μ-crystallin: NADPH-dependent T3 binding protein in cytosol

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Running title: μ-crystallin binds T3 in cytosol

key words: CRYM, thyroid hormone, 3,5,3'-triiodo-L-thyronine, CTBP, deafness, crystallin

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Disclosure statement: The authors have nothing to disclose.

Abstract

Thyroid hormone action is initiated through nuclear thyroid hormone receptors. Prior to the discovery of nuclear receptors, possible major binding sites were thought to be in cytosol because of high binding activity in crude cytosolic fraction. Among several thyroid hormone-binding proteins in cytosol, NADPH-dependent cytosolic 3,5,3'-triiodo-L-thyronine binding protein is identical to μ-crystallin, which was initially cloned as the ortholog of bacterial ornithine cyclodeaminase. The expression is developmentally regulated and cell-type specific. Recently, patients with nonsyndromic deafness were reported to be associated with point mutations in the μ-crystallin gene. Cytosolic thyroid hormone-binding proteins, especially μ-crystallin, play roles in adaptation to environmental alterations by thyroid hormone and in thyroid hormone action, which may relate to hearing function.

Introduction

The thyroid gland produces and secretes two related hormones, thyroxine (T4) and 3,5,3'-triiodo-L-thyronine (T3). These hormones play pivotal roles in cellular differentiation during development and help maintain thermogenic and metabolic homeostasis in the adult, mainly through nuclear receptors that mediate transcriptional activation in the target cells. Following secretion of the hormone from the thyroid gland, T4 binds preferentially to thyroxine-binding globulin in serum. Recent findings showed that thyroid hormones enter mainly through active transporters [1]. Cells have multiple thyroid hormone binding proteins, including nuclear thyroid hormone receptors (TRs). Protein binding analyses using radioisotopes demonstrated that several T3 binding proteins are present in the endoplasmic reticulum [2], mitochondria [3], nuclear envelope [4], and cytoplasm. In this article, we focus on the T3 binding proteins in the soluble cytosolic fraction. As there is a great deal of evidence that these proteins are functional, we review and mainly discuss the physiological roles of NADPH-dependent cytosolic T3 binding protein (CTBP), identified as μ-crystallin (CRYM).

CTBP is identical to CRYM.

Cytosolic thyroid hormone binding proteins were first described by Tata [5], who presented evidence based on paper chromatography that a specific cytosolic extract from rat skeletal muscle bound [^{131}I]T4. Hamada *et al.* first demonstrated the possibility that proteins that bind specifically to triiodothyronine are present in the soluble cytosolic fraction [6].

In 1986, it was reported that charcoal treatment of rat renal cytosol abolishes the hormone binding activity, while the addition of NADPH restores this activity [7]. In 1991, NADPH-dependent CTBP was identified and purified from the rat liver. This CTBP has a molecular mass of 76,000 Da and consists of a 38,000-Da peptide dimer with an affinity constant for L-T3 binding of 2.4 liter/nmol [8]. Two L-T3 molecules bound to a 76,000-Da unit. In contrast to

NADPH, NADP suppresses T3 binding activity. Iodothyronine analog-binding specificity is L-T3=D-T3>L-T4>Triac, which differs from that of the nuclear receptors. In 1997, CTBP was identified by protein sequencing analysis of purified human CTBP [9]. The cloned CTBP was CRYM, which had already been cloned as an ortholog of bacterial ornithine cyclodeaminase [10]. CRYM is a taxon-specific protein, which is particularly abundant in kangaroo lens [11]. The protein was also expressed in the human retina, brain, heart, and kidney.

Physiological function of CRYM as a T3 binder

Although the T3 binding of CRYM has been demonstrated *in vitro*, it has not been determined whether the expression of CRYM molecule alters the T3 concentration in living cells. To approach this issue, permanent CRYM-expressing GH3 cell lines were established [12]. The expression of CRYM increased cellular uptake of T3 and the efflux rate was also decreased by induction of CRYM. Although these findings indicate that the expression of CRYM increases the concentration of intracellular T3, CRYM expression suppressed the T3-regulated luciferase activity in a series of GH3 cell lines and suppression correlated with the expression level of CRYM. Furthermore, T3 induction of rat growth hormone mRNA was lower in cells expressing CRYM than in CRYM-null cells, suggesting that the expression of CRYM suppressed T3-mediated transactivity in CRYM-expressing cells (Fig 1). We further noted that intracellular free T3 concentration is also a crucial factor in peripheral thyroid hormone action. Because of technical problems, it is difficult to discriminate between free and bound fractions of intracellular T3. However, it is speculated that the NADPH-dependency of T3 binding activity on CRYM may affect the free T3 concentration in cytoplasm.

To further investigate the *in vivo* functions of *CRYM* gene products, we generated mice with targeted disruption of the *CRYM* gene, which abrogates the production of CRYM [13]. CRYM-knockout mice showed loss of the entire NADPH-dependent T3 binding activity in the cytosol of the brain, kidney, heart, and liver. In the euthyroid state, knockout significantly reduced the serum

concentrations of T3 and T4 despite normal growth and normal heart rate. Disruption of the gene did not alter the expression of TSHβ mRNA in the pituitary gland. In addition, disruption did not alter the mRNA expressions of glutathione S transferase alpha 2 or deiodinase 1, which are negatively and positively regulated by T3, respectively, in either the liver or kidney. When radiolabeled T3 was injected intravenously, labeled T3 entered rapidly into and then escaped from the tissues in CRYM-knockout mice. These observations suggest that, due to rapid T3 turnover, disruption of the CRYM gene decreases T3 concentrations in tissues and serum without alterations of peripheral T3 action *in vivo*.

To date, CRYM-null patients have not been reported. Based on the results of the knockout study, it is anticipated that the concentration of T3 would be low in putative CRYM-null patients. The concentration of thyroid hormone was not remarkable in a patient with CRYM mutation (K314T) (S. Suzuki *et al.* unpublished), indicating that residual intact CRYM protein expressed in the patient may function in the regulation of thyroid hormone. Pathophysiologically, we speculate that some part of the non-thyroidal illness may be caused by the alteration of CRYM expression in peripheral tissues.

Deletion analyses using bacterially expressed mutant proteins indicated that two separate domains are crucial for dimer formation [14]. K314T, which was a mutation at the 3' end of the amino acids, abolished NADPH-dependent T3 binding [15]. The molecular structure of CRYM has recently been determined by crystallographic analyses. The crystal structure of CRYM demonstrated the presence of an NADPH binding site, dimerization domains, and a putative T3 binding site in the molecule [16].

CRYM and hearing function

Microarray analysis demonstrated that CRYM is highly expressed at the mRNA level in the human inner ear [17]. Immunohistochemical analyses showed that the protein is expressed preferentially in type II fibrocytes where Na-K ATPase is enriched [15]. As hearing is one of the most important functions controlled by thyroid hormone, CRYM expression may be related to hearing

function.

There have been reports of two families with hereditary nonsyndromic deafness possessing mutations in CRYM mRNA. One mutation (K314T) eliminated NADPH-dependent T3 binding and patients with K314T showed severe deafness. The other mutation (X315Y) did not alter the binding activity, and the hearing ability in these patients was moderate. These data suggest that T3 binding properties affect the clinical symptoms of deafness [15]. However, auditory-evoked response was normal in CRYM-knockout mice, suggesting that deletion of the gene products does not affect hearing function [13]. The abnormal cellular distribution of mutant protein was demonstrated in a previous article, indicating that expression of abnormal mutant proteins may affect clinical hearing ability [17].

Expression of CRYM

As CRYM may contribute to tissue development through transportation or retention of T3 in the cytoplasm, NADPH-dependent T3 binding activity was assessed in various rat tissues at different developmental stages [18]. The affinity constant was approximately 2×10^9/M in the tissues studied. While maximal binding capacity was quite low at birth, it increased as development progressed in the kidney, liver, and heart. In the brain, NADPH-dependent T3 binding to CRYM, detected in the fetal stage, increased transiently 2 weeks after birth, then increased gradually until maturation. These observations imply that CRYM supplies T3 during the development of the central nervous system.

The expression of CRYM mRNA was also assessed. In the central nervous system, high levels of expression were observed in the telencephalon, with low levels in the brainstem and spinal cord [19]. Microarray experiments for serial analysis of gene expression demonstrated that CRYM was also expressed in the nucleus accumbens and medial striatum, but not in the lateral striatum [20]. The heart showed a high level of expression in humans. Moderate expression was detected in the cerebellum and pituitary. The protein expression pattern was similar to the distribution of mRNA expression: *i.e.*, high levels of expression in

the brain, heart, and kidney. Intriguingly, mosaic expression was observed in granule cells of the human cerebellum. In the human kidney, the renal tubes were stained in contrast to reduced staining in the glomeruli. In the pancreas, islet cells showed positive staining. Thus, the expression of this protein is not only time-specific, but also cell-type specific.

CRYM was first demonstrated in the kangaroo lens. Thus, the localization of the protein was also evaluated in the mammalian eye [21]. CRYM was shown to be expressed preferentially in rod cell outer segments of the rat retina. Proteome experiments demonstrated anti-CRYM autoantibody in sera obtained from monkeys with late-onset macular degeneration, indicating that autoimmune responses to CRYM are associated with the progression of macular degeneration in the retina [22]. These observations imply that CRYM may relate to visual function in addition to nuclear T3 receptors as described previously [23].

Regulation of CRYM expression

As expression is both time- and cell-type specific, it is proposed that multiple factors control the NADPH-dependent T3 binding activity, including the level of CRYM expression. NADPH-dependent T3 binding was positively regulated by thyroid hormones [24], activated vitamin D3 (1,25 $(OH)_2$D3) [25], insulin [26], and sodium butyrate [27] in cultured cells. In rats, a vitamin D-deficient diet suppressed NADPH-dependent T3 binding. This suppression was reversed by the addition of vitamin D. Streptozotocin-induced diabetic rats show low binding capacity of CRYM in the liver [26], and insulin treatment was shown to promote recovery of the binding capacity.

Following the development of microarray techniques, several studies demonstrated the up- and down-regulation of CRYM expression. Clinically, glucocorticoid induced CRYM mRNA in human myeloid cells [28]. The mRNA expression was elevated in the failing heart in patients who died of trauma or cardiomyopathy [29]. The level of expression in the dorsal prefrontal cortex of the brain was low in the majority of subjects with schizophrenia [30].

As thyroid hormone acts on cellular differentiation, carcinogenesis may be associated with the action of thyroid hormone through CRYM expression. All cancer cell lines examined for CRYM expression showed quite low levels of expression, with the exception of rat hepatoblastoma dRLh-84 cells [25]. CRYM showed a significantly lower mRNA level in hormone-refractory prostate tumors compared with that in primary tumors [31]. CRYM expression was significantly elevated in non-small cell lung carcinoma [32], while the expression level was low in brain tumors [33].

In rodents, several observations have suggested that expression is controlled during the cycle of cellular development. The expression level was shown to be regulated during development of hair follicles in mice [34]. CRYM expression was reduced in ovarian follicles 6 h after FSH treatment relative to that in untreated cells [35]. *In situ* hybridization studies using randomly cycling adult rats demonstrated that CRYM mRNA was highly expressed only in small growing follicles of the ovary [36].

A recent article demonstrated a high expression of μ-crystallin in the muscle of patients with facioscapulohumeral musclar dystrophy (FSHD) [37]. FSHD is a musclar dystrophy that is thought to be caused by the deletion of the megasatellite repeat on chromosome 4q35. The disorder is associated with nonskeletal muscle manifestations include high-frequency hearing loss as well as retinal telangiecatasias. Thus, a high expression of μ-crystallin protein may affect both hearing function and retinal development.

Although there is limited evidence at present that alteration of CRYM expression affects physiological or pathological responses *in vivo*, such research may identify novel molecular mechanisms in the field of thyroid hormone action.

Conclusions and Perspectives

In conclusion, CRYM plays pivotal roles in physiological regulation of thyroid hormone action *in vivo*. The CRYM-mediated retention of T3 affects thyroid hormone concentration in the extranuclear space, resulting in the

modification of thyroid hormone action in the nuclei. Clinically, although the molecular mechanisms are not well understood, the mutant proteins are known to affect normal development of the inner ear. The binding of pyridine nucleotides to the protein implies that CRYM may possess redundant functions, such as enzyme activity like that of oxidoreductases. These oxidative and reductive mechanisms may alter the free thyroid hormone concentration in the intracellular space, and may also regulate the action of the hormone. The expression of the protein was also regulated in individual cells by multiple factors, indicating that the concentration of intracellular free hormone may also be regulated by numerous factors. However, we propose that the complexity described above may be required in terms of adaptation to environmental alterations by thyroid hormone concentration and other factors that affect thyroid hormone action.

Acknowledgement

We thank Ms. Izumi Kinoshita for excellent secretarial work.

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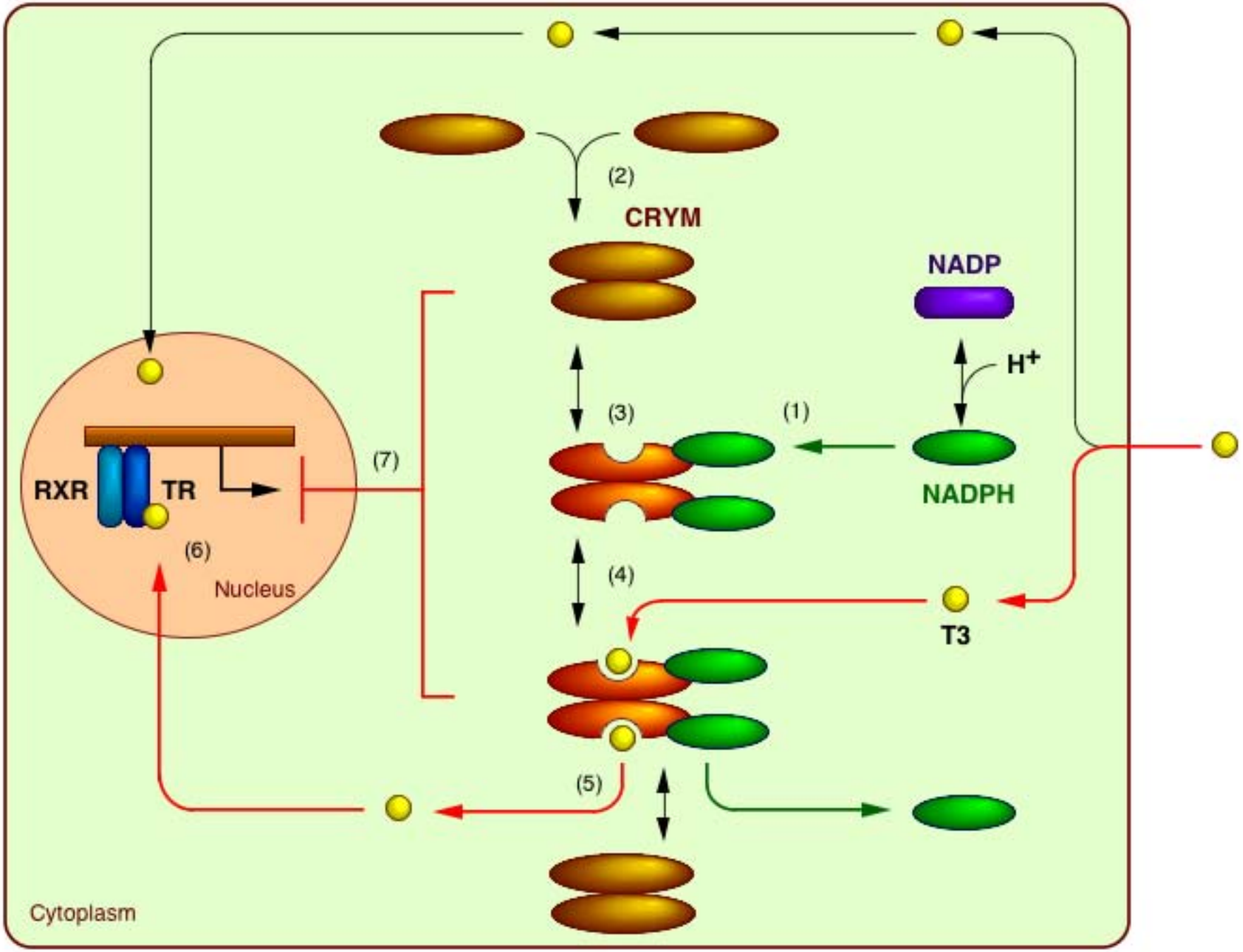
Figure Legend

Fig. 1 Molecular functions of μ-crystallin on thyroid hormone action.

T3 moves from outside the plasma membrane into the cytoplasm of the cells. Free T3 enters into the nucleus and initiates the transactivation through binding to nuclear T3 receptors. In the presence of μ-crystallin, the μ-crystallin molecule forms a dimer in the cytoplasm. Each molecule binds one molecule of T3 and NADPH. In the presence of NADPH, the T3-bound form of μ-crystallin increases the T3 concentration in the cytoplasm. Although the mechanisms have not yet been thoroughly elucidated, the expression of μ-crystallin suppresses the transcriptional activity. Regulation of T3 action through μ-crystallin is composed of (1) binding of reduced NADP *i.e.* NADPH, (2) dimerformation, (3) creation of the T3 binding site, (4) binding of T3, (5) release of T3 by dissociation of the NADPH, (6) induction of the transactivation, and (7) suppression of the transactivation

CRYM: μ-crystallin, T3: 3,5,3'-triiodo-L-thyronine, TR: thyroid hormone nuclear receptor, RXR: retinoid-x-receptor, NADP: nicotinamide adenine dinucleotide phosphate, NADPH: reduced nicotinamide adenine dinucleotide phosphate


(2)
CRYM
NADP
H+
(3)
(1)
NADPH
(7)
RXR
TR
(6)
Nucleus
(4)
T3
(5)
Cytoplasm