Detection of mRNA of Kinesin Superfamily 3A in the Cerebrum and Cerebellum: Biotin-Tyramine-Catalyzed Signal Amplification for *In Situ* Hybridization

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To improve the sensitivity of *in situ* hybridization (ISH) carried out using oligonucleotide probes without radionuclides, we employed a new method entailing amplification of ISH signals by catalyzed reporter deposition. This method is based on biotin-tyramine-catalyzed signal amplification (BT-CSA) and was used to detect expression of a relatively scarce polynucleotide, kinesin 3A mRNA, in the central nervous system. Strong signals were detected in the hippocampus and in the granular cell layer of the cerebellum, but

expression of kinesin 3A mRNA was not detected in Purkinje cells. ISH performed without the use of radionuclides has the advantage of being comparatively easy to perform, but has the disadvantage of low sensitivity. We showed that the low sensitivity can be overcome by using BT-CSA. Our method also had the advantage of enabling visualization of ISH signals via enzymatic reaction with horseradish peroxidase or by fluorescent staining.

Key words: *In situ* hybridization, Signal amplification, Tyramine, Oligonucleotide probe, Kinesin 3A

I. Introduction

The ongoing progress in the various genome projects has led to the discovery of many genes, and their sequence data are now available from databases at a number of internet sites. In many cases, however, little is known about the functions or the expression patterns of these genes. One approach to obtaining such information is to characterize the abundance and expression pattern of specific mRNAs within tissues.

Several techniques are currently available for use in determining the presence and abundance of mRNA in a tissue or cell sample; of these, Northern hybridization and reverse transcriptase polymerase chain reaction (RT-PCR) are most frequently used. However, these techniques have the basic limitation that they only provide the average mRNA level within a sample; they provide no data on the frequency or on the histological and cytological characteristics of the mRNA-expressing cells. *In situ* hybridization (ISH) enables one to visualize the distribution of mRNA at the cellular level and has, therefore, become an important additional tool for analysis of gene expression.

ISH carried out using radionuclides (R-ISH) is very often used to detect intracellular mRNA because it provides a high degree of sensitivity. While ISH without radionuclides is safer and easier to perform, it is less sensitive than R-ISH. This means that because expression of most mRNAs are relatively low as compared to expression of housekeeping genes such as β -actin mRNA [15], in many cases, it would be difficult to detect important polynucleotides using ISH [10]. For this reason, amplification procedures have been developed to overcome the low sensitivity of ISH; these include RT-PCR with subsequent ISH [5, 7, 9, 10] and catalyzed reporter deposition amplification of ISH signals (CARD-ISH) [6, 8, 10, 13, 15]. CARD-ISH is based on a method of biotin-tyramine-catalyzed signal amplification (BT-CSA), which has been previously applied to immunoassay and immuno-histochemical protocols [1, 3, 4]. In the present study, we used a combination of oligonucleotide probes with ISH and CARD-ISH to detect the relatively scarce mRNA of kinesin superfamily 3A (KIF3A).

II. Materials and Methods

Reagents

Formamide, sodium citrate trihydrate, diethyl pyrocarbonate (diethyl oxydiformate) and diaminobenzi-

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dine tetrahydrochloride dihydrate were purchased from Kanto Chemical (Tokyo, Japan); bovine serum albumin, salmon sperm DNA, tyramine-HCl and sulfosuccinimidyl-6-biotinamide hexanoate were from Sigma Chemical (St. Louis, MO, USA); proteinase K and Ficoll 400 were from Merck (Darmstadt, Germany); polyvinyl pyrrolidone was from Wako (Osaka, Japan); Dextran sulfate was from Pharmacia Biotech (Uppsala, Sweden); yeast RNA was from Ambion (Austin, TX, USA); Elite ABC kit was from Vector laboratories (Burlingame, CA, USA); fluorescein isothiocyanate-labeled streptavidin was from DAKO (Glostrup, Denmark) and ProLong Antifade kit was from Molecular Probes (Eugene, OR, USA). All other chemicals were of the highest grade available.

Specimen preparation

One-month old *BALB/cAnN* strain mice were anesthetized, and the tissues were then fixed by trans-cardiac infusion of 0.1 M phosphate buffer (pH 7.6) containing 4% paraformaldehyde for 5 min at 4°C. After resection of the cerebrum and cerebellum, specimens were immersed in the same fixative solution for 6 hr at 4°C. The samples were then washed in 0.1 M phosphate buffer (pH 7.6) and frozen in liquid N₂. Frozen sections (25 µm thick) were cut and mounted on 3-aminopropyl triethoxy silane-coated, 13-mm diameter, round cover slips (No. 1; Matsunami, Osaka, Japan).

Oligonucleotide probe

The complementary DNA (cDNA) sequences of our probes targeting the mRNA of KIF3A were obtained from the GenBank database (<http://www.ncbi.nlm.nih.gov/>). The probes were designed using GENETYX ver. 6.03 software running on a Macintosh computer as previously described [6], making certain that our probes did not hybridize with sequences from any other members of the kinesin superfamily in the database. Two sense and two antisense probes were constructed: one for the head region of the KIF3A cDNA sequence (sense, 5'-biotin-GGT-TCA-TAC-TGT-GAG-CTT-GGT-TTT-TGG-AAC-ACA-GCG-GGA-GAC-G-3'; antisense, 5'-biotin-CGT-CTC-CCG-CTG-TGT-TCC-AAA-AAC-CAA-GCT-CAC-AGT-ATG-AAC-C-3') and one for the tail region (sense, 5'-biotin-GAG-TCG-GAA-CCG-GTC-GTC-CAG-GTG-CGG-CGG-CTG-TTA-CTG-G-3'; antisense, 5'-biotin-CCA-GTA-ACA-GCC-GCC-GCA-CCT-GGA-CGA-CCG-GTT-CCG-ACT-C-3'). The oligonucleotide probes labeled with biotin at the 5' end were synthesized by TaKaRa Shuzo (Shiga, Japan). The sense probes were used for the negative control.

In situ hybridization

The specimens were first digested with proteinase K (5 µg/ml) for 10 min, post-fixed with 0.1 M phosphate buffer (pH 7.6) containing 4% paraformaldehyde, and immersed in 0.1 N HCl for 10 min. They were then immersed in the pre-hybridization solution (without

oligonucleotide probes) for 1 hr at room temperature, and subsequently incubated in 50 µl of hybridization solution in a moist chamber at 38°C overnight. The hybridization solution contained 50% deionized formamide, 10% dextran sulfate, 450 mM NaCl, 45 mM sodium citrate, 1× Denhardt's solution (0.02% bovine serum albumin, 0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone), 100 µg/ml salmon-sperm DNA, 125 µg/ml yeast RNA and 1 µg/ml of each oligonucleotide probe. After hybridization, the solution was removed by washing with 2× SSC (300 mM NaCl and 30 mM sodium citrate). The specimens were then incubated with 1× SSC and 0.5× SSC containing 50% deionized formamide at 38°C. All buffers were rendered RNase free by exposing them to diethyl pyrocarbonate (oxydiformate) at 37°C for 1 hr and then incubating them at 121°C for 40 min.

Hybridization was carried out using a technique similar to one previously used with enzyme cytochemistry [12]. The cover slips on which the specimens were mounted were placed in 12 well microplates (IWAKI, Tokyo, Japan) and washed three times with buffer (Fig. 1A). The coverslips were then placed in disposable culture dishes (Tissue Culture Dish, IWAKI, Tokyo, Japan) lined with parafilm (American National, CA, USA), where they were immersed in hybridization buffer (Fig. 1B). This method required only 3 ml of buffer to wash each cover slip (Fig. 1A), and the use of small-diameter cover slips meant

Fig. 1. Schematic diagram illustrating the method used; note that this method minimizes the amounts of buffer and probe required. **A)** Cover slips with mounted specimens are placed into 12-well microplates and washed three times by adding buffer to each well for a few minutes, after which the buffer is aspirated out and discarded. **B)** When specimens are immersed into materials that are in short supply (e.g. hybridization buffer, ABC solution or BT solution), disposable dishes lined with parafilm are used. In the example on the left, 50 µl of buffer is between the cover slip and the parafilm; whereas on the right, 100 µl of buffer is placed over the cover slip.

that only 50 to 100 μ l of hybridization buffer was needed to immerse specimens (Fig. 1B).

Amplification of ISH signals

The biotinylated probes were amplified using a modification of the procedure of Adams [1]. Briefly, the specimens were incubated with avidin-biotin complex (ABC) for 30 min, with biotinylated tyramine (BT) for 10 min, and again with ABC for 30 min. The BT stock solution was made by combining equimolar concentrations of tyramine-HCl with sulfosuccinimidyl-6-biotinamide hexanoate in 50 mM borate buffer (pH 8.0). The mixture was agitated overnight at room temperature and then filtered through a 0.45 μ m syringe filter. The concentration of BT was not measured, but the expected concentration was $\sim 7 \mu$ M [1]. The BT was diluted to a concentration of 10 μ l BT/ml phosphate buffered saline (PBS) with 0.01% H_2O_2 . For comparison, the ISH procedure was also carried out without BT amplification.

Visualization of ISH signals

Two methods were used to visualize ISH signals (Fig. 2). The first entailed avidin labeling horseradish peroxidase (HRP) using an ABC kit and then precipitating diaminobentidine (DAB) by reacting the labeled HRP with H_2O_2 . The second entailed fluorescent detection using a combination of fluorescein isothiocyanate-labeled streptavidin (FITC-SA) and the amplified biotin. In that case, the specimens were immersed in FITC-SA (10 μ g/ml) for 1 hr at room temperature and then enclosed using a ProLong Antifade kit so that fluorescence could be observed for longer periods using confocal laser microscopy (TCS-NT, Leica, Vienna, Austria and CSU10, Yokogawa, Tokyo, Japan) without photo-bleaching the specimen. In some cases, specimens were double labeled with DAB and FITC-SA enabling simultaneous detection DAB and FITC-SA signals.

Fig. 2. Schematic diagram depicting CARD-ISH with BT-CSA. HRP-ABC, horseradish peroxidase conjugated avidin biotin complex; b, biotin; b-T, biotinylated tyramine; FITC-SA, fluorescein isothiocyanate-labeled streptavidin; DAB, diaminobentidine.

III. Results

The use of BT-CSA has enabled us to reveal *in situ* expression of KIF3A mRNA in mouse cerebrum and cerebellum. DAB staining reflecting expression of KIF3A mRNA was detected in the granular cell layer of the cerebellum (Fig. 3A), but not in the Purkinje cells (Fig. 3B). When cells were labeled with FITC, ISH signals were detected in the same locations by confocal laser microscopy (Fig. 3C). No signals were detected among the negative controls (sense probes only; Fig. 3D). In contrast, no signals were detected using ISH without BT amplification (Fig. 3E). In the cerebrum, strong ISH signals showing the distribution of KIF3A mRNA were detected in the hippocampus (Fig. 3F).

KIF3A mRNA expression was readily detected using both DAB and FITC in double-labeled specimens. When specimens were sequentially stained first with DAB and then with FITC, strong DAB signals were obtained after only 3-min development (Fig. 4A), and subsequent FITC staining also yielded strong fluorescent signals (Fig. 4B). In contrast, when specimens were first labeled with FITC, which again yielded good ISH signals (Fig. 4C), more than 20 min were required for the DAB-development before the subsequent DAB-staining could be detected. Furthermore, the DAB signals followed by the observation of the FITC signals remained relatively weak even when fully developed (Fig. 4D). Thus, when double labeling with DAB and FITC, the DAB should be applied first.

IV. Discussion

In the present study, we were able to localize KIF3A mRNA within cerebellar granular cells (see Fig. 3). This represents a substantial increase in resolution over R-ISH techniques which localized mRNA only to the level of the cerebellum and the hippocampus [16].

The kinesin superfamily comprises a group of motor proteins that are involved in cellular transport in cooperation with microtubules [2]. Further, the expression of each of its many members (e.g. KIF1A, KIF1B, KIF2, KIF3A, KIF3B, KIF4, etc.) appears to localize within specific organs. Organ-specific localization of mRNA using Northern blot analysis revealed abundant *kif3a* transcripts in both brain and kidney. R-ISH then showed that the cerebellum and hippocampus are each enriched in KIF3A mRNA [16]. In the present study, we confirmed that KIF3A mRNA is expressed in the cerebellum and hippocampus and further showed that its expression is localized to cerebellar granular cells and is not present in Purkinje cells. Nonetheless, given the important role played by kinesin in cellular transport and the cell-specificity of kinesin superfamily members, it is likely that the absence of KIF3A mRNA expression in Purkinje cells only means that a different kinesin isoform is specifically expressed there.

Because they could be readily obtained from a com-

Fig. 3. KIF3A mRNA was detected using CARD-ISH with BT-CSA and visualized by staining with HRP-DAB (**A**, **B**, **D**, **E**) and FITC-SA (**C**, **F**). **A**) Cerebellum; primarily, the granular cell layer was stained. **B**) Higher magnification of cerebellum; note that the Purkinje cell was not stained (arrows). **C**) FITC-staining of cerebellum; note that the Purkinje cell was not stained (arrows). **D**) Cerebellum; negative control using only sense probes. **E**) Cerebellum; without amplification procedure (BT-CSA) using antisense probes. **F**) Cerebrum; the hippocampus was stained. The fluorescent signals in panels **C** and **F** were observed by confocal laser microscopy. Bars: **A** = 500 μm ; **B**–**F** = 50 μm .

Fig. 4. KIF3A mRNA detected in the hippocampus by CARD-ISH with BT-CSA and visualized by sequential double-labeling with DAB and FITC. When applied first, DAB staining enabled visualization of the KIF3A mRNA signal within 3-min development, and cells were well stained (A). Subsequent labeling by 1 hr immersion in FITC-SA also yielded a strong ISH signal (B). FITC also stained cells well when applied first (C). In this case, however, subsequent DAB staining required >20 min to develop visible signals, and even when fully developed, the ISH signals were comparatively weak (D). Bar = 50 μ m.

mercial source, we chose to conduct this study using oligonucleotide probes: earlier studies had shown that ISH could be successfully carried out using such oligonucleotide probes [6, 11, 14]. The sequence data of selected genes were obtained from the GenBank internet site, and the sequences of the probes are designed using commercially available computer software. To visualize the ISH signals, the probes were labeled with biotin or FITC at their 5' ends; although labeling with other materials can be arranged with the manufacturer. Because there is a 1:1 stoichiometry between the label and the probe, the sensitivity was relatively low compared to some other labeling protocols. Therefore, to increase the sensitivity, we used a cocktail of two probes that were bound to separate sites at the head and tail regions of the targeted mRNA [6].

We found that CARD-ISH with BT-CSA was highly useful for detecting KIF3A mRNA which would otherwise

not be detected by ISH without radionuclides. Thus, our findings are consistent with those of Komminoth *et al.*, who observed that CARD amplified the sensitivity of immunohistochemical signals 500–1,000 fold [10], and are indicative of the usefulness of CARD-ISH in cases where the target polynucleotides (e.g. mRNA) are present in relatively small amounts. Our CARD-ISH with BT-CSA protocol entailed simultaneous staining with DAB and FITC. The ProLong Antifade kit proved valuable for retarding photo-bleaching during confocal laser microscopy. Nonetheless, the fluorescent signals from the specimens were substantially diminished after several months of storage at the proper temperature. In this respect, DAB is advantageous since its signal does not decline during storage.

Although DAB and FITC bind to different sites -HRP and biotin, respectively- differential results were obtained

in double-labeling experiments, depending upon the labeling sequence. When nucleotides were first labeled with DAB and then with FITC, strong signals were obtained with each of the labels. In contrast, initial FITC labeling resulted in a comparatively poor DAB signal. One possible explanation for this is that the excited state elicited during observation of fluorescence adversely affects HRP molecules such that subsequent DAB staining is reduced.

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