Communication

Induction of Calmodulin Kinase IV by the Thyroid Hormone during the Development of Rat Brain*

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This communication reports the specific induction of calmodulin kinase IV by the thyroid hormone 3,3',5-triiodo-L-thyronine (T₃) in a time- and concentration-dependent manner at a very early stage of brain differentiation using a fetal rat telencephalon primary cell culture system, which can grow and differentiate under chemically defined conditions. The induction of the enzyme that can be observed both on the mRNA and on the protein level is T₃-specific, i.e., it cannot be induced by retinoic acid or reverse T₃, and can be inhibited on both the transcriptional and the translational level by adding to the culture medium actinomycin D or cycloheximide, respectively. The earliest detection of calmodulin kinase IV in the fetal brain tissue of the rat is at days E16/E17, both on the mRNA as well as on the protein level. This is the first report in which a second messenger-dependent kinase involved in the control of cell regulatory processes is itself controlled by a primary messenger, the thyroid hormone.

As one of the intracellular second messengers, calcium plays a central role in cell growth and differentiation (1). The primary receptor protein mediating the calcium signal inside the cell is calmodulin (CaM),† which regulates, among others, protein kinases and protein phosphatase(s). Next to the well studied multifunctional CaMKII (see Ref. 2 for a recent review), CaMKIV recently received considerable attention. In contrast to the ubiquitous CaMKII, CaMKIV is more restricted in its expression in different tissues. The highest levels of the enzyme in mammalian tissues can be found in brain, thymus, and, to a somewhat lower extent, in testis and spleen, whereas in other tissues the enzyme remained undetectable (3–7). The amino acid sequence of CaMKIV, which exists in two monomeric isoforms of Mr, 65,000 (α) and Mr, 67,000 (β) due to alternative splicing, has been deduced from rat, mouse, and human brain cDNAs (4, 5, 8–10), demonstrating less than 50% homology to the corresponding regions of CaMKII. Next to the rather poor sequence homology between the two CaM kinases, CaMKII and IV, the two enzymes also seem to differ in their activation mechanism. Whereas CaMKII can be efficiently activated by autophosphorylation (2), the activation of CaMKIV by autophosphorylation is rather slow and inefficient (11, 12). Recent reports seem to indicate that CaMKIV is activated by a CaMKIV kinase (13–16), reminiscent of the regulation of mitogen-activated protein kinase activity by a kinase cascade.

In comparison to CaMKII, the substrate specificity of CaMKIV seems to be more restricted. Apart from synapsin I, which is a substrate for both CaM kinases, the only other substrates reported for CaMKIV are the Ras-related GTP-binding protein Rap-1b (17), the AMP regulatory element-binding protein CREB (11, 18), the serum response factor SRF (19), and members of the Ets family of transcription factors (20, 21). The recent report of a substantial localization of CaMKIV in the nucleus (18, 22) permits the enzyme direct access to these transcription factors to regulate their function in a Ca²⁺-dependent manner. Thus, it has been reported that CaMKIV is involved in the Ca²⁺-dependent regulation of expression of immediate early genes either through CREB (Refs. 12, 18, 23, and 24; see also Ref. 25) or through SRF (19).

Since thyroid hormones have been shown to be required for normal growth and differentiation of the mammalian brain (26–28), we initiated studies to investigate the influence of the thyroid hormone 3,3',5-triiodo-L-thyronine (T₃) on the expression of a number of neuronal and glial membrane markers during development in rat brain cell cultures (29–31). Here we report the specific induction of CaMKIV by T₃ in a time- and concentration-dependent manner at a very early stage of brain differentiation using a fetal rat telencephalon primary cell culture system, which can grow and differentiate under chemically defined conditions (32). The induction is T₃-specific, i.e., the expression of the enzyme cannot be induced by either reverse T₃ or retinoic acid. The expression of CaMKIV is regulated on both the transcriptional and the translational level, since both the addition of actinomycin D as well as cycloheximide to the culture medium can prevent the T₃-dependent induction of the enzyme. In addition, the T₃-specific induction can be observed both on the mRNA and on the protein level. This is the first report in which a second messenger-dependent kinase involved in the control of cell regulatory processes is itself controlled by a primary messenger, the thyroid hormone. Preliminary accounts of part of the data presented here have been given elsewhere (33, 34).

EXPERIMENTAL PROCEDURES

Cell Culture—Serum-free, rotation-mediated aggregating cell cultures were prepared from fetal (15 days of gestation) rat (OFA/Ico/Ibm strain, Biological Research Laboratories Ltd., Basel, Switzerland) telencephalon, as described in detail previously (32). Aliquots of 6 × 10⁶ cells were transferred to DeLong flasks and maintained under constant gyratory agitation at 37°C in an atmosphere of 10% CO₂ and 90% humidified air. The culture medium used was Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with nutritional factors, vitamins, trace elements, transferrin (1 mg/liter), insulin (800 ng), and hydrocortisone 21-phosphate (20 ng). Gentamicin sulfate (25

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†The abbreviations used are: CaM, calmodulin; CaMKII, Ca²⁺-CaM-dependent protein kinase II; CaMKIV, Ca²⁺-CaM-dependent protein kinase IV or Gr; CREB, AMP response element-binding protein; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; SRF, serum response factor; T₃, 3,3',5-triiodo-L-thyronine; PBS, phosphate-buffered saline.
mg/liter) was used as an antibiotic. For analyses, the aggregates of each flask were washed twice with 5 ml of phosphate-buffered saline (PBS).

Preparation of Cell Extracts—Proteins were extracted, usually from the pellet of whole aggregates (4–5 × 10⁶ cells), by adding 200 μl of 25 mM HEPES, pH 7.5, 5 mM EDTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 mM l-tosylamido-2-phenylethyl chloromethyl ketone, 1 mM N-p-tosyl-L-lysine chloromethyl ketone, 1,10-phenanthroline, 2 mM p-amino-benzamidine (buffer A), and incubated on ice for 10 min after extensive vortexing. After centrifugation at 25,000 × g for 5 min, proteins in the supernatant were stored at −70°C.

SDS-PAGE and Electrophoretic Blotting—To 40 μl of supernatant of cellular extracts, 20 μl of loading buffer (35) was added and the proteins were separated on a 10% SDS-PAGE (35). After electrophoretic transfer onto nitrocellulose sheets for 2 h using 240 mM glycine, 30 mM Tris, 20% methanol, and 0.02% SDS as transfer buffer, the blots were incubated overnight at 4°C with 2% milk powder in PBS as blocking buffer, after which the blot was radiographed at room temperature for 3 h with 125I-CaM (36) in 2% milk powder/PBS containing either 1 mM CaCl₂ or 5 mM EDTA, washed once with 2% milk powder/PBS containing either CaCl₂ or EDTA, then with PBS containing either CaCl₂ or EDTA, and third with PBS. Each washing was done for 15 min. After drying the blot under cold air, the sheets were exposed either to x-ray sensitive films (Kodak XAR-5) at −70°C or to Phosphor imaging screen (Molecular Dynamics, Sunnyvale, CA) at room temperature and developed. Quantitation was obtained using the Phosphor imaging software as recommended by the manufacturers. CaM was purified from bovine brain as described previously (37).

Preparation and Characterization of Total RNA—For RNA preparation, cell culture aggregates (6–8 × 10⁶ cells) were grown for 5 days either in the presence or in the absence of 3 × 10⁻⁸ M T₃. They were washed three times with PBS, and quickly frozen in liquid nitrogen. Total RNA was isolated by using the UltraSpec RNA kit (Biotec Laboratories, Inc. Houston, TX) as described by the manufacturer. For the identification of CaMKIV-specific mRNA, 40 μg of total RNA was denatured and size-fractionated on a 1.5% agarose, 2.2 M formaldehyde gel. Electrophoresis was carried out at 30 V for 16 h with circulating Na₃PO₄ buffer at 4°C. Prior to blotting the gels were stained with ethidium bromide to identify 18 and 28 S RNA as markers. The RNA was transferred onto Biodye nylon membranes (ICN Biomedicals, Inc., Costa Mesa, CA) and stabilized by using UV cross-linking. Northern blots were prehybridized and hybridized at 37°C in 50% formamide as described previously (38). The probe was a 1-kilobase pair (BamHI-KpnI) fragment of the rat CaMKIV cDNA (4) and was labeled with [α-32P]dCTP according to the random oligonucleotide priming method of Feinberg and Vogelstein (39).

Purification and Characterization of p64—Aggregates of a total of 20 culture flasks (~10⁶ cells) grown for 5 days in the presence of T₃ (3 × 10⁻⁸ M) were homogenized at 4°C in three volumes of ice-cold buffer A, basically following the purification protocol for CaMKIV described in Ref. 40. Briefly, the lysates were centrifuged at 100,000 g for 30 min, and the supernatants were pooled and applied to a 15-ml DEAE-cellulose column equilibrated with 25 mM Hepes, pH 7.5, 0.5 mM EDTA, 0.5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT (buffer B). The column was washed with 10 bed volumes of buffer B, and bound proteins were eluted with a stepwise gradient using buffer B supplemented with 100 mM NaCl at each step. p64 eluted between 150 and 300 mM NaCl. The fractions containing p64 were pooled and applied onto a packed 1 ml hydroxyapatite column from Bio-Rad equilibrated with buffer B containing 200 mM NaCl (buffer C). The column was washed with five bed volumes of buffer C, and bound proteins were eluted with a linear 0–200 mM Na₃PO₄ gradient. P64-containing fractions eluting between 75 and 100 mM Na₃PO₄ were pooled, dialyzed overnight against 5 liters of 25 mM Hepes, pH 7.5, 2 mM CaCl₂, 2 mM MgCl₂, 1 mM DTT (buffer D), and applied to a 1.5 M column equilibrated with buffer D. After washing the column with 10 bed volumes of buffer D containing 10% glycerol (buffer E), followed by a wash of five bed volumes with buffer D containing 0.5 mM NaCl, p64 was eluted by a buffer containing 25 mM Hepes, pH 7.5, 2 mM EDTA, 5 mM EGTA, 1 mM DTT, 10% glycerol. Puriﬁed p64 was identiﬁed as a calmodulin-dependent kinase by autophosphorylation using the assay as described by Cruzalezuli and Means (11). Autophosphorylated CaMKIV were identiﬁed by speciﬁc antibodies, kindly provided by Dr. H. Schulman. Identiﬁcation of p64 as CaMKIV by speciﬁc antibodies was carried out by Western blotting using 2% milk powder/PBS as blocking buffer.

Fig. 1. Identification of CaMKIV in fetal rat telencephalon cell culture extracts grown in the presence or absence of T₃. Soluble proteins (A) or total RNA (B) extracted from cultures grown for 5 days in the presence (+) or absence (−) of 3 × 10⁻⁸ M T₃ were separated on either SDS-PAGE (A) or agarose gels (B), and after electroblotting onto appropriate membranes were either identified by specific antibodies, kindly provided by Dr. H. Schulman, or a CaMKIV-specific cDNA probe (B), as described in detail under “Experimental Procedures.” Molecular size standards (in kDa) are indicated at the left edge of panel A, the arrow denotes the location of CaMKIV (p64). The numbers on the right side of panel B (right panel) indicate the calculated sizes of the hybridizing bands. Migration of nucleic acid size standards are shown at the left edge of panel B (left panel), which shows the ethidium bromide staining of the same gel prior to blotting. 285 and 185 mark the levels of the corresponding ribosomal RNA species.
other CaM-binding proteins exhibiting amounts of the recently described CaMKIV kinase (13–16). The could also be indicative for the presence of contaminating nant CaMKIV. On the other hand, slow phosphorylation of p64 cerebellum, and can be observed only postnatally (6).

A specific antibodies (3 and 6,000 were identified as CaMKII by using monoclonal antibodies against CaMKII 

Determination of NaCl between 200 and 300 mM, typical for CaMKIV. (Ref. 40; see also “Experimental Procedures”). As shown in Fig. 2, p64 eluted from the DEAE-cellulose column at a concentration of NaCl between 200 and 300 mM, typical for CaMKIV. Further purification was obtained by using a hydroxyapatite column, and finally a CaM affinity column. After extensive washing with a calcium-containing buffer, p64 was eluted using an EGTA-containing buffer (2). The purified protein was identified as a CaM-dependent kinase by CaM-dependent autophosphorylation (Fig. 2B) and as CaMKIV by specific antibodies (Fig. 2A). Using immunoprecipitation CaMKIV could be identified only in cells that had been grown in the presence of T3 (data not shown). With respect to its M, of 64,000–65,000 and its prenatal appearance, p64 most likely represents the a-subunit of CaMKIV since the b-peptide has a slightly higher M, i.e. 67,000, is specifically expressed in brain only in cerebellum, and can be observed only postnatally (6).

It was noted that autophosphorylation of pure p64 was rather slow, as described by Cruzalezui and Means (11) for a recombinant CaMKIV. On the other hand, slow phosphorylation of p64 could also be indicative for the presence of contaminating amounts of the recently described CaMKIV kinase (13–16). The other CaM-binding proteins exhibiting M, values between 60,000 and 63,000 were identified as CaMKII and calcineurin A, respectively, by using monoclonal antibodies against CaMKII and by comparison with purified calcineurin. It appeared that in contrast to CaMKIV, the expression of both CaMKII and calcineurin was independent of T3 (data not shown).

The influence of T3 was studied further in a dose- and time-dependent expression of CaMKIV. As shown in Fig. 3, the enzyme was detectable already at very low concentrations of T3 (3 × 10^-10 M; Fig. 3) and increased in intensity with increasing concentrations of T3 in the culture medium, indicating that the induction of CaMKIV by T3 was dose-dependent.

The influence of T3 was studied further in a dose- and time-dependent expression of CaMKIV. As shown in Fig. 3, the enzyme was detectable already at very low concentrations of T3 (3 × 10^-10 M; Fig. 3) and increased in intensity with increasing concentrations of T3 in the culture medium, indicating that the induction of CaMKIV by T3 was dose-dependent. In addition, when cultures received T3 (3 × 10^-8 M) for various lengths of time, CaMKIV was already detectable after 6 h of stimulation (Fig. 4), and the amount of CaMKIV increased as a function of the duration of the stimulus until maximal expression was reached between 24 and 48 h, suggesting that protein synthesis played a role in the induction of this gene. This interpretation was corroborated by incubating the cell cultures with either actinomycin D or cycloheximide, respectively, to prevent transcription or protein synthesis of the inducible gene. As can be seen from Fig. 5, CaMKIV was clearly induced after exposure to 3 × 10^-8 M T3 for 24 h (Fig. 5, lane 3; see also Fig. 4, lane 4), but the protein was not detectable if the cultures had been preincubated for 1 h with either 1 μM actinomycin D (Fig. 5, lane 5) or 5 μM cycloheximide (Fig. 5, lane 4), respectively, before T3 was added. This is in contrast to the other detectable calmodulin-binding proteins, which during the time period of observation (i.e. 24 h) were independent of transcription or translation.

A further observation supporting the view that the expression of CaMKIV was a T3-specific process was the finding that next to T3 only T4 was able to induce CaMKIV (Fig. 6, lanes 2 and 3). On the other hand, neither reverse T3 nor retinoic acid could induce the expression of CaMKIV (Fig. 6, lanes 2 and 4). In addition, neither nerve growth factor nor epidermal growth factor were able to induce the expression of the kinase (data not shown), although both growth factors have been demonstrated to regulate developmental processes in these cultures (41, 42). Also, CaMKIV may be specifically expressed in neurons, since it was found at high levels in neuron-enriched aggregate cultures (data not shown), in which highly proliferating glial cells have been suppressed by the addition of 1-β-β-β-arabino-furano-syl-tyrosine (Ara-C) (31).

Recent reports suggested that CaMKIV is responsible for the Ca2+- dependent regulation of expression of a number of immediate early genes such as c-fos, due to the phosphorylation of the cAMP-responsive element-binding protein (CREB) (12, 18, 23, 25) or the serum response factor SRF (19). Our results indicate that during rat brain development the expression of CaMKIV, not detectable at the early stages of ontogenesis (i.e. at E15; data not shown) is regulated by the thyroid hormone in a time- and concentration-dependent manner. Whether this T3-dependent regulation is due to a direct interaction of the T3-Receptor with a responsive element of the CaMKIV gene (28) or whether the effect is indirect remains to be determined, but since the T3-specific induction of CaMKIV could also be observed on the mRNA level, this observation could be indicative for a T3-receptor-dependent regulation. In this respect it is of interest that in a recent abstract (43), it was reported that in a mouse embryonic stem cell-derived neuronal culture system the expression of CaMKIV was strictly dependent on the presence of the thyroid hormone receptor. In addition, it should be noted that Shakagami et al. (7) observed a rather late appear-
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