Functional studies of a glucagon receptor isolated from frog *Rana tigrina rugulosa*: implications on the molecular evolution of glucagon receptors in vertebrates

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Abstract In this report, the first amphibian glucagon receptor (GluR) cDNA was characterized from the liver of the frog *Rana tigrina rugulosa*. Functional expression of the frog GluR in CHO and COS-7 cells showed a high specificity of the receptor towards human glucagon with an EC_{50} value of 0.8 ± 0.5 nM. The binding of radioiodinated human glucagon to GluR was displaced in a dose-dependent manner only with human glucagon and its antagonist (des-His^1-[Nle^9-Ala^{11}-Ala^{16}]) with IC_{50} values of 12.0 ± 3.0 and 7.8 ± 1.0 nM, respectively. The frog GluR did not display any affinity towards fish and human GLP-1s, and towards glucagon peptides derived from two species of teleost fishes (goldfish, zebrafish). These fish glucagons contain substitutions in several key residues that were previously shown to be critical for the binding of human glucagon to its receptor. By RT-PCR, mRNA transcripts of frog GluR were located in the liver, brain, small intestine and colon. These results demonstrate a conservation of the functional characteristics of the GluRs in frog and mammalian species and provide a framework for a better understanding of the molecular evolution of the GluR and its physiological function in vertebrates.

Key words: Glucagon; Glucagon-like peptide 1; Glucagon antagonist des-His^1-[Nle^9-Ala^{11}-Ala^{16}]; Glucagon receptor; Frog

1. Introduction

Glucagon is a 29 amino acid peptide which belongs to a large family of brain-gut peptides that includes glucagon-like peptides (GLPs), gastric inhibitory polypeptide (GIP), pituitary adenylate cyclase activating polypeptide (PACAP), vasoactive intestinal polypeptide (VIP), secretin, growth-hormone releasing factor (GRF) and peptide histidine methionine (PHM). Glucagon is encoded in a precursor structure, preproglucagon, which is differentially processed to glucagon in α-cells of the pancreas, and to GLP-1 and GLP-2 in the L-cells of the small intestine [1,2]. Glucagon shows strong functional and structural conservation across vertebrate species. The major physiological functions of glucagon in mammals and fish are similar. Glucagon plays a central role in the physiology of blood glucose regulation by inducing the processes of hepatic glycogenolysis and gluconeogenesis [3,4]. However, some of the actions of glucagon have diversified during evolution. Glucagon is able to stimulate ureogenesis in mammals, but this has not been demonstrated in fish, including the ureogenic toadfish, *Opsanus beta* [5]. In addition, the redundant effects of GLP-1 and glucagon in fish [6,7]) make the functional and evolutionary relationships between GLP-1 and glucagon in vertebrates a fascinating story to investigate. Structurally, the primary amino acid sequence of glucagon is conserved across most vertebrates (Fig. 1) [7]. In addition to mammalian glucagons [8], peptides or cDNAs corresponding to bird [9], several species of frogs [10–13], bony fishes [14], cartilaginous fishes [15] and jawless fishes [16] have also been characterized. The conservation of glucagon structure indicates that there has been a strong evolutionary pressure to conserve the whole glucagon molecule in vertebrates, and this finding is consistent with its central position in the regulation of metabolism.

The actions of glucagon are mediated via its specific interaction with cell-surface receptors, hence, characterization of the glucagon receptor (GluR) is a major step towards the understanding of glucose metabolism. The glucagon receptor belongs to a subfamily of the glucagon-secretin receptors including glucagon, GLP-1 and GIP receptors. Like other family members, GluR is a glycoprotein with a large hydrophilic extracellular domain followed by seven highly conserved hydrophobic transmembrane helices. Recently, GluRs from rat [17], human [18] and mouse [19] have been cloned and characterized. Due to the central position of amphibians in vertebrate evolution, the characterization of the frog GluR is able to fill a significant gap of our knowledge with respect to the understanding of the evolutionary aspects of glucagon and GLP-1 as well as their receptors in vertebrates.

2. Materials and methods

2.1. Cloning of the full-length frog Rana tigrina rugulosa glucagon receptor cDNA

A partial cDNA clone corresponding to the transmembrane domains (TMD) 2–6 of the putative frog GluR was obtained by a two-step polymerase chain reaction (PCR) approach essentially following a protocol described earlier [20]. The partial cDNA clone was used as a probe to screen a frog liver cDNA library (0.5 million primary clones). The library was constructed using the Stratagene ZAP-Express cDNA library system. 5 μg of poly(A$^+$) RNA was used and the number of primary clones obtained was 2.5 million. A full-length cDNA clone (2.2 kb) encoding the frog GluR was isolated and then excised to the phagemid, pBK-CMV-frogGR1. The clone was sequenced from both strands using a T7 DNA sequencing kit

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(Pharmacia) by synthetic primers and by subcloning of restriction fragments. DNA sequence was analyzed by DNasis v2.0 (Hitachi, San Bruno, CA).

2.2. Functional expression of the frog glucagon receptor

2.2.1. Stimulation of intracellular cAMP in CHO cells expressing the frog glucagon receptor. The bacterial *lac* promoter was released from the construct pBK-CMV-frogGR1 by releasing a *Eco*RI/*Nhe*I restriction fragment to produce the eukaryotic expression plasmid pBK-CMV-frogGR. A permanent cell line, CHO-frogGR, expressing the putative frog GluR was obtained by transfecting 10 μg of pBK-CMV-frogGR into 1 million CHO cells using the Lipofectamine reagent (Gibco-BRL), and followed by G418 selection at 500 μg/ml (Gibco-BRL) for 2 weeks. Functional expression and cAMP assays were performed as described earlier [20]. The cAMP level was measured by radioimmunoassay using a cAMP assay kit (Amersham, Arlington Heights, IL). All peptides used in this study were purchased from Bachem Fine Chemicals, Inc. (Torrance, CA). Goldfish glucagon was synthesized by Peninsula Laboratories, Inc. (Belmont, CA) and was found to be able to stimulate cAMP production and bind specifically to the goldfish GluR (unpublished results).

2.2.2. Competitive binding experiments with the frog glucagon receptor. For the competitive binding experiments, the frog GluR was expressed transiently into COS-7 cells using the standard protocol [21]. In brief, COS-7 cells were grown to confluence into 100 mm plates, and transfected with 8 μg pBK-CMV-frogGR using the DEAE-dextran/chloroquine method. After 24 h, cells were transferred into 24 well plates and cultured for an additional 24 h prior to the binding assay. In the competitive binding experiments, ^{125}I-human glucagon (2200 Ci/mmol, receptor grade, NEN Life Science Products, Boston, MA) was incubated with the COS-7 cells expressing the frog GluR in the presence of increasing concentrations (pM–μM) of different peptides for 16–18 h at 4°C. The non-specific binding was determined in the presence of 1 μM human glucagon and/or 1 μM glucagon antagonist des-His^{1}-[Nle^{9}-Ala^{11}-Ala^{16}] [22]. Peptides were diluted from a stock solution (10 μM) in the binding buffer prepared in Hanks' balanced salt solution containing 20 mM HEPES, pH 7.4, 0.5% BSA and 0.1 mM phenylmethylsulfonyl fluoride. Each peptide concentration was added in triplicate wells. Following the incubation, the cells were washed twice with ice-cold PBS, lysed with 1 M NaOH, and radioactivity measured in a gamma counter. Total specific binding was on average 45% of the total radioactivity added (1×10^{5} cpm). The glucagon antagonist des-His^{1}-[Nle^{9}-Ala^{11}-Ala^{16}] [22] was a gift from Dr. Cecille Unson, while zebrafish glucagon was synthesized by the Protein/DNA Technology Center of the Rockefeller University. ^{125}I-zebrafish glucagon was found to bind specifically to the goldfish GluR transfected into COS-7 cells (Mojsov et al., unpublished results).

2.2.3. cAMP assays with the frog glucagon receptor expressed transiently in the COS-7 cells. The ability of glucagon and its antagonist to stimulate intracellular cAMP was assayed in parallel with the competitive binding experiments. The conditions of the incubations were the same as the ones described for the CHO cells expressing the frog GluR, with the exception that the cells were seeded into 24 well plates, instead of 6 well plates. The levels of cAMP were determined by the Enzyme Immunoassay Kit (Cayman Chemicals, Ann Arbor, MI). To assess whether the glucagon antagonist is able to inhibit the ability of glucagon to stimulate intracellular cAMP formation, the antagonist was added to each well at 100 nM concentration prior to the addition of increasing concentrations of glucagon (pM–μM). Forskolin (100 nM) was used as a positive control in triplicate wells in each plate.

2.3. Tissue distribution of the frog glucagon receptor mRNA

Tissue distribution of the frog GluR transcripts was studied by RT-PCR. 3 μg poly(A)$^{+}$ RNA from individual tissues was used for the preparation of first stand cDNA [20]. The sequences of PCR primers are: GLU-5 TCCGTGCTTGTGAATGACACCATGC and GLU-3 GTCTGTGTACCTCATCTGATGAGC. PCR conditions were 1 min at 94°C, 1 min at 55°C and 1 min at 72°C for 30 cycles. The identity of the PCR product was confirmed by Southern blotting using the partial frog GluR cDNA fragment (443 bp) as a probe.

	1				5					10					15					20					25				
Mammalian	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T
Avian (chicken)	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	S	T
Xenopus l.	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	T
Amphiuma	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	N	R	R	A	Q	D	F	I	Q	W	L	M	S	T
Bullfrog	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	S
Bufo marinus	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	S
R. sylvatica	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	S
R. tigrina R.	H	S	Q	G	T	F	T	S	D	Y	S	K	Y	L	D	S	R	R	A	Q	D	F	V	Q	W	L	M	N	S
Anglerfish I	H	S	E	G	T	F	S	N	D	Y	S	K	Y	L	E	D	R	K	A	Q	E	F	V	R	W	L	M	N	N
Anglerfish II	H	S	E	G	T	F	S	N	D	Y	S	K	Y	L	E	T	R	R	A	Q	D	F	V	Q	W	L	K	N	S
Catfish	H	S	E	G	T	F	S	N	D	Y	S	K	Y	L	E	T	R	R	A	Q	D	F	V	Q	W	L	M	N	S
Coho salmon	H	S	E	G	T	F	S	N	D	Y	S	K	Y	Q	E	E	R	M	A	Q	D	F	V	Q	W	L	M	N	S
Rainbow Trout I	H	S	E	G	T	F	S	N	D	Y	S	K	Y	Q	E	E	R	M	A	Q	D	F	V	Q	W	L	M	N	S
Zebrafish	H	S	E	G	T	F	S	N	D	Y	S	K	Y	L	E	T	R	R	A	Q	D	F	V	Q	W	L	M	N	A
Goldfish	H	S	E	G	T	F	S	N	D	Y	S	K	Y	L	E	T	R	R	A	Q	D	F	V	E	W	L	M	N	S

Fig. 1. Comparison of amino acid sequences of glucagon in vertebrate. Shaded areas represent identical amino acid residues. The chicken sequence is from [9], the *Xenopus laevis* from [11], bullfrog from [10], *Bufo marinus* from [14], and *Rana sylvatica* from [13]; the sequences from different species of teleost fishes are adapted from [7] with the exception of glucagons from goldfish [14] and zebrafish [28].

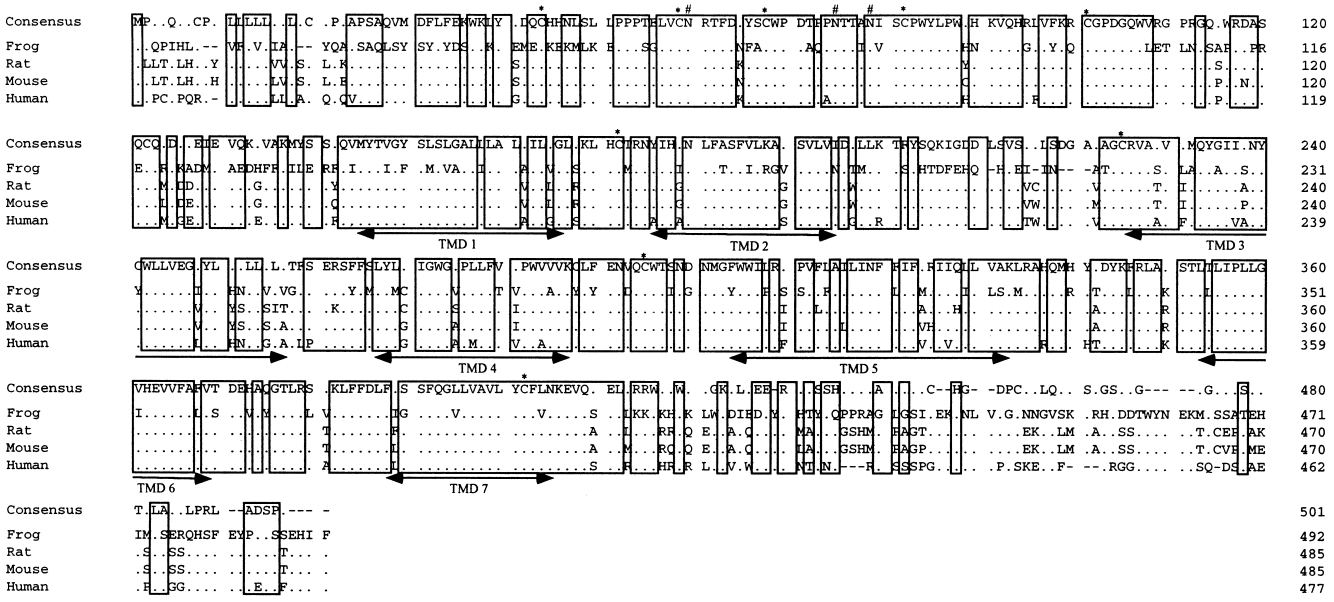

Fig. 2. Amino acid sequence alignment of frog (top), rat, mouse and human GluRs by GeneWorks (IntelliGenetics). The arrows represent the seven putative TMDs. Identical and conservative sequences are boxed. Conserved *N*-linked glycosylation sites and Cys residues among these GluRs are labeled # and *, respectively.

3. Results

3.1. Isolation of the frog glucagon receptor cDNA

The approach used to obtain partial GluR clones was based on the amplification of partial VIP1 receptor cDNAs using degenerate oligonucleotide primers [20]. The partial receptor cDNA clone was subsequently used as a probe to screen a frog liver cDNA library. A clone 2224 bp in length was obtained and DNA sequence analysis of the putative GluR cDNA clone revealed a single open reading frame of 1476 bp (from nucleotide 266 to 1741) encoding a protein of 492 amino acids (Fig. 2) with a predicted molecular weight of 56 kDa. The sequence homologies between the frog and human or rat GluRs were 57% and 62%, respectively, at the cDNA level, and 51% and 60% at the amino acid level. A Kyte-Doolittle hydrophobicity analysis of the receptor indicated that the protein is a G protein-coupled receptor with seven segments of hydrophobic amino acids presumably forming the transmembrane spanning regions and a hydrophobic signal peptide at the N-terminus (data not shown).

3.2. Functional expression of the frog glucagon receptor

3.2.1. Stimulation of intracellular cAMP levels. To demonstrate that the recombinant frog GluR expressed in mammalian CHO cells could transduce a cellular signal, cAMP responses in the presence of various peptides were measured (Fig. 3A). We used the human glucagon for the functional characterization of the frog GluR because there is only one conservative difference (Ser-29 replacing Thr-29) at the C-termini of human and *Rana tigrina rugulosa* glucagons (Fig. 1). More importantly, this residue was shown not to be involved in the binding of human glucagon to its receptor [22,26,27].

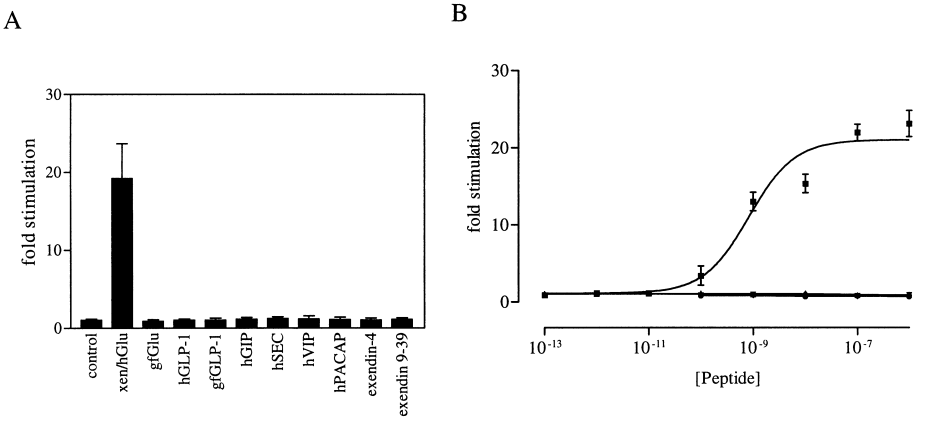

Fig. 3. Stimulation of cAMP production in CHO-frog GluR cells. A: The cells were incubated with 100 nM of human (h), *Xenopus* (xen) and goldfish (gf) glucagon and several structurally related peptides including hGLP-1, gfGLP-1, hGIP, hVIP, human secretin (hSEC), hPACAP-38, exendin-4 and exendin (9–39). cAMP levels are expressed as fold stimulation compared to that of the control (no peptide). B: cAMP production in CHO-frog GluR cells stimulated with various concentrations of hglucagon (■), hGLP-1 (♦), gfglucagon (▲), gfGLP-1 (▼) and exendin-4 (●). Data presented here were from six independent peptide stimulations and the values are the means ± S.D. and the average values of basal cAMP were 5.9 pmol/well.

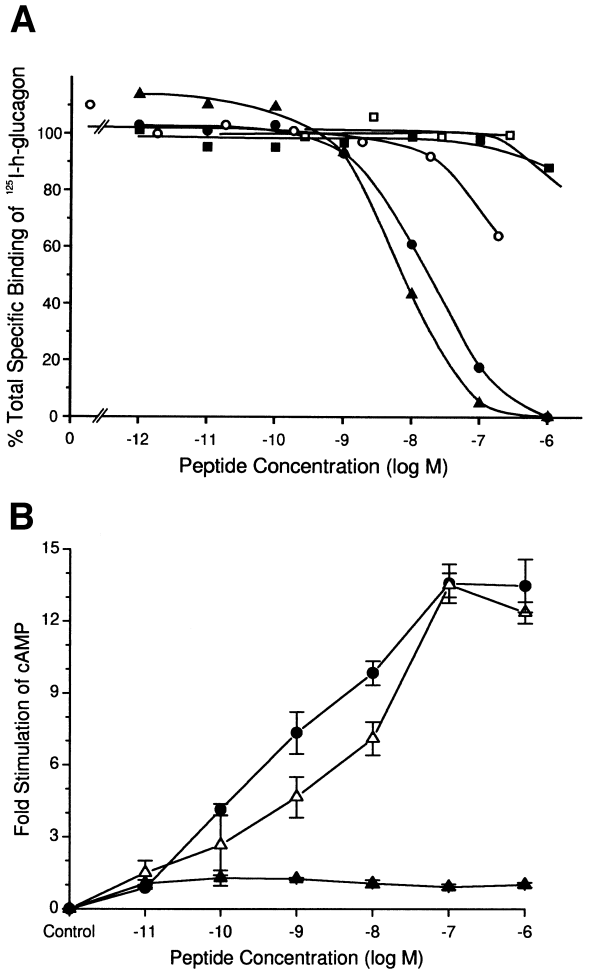

Fig. 4. Functional experiments with the frog GluR expressed transiently in COS-7 cells. A: Competitive binding experiments measuring the displacement of ^{125}I-human glucagon binding in the presence of increasing concentrations of hglucagon (●), the glucagon antagonist des-His^{1}-[Nle^{9}-Ala^{11}-Ala^{16}] (▲), gfglucagon (○), zebrafish glucagon (◆), gfGLP-1 (□), hGLP-1 (■). The results for hglucagon are an average of six independent measurements, and for the other peptides are averages of three independent measurements. Total specific binding was on average 45% of the total radioactivity added (100 000 cpm). B: Stimulation of cAMP production. The results are expressed as fold stimulation of cAMP levels in response to increased concentrations of hglucagon (○), glucagon antagonist des-His^{1}-[Nle^{9}-Ala^{11}-Ala^{16}] (▲) and hglucagon in the presence of glucagon antagonist (100 nM) (△). The cAMP stimulations were performed at least two times each in triplicate with two different batches of transfected COS-7 cells. The average values for basal cAMP levels were 15 pmol/well.

At a concentration of 100 nM, only human glucagon (Fig. 1), but not goldfish glucagon was able to stimulate intracellular cAMP production in the receptor-transfected cells. Other structurally related peptides, such as human GLP-1, goldfish GLP-1, human GIP, human secretin, human VIP, human PACAP, exendin-4 and exendin (9–39), were also unable to stimulate cAMP production. The cAMP stimulatory effect of human glucagon was found to be dose-dependent with an EC_{50} value of 0.8 ± 0.5 nM (Fig. 3B). However, even at a concentration of 1 μM, human and goldfish GLP-1s, goldfish glucagon and exendin-4 were unable to activate the receptor (Fig. 3B).

3.2.2. Competitive binding experiments. For the competitive binding experiments, ^{125}I-human glucagon was able to bind to the recombinant frog GluR transiently expressed in mammalian COS-7 cells (45% specific binding). The specific binding was displaced in a dose-dependent manner with human glucagon with an IC_{50} value of 12 ± 3 nM. The structurally related GLP-1s (human and goldfish), as well as zebrafish glucagon, were unable to displace the specific binding of the ^{125}I-human glucagon, while goldfish glucagon showed some displacement only at a high concentration of 270 nM (Fig. 4A). One of the antagonists of the human GluR, des-His^{1}-[Nle^{9}-Ala^{11}-Ala^{16}] [22], was used in the competitive binding experiments to further characterize the binding properties of the recombinant frog GluR. The antagonist was able to displace the specific binding of ^{125}I-human glucagon in the same concentration range as human glucagon with a similar IC_{50} value of 7.8 ± 1.0 nM (Fig. 4A). Based on these results, we conclude that the frog GluR exhibits similar ligand binding specificities as the human GluR.

3.2.3. Functional effects of the glucagon antagonist on the frog glucagon receptor. To extend the comparisons of the structural and functional properties of the frog and mammalian GluRs, we tested the human glucagon antagonist des-His^{1}-[Nle^{9}-Ala^{11}-Ala^{16}] and we found that it was unable to stimulate intracellular cAMP levels in the concentration range from 10 pM to 1 μM (Fig. 4B). In contrast, incubation of the COS-7 cells that transiently expressed the frog GluR with human glucagon led to a dose-dependent stimulation of intracellular cAMP levels with EC_{50} = 0.8 nM, in agreement with the results obtained from the initial functional experiments in the CHO cells. More interestingly, incubation of the cells with human glucagon in the presence of 100 nM antagonist shifted the EC_{50} values from 0.8 nM to 2.7 nM (Fig. 4B) and inhibited the stimulated cAMP levels by 43% (4.7-fold vs. 8.2-fold in the absence of the antagonist) at a concentration of 10^{-9} M. Together with the results obtained from the competitive binding experiments, we established that the frog and human GluRs share similar structural and functional properties.

3.3. Tissue distribution of frog glucagon receptor mRNA

To examine the tissue distribution of frog GluR, RT-PCR was performed using first strand cDNAs prepared from various frog tissues, including skeletal muscle, brain, liver, small intestine and colon, as templates. PCR products (443 bp) were detected in all the tissues tested except in the muscle. The authenticity of the PCR products was confirmed by Southern blotting using the partial cDNA fragment as a probe (Fig. 5).

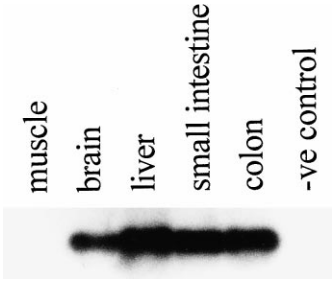

Fig. 5. Tissue distribution of frog GluR mRNA revealed by RT-PCR. PCR products obtained from muscle, brain, liver, small intestine and colon were subjected to Southern blot analysis using the partial frog GluR cDNA as a probe.

4. Discussion

In the present study, the first non-mammalian full-length GluR cDNA was isolated and characterized. The N-terminal ectodomain for this family of receptors has been shown to be largely responsible for ligand specificity. Mutational analysis of the glycine residue at position 40 of the human GluR demonstrated that Gly-40 is involved in the structural determination of the extracellular ectodomain and the sensitivity of the receptor to glucagon [23]. However, Gly-40 is not present in the frog, rat and mouse GluRs. Since all these receptors can interact with human glucagon, these data suggest that this glycine residue plays a partial role in ligand binding and receptor stabilization.

A number of other residues are also involved in ligand binding and receptor activation, such as Ser-80, Gln-142 and especially Asp-64 [24,25]. Functional characterization of rat GluR [25] indicated that replacement of Asp-64 with Glu, Asn, Lys or Gly results in the abolition of ligand binding. All three residues are conserved in these GluRs, suggesting that they are crucial to the binding of human glucagon with the frog GluR to mediate cAMP responses. Functional characterization of the human GluR [24] revealed that Ser-80 and Gln-142 are necessary for glucagon binding specificity. Both residues are conserved in the frog GluR. This observation may account for the fact that the frog GluR interacts specifically with only human glucagon, but not with other related peptides such as GLP-1 and GIP.

Several non-continuous domains that are required for high affinity binding with glucagon, including the membrane-proximal half of the amino-terminal extension, the first exoloop, the third, fourth, and sixth transmembrane domains [24], are conserved among frog and mammalian GluRs. The conservation of these ligand recognition domains between the frog and human GluRs may further explain the specificity of the frog GluR towards the structure of human glucagon.

In addition, the results of our competitive binding experiments demonstrated that the frog and human GluRs contain similar, if not identical, binding affinity towards the active site of glucagon structure. These conclusions are based on the following observations. First, the frog GluR binds the human GluR antagonist des-His^{1}-[Nle^{9}-Ala^{11}-Ala^{16}] with the same affinity as the human GluR (Fig. 4A and [22]). Second, the frog GluR did not bind to goldfish and zebrafish glucagons.

The lack of binding specificity of the frog GluR towards the goldfish and zebrafish glucagons is consistent with the proposed mechanism of the interaction of the human glucagon with the human GluR [26,27]. According to this mechanism, His-1, Asp-9 and Ser residues are essential amino acids needed for the formation of the active center of the human glucagon/glucagon-receptor system. Of the four Ser residues present in the sequence of human glucagon, Ser-8 and to a lesser extent Ser-16 are critical residues needed for the formation of the active center of glucagon. Thus, the substitution of Ser-8 with an Asn residue decreased the binding affinity of [Asn^{8}] glucagon to 8% as compared to the naturally occurring glucagon [27]. Similarly, substitution of the Ser-16 with a Thr residue led to an analog that possessed about 8% binding affinity [27]. Finally, substitution of Asp-15 with Glu resulted in an analog that contained about 80% of the binding affinity [27]. Clearly, the triple substitutions of Ser^{8}, Asp^{15} and Ser^{16} with Asn^{8}, Glu^{15}, and Thr^{16} found in the sequences of goldfish and zebrafish glucagons leads to a disruption of this active center and hence there was a complete loss of binding affinity of the frog GluR towards these fish glucagons.

As seen from Fig. 1, the same substitutions in amino residues at identical positions of the sequence are found in the glucagons in all teleost fishes analyzed so far, suggesting that the GluRs in species that existed before the appearance of the frogs would have different ligand binding specificities. Taken together, these observations indicate that evolutionary pressures manifested at the time of the emergence of the frog species may have directed simultaneous changes in the sequences of glucagon and sequences of the ligand recognition domains of GluRs. As a result, starting with the frog species, more stringent structural constraints were developed in the glucagon/glucagon-receptor system and were maintained subsequently during the evolution in the mammalian species. Further experiments and especially functional characterization of fish GluRs are needed in order to understand the structural evolution of vertebrate glucagons and their receptors.

The frog glucagon and GLP-1s may have unique functions probably by interacting with multiple glucagon/GLP-1 receptors that are expressed in a tissue-specific manner. In the present study, the tissue distribution of the GluR mRNA was investigated as a preliminary step to understand the glucagon/GLP-1 system in *Rana tigrina rugulosa*. Similar to other species, GluR mRNA is present at high levels in the liver, gastrointestinal tract and brain. The expression of GluR transcripts in the liver is consistent with the major functions of glucagon, which are the stimulation of hepatic glycogenolysis and gluconeogenesis. In summary, our results demonstrate a conservation of the functional characteristics of the GluRs in frog and mammalian species and provide a framework for a better understanding of the molecular evolution of the GluR and its physiological function in vertebrates.

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