Summary. The unique cytoarchitecture of glomerular podocytes is conserved in vertebrate evolution. Actin filaments play a crucial role in the formation of the conserved cytoarchitecture, though several isoforms of cytoplasmic actin have been found in vertebrates. The present study examined the expression and subcellular distribution of the \(\beta\)-cytoplasmic actin (\(\beta\)-actin) isoform in the podocytes of six vertebrate species by means of immunohistochemical techniques to reveal whether the \(\beta\)-actin isoform is involved in the formation of podocyte cytoarchitecture throughout vertebrates. \(\beta\)-actin was predominantly localized at the foot processes in carp, turtle, quail, and rat podocytes in addition to actin filament condensations, which were found only in carp and rat podocytes. The actin filament condensations in rats were in direct contact with the basal plasma membrane, but those in carp were found at the cell body and separated from the basal plasma membrane. In contrast with the above four species, \(\beta\)-actin was not detected in podocytes in two amphibians—newt and frog, although podocyte foot processes are actin-filament based cytoplasmic protrusions in these species as well as in other vertebrates. In conclusion, the \(\beta\)-actin isoform is involved in the formation of the podocyte actin cytoskeleton in vertebrates except for amphibians. Several kinds of unconventional cytoplasmic actins other than \(\beta\)- and \(\gamma\)-cytoplasmic actins are known to be expressed in amphibians, making it highly likely that one of these isoforms, instead of \(\beta\)-actin, constructs actin filaments in the foot processes of newt and frog podocytes.

Introduction

Podocytes, an epithelial cell type specialized for glomerular filtration, are found in all vertebrate species except for aglomerular teleost fishes. This cell type consists of three kinds of subcellular compartments: the cell body, the primary processes, and the foot processes (Mundel and Kriz, 1995; Pavenstadt et al., 2003). Podocytes mainly adhere to the glomerular basement membrane (GBM) with their numerous foot processes, which are separated from each other by a filtration slit and bridged with the slit diaphragm. The cell bodies of podocytes are separated from the GBM via the subpodocyte space (Neal et al., 2005), and the primary processes connect the foot processes to the cell body. The basic cytoarchitecture of podocytes is conserved in vertebrate evolution, although podocytes exhibit a wide variety of structural modifications among the classes (Youson and McMillan, 1970; Davis et al., 1976; Schwarz and Radke, 1981; Zuasti et al., 1983; Meseguer et al., 1987; Takahashi-Iwanaga, 2002; Møbjerg et al., 2004; Ojeda et al., 2006).

There is little doubt that the unique morphology of podocytes is structurally supported by the cytoskeleton. In vertebrates, the major cytoskeletal component in the foot processes is actin filaments, which are organized into three kinds of actin cytoskeletons in the processes (Andrews and Bates, 1984; Vasmant et al., 1984; Drenckhahn and Franke, 1988; Ichimura et al., 2003, 2009).
The cortical actin network, which is a meshwork of actin filaments, is an essential actin cytoskeleton for the formation and maintenance of the foot processes. The actin bundle, which is a prominent bundle of actin filaments, is found only in the foot processes of avian and mammalian podocytes. In birds and mammals, the glomerular capillary wall is thinner even though the intraglomerular pressure is higher. The actin bundle therefore contributes to the mechanical protection of the thinner glomerular capillary wall from this higher intraglomerular pressure.

A number of recent studies have investigated the composition and regulation of the podocyte actin cytoskeleton (Faul et al., 2007). In mammals, six kinds of actin isoform have been identified and classified into two cytoplasmic and four muscle types on the basis of their N-terminal amino acid sequence (Khaitlina, 2001). The muscle actin isoforms are specifically expressed in muscle cell types and constitute their contractile apparatus, whereas the cytoplasmic actin isoforms, β- and γ-cytoplasmic actins, are ubiquitously expressed. In non-mammalian species, several kinds of cytoplasmic actin isoforms including β- and γ-cytoplasmic actins were previously reported (Vandekerckhove et al., 1981; Bergsma et al., 1985; Cross et al., 1988; Venkatesh et al., 1996), though little is known about the expression and distribution pattern of these.

The purpose of this study was to reveal whether the β-cytoplasmic actin (β-actin) isoform is involved in the formation of the podocyte cytoarchitecture conserved throughout vertebrates, examining the expression and subcellular distribution of β-actin in the podocytes of six animal species from teleost fish to mammals.

### Materials and Methods

#### Animals

Six kinds of vertebrate, as listed in Table 1, were examined in the present study. The Wistar strain of Norway rats was purchased from Charles River Japan (Yokohama). Reeves’ turtles and Japanese red-bellied newts were obtained from the Reptile Shop (Tokyo). Bullfrogs and common quails came from the Saitama Experimental Animal Supply (Saitama). Common carp were from the San-no-miya Fish Farm (Niigata). The animals were sexually matured except for the turtles (one to two years of age). All animal experiments were carried out in compliance with the Guidelines for Animal Experimentation of Juntendo University.

#### Antibodies

The mouse monoclonal anti-β-actin antibody (clone AC-74) was obtained from Sigma-Aldrich (St. Louis, MO). AC-74 recognizes the N-terminus of the β-actin protein, and the amino acid sequence of this region is highly conserved among vertebrates (Khaitlina, 2001). FITC-conjugated donkey anti-mouse IgG F(ab')₂ fragments, HRP-conjugated donkey anti-mouse IgG F(ab')₂ fragments, and normal goat serum were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The 5-nm-colloidal-gold-conjugated goat anti-mouse IgG F(ab')₂ fragments were from British BioCell (Cardiff, UK).

#### Immunoblot analysis

SDS-PAGE and immunoblot analyses were performed as described previously (Ichimura et al., 2003). In brief,

| Class          | Abbreviated name used | Common name          | Scientific name                  |
|----------------|-----------------------|----------------------|----------------------------------|
| Osteichthyes   | Carp                  | Common carp          | Cyprinus carpio (L.)             |
| Amphibia       | Newt                  | Japanese red-bellied newt | Cynopus pyrrhogaster (Boie)     |
| Frog           | Bullfrog              |                      | Rana catesbeiana (Shaw)         |
| Reptilia       | Turtle                | Reeves' turtle       | Chinemys reevesii (Gray)         |
| Aves           | Quail                 | Common quail         | Coturnix japonica (Temminck et Schlegel) |
| Mammalia       | Rat                   | Norway rat (Wistar strain) | Rattus norvegicus (Berkenhout)   |
knife and mounted on silane-coated glass slides. The sections were washed with PBS, blocked with 0.1% bovine serum albumin (BSA, Fraction V, Sigma-Aldrich) in PBS, and incubated for 2 h at RT with the primary antibody diluted 1:100 with 1% BSA in PBS. After washing with PBS containing 0.1% BSA, the sections were incubated for 1 h at room temperature (RT). Bound antibodies were detected by the ECL Western blotting detection system (Amersham Biosciences, Arlington Heights, IL).

**Immunofluorescence microscopy**

Animals were perfused with physiological saline and subsequently a 2.5% glutaraldehyde (GA) fixative buffered with 0.1 M phosphate buffer (PB, pH 7.4) under anesthesia. The perfused kidneys were cut into small blocks and immersed in the same fixative for several days. The blocks were cut into 250-μm-thick sections with a DTK-1000 Microslicer (Dosaka EM, Kyoto), and the sections were processed by a modified cold dehydration method. This method enabled the detailed morphological observation of the actin cytoskeleton (Sakai and Kriz, 1987; Elger et al., 1998). In brief, the samples were successively immersed in 0.4% OsO4 in 0.1 M PB for 1 h, 2% low molecular weight tannic acid (Electron Microscopy Sciences, Hatfield, PA) in a 0.05 M maleate buffer for 3 h, and 1% uranyl acetate in a 0.05 M maleate buffer for 3 h. The samples were then dehydrated with a graded series of acetone at 0°C to −30°C before embedment in Epon 812. Ultrathin silver-gold sections were processed with a diamond knife, transferred to copper grids (50 mesh) which had been coated with a Formvar membrane, stained with a uranyl acetate and lead citrate, and observed with a JEM1230 transmission electron microscope (JEOL, Tokyo).

**Immunogold electron microscopy**

Ultrathin gold sections of the LR-white-resin-embedded samples were produced with a diamond knife, and then transferred to nickel grids (150 mesh) which had been coated with a Formvar membrane. After blocking with 1% normal goat serum in PBS, the sections were
Fig. 2. Legend on the opposite page.
Fig. 2. Immunofluorescence labeling for β-actin in the glomeruli from the carp (A), turtle (B), quail (C), and rat (D). In these four species, the labeling for β-actin is found predominantly at the foot processes (A', B, C, and D). The labeling for β-actin is also found along the surface membrane of the cell body (arrowheads in A, B, C', and D). CB: cell body of podocyte, CL: capillary lumen, EC: glomerular endothelial cell, GBM: glomerular basement membrane, N: nucleus of podocyte, PP: primary process, US: urinary space. Bars: 200 nm

Fig. 3. Immunogold labeling for β-actin in the podocytes from the carp (A and A'), turtle (B), quail (C, and C'), and rat (D). In these four species, the labeling for β-actin is found predominantly at the foot processes (A', B, C, and D). The labeling for β-actin is also found along the surface membrane of the cell body (arrowheads in A, B, C', and D). CB: cell body of podocyte, CL: capillary lumen, EC: glomerular endothelial cell, GBM: glomerular basement membrane, N: nucleus of podocyte, PP: primary process, US: urinary space. Bars: 200 nm
for 5 min, and observed with a JEM1230. The primary antibodies were omitted from the incubation solution as a negative control, and no non-specific staining of the secondary antibody was found in the kidney sections.

incubated overnight with the anti-β-actin antibody (1:50) diluted with 1% BSA in PBS for 12 h at 4°C. They were subsequently incubated with colloidal-gold-conjugated secondary antibodies diluted 1:100 with 1% BSA in PBS for 1 h at RT, contrasted with 4% uranyl acetate for 5 min, and observed with a JEM1230. The primary antibodies were omitted from the incubation solution as a negative control, and no non-specific staining of the secondary antibody was found in the kidney sections.
Results

Expression of β-actin protein in kidney

Immunoblot analysis showed that two protein bands immunoreactive to the anti-β-actin antibody appeared at molecular masses of ~42 kDa in kidney samples from the six vertebrate species (Fig. 1). The higher protein bands was considered to represent a complex of β-actin and a certain actin-associated protein. The anti-β-actin antibody easily detected ~42-kDa protein bands in the carp, turtle, quail, and rat (Fig. 1A), but in two amphibians, the newt and frog, the antibody required three times the amount of protein for the detection of similar ~42 kDa protein bands (Fig. 1B). These findings indicated that the expression level of β-actin protein was much lower in the newt and frog kidneys than in those of the other species.

Localization of β-actin in glomerulus

Localization of β-actin in the glomerulus was quite different between the two amphibians (newt and frog) and the four non-amphibian species (carp, turtle, quail, and rat).

Carp, turtle, quail, and rat

In the glomerulus, a prominent immunofluorescence signal for β-actin was recognized along the glomerular capillary wall as a linear signal (Fig. 2A–D). A similar linear signal is also found in the immunofluorescence labeling for the proteins which are predominantly localized at the foot processes, e.g. ZO-1 and nephrin (Schnabel et al., 1990; Kurihara et al., 1992; Hirabayashi et al., 2005), suggesting that β-actin is mainly localized at the foot processes of podocytes. As expected, immunogold labeling for β-actin was predominantly found at the foot processes (Fig. 3A’, B, C, D).

A faint immunofluorescence signal for β-actin also appeared along the margin of the podocyte cell body (arrowheads in Fig. 2A–D), and immunogold labeling for β-actin was not found in the podocyte foot processes but is intensely found at the mesangial cell processes (M). Small numbers of immunogold particles are also found at the glomerular endothelial cells (arrowheads in A and B). CL: capillary lumen, GBM: glomerular basement membrane, PP: primary process, SES: subendothelial space, US: urinary space. Bars: 200 nm
Fig. 6. Diagram showing the distribution of $\beta$-actin (green dots) in glomeruli. A: Rat. $\beta$-actin is predominantly localized at the foot processes and AFC. The distribution pattern of $\beta$-actin is common to that of the carp, turtle, and quail. B: Newt and frog. $\beta$-actin is not detected in the podocytes of two amphibians species—the newt and frog. Instead of $\beta$-actin, other type(s) of cytoplasmic actin isoforms (blue dots) may be predominantly localized at the foot processes of newt and frog podocytes. AFC: actin filament condensations, EC: glomerular endothelial cell, M: mesangial cell, MP: mesangial cell process, SES: subendothelial space, P: podocyte.

$\beta$-actin was found beneath the apical surface membrane of the cell body and primary processes (arrowheads in Fig. 3A, B, C, D). $\beta$-actin molecules beneath the surface plasma membrane presumably correspond to the actin filament meshwork of the cell cortex.

Electron dense condensations of actin filaments (AFC) were found only in rat and carp podocytes (Fig. 4). Immunogold labeling for $\beta$-actin was densely found in the AFC of both rat and carp podocytes (asterisks in Fig. 4C, F), but their relative position to the GBM differed between the rat and carp. In rat podocytes, the AFC were mainly found in the irregularly-shaped thick processes which occupied the space between two adjacent capillary loops; they were in direct contact with the basal plasma membrane (Fig. 4A–C). On the other hand, in carp podocytes, the AFC were found mainly in the cell body and were separated from the basal plasma membrane (Fig. 4D–F).

In the mesangial region, a prominent immunofluorescence signal for $\beta$-actin was also recognized in the carp, quail, and rat (Fig. 2A, C, D), as well as in the turtle (data not shown). Immunogold labeling for $\beta$-actin was also found in the mesangial cell processes and the glomerular endothelial cells (data not shown).

Newt and frog

The glomerular capillary wall was much thicker in the newt and frog than in the above four species since the mesangium spreads between the endothelial cell layer and the GBM to form a conspicuous subendothelial space in amphibians, as shown by previous studies (Schaffner and Rodewald, 1978; Sakai and Kawahara, 1983; Sakai et al., 1988; Meseguer et al., 1996).

In the newt, immunofluorescence signals for $\beta$-actin were recognized along the glomerular capillary wall as discontinuous lines (Fig. 2E), while in frog, $\beta$-actin signals were recognized as numerous dots (Fig. 2F). The localization patterns of $\beta$-actin were reminiscent of that of an actin-binding protein $\alpha$-actinin, which is localized in the mesangial cells of the newt and frog (Ichimura et al., 2007).

In both the newt and frog, immunogold labeling for $\beta$-actin was predominantly found at the mesangial cell processes which existed in the mesangium, including the subendothelial space (Fig. 5). Labeling for $\beta$-actin was also found in the glomerular endothelial cells (arrowheads in Fig. 5). Only scarce labeling—or none at all—for $\beta$-actin was found in the podocyte cytoplasm, including the foot processes (Fig. 5).
**Discussion**

The present study showed the distribution of $\beta$-actin in podocytes from teleost fish to mammals (summarized in Fig. 6). $\beta$-actin was predominantly localized at the foot processes in the carp, turtle, quail, and rat in addition to the AFC in the carp and rat. In contrast, no $\beta$-actin protein was detected in newt and frog podocytes.

Immunoblot analysis confirmed that the anti-$\beta$-actin antibody used in this study recognized $\sim$42-kDa protein bands—which correspond to actin—in the kidney samples from all of the six vertebrate species examined. We thus consider that this antibody is able to cross-react with the $\beta$-actin of newts and frogs, and that their podocytes either do not express $\beta$-actin or its expression is below the minimum detectable quantity. However, our previous study showed that the foot processes of podocytes are rich in actin filaments in the newt and frog as well as in other vertebrates (Ichimura et al., 2007). These findings indicate that other kinds of actin isoforms, instead of $\beta$-actin, are predominantly localized in the foot processes of newt and frog podocytes. Vandekerckhove and colleagues (1981) reported that several amphibian species possess four kinds of novel cytoplasmic actin isoforms (types 3, 4, 5, and 7 acts) in addition to $\beta$- and $\gamma$-cytoplasmic actin isoforms (type 1 and type 8 acts), by means of an amino acid sequencing technique. In newt and frog podocytes, one of these isoforms other than $\beta$-actin (type 1 actin) is almost certain to construct the actin filaments of foot processes. Newts and frogs are representative species of urodel and anuran amphibians, so this characteristic phenomenon is likely to be common to many kinds of amphibians.

Functional and biochemical properties are believed to differ among the cytoplasmic actin isoforms (Khaitlina, 2001). Unlike podocytes, mesangial and glomerular endothelial cells expressed $\beta$-actin in all of the vertebrate species examined, suggesting that the biochemical properties of $\beta$-actin are important for the maintenance of the structures and functions in the two kinds of glomerular cells. Karakozova et al. (2006) reported that approximately 40% of $\beta$-actin molecules are posttranslationally modified by N-terminal arginylation in mouse embryonic fibroblasts and that such arginylation is not found on $\gamma$-cytoplasmic actin. The N-terminal arginine of $\beta$-actin provides positive charges onto the surface of the actin filament, and the positive charges are thought to be involved in specialized biochemical properties of $\beta$-actin (Kashina, 2006).

The function of AFC presumably differs between carp and rat podocytes, judging from its localization. In the rat, the AFC connect the GBM of adjacent capillary loops, and this connection is believed to stabilize the folding pattern of the GBM (Kriz et al., 1995; Kriz and Endlich 2005). Such a type of AFC was only found in rat podocytes among the six vertebrate species examined and was considered to play a role in the formation and maintenance of the mammalian-type glomerulus, which exhibits a highly folded pattern of the GBM enclosing well-developed capillary loops and an arborized mesangium. On the other hand, the AFC of carp podocytes was mainly found in the cell body, and was not linked to the GBM. They are therefore not likely to contribute to any mechanical function forming the glomerular architecture.

In conclusion, the basic cytoarchitecture of podocytes is highly conserved in vertebrate evolution, and $\beta$-actin is involved in the formation of the podocyte actin cytoskeleton in vertebrates except for amphibians. Several isoforms of unconventional cytoplasmic actins are expressed in amphibians, and one of these isoforms presumably constructs the actin cytoskeleton in amphibian podocytes.

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