Parvalbumin Isoforms in Chicken Muscle and Thymus. Amino Acid Sequence Analysis of Muscle Parvalbumin by Tandem Mass Spectrometry†,‡

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ABSTRACT: Parvalbumins are high-affinity Ca^{2+}-binding proteins characterized by an EF-hand structure. Muscles of lower vertebrates contain up to five isoparvalbumins whereas higher vertebrates were believed to contain only one isoform per species. Recently Brewer et al. [Brewer, J. M., Wunderlich, J. K., & Ragland, W. (1990) *Biochimie 72*, 653–660] purified and sequenced a protein that they named avian thymic hormone, from chicken thymus. This protein, promoting immunological maturation of bone marrow cells in culture, was identified as a parvalbumin. The amino acid composition of this thymic parvalbumin was, however, considerably different from those of chicken muscle parvalbumin [Strehler, E. E., Eppenberger, H. M., & Heizman, C. W. (1977) *FEBS Lett. 75*, 127–133], suggesting the existence of two tissue-specific parvalbumins in chicken. We purified parvalbumin from chicken muscle, determined its complete amino acid sequence by tandem mass spectrometry, and showed that this protein is rather homologous to muscle parvalbumins from other species but different in 45 positions from the thymic parvalbumin. We discuss the possibility that a parvalbumin gene family might exist in higher vertebrates, expressed in a tissue-specific and developmentally regulated manner.

Parvalbumins are high-affinity Ca^{2+}-binding proteins that contain an EF-hand structural motif (Kretsinger et al., 1991; Heizmann & Hunziker, 1991). They were first isolated from muscles of lower vertebrates, and their primary structures and biochemical properties are well documented [for reviews see Wnuk et al. (1982) and Gerday (1988)]. Muscles of lower vertebrates were found to contain up to five different isoparvalbumins. In higher vertebrates (chicken, mouse, rat, cat) and in humans, however, only one isoform per species has been detected so far (Heizmann, 1984; Heizmann & Berchtold, 1987; Berchtold, 1989a; Heizmann & Braun, 1990). Some evidence has accumulated in the past years that there might be more than one parvalbumin in higher vertebrates, and this view is mainly based on the following observations. In the course of the purification of parvalbumin from chicken muscle, several other parvalbumin-like proteins were also detected but they were not further characterized (Strehler et al., 1977; Heizmann et al., 1977; Heizmann & Strehler, 1979). A stronger indication that there might be more than one parvalbumin protein in chicken comes from more recent observations by Brewer et al. (1989, 1990) and Palmisano and Henzl (1990). They isolated a protein named avian thymic hormone (ATH) from chicken thymus. This protein was shown to promote immunologic maturation of chicken bone marrow cells in culture. ATH was sequenced and shown to be a parvalbumin. However, the amino acid composition of ATH, derived from its sequence (Brewer et al., 1990), was found to be significantly different from that reported from chicken leg muscle parvalbumin (Strehler et al., 1977), suggesting the existence of two tissue-specific parvalbumins in chicken.

To investigate this possibility, we purified parvalbumin from chicken muscle and determined its complete amino acid sequence by tandem mass spectrometry and Edman degradation. Chicken muscle parvalbumin was found to be rather homologous to other muscle parvalbumins (e.g., those from mouse, rat, and human) but considerably different from chicken thymus parvalbumin. We discuss the possibility that a parvalbumin gene family might exist in higher vertebrates, expressed in a tissue-specific and developmentally regulated manner. Similar results have already been obtained for other Ca^{2+}-binding proteins such as the S-100 protein family, calbindin-D_{28K}, calretinin, and calpains as well as visinin and frequinin [for a review see Heizmann and Hunziker (1991)].

Materials and Methods

Purification of Chicken Muscle Parvalbumin. Parvalbumin was isolated from chicken leg muscles as described previously (Strehler et al., 1977) and then purified on a reversed-phase column (RP-300/CO3-GU, Brownlee Labs, 30 × 4.6 mm, 7 μm) with the use of the HPLC system (625-LC) of Waters. A gradient system was used: buffer A (25 mM Tris, 1 mM EGTA, pH 7.5) and buffer B (same buffer as buffer A but with 60% acetonitrile). Flow rates of 1 mL/min at room temperature were standard. Muscle parvalbumin eluted as a single peak at 49% buffer B, migrated as a single band on 1D PAGE (M_r 12000; p*I* 5.2) and on an isoelectric focusing gel (Immobiline, Pharmacia).

Proteolytic Cleavage Reactions. Trypsin: Chicken muscle parvalbumin (25 μg; 2 nmol) was dissolved in 150 μL of freshly prepared 50 mM ammonium bicarbonate containing 1 mM ethylene glycol bis(β-aminoethyl)-*N,N,N′,N′*-tetraacetic acid (EGTA) and digested with 1 μg of bovine TPCK–trypsin (Fluka, Switzerland) at 37 °C for 4 h. Proteolysis was terminated by the addition of acetic acid. The resulting mixture was concentrated to ca. 50 μL by Speed-Vac centrifuge and

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‡ The accession number P80026 for chicken muscle parvalbumin is included in the Swiss-PROT protein sequence data base, the MBL network file-server, and the PIR international protein sequence data bases.

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Table I: Peptide Fragments from Chicken Muscle Parvalbumin Characterized by Tandem Mass Spectrometry

peptide no.	cleavage method	HPLC fraction no.	$(M + H)^+$ nominal mass	residue[a] no.	sequence[b]
1	trypsin	T 54	1334	1–12	AcAMTDVXSAEDXK
2	trypsin	T 46	1589	14–28	AVGAFSAAESFNYKK
3	trypsin	T 55	970	29–36	FFEMVGXK
4	trypsin	T 28	674	39–44	SPEDVK
5	trypsin	T 45	871	46–52	VFHXXDK
6	trypsin	T 42	1142	46–54	VFHXXDKDR
7	trypsin	T 44	1180	55–64	SGFXEEEEXK
8	trypsin	T 38	506	65–68	FVXK
9	trypsin	T 33	749	69–75	GFTPDGR
10	trypsin	T 29	935	76–83	DXSDKETK
11	trypsin	T 33	1230	84–96	AXXAAGDKDGDGK
12	trypsin	T 53	1340	97–109	XGADEFATMVAES
13	Glu-C	E 14	978	1–9	AcAMTDVXSAE
14	Glu-C	E 12	1306	10–22	DXKKAVGAFSAAE
15	Glu-C	E 18	1209	23–31	(SFNYKKFFE)
16	Glu-C	E 9	1116	32–41	MVGXKKKSPE
17	Glu-C	E 18	2532	42–62	[DVKKVFHILDKDRSGFIEEEE]
18	Glu-C	E 21	2164	63–81	[LKFVLKGFTPDGRDLSDKE]
19	Glu-C	E 10	1944	82–101	[TKALLAAGDKDGDGKIGADE]
20	Glu-C	E 10	855	102–109	FATMVAES

[a] Peptides containing residues marked X were subjected to Edman degradation to differentiate between Ile and Leu. [b] Sequences displayed in brackets were determined by a combination of collision-activated dissociation and Edman degradation (see Discussion in text). The peptide set in parentheses, corresponding to the Edman sequence, showed a weak FAB signal, insufficient for collision-activated dissociation.

fractionated by HPLC (system A, see below).

Endoproteinase Glu-C: Chicken muscle parvalbumin (25 μg, 2 nmol) dissolved in 100 μL of 50 mM ammonium bicarbonate containing 1 mM EGTA and 1 μg of endoproteinase Glu-C from *Staphylococcus aureus* V8 (Boehringer, Mannheim, FRG) was allowed to proceed at 37 °C for 15 h. Digestion was stopped by freezing the reaction mixture at −70 °C; separation of the oligopeptide products was performed by HPLC (system B, see below).

High-Performance Liquid Chromatography. System A: Fractionation of tryptic peptides was performed on an Applied Biosystems Model 130A separation system. The sample was injected onto a microbore RP300 Aquabore column (1 mm × 25 cm), and the peptides were eluted with a 120-min linear gradient of 0–70% acetonitrile. Solvent A: 0.1% trifluoroacetic acid (TFA). Solvent B: 70% acetonitrile in 0.08% TFA. The column effluent was monitored at 214 nm, and 1-min fractions were collected automatically.

System B: Separation of Glu-C-digested peptides was performed on a Waters 625 LC system. The sample (2 nmol in 150 μL) was injected directly onto a C8 reversed phase column (Nucleosil 300-7; 25 cm × 4 mm) and eluted with a 60-min linear gradient from 0.1% TFA to 60% acetonitrile in 0.1% TFA. The column effluent was monitored at 220 nm, and fractions were collected manually. The solvent was then evaporated by Speed-Vac centrifuge.

Methyl Ester Formation. A 2 N HCl in methanol mixture was prepared by slowly adding 160 μL of acetyl chloride under stirring to 1 mL of methanol (kept on ice). Aliquots of dissolved HPLC fractions were evaporated to dryness and mixed with 25 μL of 2 N HCl in methanol. Conversion to methyl esters was allowed to proceed for 2 h at room temperature, and then the solvent was removed by Speed-Vac and redissolved in 5 μL of 0.1% TFA. Aliquots of 0.5–1.0 μL were used for mass analysis on the triple-quadrupole instrument.

Mass Spectrometry. Mass spectra were recorded on a TSQ-70 triple-quadrupole instrument (Finnigan-MAT, San Jose, CA) equipped with a cesium ion gun (Antek, Palo Alto, CA). The methodology for sequence analysis of peptides by collision-activated dissociation was performed according to Hunt et al. (1986, 1989) and Gabrielides et al. (1991), with minor modifications. Collision energies varied from 10 eV (mass 500) to 30 eV (mass 2600); the collision gas was argon at a pressure of 2 m torr. The samples for mass analysis on the triple-quadrupole instrument were prepared by dissolving the HPLC fractions in 5–15 μL of 0.1% TFA. A 0.5–1.0-μL aliquot of these solutions, containing peptides in concentrations of 20–200 pM levels, was added to 1 μL of thioglycerol on a gold-plated stainless steel probe with a 2-mm diameter. Peptides were ionized and transferred to the gas phase by bombarding the sample matrix with 5–10-keV Cs^+ particles.

NH_2-Terminal Sequence Determination. This was carried out with a pulse liquid-phase microsequencer, model 477A, from Applied Biosystems (Foster City, CA).

Results

In order to determine if muscle parvalbumin is different from thymus parvalbumin, we sequenced the muscle protein by tandem mass spectrometry. Direct sequences of the intact muscle parvalbumin by Edman degradation failed, indicating a possibly blocked N-terminus. Therefore, the protein was digested with either trypsin or endoproteinase Glu-C, and after fractionation on reversed-phase HPLC the peptides were sequenced by tandem mass spectrometry and interpreted by hand. Table I lists the peptides sequenced by tandem mass spectrometry. All fractions were analyzed in native form. In addition, they were converted to their respective methyl esters. This results in a mass shift of 14 for each acidic residue and allows the determination and localization of aspartic and glutamic acid and greatly simplifies the assignment of the various b- and y-ions (Roepstorff et al., 1984; Biemann, 1988). The Xs in the sequences indicate the respective residue to be either Leu or Ile. Since these two amino acids have identical masses they cannot be distinguished by low collision energy tandem mass spectrometry. Therefore, all peptides containing an X were further subjected to Edman degradation to determine the respective amino acids. The X in peptide 13 has been determined by cyanogen bromide cleavage prior to Edman degradation.

The assignment of the N-terminal residue as acetyl-Ala rather than Leu or Ile was effected by acetylation of the tryptic peptide (methanol/acetic anhydride 3:1 and a trace of pyridine for 1 min on the tip). An $(M + H)^+$ ion shift to a higher mass by 42 mass units was observed, consistent with the introduction

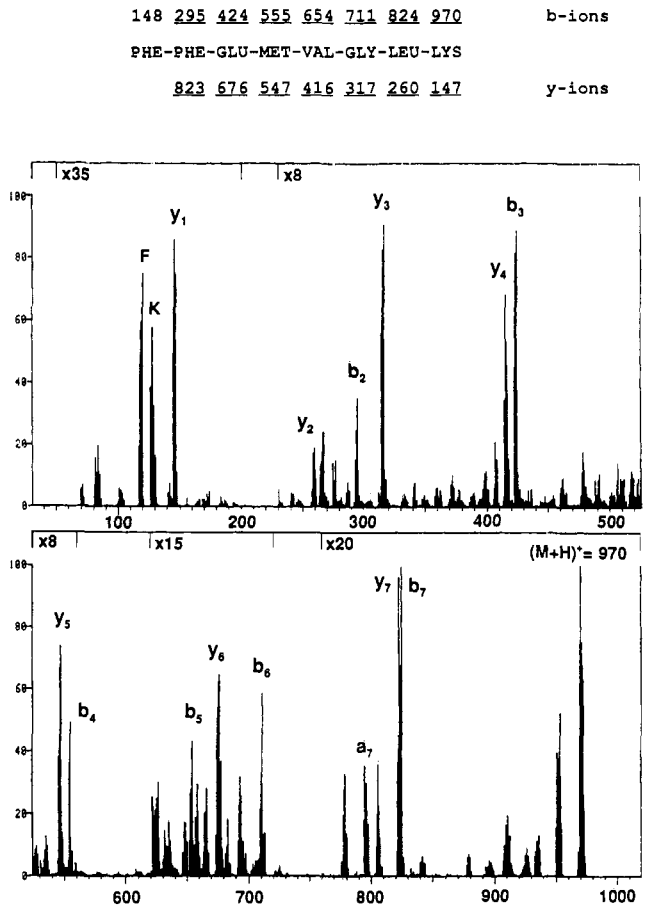

FIGURE 1: Collision-activated dissociation mass spectrum recorded on the $(M + H)^+$ ion of peptide 3 from chicken muscle parvalbumin.

of one acetyl group. The peptide therefore contains a single amino group, a condition that is fulfilled by the side chain of the C-terminal lysine.

Additional support for the acetyl-ala N-terminus was obtained by treating the smaller Glu-C fragment 13 (Table I) in a similar way. Here no mass 42 shift was observed, in agreement with the absence of free amino groups.

All other parvalbumins were also found to be acetylated at the N-terminus. This was also suggested, however not proven, for chicken thymus parvalbumin, with the postulated N-terminal tetrapeptide (acetyl)-Ala-Ile-Thr-Asp-COOH (Brewer et al., 1990).

Peptides 16–18 (Table I) exhibit relatively high molecular weights; therefore, it was not possible to fully sequence them by mass spectrometry. Since all of them contained an X they were subjected to Edman degradation for sequence information. An alternative approach would be cleavage of these larger peptides into smaller fractions that could be sequenced then by mass spectrometry. Figure 1 shows the collision-activated dissociation mass spectrum of the $(M + H)^+$ ion originating from peptide 3 (Table I). Masses for fragment ions of the b- and y-type predicted for the sequence FFEMVGXK are given above and below the proposed structure on Figure 1, and the respective peaks in the spectrum are marked by b and y.

The amino acid sequence of chicken muscle parvalbumin is compared to chicken thymus parvalbumin and to all other known mammalian parvalbumins, as well as to fish and *Xenopus* parvalbumin and to oncomodulin (Figure 2).

Chicken muscle parvalbumin is homologous to other mammalian parvalbumins. For example, chicken muscle parvalbumin differs only in 23 positions from human parvalbumin, in 25 from rat, in 20 from rabbit, and in 25 from mouse parvalbumins, but in 45 positions from chicken thymus parvalbumin. Chicken thymus parvalbumin (as parvalbumins from carp *Xenopus* and rat oncomodulin) contains only 108 amino acid residues whereas chicken muscle parvalbumin and mammalian parvalbumins contain 109 residues.

Parvalbumins are three-domain Ca^{2+}-binding proteins. They diverged probably late during evolution from a four-domain ancestor protein by deletion of the N-terminal domain. The first Ca^{2+}-binding loop (19–28) has lost its Ca^{2+}-binding capacity due to deletion of two amino acids (Kretsinger et al., 1991; Heizmann & Hunziker, 1991). This region is quite conserved between chicken muscle and thymus parvalbumins; however, it shows some differences from the other parvalbumins. As expected, a strong conservation is observed in the

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                        10         20         30         40         50
                        .          .          .          .          .
chicken muscle      AMTDVLSAED IKKAVGAFSA AESFNYKKFF EMVGLKKKSP EDVKKVFHIL
chicken thymus[a]    I  I   K  ES LSSCQ   D     S   ST   SS T  DQI    G
human[b]            S   L N              TD  DH     Q        A D       M
rat[c]              S   L          I   T  D  DH     Q        A D
rabbit[d]              EL N        I   A     DH     Q        T
mouse[e]            S              I   A  D  DH     Q        N P D
carp[f]              FAG  NDA   AA LE CK   D   H A   AK   TS  A D    A A I
Xenopus[g]           FGGI  EA   SA LQNCQ   D   F T   AQS  SS  A D   N  A
rat om[h]           SI  I       AA LQECQD PDT EPQ    QTS  S M A SQ  DI RFI

                        60         70         80         90         100
                        .          .          .          .          .
chicken muscle      DKDRSGFIEE EELKFVLKGF TPDGRDLSDK ETKALLAAGD KDGDGKIGAD EFATMVAES
chicken thymus       Q K         QLF  N  SSSA V TSA     F       T       VE   QSL KA-
human                  K       D  G I    S  A    A      M M             V    S L
rat                    K       D  GSI    SS A    A      T M             VE   S L
rabbit                 K          G I    S  A    V      T M                  S L S
mouse                  K       D  GSI    SS A    A      T                VE   S L   T
carp                 Q K       D    LF QN  KA A A T G    TF K    S       V    TAL KA-
Xenopus              Q           AF QN  SASA A T A     F      S       VE   QSL KP-
rat om               N Q  YLDG D   YF QK  QS A E TES    S MD A  N            QE  HS-

                     X Y Z-X-Y  -Z                          X  Y Z-X-Y  -Z
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FIGURE 2: Amino acid sequence data obtained on chicken muscle parvalbumin by tandem mass spectrometry and Edman sequencing. Alignment with chicken thymus parvalbumin, other mammalian parvalbumins, carp 4.25, *Xenopus* parvalbumin, and rat oncomodulin (om is a β-parvalbumin). X, Y, and Z refer to the calcium-binding ligands established by Kretsinger (1980) extended and refined by Kretsinger et al. (1991). Superscripts: (a) Brewer et al. (1990) and Palmisano and Henzl (1991); (b) Berchtold (1989b); (c) Berchtold et al. (1982); (d) Enfield et al. (1975); (e) Zühlke et al. (1989); (f) Coffee and Bradshaw (1973); (g) Kay et al. (1987); (h) Gillen et al. (1987).

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                          10         20         30         40         50
ß-parvalbumin             .          .          .          .          .
chicken thymus[a]  AITDILSAKD IESALSSCQA ADSFNYKSFF STVGLSSKTP DQIKKVFGIL
ß-parvalbumin[b]   S       E   AA  QE  D P T EPQK   Q S   KMSA S V DI RFI
rat(om)

                          60         70         80         90         100
ß-parvalbumin             .          .          .          .          .
chicken thymus     DQDKSGFIEE EELQLFLKNF SSSARVLTSA ETKAFLAAGD TDGDGKIGVE EFQSLVKA
ß-parvalbumin       N Q  YLDG D  KY  QK  Q D  E  ES    SLMD A  N       AD    EM HS
rat(om)
                   X Y Z-X-Y  -Z                              X  Y Z-X-Y  -Z
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FIGURE 3: Alignment of the amino acid sequences of two β-parvalbumins, chicken thymus parvalbumin, and rat oncomodulin (om). Superscripts: (a) Brewer et al. (1990) and Palmisano and Henzl (1991); (b) Gillen et al. (1987).

first Ca^{2+}-binding loop (positions 51–62) and the second Ca^{2+}-binding loop (90–101).

The regions with the highest sequence variations in all parvalbumins are before the first (nonfunctional) loop (12–19), in the linker region between the two Ca^{2+}-binding loops (64–80), and at the C-terminal regions. Less pressure to maintain the original structure was exerted on these parts of the parvalbumin during evolution. These variable regions might be suitable for the synthesis of specific peptides and after coupling to keyhole hemocyanide the production of species-specific antibodies.

One region within the second Ca^{2+}-binding loop (92–98) was found to be identical in all parvalbumins. Production of antibodies against a synthetic peptide corresponding to that sequence may recognize all parvalbumins in higher as well as in lower vertebrates.

A region within the first Ca^{2+}-binding loop (55–60) is identical in all parvalbumins except for oncomodulin.

Two subclasses of parvalbumins have been described, α (p*I* above 5.0) and β (p*I* below 4.5) (Goodman & Pechère, 1977). With a p*I* of 5.2, chicken muscle parvalbumin would qualify as an α-parvalbumin. The p*I* of the chicken thymus parvalbumin was 4.3 (Palmisano & Henzl, 1991), indicating that chicken thymus parvalbumin is a β-parvalbumin. The presence of Cys 18 and Phe 66 in thymus parvalbumin supports this β assignment. This is further substantiated by the best sequence alignment fit of thymus parvalbumin with map turtle β-parvalbumin (Brewer et al., 1989). The complete primary structures of both proteins are identical at 90 of 108 residues (83% sequence identity). Therefore, chicken contains an α- and a β-parvalbumin, probably coded by two genes. A similar result has been found in the rat but not in other species. In the rat an α-parvalbumin (p*I* 4.9; Heizmann, 1984; Berchtold et al., 1987) and a β-parvalbumin (oncomodulin, p*I* 3.9) (MacManus et al., 1987; Furter et al., 1989; Banville & Boie, 1989), coded by two different genes, were found.

Alignment of rat muscle α-parvalbumin with rat β-parvalbumin (or oncomodulin) shows differences in 53 positions. Both proteins in the rat were found to be expressed in a tissue-specific and cell type specific manner and regulated differently during development (parvalbumin; Heizmann, 1984; Heizmann & Braun, 1990) (oncomodulin; MacManus et al., 1987). Similar results were obtained for the chicken counterparts where α-parvalbumin was found mainly in muscle and brain (Strehler et al., 1977) and the β-parvalbumin was localized within certain cell types of the thymus (in large epithelial cells and some small thymocytes in the cortex) (Murthy et al., 1984; Hall et al., 1991). Chicken muscle α-parvalbumin was found to be very homologous to rat muscle α-parvalbumin (Figure 2), differing only by 25 amino acids. The sequences of chicken thymus β-parvalbumin and rat β-parvalbumin (or oncomodulin), the only two β-parvalbumins found so far in higher vertebrates, were quite distinct, differing in 54 positions (Figure 3). This indicates that thymus parvalbumin in chicken is a β-isoform distinct from rat β-parvalbumin (or oncomodulin). This is further supported by their distinct tissue specificities.

Discussion

Until very recently it was believed that higher vertebrates (in contrast to lower vertebrates) contain only one parvalbumin protein coded by one gene. This report confirms the existence of two parvalbumin proteins in chicken, one expressed in muscle and one in thymus.

We chose tandem mass spectrometry to sequence chicken muscle parvalbumin (after proteolytic digestion) since this method is capable of (a) rapid sequencing of peptides even in mixtures, (b) sequencing of peptides with blocked N-termini, (c) detecting any posttranslational modifications, and (d) affording optimal use of Edman sequencing. Such modifications of parvalbumin have not been examined previously although the phosphorylation of P14 (a member of the S-100 protein family; Edgeworth et al., 1989), crystallins (Voorter et al., 1989), and β-centrin (Bazinet et al., 1991), the methylation of calmodulin (Siegel et al., 1987), and the sialylation of calcyclin, a member of the S-100 protein family (Gabius et al., 1989), have been reported. We did not find any indication of a modification in chicken muscle parvalbumin. This does not exclude, however, the possibility that parvalbumin might be modified in vivo. For example, it has been demonstrated that the level of phosphorylated Ca^{2+}-binding protein P14 in both monocytes and neutrophils is dependent on $[Ca^{2+}]_i$ (Edgeworth et al., 1989). In the nonactivated cells the protein seems to be dephosphorylated.

Thymus parvalbumin was detected in thymus and blood but not in muscle. It was postulated to have an extracellular and hormone-like activity, apparently promoting the maturation of the immune system (Murthy et al., 1984). This implies that thymic parvalbumin may exert its action through membrane receptors. In this respect the findings of Permyakov et al. (1989) might be of interest. They reported a Ca^{2+} ion inhibited binding of mellitin to parvalbumin, suggesting a regulatory role through cellular targets.

On the other hand, parvalbumin in the skeletal muscle of the rat (this might also be true for the skeletal muscle of chicken) is believed to act intracellularly as a relaxing factor in the cytosol of fast-twitch fibers or as a Ca^{2+} buffer/Ca^{2+} shuttle in the cytosol of inhibitory neurons and specific cells of endocrine glands [for reviews see Heizmann (1984, 1990) and Heizmann and Braun (1990)].

In conclusion, the findings of two parvalbumins, specifically expressed in thymus and muscle of the chicken, will initiate the search for further members of the parvalbumin gene family (and for its secondary effector proteins) in mammals and man. An important issue will also be to examine possible extracellular functions and to search for possible secondary effector proteins as have been found for members of the S-100 protein family [for reviews see Donato (1986), van Eldik and Zimmer

(1988), and Hilt and Kligman (1991)].

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