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Antibodies for Growth Hormone and Prolactin Using Multiple Antigen Peptide Immunogens

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Abstract: Antibodies elicited by novel synthetic peptide antigens derived from a highly conserved domain of the growth hormone (GH) and prolactin (PRL) of vertebrates were developed using the multiple antigen peptide approach. The sequence of the antigens is located near the carboxy-terminus in the D domain of the GH and PRL in a cluster of 11 and 10 conserved amino acids, respectively, within a sequence of 18 residues. The synthetic peptides were manually synthesized, purified by high-performance liquid chromatography, and the corresponding antibodies, elicited in rabbits, were cross-reacted with the GH and PRL of a variety of mammalian (human, bovine, ovine, pig, and equine) and nonmammalian (chicken, coho salmon, chum salmon, rainbow trout, catfish and striped bass) vertebrates. The cross-reactivity between the immunogen and its corresponding antigen was tested by immunobloting using either GH or PRL. The GH and PRL of the organisms tested cross-reacted specifically with the corresponding antibody. Chicken and fish GH and PRL showed stronger antibody cross-reactivity than that observed in mammalian sources. These results demonstrate the utility of peptide-derived polyclonal antibodies in the detection of native and recombinant GH and PRL of a variety of vertebrates.

Key words: Multiple peptide antigens, polyclonal antibodies, growth hormone, prolactin, fish.

INTRODUCTION

Antibody molecules are extremely useful biological reagents for the identification, measurement, purification, and characterization of the cognate or parent protein. Although several amino acids on the protein's surface interact with the antibody binding site, these residues are usually from different segments of the primary structure. Thus short linear peptides can also induce useful cross-reactive antibodies, as a result of induced fit (Sutcliffe et al., 1983). Antibodies raised against short peptides are specially valuable when the parent protein is rare or difficult to purify as is the case for the native forms of growth hormone (GH) and prolactin (PRL).

Growth hormone and PRL are produced by the somatotropes and lactotropes, respectively, in the anterior portion of the pituitary gland. Thus a large amount of pituitary tissue from any vertebrate species is usually required to obtain an adequate yield for protein purification and antibody production. For example, in teleost fish (e.g., carp, catfish, coho salmon) about 30 mg of purified GH can be obtained from 6 g of fresh pituitary glands, which implies

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the sacrifice of about 1000 adult specimens. Consequently, purification of GH and PRL from fresh tissue, to prepare antibodies, is impractical. As an alternative to using large amounts of tissue, synthetic peptides also can be used to raise antibodies. Several peptide sequences of the GH molecule have been shown to be immunoreactive and antibody specific, and to have somatogenic effect (Bomford and Aston, 1990; Wang et al., 1990; Aston et al., 1991). Thus peptides containing a particular epitope specificity could be useful for the preparation of antibodies or as potentiators of a biological activity.

One way to prepare antibodies is to follow the simple approach of using a peptide segment with the desired epitopes of a protein, which in theory provides the desired selectivity (Milich, 1989; Ada, 1990; Brown, 1990). Accordingly, synthesis of peptides for immunization and preparation of site-specific antibodies has become a well-accepted approach, and has been widely used in the characterization of the parent protein by mimicking one of the protein's epitopes, allowing the production of cross-reacting antibodies.

Although the traditional approach of antibody preparation requires modification and presentation of the peptide (i.e., peptide conjugation to a protein carrier such as albumin, ovalbumin, or keyhole limpet hemocyanin (KLH) and polymerization in the presence of adjuvants, the multiple antigen peptide (MAP) approach (Posnett et al., 1988; Tam, 1988) provides well-defined structures which are desirable for the preparation of site-specific antibodies. The MAP approach uses a core matrix comprising oligomeric lysines attached at their carboxy-terminal residues replacing the protein carrier and eliminating the problems associated with it. This unique presentation provides peptide epitopes unambiguously in multiple copies. As a result, the MAP system provides a very high density of the desired peptide epitopes at the surface of the construct, with a small nonprotein core matrix as a scaffold (Tam, 1988). Following the same approach, antibodies specific for GH and PRL were prepared using linear peptides attached to a lysine core matrix. Because of the importance of GH and PRL in medicine and their commercial value in animal husbandry and aquaculture, the antibodies developed were tested against recombinant and native GH and PRL hormones of a variety of higher vertebrates and fish species.

The specificity of the GH and PRL MAP-derived antibodies toward their corresponding hormones makes them useful in biochemical and medical applications. The antibodies could be useful in the detection of GH and PRL in gene expression studies of different animal species, in serodiagnosis, in studies with transgenic animal models for GH and PRL, and in animal disease studies. Other applications of the MAPs and their corresponding antibodies include the production of monoclonal and polyclonal antibodies, immunoassays, screening procedures, epitope mapping, inhibitors, purification methods, and the evaluation of kinetics, quantity, and specificity of humoral responses.

Several GH and PRL structural characteristics were considered in the selection of the peptide segment used for synthesizing the multiple antigen peptide. Amino acid sequences for GHs and PRLs of higher and lower vertebrates have revealed that they are single-chain polypeptides of about 21 to 22.5 kDa. Alignment of their amino acid sequences resulted in the identification of four domains (A_{GH}, B_{GH}, C_{GH}, D_{GH} and A_{PRL}, B_{PRL}, C_{PRL}, D_{PRL}), respectively, that are highly conserved throughout evolution and separated by variable regions and deletions (Kawauchi and Yasuda, 1989; Chen et al., 1994). The D domain, located near the carboxy-terminus of the GH and PRL molecule, contains a cluster of 11 and 10 conserved amino acids, respectively, within a sequence of 18 residues. Surface probability plot analysis indicates it is likely that near the carboxy-terminus of GH, the α-helix in the D domain emerges at the surface of the protein around amino acids CFKKDMH and then returns toward the protein's interior. This region contains several amino acids, at the surface, involved in receptor binding and in maintaining the protein's structural conformation (Chen et al., 1994). A similar structural region is observed in PRL, suggesting that some amino acids located near the carboxy-terminus, in the D domain, also emerge at the surface of the peptide as in GH. These structural features were considered as desirable to synthesize the MAPs. Evidence that synthetic peptides derived from a chain terminus, often located at the surface of the protein, are good candidates for raising antipeptide antibodies has been shown by Westhof et al. (1984) and Thornton and Sibanda (1983). Such synthetic peptides usually correspond to predicted turns that correlate well with antigenicity (Levitt, 1978).

MATERIALS AND METHODS

Synthetic Peptides for Growth Hormone and Prolactin

Two synthetic peptides derived from a unique region of a domain located near the carboxy-terminus of teleostean GH

and PRL, respectively, were synthesized using the MAP system (Posnett et al., 1988; Tam, 1988) described below. The synthetic peptides identified by the letter code YELLACFKKDMHKVETYL and FRRDSHKIDSFLKVLRCR correspond to the amino acid residues Tyr-Glu-Leu-Leu-Ala-Cys-Phe-Lys-Lys-Asp-Met-His-Lys-Val-Glu-Thr-Tyr-Leu and Phe-Arg-Arg-Asp-Ser-His-Lys-Ile-Asp-Ser-Phe-Leu-Lys-Val-Leu-Arg-Cys-Arg of the GH and PRL D domains, respectively, and are located near the carboxy-termini of the molecules. These 18 amino acid synthetic peptides correspond to positions 172 (Tyr) to 189 (Leu), and 176 (Phe) to 193 (Arg), of teleostean GH and PRL peptides, respectively.

Synthesis of Immunogenic Peptides by the MAP System

Each 18-residue octameric MAP was synthesized manually by a conventional stepwise solid-phase procedure (Tam et al., 1983; Merrifield, 1986) in which multiple attachment of each peptide to a polylysine scaffolding was achieved. A core matrix with peptide antigen attached was accomplished on 1 g of Boc-β-Ala-OCH_2-Pam resin swelled in 10% acetic anhydride (Mitchell et al., 1978). Briefly, the Boc group was removed by treatment with trifluoroacetic acid (TFA) and dichloromethane (DCM) at a ratio of 1:1 (vol/vol). The resulting salt was neutralized with 5% diisopropylethylamine in DCM. Qualitative and quantitative ninhydrin assays were used to check each coupling reaction and the uncoverage of free amine sites after each deprotection step of the resin, respectively. The first level of the carrier core to form Boc-Lys(Boc)-β-Ala-OCH_2-Pam resin was synthesized using preformed symmetrical anhydride of Boc-Lys (Boc) (0.52 mM) in dimethylformamide followed by a coupling via dicyclohexylcarbodiimide in DCM. The second or third level were synthesized by the same protocol to give the tetramer or octamer core matrix for PRL and GH MAPs, respectively. The protecting groups for the synthesis of the peptide antigen were Boc groups for the α-amino-termini, and benzyl alcohol derivatives for most side-chain amino acids. All free amino-terminal amino groups of the branched peptide oligolysine were acetylated in the presence of dimethylformamide, acetic anhydride, and triethylamine. The protective group from histidine was removed by thiolysis using thiophenol, which was then removed by gel filtration on G-25 Sephadex in 10% acetic acid. The peptide was removed from the resin by acid cleavage using 100 μl trifluoromethanesulfonic acid (TFMSA), 1 ml of TFA, dimethylsulfide and *p*-cresol, mixed by vigorous shaking in a solution containing anhydrous ether and β-mercaptoethanol, and extracted in 0.1 M Tris buffer, pH 8.0, containing 8 M urea and 0.2 M dithiothreitol. Aromatic by-products were removed by dialysis in a buffer containing 8 M urea, 0.1 M ammonium bicarbonate, pH 8.0 with 0.1 M β-mercaptoethanol at 0°C for 24 hours. The dialysis was continued in 8 M urea and then in 2 M urea in the same buffer for 12 hours and then sequentially in water and HOAc to remove the urea.

MAPs Purification and Amino Acid Analysis

The multiple antigenic peptides were lyophilized and purified batchwise by gel permeation chromatography with 2% to 5% acetic acid as solvent, and assessed by high-performance liquid chromatography (HPLC) using solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile; gradient from 5% B to 50% B in 45 minutes with a 1 ml min^{-1} flow rate. A Vydac column (218TP54), 4.6 × 250 mm was used, and detection was carried out at 215 nm (UV). Amino acid analyses of the MAPs were accomplished using Waters Picotag HPLC system (Millipore Corp.).

Immunization Procedure

Four New Zealand White 3-month-old female rabbits were bled (preimmune) and immunized subcutaneously with soluble antigenic peptides at 1 mg for the first injection and 0.5 mg for booster injections, per immunization per animal. Two rabbits were injected with the MAP containing GH sequences and two with the MAP containing PRL sequences. Rabbits showing high antibody titers for either GH or PRL were chosen in this study. The antigenic peptides were dissolved in a final volume of 1 ml in phosphate-buffered saline (PBS) and complete Freund's adjuvant (1:1) for the first injection (day 0), and with incomplete Freund's adjuvant (1:1) for booster injections on days 13, 26, 56, and 84. Rabbits were bled (about 20 ml per rabbit) on days 20, 33, 41, 46, 60, 74, 83, 94, 108, and 117. The polyclonal antibodies were purified and used as immunoreagents for Western blot. Rabbits' care, maintenance, and immunization protocols were carried out according to procedures established by the American Veterinary Medical Association and the USDA Animal Welfare Act.

Antibody Purification

The antiserum was purified by precipitation with caprylic acid (octanoic acid) (Russo et al., 1983; McKinney and Par-

kinson, 1987) followed by affinity chromatography on protein A agarose. Briefly, the bulk of proteins from serum were precipitated with caprylic acid without affecting IgG, and the accumulation of aggregates was eliminated. Twenty milliliters of 0.06 M acetate buffer, pH 4.0 was added to 10 ml of serum. The pH was adjusted to 4.8 by addition of caprylic acid. The solution was stirred for 30 minutes at room temperature and centrifuged at 10,000 *g* for 30 minutes at 10°C. The supernatant was adjusted to pH 5.7 and dialyzed against 0.05 M acetate buffer. The dialyzed sample was purified by affinity chromatography on Affinica Protein A Agarose (Schleicher & Schuell). The fraction containing the purified immunoglobulins was divided into small aliquots and stored at −80°C.

Antibody Titer

The titer of the antibodies derived from the GH and PRL MAPs was evaluated by an indirect enzyme-linked immunosorbent assay (ELISA). Immune and control sera were serially diluted 10-fold (1:10 to $1:10^6$) and added in duplicate to an antigen-coated microtiter plate (Groome, 1994). To rule out nonspecific binding, the microtiter wells were treated with a high concentration of albumin to block the remainder of binding sites, after adsorbing wells with the antigen. After a 2-hour incubation with the corresponding antibody, the plates were washed and reacted with horseradish-peroxidase-labeled antibody specific for the primary antibody. After incubation, the plates were washed again and reacted with the chromogenic substrate. The resulting color change was recorded (optical density 492 nm) using a standard plate reader. The preimmune control serum was run in parallel to evaluate nonspecific binding. The GH antibody was also evaluated by immunobloting with the corresponding MAP as substrate. Preimmune and control sera were serially diluted in PBS in steps of 50 from 1:10 to $1:10^5$ and added in duplicate to nylon membranes (Immobilion-P Millipore) containing 1 μg of purified peptide, which had been loaded with a slot blot apparatus. The membranes were processed as outlined below.

Sources of Growth Hormone and Prolactin

The hormones used in these studies included the recombinant forms of GH of bovine from Elly Lilly Labs: human, porcine, and chicken from Dr. Cavari, Institute of Oceanographic and Limnological Research, Haifa, Israel; native striped bass, catfish, and rainbow trout GH were from Dr. Chen, Center of Marine Biotechnology, University of Maryland; recombinant coho salmon was from Dr. Gonzalez, Technology Enterprise Center, University of Maryland; and native chum salmon GH was from Dr. Kauchi, Department of Fisheries, Kitasato University, Japan. The native forms of PRL included catfish from Kitasato University, Japan; rainbow trout from Dr. Chen, Center of Marine Biotechnology, University of Maryland; and human, bovine, equine, and chicken from USDA, Animal Hormone Program, Beltsville, Md.

SDS Gel Electrophoresis and Immunoblot Analysis

The purified GHs and PRLs from several vertebrate sources (fishes, chicken, rat, porcine, pig, equine, bovine, and human) were electrophoresed using 14% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nylon membranes (Immobilon Millipore) using a semidry transfer cell set (BioRad) at 22 V for 1 hour. The membrane was then blocked in nonfat dry milk and incubated in the presence of the purified antibody (at 500-fold dilution), followed by incubation in goat antirabbit alkaline phosphatase conjugate. The bound conjugate was reacted with a chromogen to visualize the protein bands (Harlow and Lane, 1988).

Results

Design of MAP Antigens

Table 1 shows the letter code of the 18 amino acid peptide sequence derived from the unique regions of the D domain of growth hormone and prolactin of different vertebrates. We selected the conserved residues YELLACFKKDMHKVETYL and FRRDSHKIDSFLKVLRCR from teleost GH and PRL to design the MAPs and produce the antibodies. Although the D domain of both hormones are highly conserved in vertebrates, amino acid sequence variations exist between different phylogenetic groups. For instance, the 18-residue peptide of GH is identical in seven fish species. However, the peptide shows some amino acid variations when compared with other vertebrate species (see Table 1).

Purification of MAP Antigens

Table 2 shows the amino acid analysis of both HPLC-purified GH and PRL MAP peptides. The predicted and observed amino acid values were similar in each case.

Table 1. Unique Conserved Amino Acid Sequences in the D Domain of Growth Hormone and Prolactin in Vertebrates

Animal vertebrate sources	Partial D domain sequences
Growth hormone	
Human	**Y G L L** Y **C F** R **K D** M D **K** V **E T** F **L**
Bovine	**Y G L L S C F** R **K D L H K** T **E T Y L**
Ovine	**Y G L L S C F R K D L H K** T **E T Y L**
Porcine	**Y G L L S C F K K D L H K** A **E T Y L**
Rat	**Y G L L S C F K K D L H K** A **E T Y L**
Chicken	**Y G L L S C F K K D L H K** V **E T Y L**
Turtle	**Y G L L S C F K K D L H K** V **E T Y L**
Eel	**Y G L L** A **C F K K D** M **H K** V **E T Y L**
Flounder	**– E L** F **A C F K K D M H K V E T Y L**
Common carp	F R **L L A C F K K D M H K V E T Y L**
Grass carp	F R **L L A C F K K D M H K V E T Y L**
Coho salmon	**Y E L L A C F K K D M H K V E T Y L**
Rainbow trout	**Y E L L A C F K K D M H K V E T Y L**
Red sea bream	**Y E L L A C F K K D M H K V E T Y L**
Chum salmon	**Y E L L A C F K K D M H K V E T Y L**
Atlantic salmon	**Y E L L A C F K K D M H K V E T Y L**
Tuna	**Y E L L A C F K K D M H K V E T Y L**
Yellow tail	**Y E L L A C F K K D M H K V E T Y L**
Prolactin	
Human	**L R R D S H K I D N Y L K** L **L K C R**
Ovine	**L R R D S** S **K I D** T **Y L K** L **L** N **C R**
Rat	**L R R D S H K** V **D N Y L K** F **L R C** Q
Chicken	H **R R D S H K I D N Y L K V L K C R**
Turtle	**L R R D S H K I** E **N Y L K** L **L K C R**
Bullfrog	**L R R D S H K I D N Y L K L L K C R**
Tilapia	**F R R D S H K I D S F L K V L R C R**
Carp	**F R R D S H K I D S F L K V L R C R**
Chum salmon	**F R R D S H K I D S F L K V L R C R**
Catfish	**F R R D S H K I D S F L K V L R C** D

Antibody Titer

ELISA and immunoblot assays were used for determining the specificity and binding potency of PRL and GH antisera to the corresponding antigens. The titers of the antisera were above 2.0×10^5 ($OD_{1/2\ max} = 1.04$) and 10^6 ($OD_{1/2\ max} = 0.22$) for GH and PRL, respectively ($OD_{1/2\ max}$ is the titer defined as the antiserum dilution at half-maximal optical density in the ELISA). Figures 1A and 1B show the ELISA titration curves of GH and PRL antisera, respectively. Both GH and PRL MAPs were also found to be strong immunogens when they were reacted with their corresponding antibodies by Western blot, and they do not cross-react with each other (data not shown). No background was observed with any of the preimmune serial dilutions. When antibody dilutions were tested against pituitary extracts of rainbow trout, GH and PRL generated clear signals at 1:250-fold and 1:500 antibody dilutions (Figure 2A). At higher antisera dilutions the signal was gradually reduced. This is reasonable to expect because antibody prepared against a peptide that accounts for only 10% of the size of the native protein should have a recognition pattern (binding site) that is dependent on the presence and exposure of the sequence (epitope) chosen in the whole protein. In general, synthetic peptides as immunogens are known to give lower affinity than when the whole protein is used. Growth hormone antibody dilutions tested against purified GH of bovine and chum salmon (Figure 2B) also generated clear signals at 1:500 dilution.

Immunoblot Analysis

The ability of the GH and PRL antisera to discriminate between PRL and GH hormones from the background of complex protein mixtures is shown in the Western blot of Figure 2A. The GH and PRL antisera specifically detected the corresponding hormones from rainbow trout pituitary lysate. Similarly, the ability of the corresponding antibodies to cross-react with purified recombinant and native GH and PRL of a variety of vertebrate species is shown in the immunoblots of Figures 3A and 3B and Figure 4. The GHs of pig, chicken, bovine, coho salmon, chum salmon, striped bass, and rainbow trout were reacted with the antibody raised against MAP of GH. Antigen-antibody bands were observed in all cases. Although variations in the purity of the GH preparations may account for the differences in the thickness of the bands observed, the cross-reactivity of the antibody was strong with all the GHs tested (Figure 3A lanes 1 to 6, and B lanes 1, 3, 5, 6, 7, and 8). Approximately 15 μg of purified recombinant bovine, chicken, coho salmon, chum salmon, and rainbow trout, GH, and about 5 μg of striped bass GH were cross-reacted with the antibody raised against the MAP of GH (Figure 3A).

The antibody cross-reactivity was similar in all the GHs tested, but chicken and rainbow trout GHs showed the strongest antigen-antibody bands (Figures 3A, lanes 2 and 6). Similarly, equal concentrations (~10 μg each) of purified native GHs of bovine, catfish, coho salmon, pig, and striped bass were electrophoresed, transferred onto a nylon membrane, and cross-reacted with the antibody raised against

Table 2. Amino Acid Analysis of Purified Prolactin and Growth Hormone Multiple Antigen Peptides

PRL-MAP*			GH-MAP†		
Amino Acid	Theoretical	Experimental	Amino Acid	Theoretical	Experimental
Arg	16	13.20			
Asp	8	7.81	Asp	8	7.29
Cys‡	4	Presence confirmed	Cys‡	8	Presence confirmed
Glu			Glu	16	15.18
β-Ala	1	0.75	β-Ala	9	6.23
His	4	3.51	His	8	9.45
Ile	4	4.0			
Leu	8	8.25	Leu	24	21.83
Lys	11	11.29	Lys	31	30.85
Phe	8	7.52	Phe	8	6.18
Ser	8	7.93			
Val	4	4.14	Val	8	8.25
Gly			Gly	24	33.42
Thr			Thr	8	8.85
Tyr			Tyr	16	14.20
Met			Met	8	6.24

*Tetra-branched lysine carrier.

†Octa-branched lysine carrier with a triglycyl linker. Numbers represent the theoretical vs experimental residues.

‡Cysteine undergoes extensive decomposition during the hydrolysis of the peptide. Hence, Cys is recovered in low yields. However, its presence was confirmed.

the MAP of GH. The native GHs (Figure 3B) of bovine (lane 1) and fish (lanes 3, 5, 6, and 8) had similar antibody cross-reactivities, as indicated by the equal intensity of the bands. The pig GH has somewhat less affinity for the antibody, as indicated by a weaker antigen-antibody band (Figure 3B, lane 7). This could be due to structural differences in the pig immunogen, which differs by a few amino acid residues (Table 1) from that of fish and bovine. In order to determine the level of cross-reactive specificity of the MAP-derived GH antibody with the corresponding proteins, highly purified PRLs of bovine and catfish were included in the immunoblot (Figure 3B, lanes 2 and 4) and cross-reacted with the antibody raised against the MAP of GH. The lack of antigen-antibody bands for these PRLs demonstrates that the GH antibody does not cross-react with the PRL proteins.

The affinity of the antibody raised against the MAP of PRL was also tested. The native PRLs of catfish, human, rainbow trout, bovine, ovine, equine, and chicken were cross-reacted with the antibody (Figure 4, lanes 1 to 8, respectively). Although antigen-antibody bands were observed with all PRLs, slight differences in the purity of the PRL preparations could account for the differences in the intensity of the bands. Nevertheless, the level of reactivity of the antibody with the PRLs was catfish > rainbow trout > human > ovine > equine > bovine > chicken. In summary, both polyclonal antibodies cross-reacted with their respective GH and PRL of several vertebrate species. The differences observed between the antigen-antibody signal intensities could be attributed to variations in hormone concentration. However, such variations could also be due to differences in the antibody binding affinities. This is expected because the amino acid sequence that is within the cluster of conserved residues (where the synthetic peptides were derived) in the D domains near the carboxy-termini of GH and PRL, respectively, shows variation among vertebrates (see Table 1). Further studies on the determination of the residues involved in antibody recognition using point substitution may be useful to clarify the differential recognition of hormones.

The specificity of the antibody raised against the MAP or PRL was also determined by cross-reacting purified na-

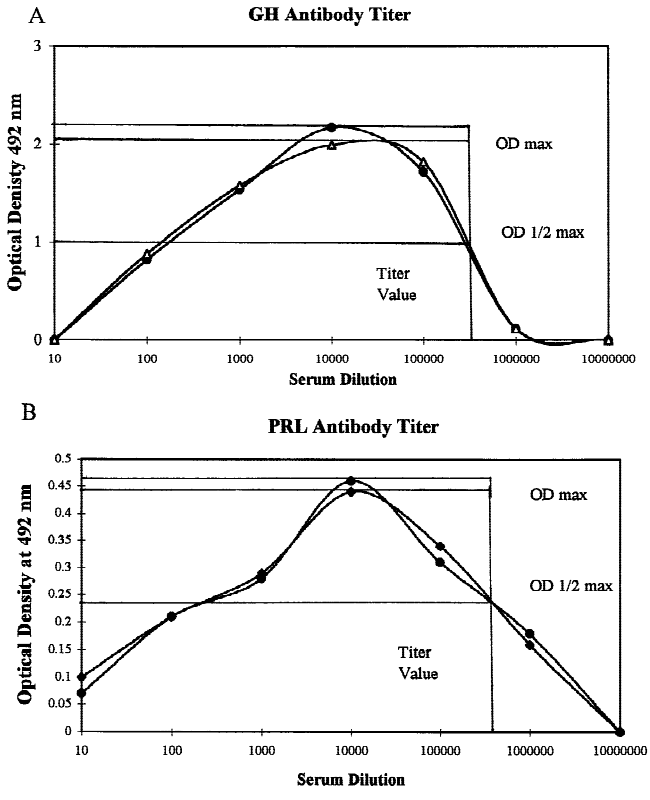

Figure 1. Titration curves of GH and PRL antisera by ELISA. **A:** GH antibody titer curve showing the optical density at 492 nm of seven 10-fold serial dilutions. The GH antibody titer was above 2.0 $\times$ 10^5 with an $OD_{1/2\ max}$ = 1.04. **B:** PRL antibody titer curve showing the optical density at 492 nm of seven 10-fold serial dilutions. The PRL antibody titer was above 1 $\times$ 10^6 with an $OD_{1/2\ max}$ = 0.22.

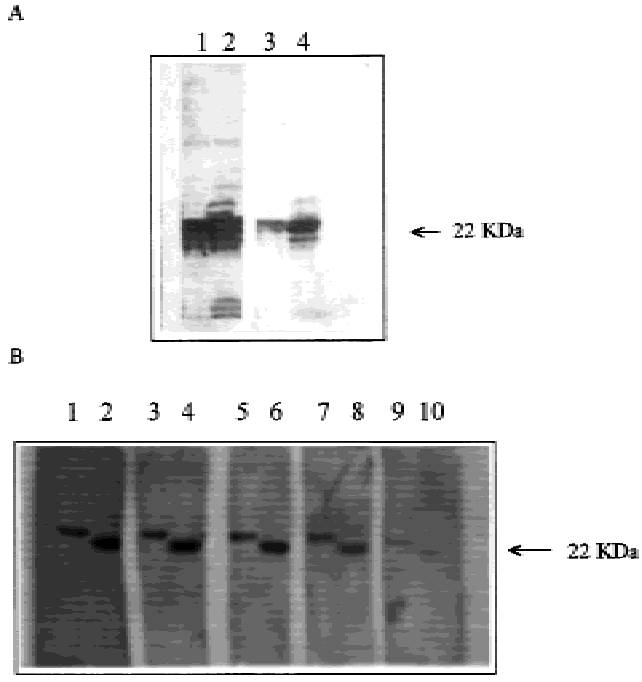

Figure 2. A: Western blot analysis of pituitary extract. Samples of rainbow trout pituitary lysates were subjected to 14% SDS–polyacrylamide gel electrophoresis, transferred to nylon membrane, reacted with the MAP-derived GH or PRL antibody, and incubated with goat antirabbit alkaline phosphatase conjugate and substrate. Lanes 1 and 2 show a nylon strip containing 10 and 20 µl of rainbow trout pituitary lysate reacted with the GH MAP antibody. Lanes 3 and 4 show a nylon strip containing 10 and 20 µl of rainbow trout pituitary lysate reacted with the PRL MAP antibody. **B:** Serial dilutions of GH MAP antibody reacted against purified GHs. Lanes 1, 3, 5, 7, and 9 show 5 µg each of purified recombinant bovine GH that has been reacted with GH MAP antibody at 1:60, 1:120, 1:250, 1:500, and 1:1000 serial dilutions, respectively. Lanes 2, 4, 6, 8, and 10 show 5 µg each of purified recombinant chum salmon GH that has been reacted with GH MAP antibody at 1:60, 1:120, 1:250, 1:500, and 1:1000 serial dilutions, respectively.

tive GH of chum salmon (Figure 4, lane 1, labeled as GH). A very weak band was observed in the blot, probably owing to traces of PRL in the preparation as native PRL is usually purified from the same tissue source. Lack of GH antibody cross-reactivity was observed with recombinant PRL (data not shown). These results indicate the high specificity of the antibody raised against the MAP of PRL.

Discussion

Synthetic peptides for GH and PRL were designed and prepared by the MAP approach and used for antibody production. The polyclonal antibodies raised against such MAPs cross-reacted with native and recombinant GH and PRL, respectively, of lower and higher vertebrates. Although it is sometimes desirable to have different peptide conformations in the immunogen to mimic peptide structure in the context of the whole protein, MAP-derived antigenic peptides were selected because they offer several advantages: i.e., elimination of undesirable immunologic reactions; elimination of conformational changes, owing to their symmetrical shape, uniform antigenic characteristics, and lack of immunogenecity of the small noncationic core matrix; presentation of multiple copies of the same or different epitopes, resulting in a much stronger cellular response; and

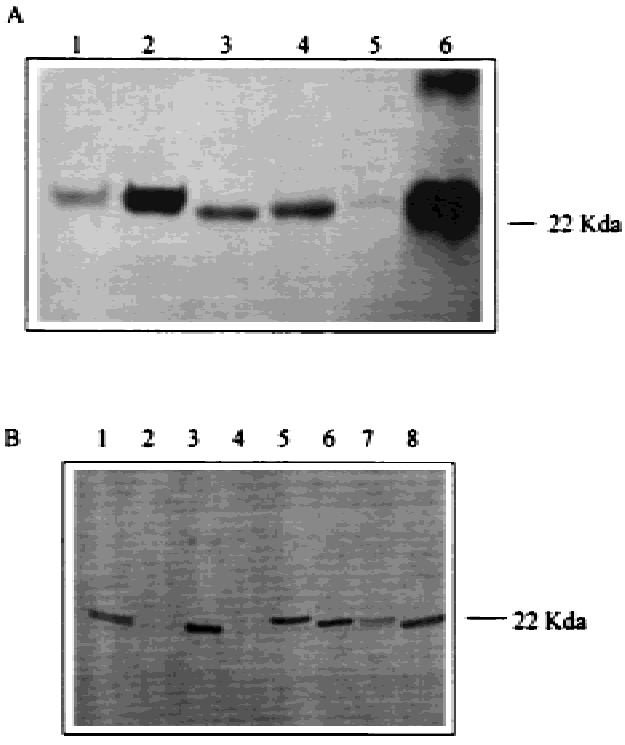

Figure 3. Western blot analysis of vertebrate GHs. Samples of recombinant or native purified growth hormones from various vertebrates were subjected to 14% SDS-PAGE, transferred to nylon membranes, reacted with the MAP-derived GH antibody, and incubated with goat antirabbit alkaline phosphatase conjugate and substrate. **A:** Purified recombinant GHs (~15 μg) of bovine, chicken, coho salmon, chum salmon, striped bass, and rainbow trout (lanes 1–6, respectively). **B:** Purified native GHs (~10 μg) of bovine, catfish, coho salmon, chum salmon, pig, and striped bass (lanes 1, 3, 5, 6, 7, and 8, respectively), and purified native PRLs of bovine and catfish (lanes 2 and 4, respectively).

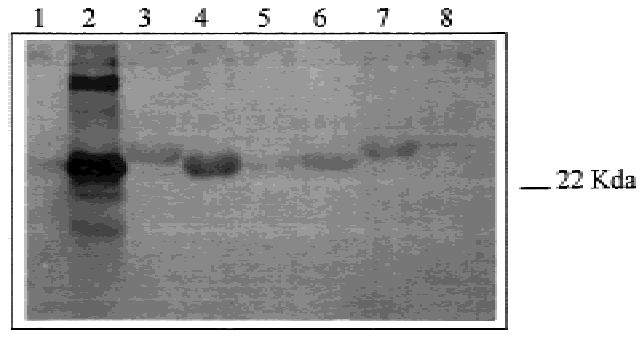

Figure 4. Western blot analysis of vertebrate PRLs. Samples of native purified prolactins from various vertebrates were subjected to 14% SDS-PAGE, transferred to a nylon membrane, reacted with the MAP-derived PRL antibody, and incubated with goat antirabbit alkaline phosphatase conjugate and substrate. Chum salmon GH (lane 1) and purified native PRLs (~10 μg) of catfish (lane 2), human (lane 3), rainbow trout (lane 4), bovine (lane 5), ovine (lane 6), equine (lane 7), and chicken (lane 8).

the ability to represent the MAP structure unequivocally by a formula and verify it by analytic methods (Tam, 1994).

Because the immunogenecity of MAPs also depends on the number of amino acid residues in the peptide, the location of the dominant epitope in the molecule, and the animal used for immunization, several factors were considered in the design of the MAP-derived antigenic peptides. First, although peptides of various lengths (5 to 37 residues) have been used to produce MAPs, a suitable length is 10 to 20 residues. Although amino acid residues representing the distal end constitute the epitope recognized by the antibodies in the polyclonal sera, peptides with 15 or fewer residues usually have decreased antigenicity from the distal end (flexible part) to the proximal end (near the core matrix) (Posnett et al., 1988; Schaaper et al., 1990; Tam, 1994). MAPs containing long peptides elicit polyclonal antibodies of high specificity (Tam, 1994). Thus an 18 amino acid residue cluster located near the carboxy-terminus domain of GH and PRL, respectively, was selected to synthesize the corresponding peptide using the MAP approach. Second, the MAP antigens were selected to contain highly conserved sequences such that the antibodies produced would react with the GH or PRL of a variety of vertebrates, as it was demonstrated. Third, the MAPs prepared for GH and PRL produced a reliable antibody response because they cross-reacted with all the species tested. Finally, rabbits rather than mice were selected to produce the PRL-specific and GH-specific antibodies because they produce larger amounts of immunoglobulin (Tam, 1988, 1994).

Another important feature in the design of MAPs is that putative immunogenic regions are found in the exposed contiguous hydrophilic regions in proteins. Similarly, the amino-terminal and carboxy-terminal regions of many proteins are also exposed at the surface and have been used to select peptides for raising antibodies. In this study, a peptide sequence located near the carboxy-terminal region of either GH or PRL, in solution, was selected for synthesis. The antibodies produced reacted with both the peptide antigen and the corresponding native and recombinant GH or PRL, respectively, of several fish species and vertebrates from different phylogenetic levels including chicken, porcine, equine, bovine, and human. Although we demonstrated that both antibodies cross-reacted with all their respective antigens tested, it appears that antibody cross-

reactivity differences observed between species might be attributed to the amino acid sequence variation that exists among the species in the cluster of conserved residues of both GH and PRL carboxy-terminal domains as shown in Table 1.

It has been argued that MAPs are not necessarily desirable immunogens, specially those containing carboxy-terminal epitopes, because they may assume an unusual conformation that does not mimic the structure of the parent protein, resulting in lack of cross-reactivity (Briand et al., 1992). In this study, we have shown that both MAP-derived antibodies cross-reacted with all the recombinant or native proteins tested, and that the levels of cross-reactivity observed were reasonably high. These antibodies can indeed be useful tools for the general detection of recombinant and native vertebrate GHs and PRLs during the process of expression, or isolation and purification using ELISA and Western blot assays, as demonstrated in this study. Other antibody-based detection systems using a variety of other substrates, including chemiluminescence, could also be used. It can be expected that more polyclonal antibodies derived from MAP immunogens will be prepared for developing diagnostic and detection reagents that can be used in a variety of biological systems. In fact, recent advances in MAP chemistry and sophisticated new methodologies have permitted a broad range of applications for serodiagnostics and drug discovery (Tam, 1996). For example, structurally modified MAPs have been used to raise strong humoral responses, to construct vaccine models for hepatitis and HIV, and for targeting and delivery of immunogens to elicit mucosal immunity. Tam (1996) provides an excellent review on MAP advances and their applications.

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