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EXPRESSION OF CELLULAR RETINOIC ACID BINDING PROTEIN II (CHICK-CRABP II) IN THE CHICK EMBRYO

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We previously demonstrated the presence of cellular retinoic acid binding protein II, chick-CRABP II, in chick embryos. In the present study, we investigated the distribution of chick-CRABP II in 14-day chick embryos by means of immunoblot analysis. Chick-CRABP II was expressed in skin, muscle, bone with tendon of the embryos, but not expressed in the nervous system. In adult chick tissues, chick-CRABP II was not detected on immunoblotting; Chick-CRABP II in adults amounts to less than 10 ng/mg soluble protein. These observations suggest that chick- CRABP II is an embryonic protein involved in the development of specific tissues, such as bone, muscle and skin.

Recently, retinoic acid has been attracting increasing attention because of its striking effects on the pattern formation in developing and regenerating limbs, and it was identified as a natural morphogen (1-4). Cellular retinoic acid binding protein (CRABP), which is considered to transfer retinoic acid into nuclei (5,6), is abundantly present in chick limb buds during early developmental stages (7). Spatial expression of CRABP has been demonstrated in chick limb buds during stages 23-25, i.e., when the digit pattern is determined in limb buds (8). Very recently, we found that there are two types of CRABP (CRABPs I and II) in chick 14-day embryos (9); CRABP I, the major CRABP, is expressed in adult testis, and CRABP II, a novel form that shows more than 83 % homology to the N-terminal region of CRABP I, is expressed in the embryonal stages, but not in adult testis. Bailey et al. also discovered a novel retinoic acid binding protein in neonatal rat and named it as

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CRABP II (10). Since rat neonatal CRABP II is entirely different molecule from chick CRABP II (9), we referred to our CRABP II as chick-CRABP II in the present study.

Here we demonstrate that chick-CRABP II is specifically expressed in skin, muscle and bone of 14-day old chick embryos.

MATERIALS AND METHODS

Preparation of a crude extract from chick embryos

Chick embryos and adult chickens (White Leghorn) were sacrificed and each tissue was excised. Skin and muscle were obtained from the chest. They were rinsed with ice-cold phosphate-buffered saline and the homogenized with 2 volumes of 10 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl and 1 mM phenylmethylsulfonyl fluoride. Bone was crushed before being homogenized. The homogenates were centrifuged at 100,000 x g for 1 hr at 4 ^{o}C. The supernatants were stored at -80 ^{o}C until the analysis.

Immunoblot analysis

Chick CRABPs I and II were purified from 14-day embryos as described previously (9). The antiserum against porcine testis CRABP (anti-CRABP), which specifically reacts with CRABP, i.e., with neither cellular retinol binding protein (CRBP) nor bovine P2 protein (11), was used for the detection of chick-CRABPs I and II. The antiserum against chick embryonal CRABP II (anti-CRABP II) was raised in New Zealand White rabbits.

Proteins of each tissue of chick embryos and adult chicks were subjected to SDS polyacrylamide gel electrophoresis using 15% gels, as described by Laemmli (12). For immunological detection of CRABP, the proteins in the gels were transferred to nitrocellulose sheets according to the method described by Towbin et al.(13). The sheets were incubated with blocking buffer (0.5 % skim milk, 50 mM Tris-HCl (pH 7.5), 0.125 M NaCl) for 30 min and then incubated with 500 times diluted anti-CRABP II serum or anti-CRABP serum in the blocking buffer for 1 hr. After the sheets had been washed with the blocking buffer, the immunoreactivity on the sheets were examined with peroxidase-conjugated protein A and 4-chloro-1-naphthol as reagents.

Protein estimation

Protein concentrations were estimated with a Bio Rad Protein Assay kit with chicken ovalbumin as a standard.

RESULTS

Specificities of antisera

The antisera raised against porcine testis CRABP (anti-CRABP) and chick embryo CRABP II (anti-CRABP II) were tested as to their specificities for chick-CRABPs I and II by immunoblot analysis. The anti-CRABP reacted with both chick-CRABPs I and II. The reactivity with chick-CRABP II was a little weaker than that with chick-CRABP I (Figure 1B). On the other hand, the anti-CRABP II specifically reacted with chick-CRABP II, i.e., not

with chick-CRABP I (Figure 1C). Thus, the anti-CRABP II was suitable for the detection of CRABP II in chick tissues, while the anti-CRABP was suitable for detecting both chick-CRABPs I and II.

Distribution of CRABP II in embryonic tissues

The presence of chick-CRABP II in embryonic tissues was investigated by immunoblot analysis. CRABP was detected in most tissues, including brain, eye, spinal cord, skin, muscle, bone

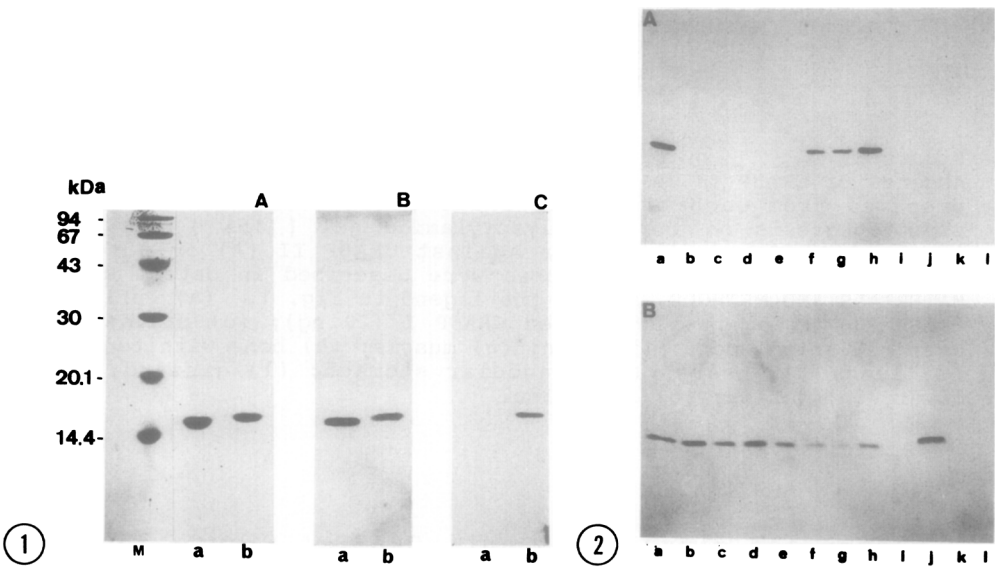

Figure 1.
Specificities of antisera. (A) 5 ug each of purified CRABP I (a) and CRABP II (b) was subjected to electrophoresis on an SDS-polyacrylamide gel (15%), and protein bands were stained with Coomassie brilliant blue. Lane M contains the molecular weight standards; phosphorylase b (94 kDa), serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa). (B), (C) 50 ng each of purified CRABP I (a) and CRABP II (b) was subjected to electrophoresis on an SDS-polyacrylamide gel (15%) and then proteins were transferred to nitrocellulose membranes according to the method described by Towbin et al. (12). Immunostaining was performed with antiserum against porcine testis CRABP (B) and antiserum against CRABP II (C). Peroxidase conjugated protein A, 4-chloro-1-naphthol and hydrogen peroxide were used for the color development.

Figure 2.
Distribution of CRABP II in tissues of 14-day chick embryos. 40 μg of soluble protein prepared from each tissue of 14-day chick embryos was subjected to electrophoresis on an SDS-polyacrylamide gel (15%) and then immunoblotted using antisera against CRABP II (A) or porcine testis CRABP (B). The procedures were described in detail under MATERIALS AND METHODS, and in the legend to Fig. 1. (a) Purified CRABP II (20 ng); (b) purified CRABP I (20 ng); (c) brain; (d) eye; (e) spinal cord; (f) skin; (g) muscle; (h) bone with tendon; (i) glandular stomach; (j) gizzard; (k) heart; (l) liver.

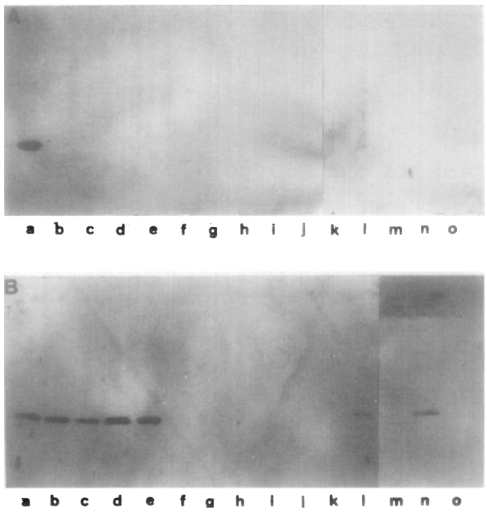

Figure 3.
Absence of CRABP II in adult tissues. 100 μg of soluble protein prepared from each tissue of adult chicken was subjected to electrophoresis on an SDS-polyacrylamide gel (15%) and then immunoblotted using antisera against CRABP II (A) or porcine testis CRABP (B). The procedures were described in detail under MATERIALS AND METHODS, and in the legend to Fig. 1. (a) Purified CRABP II (20 ng); (b) purified CRABP I (20 ng); (c) brain; (d) eye; (e) spinal cord; (f) skin; (g) muscle; (h) bone with tendon; (i) lung; (j) heart; (k) glandular stomach; (l) gizzard; (m) liver; (n) testis; (o) intestine.

with tendon and gizzard, by anti-CRABP (Figure 2B). The expression of chick-CRABP II was more restricted. Chick-CRABP II was not detected in either the nervous system or the internal organs. Among the tissues, chick-CRABP II was detected most abundantly in bone with tendon, that in muscle and skin being next in quantity (Figure 2A).

CRABP II was not detected in adult tissues

The expression of chick-CRABP II in adult tissues was investigated by immunoblotting. Using anti-CRABP, CRABP was clearly detected in brain, eye, spinal cord and testis (Figure 3B), while chick-CRABP II was not detected in any of the tissues analyzed (Figure 3A).

DISCUSSION

In this study, we prepared antiserum to a novel CRABP (CRABP II), which specifically recognizes chick-CRABP II, i.e., not chick-CRABP I. This clearly showed that chick-CRABP II has distinct antigenic determinants from those of chick-CRABP I,

though they share some antigenic determinants, since the anti-CRABP recognized both chick-CRABPs I and II.

Using this antibody, we could not detect chick-CRABP II in adult tissues including brain, eye, spinal cord and testis, in which CRABP was detected with the anti-CRABP antiserum. Since 20 ng of purified chick-CRABP II was applied to lane(a) in Fig. 3-A and about 1 ng of chick-CRABP II could be detected with this system (data not shown), the chick-CRABP II content in an adult was estimated to be not more than 1 ng/100 μg protein, that is, 10 ng/mg protein. In contrast to the case of adults, 14-day embryos contained abundant chick-CRABP II. At this stage, the expression of chick-CRABP II is restricted to skin, muscle and bone with tendon, no being expressed in the central nervous system or the internal organs.

Recently, we demonstrated that a large amount of CRABP is expressed in the central nervous system as well as limb buds of chick embryos (14,15). In that case, anti-CRABP, which was shown to cross react with chick-CRABPs I and II, was used for the detection of CRABP. Therefore, the CRABP in the central nervous system seems to be chick-CRABP I, not chick-CRABP II. On the other hand, chick embryo skin and tendon were already known to contain large amounts of CRABP (16,17). Our data suggest that the CRABP detected in these tissues of chick embryos is CRABP II, not CRABP I. Thus, two types of CRABP, chick-CRABPs I and II, may exhibit distinct distributions during development. Chick-CRABPs I and II may be involved in the development of the tissues in which they are localized.

Era-1 gene, which has been identified as Hox-1.6 vertebrate homeo box gene (18), is rapidly induced in F9 teratocarcinoma cells upon retinoic acid treatment in vitro (19). Expression of homeo box genes are specifically restricted to the central nervous system and limb bud during early development (20). Homeo-box genes are expressed in patterns that are at least consistent with the position-dependent expression of CRABPs in the central nervous system (14, 15) and the limb bud (8) in chick embryo. Retinoic acid may regulate the expression of homeo box genes and have diverse effects on the morphogenesis via these chick- CRABPs I and II during development.

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