Journal of Cellular Biochemistry 80:274–284 (2000)

Horseradish Peroxidase Binding to Intestinal Brush-Border Membranes Of *Cyprinus carpio*. Identification of a Putative Receptor

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Abstract Morphologic studies have shown that the classic endocytosis tracer horseradish peroxidase (HRP) is actively internalized by vesicular transport in the carp intestine, suggesting the existence of specific binding sites in the apical membrane of enterocytes. The aim of the present study was to develop an in vitro binding assay using isolated carp intestinal brush-border membranes (BBM) to demonstrate and characterize these specific HRP binding sites. The results obtained show that HRP binding to BBM exhibits a saturable mode and high affinity (K_d = 22 nM). In addition, HRP binding sites are highly enriched in BBM compared to basolateral membranes. On the other hand, HRP interaction with these sites is apparently of an ionic character because binding increased concomitantly with decreasing NaCl concentrations in the assay, reaching a maximum in the absence of NaCl. Other proteins that are also internalized in carp intestine did not significantly inhibit HRP binding to BBM. A lectin-type of interaction was discarded because neither manan nor ovoalbumin inhibited HRP binding. Proteinase K treatment of BBM reduced HRP binding by 70%, suggesting a proteic nature for this binding site. Finally, ligand blotting assays showed that HRP binds specifically to a 15.3-kDa protein. Taken together, these results are consistent with the existence of a functional receptor for HRP in carp intestinal mucosa that could mediate its internalization. J. Cell. Biochem. 80:274–284, 2000.

Key words: horseradish peroxidase; brush-border membranes; binding site; protein absorption; endocytosis; intestine; carp; fish

Intestinal internalization of small amounts of intact proteins that escape gastrointestinal digestion is considered a normal physiologic process [Stern and Walker, 1984; Gardner, 1988]. In fact, the ability to absorb intact macromolecules has been demonstrated in different vertebrate organisms, both in newborn and adult animals [Gardner, 1988; Heyman and Desjeux, 1992; Sire and Vernier, 1992].

The internalization across the intestinal epithelium of a wide variety of intact proteins (e.g., peroxidase [Rombout et al., 1985; McLean and Ash, 1986; McLean and Ash, 1987]; human, bovine, and salmon growth hormone [Le Bail et al., 1989; Moriyama et al., 1990; Hertz et al., 1991]; insulin [Hertz et al., 1992; Vera et al., 1993]; IgG [Nakamura et al., 1990; Nakamura et al., 1998]; apolipoprotein A-I [Vera et al., 1992]; and lactoferrin [Sakai et al., 1995]) has been demonstrated in several teleost fish species. Most of these studies were designed to demonstrate that after oral or anal administration of several proteins of biologic interest, they could reach the systemic circulation retaining their biologic activities. The purpose of several other studies performed in adult fish, has been the elucidation of the mechanism(s) of transepithelial transport of proteins. Using morphologic approaches, it has been demonstrated that this process occurs primarily via a transcellular route and it is initiated by endocytosis of the protein in the columnar epithelial cells [Noaillac-Depeyre and Gas, 1973; Rombout et al., 1985]. On the other hand, in mammals, the endocytosis of proteins takes place mainly in specialized cells overlying Peyer's patches, called the M-cells [Kimm et al., 1994; Neutra et al., 1996]. In polarized epithelia of higher vertebrates, this pathway of internalization is mediated by the binding of the macromolecules to

Grant sponsor: FONDECYT; Grant number: 1980993; Grant sponsor: DID-UACH; Grant number: S-95-23.

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Received 28 April 2000; Accepted 7 July 2000

specific sites in the apical membrane [Lamaze and Schmid, 1995]. However, in fish intestinal mucosa, the presence of such binding sites has not been demonstrated yet.

In cellular morphologic studies, horseradish peroxidase (HRP) is commonly used as an endocytosis tracer because it can be easily detected by histochemical methods [Yamaguchi et al., 1993; Stoorvogel, 1998]. HRP is a mannose-terminated glycoprotein and therefore its uptake in macrophages and hepatic nonparechymal cells is mediated by a mannose receptor. On the other hand, in other cell types, HRP is mainly taken up by fluid-phase endocytosis [Stoorvogel, 1998]. For example, this is the case for rat hepatocytes, when high concentrations of HRP are used. However, at lower concentrations of the enzyme, the uptake is mediated by a saturable mechanism, probably involving a low specificity mannose-binding site [Yamaguchi et al., 1993].

Intestinal HRP internalization in fish (e.g., carp, goldfish, and rainbow trout) occurs by endocytosis and vesicular transport [Noaillac-Depeyre and Gas, 1973; Rombout et al., 1985; Georgopoulou et al., 1988; Abaurrea et al., 1993]. In carp, the rapid appearance of this enzyme in the circulation after oral administration suggests an efficient absorption mechanism [Hertz et al., 1991; McLean and Ash, 1986]. In fact, after 5 min of oral intubation, HRP was readily detected bound to the apical membranes and in apical vesicles of the enterocytes [Rombout et al, 1985]. From electron-microscopic observations, HRP appears to be transported through a selective pathway resembling the transfer of IgG in neonatal rat [Abrahamson and Rodewald, 1981; Rombout et al., 1985]. Taken together, the above data suggests the existence of specific binding sites for HRP in the apical membranes of the carp intestinal epithelia. In the present study we have developed an in vitro binding assay using isolated carp intestinal brush-border membranes that allowed us to demonstrate for the first time the existence of HRP-specific binding sites in absorptive intestinal cells. It also allowed us to identify and characterize a putative receptor protein.

MATERIALS AND METHODS

Animals

Common carp (*Cyprinus carpio*) were caught in the Cayumapu river and maintained in an outdoor tank with running river water. Fish weighing 800–1,200 g were acclimated at 20 ± 2°C with a photoperiod of 14L:10D, for at least three weeks before they were killed. Fish were fed to satiation twice every day.

Membrane Fractionation Procedure

Brush-border membranes (BBM) and basolateral membranes (BLM) were isolated from intestinal mucosa by the divalent cation precipitation procedure described for carp intestine [Lee and Cossins, 1990]. The isolated membrane fractions were stored frozen in aliquots at −80°C. The purity of the isolated membranes relative to the crude mucosa homogenate was assessed by estimating the specific activity of intestinal alkaline phosphatase (IAP) and ouabain-sensitive Na^+/K^+ ATPase for BBM and BLM, respectively [Villanueva et al., 1997]. The protein content was determined by the bicinchoninic acid method [Smith et al., 1985] using bovine serum albumin as standard. Typically, a 10-fold enrichment for alkaline phosphatase and a 5.5-fold enrichment of ouabain-sensitive Na^+/K^+ ATPase were obtained. IAP specific activity of the BBM preparations used in this study was in the range of 1.7–2.1 U/mg protein.

Binding of HRP to Isolated BBM

Standard binding assays were carried out at 25°C for 15 min in a final volume of 25 μl, containing 25 mM Tris-HCl, pH 7.5; 100 nM HRP (Type VI, Sigma, St. Louis, MO); and 0.6 mg/ml of membrane protein. After incubation, the reaction mixture was cooled on ice and layered onto 400 μl of a sucrose cushion containing 25 mM Tris-HCl, pH 7.5 and 250 mM sucrose. Bound and unbound HRP were separated by centrifugation at 14,000*g* for 30 min at 4°C. The supernatant was discarded and the pellet containing HRP bound to BBM was resuspended in 100 μl of 100 mM sodium citrate, pH 4.5. The amount of bound HRP was determined by measuring its catalytic activity, using a standard kinetic assay at 30°C, with o-phenylenediamine as substrate. The HRP activity was expressed as ΔA_{450}/min.

Nonspecific binding of HRP to BBM was determined by addition of a 100-fold excess of inactivated HRP (HRPi) to the assay. Inactivated HRP was prepared according to Ortiz de Montellano et al. [1988]. Specific modifications

and/or additions to the standard binding assay are indicated in the legends to the figures. All the assays were done in triplicate.

The dissociation constant (K_d) and the binding capacity (B_{max}) were estimated by Scatchard plot analysis and saturation binding curve adjustments using the built-in equation from the software SigmaPlot 3.0.

Enzymatic Treatments of BBM

Purified brush-border membranes were independently treated with three different enzymes: phosphatidyl inositol phospholipase C (PIPL-C), generously provided by Dr. S. Udenfriend (Roche Research Center, Nutley, NJ, USA); hialuronidase; and proteinase K (both from Merck, Darmstadt, Germany). PIPL-C treatment was carried out for 30 min at 25°C in a final volume of 100 μl containing 25 mM Tris-HCl, pH 7.5; 5 mg/ml of BBM protein; and 15 U of the enzyme. Hialuronidase and proteinase K treatments were performed essentially as described for PIPL-C, except for the enzyme concentrations used were 0.4 mg/ml and 0.075 mg/ml, respectively. In addition, the incubation with proteinase K was shorter (10 min). For each treatment a control tube was incubated in parallel under the conditions already detailed, but omitting the enzyme. Afterwards, all the reaction mixtures were cooled on ice and layered onto 400 μl of a sucrose cushion containing 25 mM Tris-HCl, pH 7.5, and 250 mM sucrose. Brush-border membranes were recovered by centrifugation at 14,000*g* for 30 min at 4°C. The pellets were resuspended in 500 μl of 25 mM Tris-HCl, pH 7.5, and centrifuged as described above. This washing step was repeated once more and the final pellets were resuspended in 75 μl of 25 mM Tris-HCl, pH 7.5. Finally, protein concentration and IAP activity were determined as previously described.

Western Blot Analyses

The pellet recovered from the binding assay, containing HRP bound to BBM, was resuspended in reducing sodium dodecyl sulfate (SDS) sample buffer and loaded on 12% polyacrylamide-SDS slab gels. Electrophoresis was conducted according to Laemmli [1970]. The proteins were transferred electrophoretically to nitrocellulose membranes using a semi-dry blotter unit. Membranes were blocked with 5% (w/v) nonfat dry milk in phosphate-buffered saline/Tween-20 (0.1% v/v). Horseradish peroxidase was detected by incubation with a polyclonal anti-HRP antibody (Sigma, St. Louis, MO) diluted 1:3,000 followed by incubation with alkaline phosphatase-conjugated antibody (Gibco BRL) diluted 1:3,000. Finally, alkaline phosphatase activity was developed incubating the membrane at room temperature for 20 min in 0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl; 5 mM $MgCl_2$ containing 0.16 mg/ml 5-bromo-4-chloro-3-indolyl phosphate; and 0.33 mg/ml nitroblue tetrazolium.

Ligand Blot Analyses

Brush-border membrane proteins were separated by electrophoresis in 15% polyacrylamide-SDS gels under nonreducing conditions using the discontinuous buffer system described by Laemmli [1970]. The proteins were then electroblotted to Immobilon-P membranes (Millipore, Inc., Bedford, MA) and blocked with 2% (v/v) Tween-20 in 25 mM Tris-HCl, pH 7.5 [Bolte et al., 1997]. After extensive washing with 25 mM Tris-HCl, pH 7.5, the membranes were incubated overnight at 4°C with 100 mM HRP in 25 mM Tris-HCl, pH 7.5. After final washings, the peroxidase activity was detected by incubation with 50 mM Tris-HCl, pH 7.5, containing 0.5 mg/ml 3,3'-diamino benzidine and 0.03% (v/v) H_2O_2.

RESULTS

During the optimization of the in vitro binding assay, we established that HRP binding to BBM follows a very fast kinetics, reaching equilibrium in less than 5 min and remaining constant for at least 30 min (data not shown). Therefore, all the binding assays were performed with an incubation time of 15 min. Because the quantification of HRP binding to BBM is based on measuring the enzymatic activity, it was important to verify the integrity of peroxidase during the incubation period with BBM. No degradation of HRP was observed by Western blot analysis. As shown in Figure 1, a single band corresponding to HRP was detected both in the binding assay media and in the pellet recovered after the incubation period, indicating the absence of proteolytic degradation (Fig. 1, lanes 2 and 3, respectively). Also, HRP activity remained stable during a 2-h incubation period performed at 25°C with

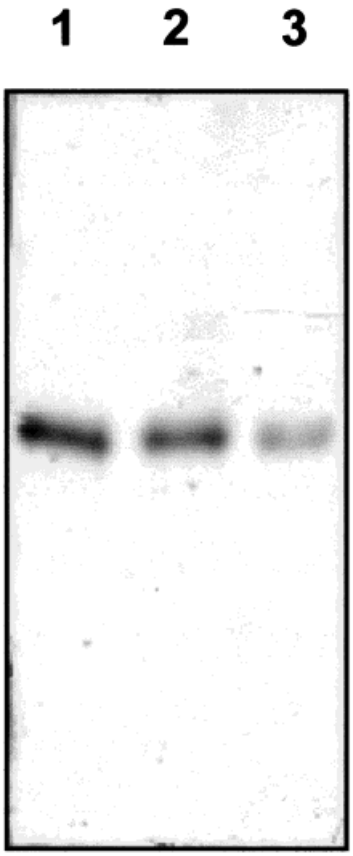

Fig. 1. Western blot analysis of the integrity of horseradish peroxidase (HRP) in the binding assay. Ten nanograms of control HRP (**lane 1**), 10 μl aliquot of a binding assay (**lane 2**), and pellet recovered from the binding assay containing HRP bound to brush-border membranes (BBM) (**lane 3**). Samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, incubated with anti-HRP antibody followed by an alkaline phosphatase-labeled antibody as described in Materials and Methods.

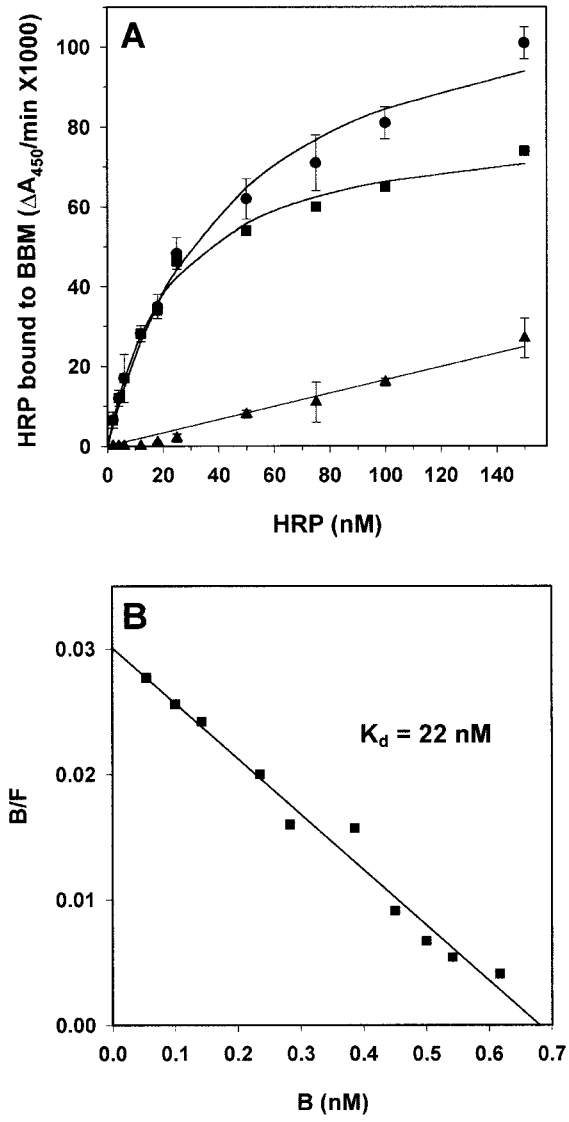

Fig. 2. Concentration-dependent binding of horseradish peroxidase (HRP) to brush-border membrane (BBM). **A:** Brush-border membranes were incubated with varying concentrations of HRP. The activity of bound HRP was determined as described in Materials and Methods. Total HRP binding (●) and non-specific binding was determined by adding 100 times the molar ratio of inactivated HRP to the binding assay (▲). Specific binding was calculated by subtracting the nonspecific binding from the total binding (■). Values represent means ± standard error measurements of triplicate determinations. **B:** Scatchard plot for the specific binding. Estimated K_d = 22 nM and B_{max} = 0.68 nM.

BBM, prior to the enzyme kinetic assay. In addition, a linear correlation between bound HRP detected by Western blot analysis and activity measurements was observed when different HRP concentrations were used in the binding assay (data not shown). Taken together, these results validate the binding assay used.

As shown in Figure 2, when a fixed amount of BBM was incubated with increasing concentrations of HRP, the specific binding exhibited a saturable mode, whereas nonspecific binding increased in a linear way at a significantly lower level. Binding analysis by Scatchard plot (Fig. 2, panel B) revealed the existence of one population of binding sites with a K_d of 22 nM and a B_{max} of 0.68 nM.

The internalization of proteins from the intestinal lumen into the enterocyte is a vectorial process and therefore one should expect more binding sites in the apical than in the basolateral membrane. Thus, it was important to evaluate the binding of HRP to both BBM and BLM

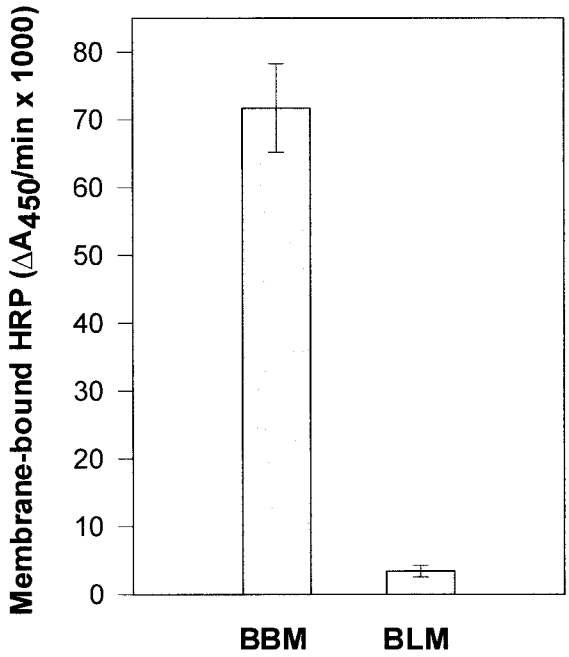

Fig. 3. Selective binding of horseradish peroxidase (HRP) to brush-border membranes (BBM). The binding of HRP was assayed using 0.6 mg/ml of membrane protein from either BBM or basolateral membranes (BLM). Mean ± standard error of three determinations.

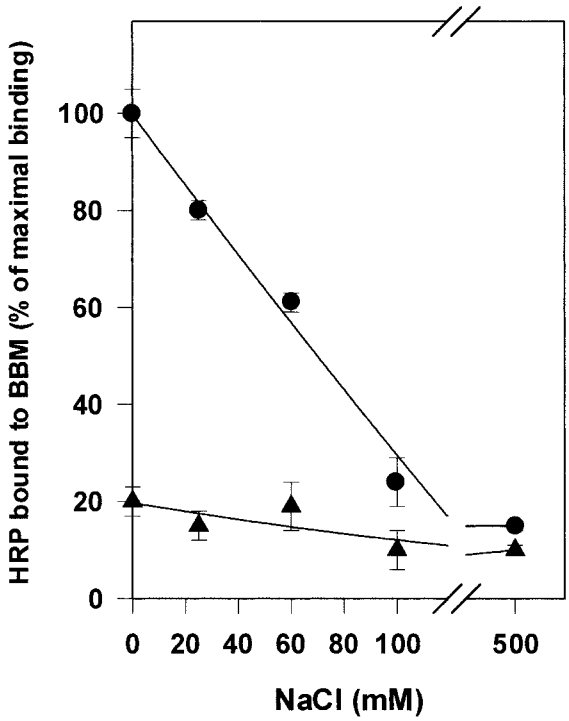

Fig. 4. NaCl effect on horseradish peroxidase (HRP) binding to brush-border membranes (BBM). Binding assays were performed as described, in the presence of increasing NaCl concentrations. Results were expressed as percentage of binding. Maximal binding (100%) represents HRP binding in the absence of NaCl. The figure presents the mean of three experiments. Bars show the standard error of the mean.

preparations. As expected, the results shown in Figure 3 were obtained using equal amounts of protein for each membrane preparation in the binding assay, indicating that HRP binds 20 times more to BBM than to BLM.

With the aim to characterize the nature of the interaction between HRP and its binding sites, we analyzed the effect of NaCl and different competitors in the binding assay. As shown in Figure 4, total HRP binding to BBM was markedly reduced when increasing NaCl concentrations were used. On the contrary, nonspecific binding remained invariable within the salt concentration range tested. At >200 mM NaCl, the total binding almost dropped to nonspecific binding levels. The maximum HRP binding was obtained in the absence of NaCl in the assay (Fig. 4). Similar results were obtained when KCl instead of NaCl was added to the assay (data not shown). Also, the addition of the polyions, heparin and poly-L-lysine (2,5 μM each) to the binding assay inhibited HRP binding to BBM in 80% and 83%, respectively (data not shown).

Competition between HRP and other proteins, some of which are also internalized by the carp enterocyte, was examined by incubating BBM with HRP in the presence of an excess molar concentration of the different proteins detailed in the legend to Figure 5. The results obtained indicate that only ferritin and IgG compete to some extent, diminishing HRP binding to BBM by 38% and 25%, respectively. All the other proteins tested had no significant effect on HRP binding, not even ovoalbumin, which has been frequently used as an HRP competitor based on its similarities with respect to molecular weight and type and degree of glycosylation. To analyze whether the mannose terminal glycosylations of HRP are involved in its binding to BBM, competition with mannan, a mannose polymer, was evaluated. As shown in Figure 5, mannan did not compete with HRP binding.

To characterize the nature of these HRP binding sites, brush-border membranes were pretreated with several enzymes that affect different components of the membrane surface.

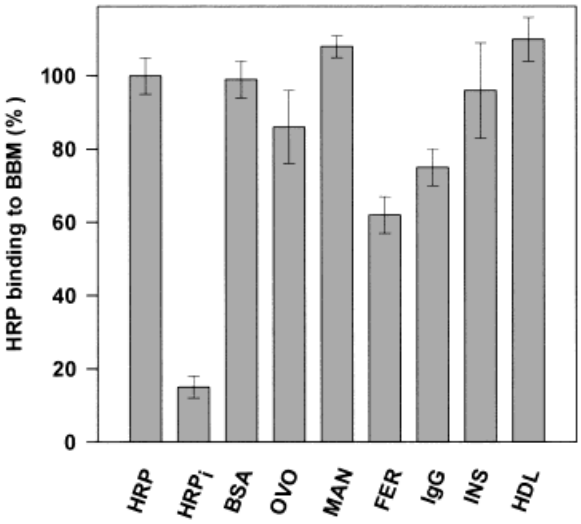

Fig. 5. Effect of different competitors on horseradish peroxidase (HRP) binding to brush-border membranes (BBM). Binding assays were performed using 50 nM HRP, in the presence of the following competitors: inactivated HRP (HRP_i), bovine serum albumin (BSA), ovoalbumin (OVO), mannan (MAN), horse spleen ferritin (FER), rabbit immunoglobulin G (IgG), porcine pancreas insulin (INS), and carp high density lipoprotein (HDL). Final concentration of all competitors was 2.5 μM. Results were expressed as percentage of binding where 100% represents HRP binding in the absence of competitor. Data from three separate experiments (mean ± standard error).

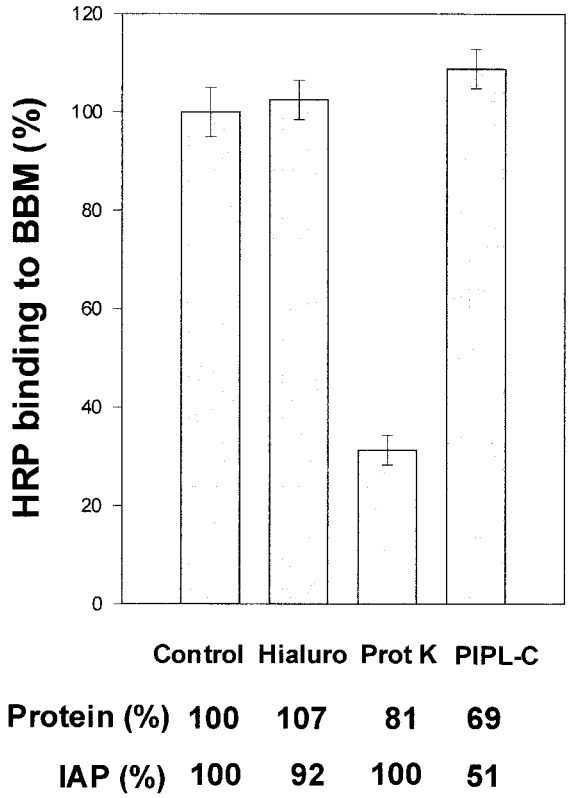

Fig. 6. Horseradish peroxidase (HRP) binding to treated brush-border membranes (BBM). Enzymatic treatments of BBM were performed previous to the binding assay as described in Materials and Methods. HRP binding, protein content, and intestinal alkaline phosphatase (IAP) activity in the BBM were expressed as percentages. One hundred percent represents the respective values obtained for control BBM. Data represent mean ± standard error from three independent experiments. Hialuro, hialuronidase; Prot K, proteinase K; PIPL-C, phosphatidyl inositol phospholipase C.

As depicted in Fig. 6, treatment of BBM with hialuronidase, which degrades mucopolysaccharides, did not affect HRP specific binding. Also, a 50% reduction of glycosyl-phosphatidilinositol (GPI)-anchored membrane protein in BBM, estimated through the remnant IAP activity after PIPL-C treatment, had no effect on HRP binding. However, treatment with proteinase K, which only diminished by 20% the total membrane protein associated to BBM, produced a 70% reduction of the initial HRP binding. These results clearly indicate that the specific HRP binding site probably corresponds to a membrane-associated protein. To visualize and further characterize the putative protein corresponding to this binding site, a ligand blot assay was devised. As shown in Figure 7, among all the BBM proteins (lane 3), HRP binds exclusively to a single protein band that was designated as mp15 based on its apparent molecular weight of 15.3 kDa (lane 1). The specificity of the ligand blot conditions used was demonstrated by the complete abolition of HRP binding after incubation with a 50-fold excess of HRPi.

DISCUSSION

Different approaches have been used to study the interaction of macromolecules and the apical membrane of enterocytes. Beside histochemical studies [Straus, 1983], other systems based on the utilization of isolated cells [Mackenzie et al., 1983; Kagami et al., 1984; Colyer et al., 1987], gut sacs [Stern and Walker, 1984], and BBM vesicles [Wallace and Rees, 1980; Kagami et al., 1984] have been used. Assays based on ligand binding to BBM vesicles followed by centrifugation or filtration steps are widely used due to their simplicity [Wallace and Rees, 1980; Kagami et al., 1984; Bolte et al., 1998a]. Recently a dot blot chemiluminescence assay was developed to study food protein binding to small intestinal BBM

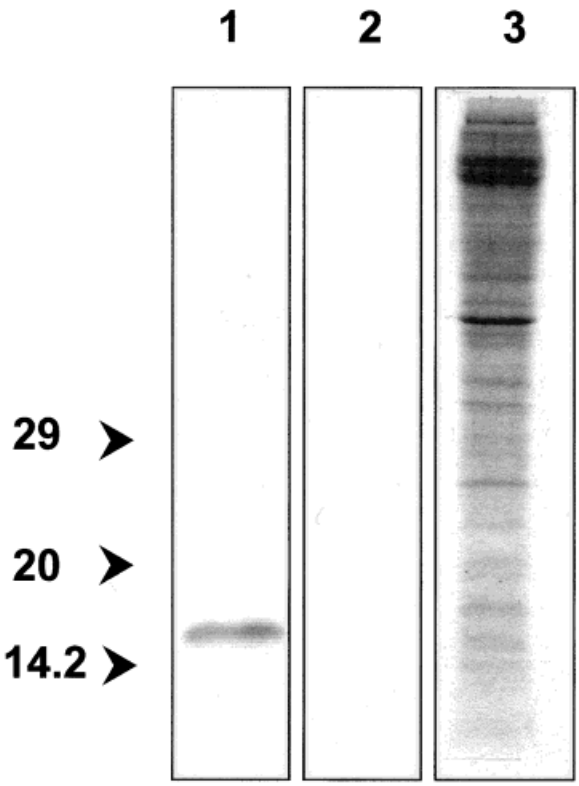

Fig. 7. Identification of horseradish peroxidase (HRP) binding sites in brush-border membranes (BBM) by ligand blot analyses. BBM proteins were separated by SDS-PAGE and electrotransferred to Immobilon-P membranes. After the blocking step, the membranes were incubated with 100 nM HRP **(lane 1)** and 100 nM HRP plus 5 μM inactivated HRP **(lane 2)**. The HRP activity was developed using 3,3′-diamino benzidine/H_2O_2. **Lane 3** shows BBM proteins stained with Coomasie blue. Arrows indicate protein molecular weight standards (Mr) in kDa.

[Bolte et al., 1997]. Although this assay was more sensitive for binding studies than the centrifugation assay, it presents an important disadvantage that should be considered. During immobilization of the BBM, the membrane structures become disrupted [Bolte et al., 1997; 1998b] and as a consequence, intravesicular proteins (such as villin and actin) might become accessible to interact with the ligand tested. Therefore, we preferred to use BBM vesicles in a soluble binding assay. On the other hand, the utilization of a ligand of high specific activity such as HRP fulfilled the requirements for sensitivity in our binding assay.

Lee and Cossins [1990] established an isolation procedure to obtain crude preparations of BBM and BLM from the same carp intestinal homogenate, with reasonable purity and limited cross-contamination. These authors also determined that marker enzymes for mitochondria, lysosomes, and endoplasmic reticulum showed no enrichment either in BBM or in BLM fractions. However, these carp BBM preparations have never been evaluated for the presence of possible protease activities. This could be a problem for the proper validation of binding assays utilizing these preparations. In fact, in rabbit, the degradation of β-lactoglobulin by proteases in ileum BBM preparations has been demonstrated [Caillard and Tomé, 1994]. In spite of this possible complication, our results (Fig. 1) indicate that the addition of protease inhibitors such as benzamidine and phenylmethylsulfonyl fluoride to BBM preparations prevented any detectable degradation of the ligand (HRP) in our binding assays. Moreover, the activity of the enzyme also remained unaffected after incubation with BBM preparations.

Horseradish peroxidase binding was measured using a protein concentration range from 2 to 150 nM. The results indicate that saturation of BBM binding sites occurs with an HRP concentration higher than 100 nM. The corresponding dissociation constant and maximal binding was estimated expressing these data in the form of a Scatchard plot. The K_d (22 nM) obtained indicates that this HRP binding site has comparable or higher affinity than other BBM protein binding sites from similar systems, e.g., the binding of IgG to BBM ($K_d \approx 10^{-8}$ M) [Wallace and Rees, 1980] or lactotransferrin to rabbit BBM (K_d 1.8 μM) [Mazurier et al., 1985]. In a recent study, the saturable binding of proteins to BBM was also demonstrated, but unfortunately no dissociation constants were noted [Bolte et al., 1998a].

In fish as well as in mammals, the columnar cells of the intestinal epithelium are held together at their apical boundary by a continuous network of tight junctions acting as a barrier to the passive diffusion of ions and macromolecules through a paracellular route [Weinberg, 1976; Kiliaan et al., 1996]. Thus, the protein to be internalized must interact with the apical membrane (BBM) of the enterocyte. Therefore, our results showing an enrichment of specific HRP binding sites in BBM compared with BLM is consistent with this proposed mechanism for HRP internalization and also with the polarized nature of the enterocytes. On the other hand, these results are also in agreement with the morphologic evidence that demon-

strate the early interaction of HRP with the apical membrane of the intestinal epithelium [Noaillac-Depeyre and Gas, 1973; Rombout et al., 1985]. All the above data suggest that the specific HRP binding to the carp intestinal brush-border membranes is the necessary first step that will lead to its endocytosis and transepithelial transport during the internalization process.

Seven horseradish peroxidase isoenzymes have been isolated and characterized [Shannon et al., 1966]. The isoenzyme used in this study is basic [Oliver et al., 1989] and therefore is positively charged at the pH used in the binding assay. The existence of anionic sites in different cell membranes, including the apical membrane of mammalian enterocytes, has been demonstrated using cationized ferritin and other chemically modified proteins as ligand [Danon et al., 1972; Triguereo et al., 1989; Sanderson and Walker, 1993; Lehr, 1994]. The ionic interaction of a ligand with these anionic sites is usually associated with a nonreceptor mediated endocytosis that segregates the ligand to a degradative pathway within the cell [Gómez-Pascual et al., 1995]. Our results show that increasing NaCl concentrations in the binding assay only affected the specific binding, suggesting that HRP is not interacting with the broad family of anionic sites. In fact, the Scatchard analysis gave a linear plot, which is consistent with the presence of a homogeneous population of binding sites. Nevertheless, the specific interaction of HRP with its binding sites, under the in vitro conditions used, presents an ionic character. It must be considered that these conditions do not necessarily represent the in vivo interaction of HRP with the apical membrane of enterocytes because isolated BBM vesicles lack important parts of the mucosal barrier [Sanderson and Walker, 1993].

It is clear from the results presented here that the binding of HRP to carp BBM satisfies all the criteria required for a receptor molecule. The interaction is saturable, has a high binding affinity, and is specific for HRP. In fact, only two of six proteins tested, IgG and ferritin, partially compete for HRP binding to BBM. Although most of the competitor proteins used are structurally unrelated to HRP, all of them meet the criteria of being internalized when administered orally to the carp. Bolte et al. [1998a], studying the binding of food proteins to rat small intestinal BBM, also tested the specificity using different unrelated dietary proteins. In this study they demonstrated that all the proteins tested were able to compete with each other, supporting the idea of common or at least adjoining binding sites for different food proteins in rat BBM. On the contrary, our results are consistent with a specific and independent binding site for HRP.

Horseradish peroxidase is a mannose-terminated glycoprotein [Clarke and Shannon, 1976] and therefore its uptake by fluid phase endocytosis is considered to be receptor-mediated via a mannose receptor in macrophages and hepatic nonparenchymal cells [Yamaguchi et al., 1993]. In addition, mannose-specific binding sites for HRP have been demonstrated in several other cell types [Straus, 1983]. Taking in consideration that the HRP binding to carp BBM was inhibited by neither mannan nor ovoalbumin, a glycoprotein that also exhibits mannose terminal groups, the involvement of a lectin-type of interaction is very unlikely.

To analyze the nature of the HRP binding sites, brush-border membranes were pretreated with several enzymes to affect different components of the membrane. Hialuronidase treatment did not affect HRP specific binding to carp BBM. This treatment had been previously used to remove components of the glycocalyx in rat BBM where it also effectively reduced nonspecific binding, thereby enhancing the IgG-specific binding [Wallace and Rees, 1980]. A second treatment that also did not affect HRP binding was performed with PIPL-C, an enzyme that partially removes GPI-anchored proteins from the membranes. This result suggests that most probably the putative receptor does not correspond to this class of membrane protein. Nevertheless, it is important to note that some GPI-anchored proteins are resistant to PIPL-C [Wong and Low, 1992]. Protease treatment is a standard procedure used to demonstrate the proteic nature of a macromolecule with biologic activity. Treatments of BBM with proteinase K, which produces a limited proteolysis of the outer leaflet of the vesicle, reduced the HRP binding by 70%. Taken together, these results strongly suggest that the putative receptor for HRP is a protein.

With the ligand blotting technique, different membrane receptors have been identified [Ma-

zurier et al., 1985; Graham and Oram, 1987; Semenkovich et al., 1990]. Using this technique we were able to demonstrate specific HRP binding to a single band. Based on its electrophoretic mobility on 0.1% SDS slab gels in the absence of reducing agents (15.3 kDa), this putative HRP receptor was designated as mp15. Although this receptor retained its ability to bind HRP in the presence of 0.1 % SDS, previous treatment of the sample with heat (above 50°C) or reducing agents, such as 140 mM β-mercaptoethanol, completely abolished the ligand binding. These results give additional support to the idea that the HRP binding site in carp BBM is proteic in nature. This optimized ligand blot assay will constitute an important tool to proceed with the purification and further characterization of this putative HRP receptor.

Several hypotheses have been proposed to explain the possible physiologic significance of the ability of the fish gut to absorb intact proteins. One of these hypotheses suggests that the nonselective absorption of proteins may represent an extension of the intestinal digestive capacity by intracellular degradation [Ezeasor and Stokoe, 1981; Georgopoulou et al., 1986]. In addition, McLean and Ash [1987] suggested that in agastric teleosts this process could constitute a compensatory mechanism for their absence of stomach. Another proposed function for this internalization process is that it would be required for the existence of a putative entero-pancreatic recycling system for digestive enzymes. It has been argued that such a system would not only prevent waste of active enzymes, but it would also save the energetic costs involved in the hydrolysis and de novo synthesis of these enzymes [Hofer and Schiemer, 1981; Hofer, 1982].

On the other hand, Davina et al. [1982] and Dorin at al. [1993] considered that the absorption of intact proteins by the fish enterocytes might provide an antigen sampling system similar to that associated with M-cell specialization in higher vertebrates.

Efforts to elucidate the molecular mechanisms involved in protein internalization using different tracer macromolecules will be helpful to clarify the real physiologic meaning of this process. Although HRP is not a normal component of carp food, it has proven to be an excellent tracer macromolecule to dissect the mechanisms underlying the internalization process. The mp15 protein, which apparently is responsible for the HRP specific binding sites characterized in this study, most probably constitutes a subunit of an oligomeric type of receptor whose natural ligand and physiologic function are still unknown. One could also speculate that binding of HRP, or the natural ligand, to this functional receptor in the carp intestine could trigger cellular signals producing the endocytosis of the receptor-ligand complex. However, further studies will be needed to test any of these hypotheses.

REFERENCES

Abaurrea MA, Nuñez MI, Ostos MV. 1993. Ultrastructural study of the distal part of the intestine of *Oncorhynchus mykiss*. Absorption of dietary protein. Micron 24:445–450.

Abrahamson DR, Rodewald R. 1981. Evidence for the sorting of endocytic vesicle contents during the receptor-mediated transport of IgG across the newborn rat intestine. J Cell Biol 91:270–280.

Bolte G, Knauss M, Metzdorf I, Stern M. 1997. Dot blot chemiluminescence assay for studying food protein binding to small intestinal brush border membranes in vitro. J Biochem Biophys Methods 34:189–203.

Bolte G, Knauss M, Metzdorf I, Stern M. 1998a. Postnatal maturation of rat small intestinal brush border membranes correlates with increase in food protein binding capacity. Dig Dis Sci 43:148–155.

Bolte G, Wolburg H, Beuermann K, Stocker S, Stern M. 1998b. Specific interaction of food proteins with apical membranes of the human intestinal cell lines Caco-2 and T84. Clin Chim Acta 270:151–167.

Caillard I, Tomé D. 1994. Modulation of β-lactoglobulin transport in rabbit ileum. Am J Physiol 266:G1053–1059.

Clarke J, Shannon LM. 1976. The isolation and characterization of the glycopeptides from horseradish peroxidase isoenzyme C. Biochim Biophys Acta 427:428–442.

Colyer J, Kumar PJ, Waldron NM, Clark ML, Farthing MJG. 1987. Gliadin binding to rat and human enterocytes. Clin Sci 72:593–598.

Danon D, Goldstein Y, Marikovsky Y, Skutelsky E. 1972. Use of cationized ferritin as a label of negative charges on cell surfaces. J Ultrast Res 38:500–510.

Davina JHM, Parmentier HK, Timmermans LPM. 1982. Effect of oral administration of *Vibrio* bacteria on the intestine of cyprinid fish. Dev Comp Immunol Suppl 2:157–166.

Dorin D, Martin P, Sire MF, Smal J, Vernier JM. 1993. Protein uptake by intestinal macrophages and eosinophilic granulocytes in trout: an *in vivo* study. Biol Cell 79:37–44.

Ezeasor DN, Stokoe WM. 1981. Light electron microscope studies of the absorptive cells of intestine, caeca and rectum of the adult rainbow trout *Salmo gairdneri*, Rich. J Fish Biol 18:527–544.

Gardner MLG. 1988. Gastrointestinal absorption of intact proteins. Ann Rev Nutr 8:329–350.

Georgopoulou U, Vernier JM. 1986. Local immunological response in the posterior intestinal segment of the rainbow trout after oral administration of macromolecules. Dev Comp Immunol 10:529–537.

Georgopoulou U, Dabrowski K, Sire MF, Vernier JM. 1988. Absorption of intact proteins by the intestinal epithelium of trout, *Salmo gairdneri*. A luminescence enzyme immunoassay and cytochemical study. Cell Tissue Res 251:145–152.

Gómez-Pascual A, Londoño I, Ghitescu L, Desjardins M, Bendayan M. 1995. Immunocytochemical investigation of the in vivo endocytosis by renal tubular epithelial cells. Microsc Res Tech 31:118–127.

Graham DL, Oram JF. 1987. Identification and characterization of a high-density lipoprotein-binding protein in cell membranes by ligand blotting. J Biol Chem 262: 7439–7442.

Hertz Y, Techelet A, Madar Z, Gerter A. 1991. Absorption of bioactive human growth hormone after oral administration in the common carp (*Cyprinus carpio*) and its enhancement by deoxycholate. J Comp Physiol B 161: 159–163.

Hertz Y, Shechter Y, Madar Z, Gertler A. 1992. Oral absorption of biologically active insulin in common carp (*Cyprinus carpio L.*). Comp Biochem Physiol 101A:19–22.

Heyman M, Desjeux JF. 1992. Significance of intestinal food protein transport. J Pediatr Gastroenterol Nutr 15: 48–57.

Hofer R. 1982. Protein digestion and proteolytic activity in the digestive tract of an omnivorous cyprinid. Comp Biochem Physiol 72A:55–63.

Hofer R, Schiemer F. 1981. Proteolytic activity in the digestive tract of several species of fish with different feeding habits. Oecologia 48:342–345.

Kagami A, Fidge N, Suzuki N, Nestel P. 1984. Characteristics of the binding of high-density lipoprotein-3 by intact cells and membrane preparations of rat intestinal mucosa. Biochim Biophys Acta 795:179–190.

Kiliaan AJ, Scholten G, Bijlsma PB, Dekker K, Groot JA. 1996. Influence of forskolin and carbachol on intestinal absorption of horseradish peroxidase in the goldfish (*Carassius auratus*). Cell Tissue Res. 285:51–56.

Kimm MH, Curtis GH, Hardin JA, Gall DG. 1994. Transport of bovine serum albumin across rat jejunum: role of the enteric nervous system. Am J Physiol 266:G186–G193.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, Lond. 227:680–685.

Le Bail PY, Sire MF, Vernier JM. 1989. Intestinal transfer of growth hormone into the circulatory system of the rainbow trout, *Salmo-gairdneri*: interference by granule cells. J Exp Zool 251:101–107.

Lee JAC, Cossins AR. 1990. Temperature adaptation of biological membranes: differential homeoviscous responses in brush-border and basolateral membranes of carp intestinal mucosa. Biochim Biophys Acta 1026:195–203.

Lehr CM. 1994. Bioadhesion technologies for the delivery of peptide and protein drugs to the gastrointestinal tract. CRC Crit Rev Therap Drug Carrier Syst 11:119–160.

Lamaze C, Schmid SL. 1995. The emergence of clathrin-independent pinocytic pathways. Curr Opin Cell Biol 7:573–580.

Mackenzie NM, Morris B, Morris R. 1983. The binding of proteins to isolated enterocytes from the small intestine of the neonatal rat. Immunology 48:489–496.

Mazurier J, Montreuil J, Spik G. 1985. Visualization of lactotransferrin brush-border receptors by ligand-blotting. Biochim Biophys Acta 821:453–460.

McLean E, Ash R. 1986. The time-course of appearance and net accumulation of horseradish (HRP) presented orally to juvenile carp *Cyprinus carpio* (L.). Comp Biochem Physiol 84A:687–690.

McLean E, Ash R. 1987. The time-course of appearance and net accumulation of horseradish peroxidase (HRP) presented orally to rainbow trout *Salmo gairdneri* (Richardson). Comp Biochem Physiol 88A:507–510.

Moriyama S, Takayashi M, Hirano T, Kawauchi H. 1990. Salmon growth hormone is transported into the circulation of rainbow trout, *Onchorhynchus mykiss*, after intestinal administration. J Comp Physiol 160B:251–257.

Nakamura O, Kobayashi M, Suzuki Y, Aida K, Hanyu I. 1990. Transport of orally administered rabbit IgG into blood circulation of goldfish. Nippon Suisan Gakkaishi Bull Jap Soc Sci Fish 56:1749–1753.

Nakamura O, Suzuki Y, Aida K. 1998. Humoral immune response against orally administered human gamma globulin in the carp. Fisheries Science 64:558–562.

Neutra MR, Frey A, Kraehenbuhl JP. 1996. Epithelial M cells: gateways for mucosal infection and immunization. Cell 86:345–348.

Noaillac-Depeyre J, Gas N. 1973. Absorption of protein macromolecules by the enterocytes of the carp (*Cyprinus carpio* L). Z Zellforsch 146:525–541.

Oliver C, Tolbert CL, Waters JF. 1989. Internalization of horseradish peroxidase isozymes by pancreatic acinar cells in vitro. J Histochem Cytochem 37:49–56.

Ortiz de Montellano PR, David KS, Ator MA, Tew D. 1988. Mechanism-based inactivation of horseradish peroxidase by sodium azide. Formation of meso-azidoprotoporphyrin IX. Biochemistry 27:5470–5476.

Rombout JHWM, Lamers CHJ, Helfrich MH, Dekker A, Taverne-Thiele AJ. 1985. Uptake and transport of intact macromolecules in the intestinal epithelium of carp (*Cyprinus carpio* L.) and the possible immunological implications. Cell Tissue Res 239:519–530.

Sakai M, Kobayashi M, Yoshida T. 1995. Activation of rainbow trout, *Oncorhynchus mykiss*, phagocytic cells by administration of bovine lactoferrin. Comp Biochem Physiol 110B:755–759.

Sanderson IR, Walker WA. 1993. Uptake and transport of macromolecules by the intestine: possible role in clinical disorders (an update). Gastroenterology 104:622–639.

Semenkovich CF, Ostlund JrRE, Olson MOJ, Yang JW. 1990. A protein partially expressed on the surface of HepG2 cells that binds lipoproteins specifically is nucleolin. Biochemistry 29:9708–9713.

Shannon LM, Kay E, Lew JY. 1966. Peroxidase isozymes from horseradish roots. I. Isolation and physical properties. J Biol Chem 241:2166–2172.

Sire MF, Vernier JM. 1992. Intestinal absorption of protein in teleost fish. Comp Biochem Physiol 103A:771–781.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85.

Stern M, Walker WA. 1984. Food proteins and gut mucosal barrier I. Binding and uptake of cow's milk proteins by adult rat jejunum in vitro. Am J Physiol 246:G556–562.

Stoorvogel W. 1998. Analysis of the endocytic system by using horseradish peroxidase. Trends Cell Biol 8:503–505.

Straus W. 1983. Mannose-specific binding sites for horseradish peroxidase in various cells of the rat. J Histochem Cytochem 31:78–84.

Triguereo D, Buciak J, Yang J, Padridge W. 1989. Blood-brain barrier transport of cationized immunoglobulin G. Enhanced delivery compared to native protein. Proc Natl Acad Sci 86:4761–4766.

Vera MI, Romero F, Amthauer R, Figueroa J, Goicoechea O, Leon G, Krauskopf M. 1992. Carp apolipoprotein A-I intestinal absorption and transfer into the systemic circulation during the acclimatization of the carp (*Cyprinus carpio*). Comp Biochem Physiol 101A:573–581.

Vera MI, Romero F, Figueroa J, Amthauer R, Leon G, Villanueva J, Krauskopf M. 1993. Oral administration of insulin in winter-acclimatized carp (*Cyprinus carpio*) induces hepatic ultrastructural changes. Comp Biochem Physiol 106A:677–682.

Villanueva J, Vanacore R, Goicoechea O, Amthauer R. 1997. Intestinal alkaline phosphatase of the fish *Cyprinus carpio*: regional distribution and membrane association. J Exp Zool 279:347–355.

Wallace KH, Rees AR. 1980. Studies on the immunoglobulin-G Fc-Fragment receptor from neonatal rat small intestine. Biochem J 188:9–16.

Weinberg S. 1976. Morphology of the intestine of goldfish (*Carassius auratus*). Bijdragen Tot de Dierkunde 46:35–46.

Wong YW, Low MG. 1992. Phospholipase resistance of the glycosyl-phosphatidylinositol membrane anchor on human alkaline phosphatase. Clin Chem 38:2517–2525.

Yamaguchi Y, Dallemolle E, Hardison WGM. 1993. Hepatocyte horseradish peroxidase uptake is saturable and inhibited by mannose-terminal glycoproteins. Am J Physiol 264:G880–G885.