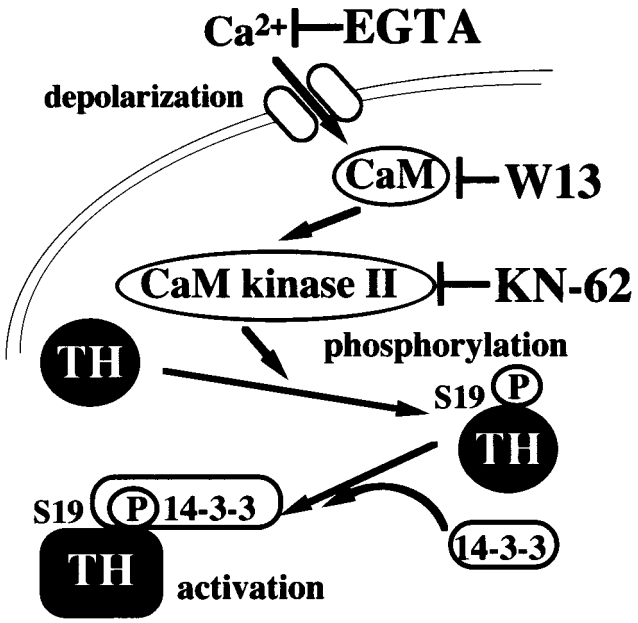

FIGURE 7: Schematic illustration of the proposed mechanism of depolarization-evoked activation of TH.

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