ELSEVIER

Biochimica et Biophysica Acta 1510 (2001) 321–329

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

# Characterization of an insulin receptor-related receptor in *Biomphalaria glabrata* embryonic cells

Vinca Lardans, Jean-François Coppin, Jérôme Vicogne, Eric Aroca, Melaine Delcroix, Colette Dissous *

*Unité INSERM 167, Institut Pasteur, 1 rue du Prof. A. Calmette, 59019 Lille, France*

Received 1 August 2000; received in revised form 1 November 2000; accepted 7 November 2000

**Abstract**

Tyrosine kinase receptors play a key role in the communication of cells with their environment. Growth hormone receptors, such as insulin receptors, are involved in the regulation of cell growth, differentiation and metabolism in multicellular organisms. Insulin-related peptides and members of the insulin receptor subfamily have been described in a wide variety of invertebrates, including freshwater molluscs. In this paper, we describe the metabolic effect of insulin on a mollusc cell line (Bge) derived from embryos of the snail *Biomphalaria glabrata*. Using a PCR strategy, we have cloned from Bge cells a cDNA encoding a protein (BgIR) homologous to, and exhibiting all of the typical features of insulin receptors. Northern blot analysis confirmed the expression of BgIR in *B. glabrata* snails and suggested its wide distribution in the snail body. Bge cells have been shown to provide the environmental conditions necessary for the in vitro development of the sporocysts of *Schistosoma mansoni*, a trematode parasite that uses *B. glabrata* as an intermediate host. The possible implication of BgIR in the activating and proliferating processes observed in Bge cells during their coculture with *S. mansoni* larvae is discussed. 

*Keywords:* Insulin; Receptor; Mollusc; *Biomphalaria glabrata*

## 1. Introduction

Insulin-related peptides (IRPs) are present in a wide variety of invertebrates. In insect and mollusc species, there is evidence that insulin peptides play, as in vertebrate organisms, a major role in development and metabolism. For example, human insulin has been shown to stimulate proliferation and differentiation of *Drosophila* embryonic neural cells [1] and to regulate trehalose and glucose levels in blowflies [2]. In the mollusc *Haliotis tuberculata*, insulin promotes the growth of primary cultures of hemocytes [3]. Structural data are now available for insect IRPs (bombyxins of *Bombyx mori* [4] and LIRP of *Locusta migratoria* [5] and for molluscan insulin-like peptides (MIPs). Five members (I, II, III, V and VII) of the MIP family have been characterized from the pulmonate snail *Lymnaea stagnalis* [6–10]. The peptides are essentially produced by neuroendocrine cells of the central nervous system (CNS), the light green cells (LGC) that are involved in body growth and metabolism of the mollusc [11]. More recently, a tyrosine kinase protein homologous to mammalian insulin receptors (IRs) has been cloned that potentially

* Corresponding author. Fax: +33-3-20-87-78-88;
E-mail: colette.dissous@pasteur-lille.fr

0005-2736/01/$ – see front matter 
PII: S0005-2736(00)00364-3

represents the receptor for *L. stagnalis* MIPs (MIPR) [12].

The unique cell line established from molluscs is derived from *Biomphalaria glabrata*, a snail that is a required intermediate host for the human parasite *Schistosoma mansoni*. *B. glabrata* embryonic (Bge) cells were established by Hansen in 1976 [13] with the objective of providing a culture system adequate for schistosome development. These cells share some features with snail hemocytes such as the emission of filopodia and capacities of adherence and encapsulation. Recent studies have demonstrated their potential effect on the viability and growth of the intramolluscan stages of several schistosome species [14,15]. During recent years, efforts have aimed at a better knowledge of the biochemical composition of these cells in order to identify some of the molecular signals involved both in Bge cell activation and parasite development. Adhesion molecules such as selectin [16] and β-integrin-like proteins [17] have been characterized that may be implicated in the cell activation processes consecutive to sporocyst adhesion. Like many cell lines, Bge cells require addition of fetal calf serum in culture medium that is supposed to furnish growth factors essential for synthesis and division. The stimulating effect of insulin demonstrated on *Haliotis* hemocytes [3] prompted us to consider the effect of insulin on the metabolism of Bge cells. Preliminary results showed that insulin could indeed act as a positive signal on Bge cells, suggesting therefore the presence of an insulin receptor. A gene encoding a protein homologous to the members of the insulin receptor family has been characterized in *B. glabrata* that is expressed in Bge cells.

## 2. Materials and methods

### 2.1. Biological material

An albino strain of *B. glabrata* was maintained in the laboratory. Feet and digestive glands were dissected from juvenile ($<7$ mm diameter) and adult ($>15$ mm diameter) snails. Bge cells (ATCC CRL 1494) were grown at 27°C in Bge medium supplemented with 10% heat-inactivated fetal calf serum (Myoclone, Gibco) and antibiotics [13].

### 2.2. Metabolic labeling of Bge cells

Metabolic labeling was performed in sterile snail saline (SSS) medium [18]. Radioactive precursors were obtained from Amersham Pharmacia Biotech. 100 μl of cell suspension (equivalent to $5\times10^4$ cells) were aliquoted in 96-well microtiter plates and incubated with 2 μCi [$^{35}$S]methionine ($>1000$ Ci/mmole) or 1 μCi [*methyl*-$^3$H]thymidine (55 Ci/mmole). Following 24 h of incubation at 27°C, cells were collected and the amount of $^{35}$S radioactivity was determined in the TCA-precipitable material. [$^3$H]-Thymidine incorporation was determined following cell filtration using a combi cell harvester (Skatron). $^{32}$P labeling was performed in similar conditions with the addition of 2 μCi [γ-$^{32}$P]ATP (3000 Ci/mmole) per well. Labeling was stopped by the addition of 100 μl SDS 2% to each sample and boiling for 2 min. Proteins were precipitated by 5% TCA and radioactivity was measured using an LKB scintillator counter.

### 2.3. RNA extraction

Total RNA was extracted from digestive glands and cephalopedial tissues of snails or from Bge cell pellets using the method of Chirgwin et al. [19] and purified by centrifugation through a cesium chloride gradient. The poly(A)$^+$RNA was purified by passage of total RNA through an oligo(dT)-cellulose column (Pharmacia, Sweden).

### 2.4. Cloning of BgIR

Single stranded cDNA was synthesized from 1 μg of poly(A)$^+$RNA in the presence of the SuperScript II reverse transcriptase (Gibco) and 0.5 μg oligo(dT) for 50 min at 42°C. RT-PCR experiments were performed using degenerate primers TK1 (5′-GGNTC-NTTYGGNATGGTDTAYVARGG-3′) and TK2 (5′-ATRTCHCKDGYCATDCCRAARTCDCC-3′) complementary to conserved sequences present in the tyrosine kinase (TK) domains of IR sequences from various species. RT-PCR was carried out using the Taq Gold polymerase (Perkin Elmer) in the following conditions: 10 min at 94°C, 40 cycles (1 min at 94°C, 1 min at 50°C, 1 min at 72°C). PCR products were cloned into the pTag vector (R&D systems) and

the sequence was determined. A Bge cell cDNA library was constructed from 1 μg of poly(A)$^+$RNA using the Marathon cDNA amplification kit (Stratagene). Double stranded cDNAs were ligated to cDNA adaptors according to the manufacturer's instructions. Random amplification of cDNA ends (RACE) was performed using the AP-1 adaptor primer and gene specific primers BgTK1 (sense, 5′-ACCAGATGAGGAACATCCATTTGCTACACCTCC-3′) and BgTK2 (antisense, 5′-CCAGCAACCATACAGTTCCTTGCCGCCAGG-3′) with the TaqPlus Long PCR system (Stratagene). Nested PCR reactions were performed using AP-2 and BgTK3 (sense, 5′-CATAGAGACCTGGCGGCAAGG-3′) or BgTK4 (antisense, 5′-CAGCCACAGGGATTTCTTCCTCAGG-3′) nested primers. Sequencing reactions were performed using the dye terminator cycle sequencing kit and analyzed on an ABI Prism 377 DNA sequencer (Perkin Elmer Biosystems). Sequence analysis was performed using the DNASTAR program. Sequence alignments were obtained by the Clustal method.

### 2.5. Northern blot analysis

Total RNA (30 μg) was separated on a formaldehyde agarose gel, blotted to Hybond-N$^+$ nylon charged membrane (Amersham), fixed by alkali treatment (NaOH 50 mM) and prehybridized at 65°C for 3 h. The probe was made from random primed (Megaprime DNA labeling system kit, Amersham) $^{32}$P-labeled full-length BgIR cDNA. Hybridization was performed at 42°C for 18 h in 5×SSC, 5×Denhardt's reagent, 0.5% SDS, 0.1 mg/ml denatured salmon sperm DNA, 50% formamide. The membrane was washed twice in 2×SSC, 0.1% SDS for 10 min at room temperature, once in 0.1×SSC, 0.1% SDS for 10 min at 42°C and then autoradiographed.

### 2.6. Production of antibodies to the catalytic domain of BgIR

The TK domain of BgIR was subcloned in the expression vector pQE30 (Qiagen) and expressed in *Escherichia coli*. Antisera were produced in mice immunized with the recombinant protein in the presence of complete Freund's adjuvant [20].

### 2.7. Immunostaining of Bge cells

Bge cells were allowed to adhere to glass slides, then washed in diluted (1/3) phosphate-buffered saline (PBS, 10 mM Na phosphate, pH 7.4, 150 mM NaCl), fixed with 1.7% paraformaldehyde for 10 min and permeabilized by a 4 min treatment with Triton X-100 at 0.1%. Slides were saturated for 90 min with PBS containing 1% bovine serum albumin (BSA) and normal goat serum (1:50) at room temperature. This blocking step was followed by a 2 h incubation with mouse anti-BgIR serum (diluted at 1/100 in PBS-BSA 1%). After three washings, slides were allowed to react for 90 min at room temperature with alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma). Slides were stained with New Fuschin (Dako) and counterstained with hematoxylin (Fluka).

## 3. Results and discussion

### 3.1. Effect of insulin on Bge cell metabolism

Experiments presented in Fig. 1 indicated that

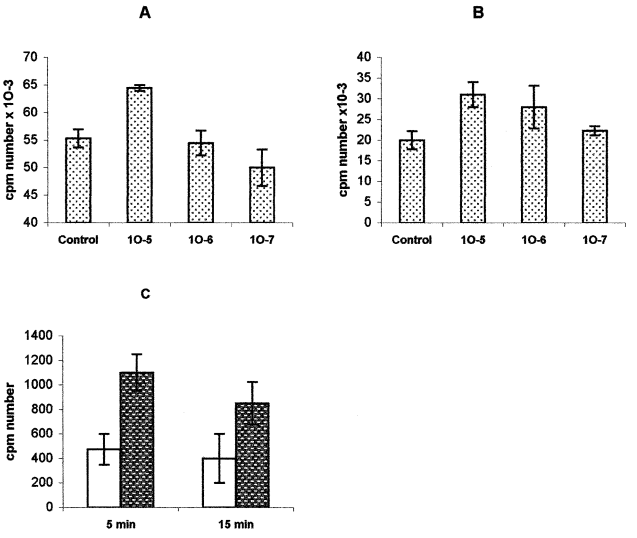


Fig. 1. Effect of insulin on the metabolic activity of Bge cells. [$^{35}$S]Methionine (A) and [$^3$H]thymidine (B) incorporation in the absence (control) or in the presence of $10^{-5}$, $10^{-6}$, $10^{-7}$ M bovine insulin. Amount of $^{32}$P (C) incorporated after 5 or 15 min incubation without or with (shaded areas) $10^{-5}$ M insulin. Results are expressed as means ± S.E.M. of triplicate values obtained in one representative experiment.

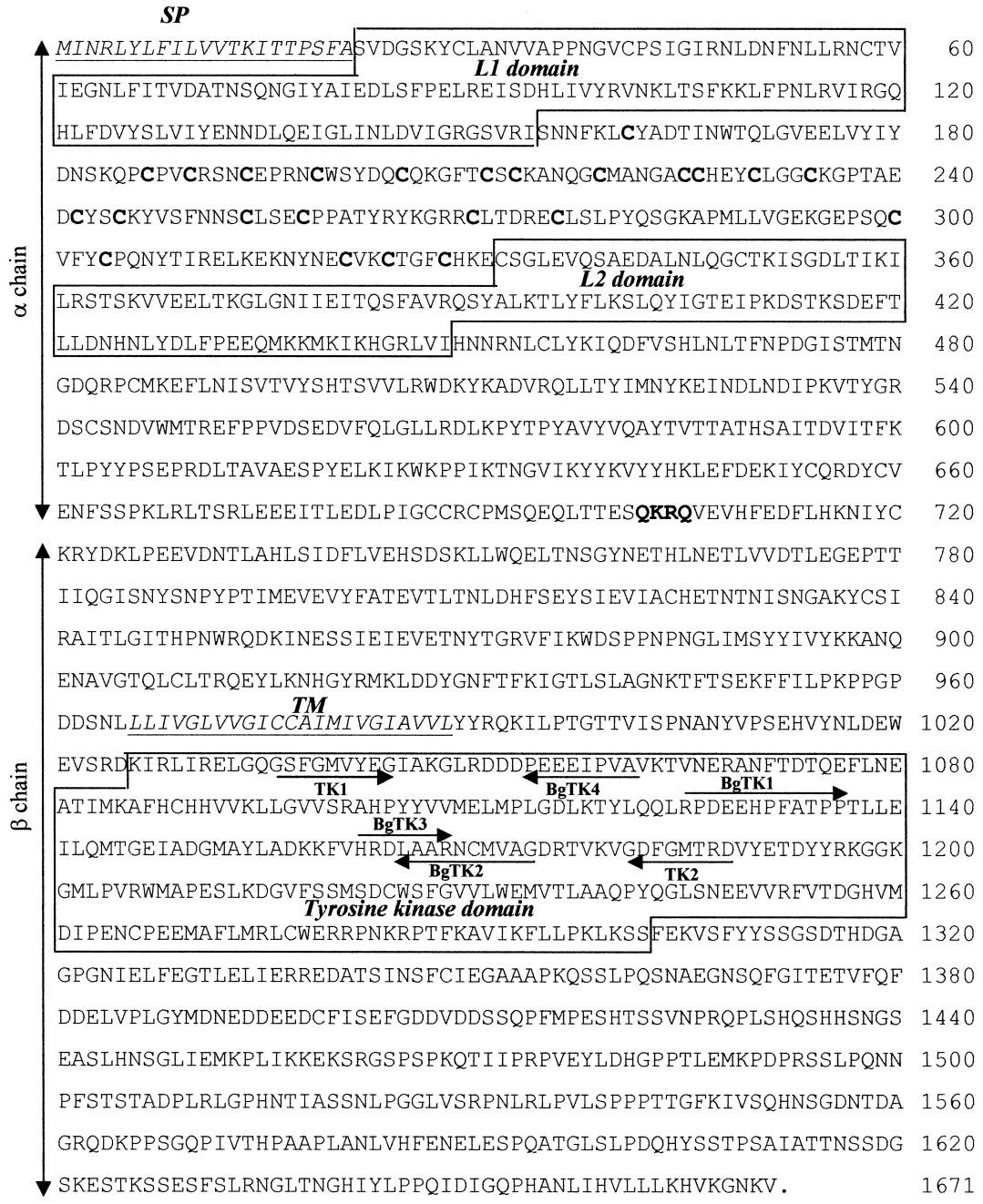


Fig. 2. Amino acid sequence of BgIR and structural analysis. The basic site QKRQ (in bold letters) could potentially separate the α chain containing the L1 and L2 binding domains and the β chain containing the tyrosine kinase domain. The putative signal peptide (SP) and transmembrane domain (TM) are underlined.

bovine insulin was able to stimulate both [$^{35}$S]methionine and [$^{3}$H]thymidine incorporation into Bge cells in a dose-dependent manner. High doses of insulin (0.6–60 μg/ml) were required to obtain a significant effect on protein or DNA synthesis. These concentrations were comparable to those required to activate *H. tuberculata* hemocytes [3]. The maximal concentration ($10^{-5}$ M) stimulated a moderate increase in Bge cell metabolism but more remarkably, it stimulated a rapid protein phosphoryla-

tion in cells. Following short periods (5 and 15 min) of incubation with $10^{-5}$ M insulin, we observed a two-fold increase in the level of $^{32}P$ incorporation as compared to the controls, suggesting that insulin could activate a signaling pathway through a protein kinase receptor in Bge cells. These data prompted us to search for the existence of a member of the insulin receptor family in these cells.

### 3.2. Isolation of a putative insulin receptor cDNA

The insulin receptor family belongs to class II of the receptor tyrosine kinase (RTK) superfamily and in mammals, includes the IR, the insulin-like growth factor receptor (IGF1R) and the insulin receptor-related receptor (IRR). IRs have been conserved throughout evolution. They comprise two α-subunits with the extracellular domain for ligand binding and two β-subunits which contain a tyrosine kinase catalytic domain which is highly conserved in vertebrates and invertebrates. On the basis of this identity, we designed degenerate oligonucleotides (TK1 and TK2, see Section 2) to conserved sequences delimiting the TK domain of human, amphioxus, nematode, insect and mollusc IR sequences. TK1 and TK2 were used as primers to amplify a 465 bp fragment from Bge cell RNA by RT-PCR, that was homologous (more than 50% identity) to the TK domains of other IR members. Further PCR experiments performed on a Marathon library of Bge cell RNA using *B. glabrata* specific primers (BgTK1–4) allowed us to obtain a complete cDNA sequence (BgIR) of 5600 bp containing a C-terminal poly(A) tail (GenBank accession No. AF101195) and with a unique and continuous open reading frame of 1672 amino acid residues (Fig. 2) starting at the ATG codon (position nt 478–480). The encoded sequence showed significant overall homology with other IRs and displayed many of their typical structural features. Southern blot experiments performed with mollusc genomic DNA confirmed the *B. glabrata* origin of BgIR (results not shown).

### 3.3. Structural analysis of BgIR

Many of the typical characteristics of IRs are present in BgIR (Fig. 2). Hydropathic analysis confirmed the presence of two highly hydrophobic regions in the molecule. The first region found at the N-terminus of the protein (first 21 residues) could represent the hydrophobic signal peptide (SP) of the precursor [21]. The second hydrophobic region comprised residues 965–988 and is the putative transmembrane (TM) domain of the receptor [22]. This region was located downstream from a tetrabasic sequence ($Q_{702}$, $K_{703}$, $R_{704}$, $Q_{705}$) that was therefore predicted to be the recognition site for the precursor processing enzyme. Further structural analyses were based on the alignment of the putative α-chain (residues 22–701) and β-chain (residues 702–1671) with their vertebrate and invertebrate counterparts.

The first requisite for the identification of BgIR as an insulin-related peptide receptor was to assess the conservation of its TK catalytic domain. Alignment of the different IRβ chains provided evidence of the presence in BgIR of the highly conserved region corresponding to the TK domain and lying between residues 1026 and 1302 (Fig. 3). This region contains most of the conserved amino acids expected to play an important role in protein kinase activity. Near the amino terminus of the TK domain, the consensus sequence $G_{1034}$-X-G-X-X-$G_{1039}$ is present that is essential for ATP binding, as well as the invariant $V_{1041}$ necessary for the correct positioning of conserved glycines and their interaction with the ATP molecule. We could also note the presence of the invariant $K_{1063}$ essential for enzyme activity and directly involved in the phospho-transfer reaction [23]. The central core of the catalytic domain (from $P_{1135}$ to $G_{1244}$) known to be highly conserved between species, effectively exhibited the greatest frequency of identical residues. This region contains several motifs that play a role in the recognition of the specific amino acid and can be used to discriminate tyrosine from serine/threonine kinases. The presence in BgIR of both the consensus sequences $D_{1164}$LAARN$_{1169}$ and $P_{1204}$VRWMAPE$_{1211}$ that are strong indicators of a tyrosine substrate specificity confirmed the tyrosine kinase nature of the mollusc receptor [23]. The signature pattern for class II RTKs ($D_{1188}$-V-Y-x(3)-Y-Y-$R_{1195}$) that includes the putative site of autophosphorylation, is also found in the TK domain [24].

Alignment of the N-terminal part of BgIR indicated significant homology with α chains from other IR species. IRs are known to exhibit in the first half of their ectodomain a cysteine-rich region, composed

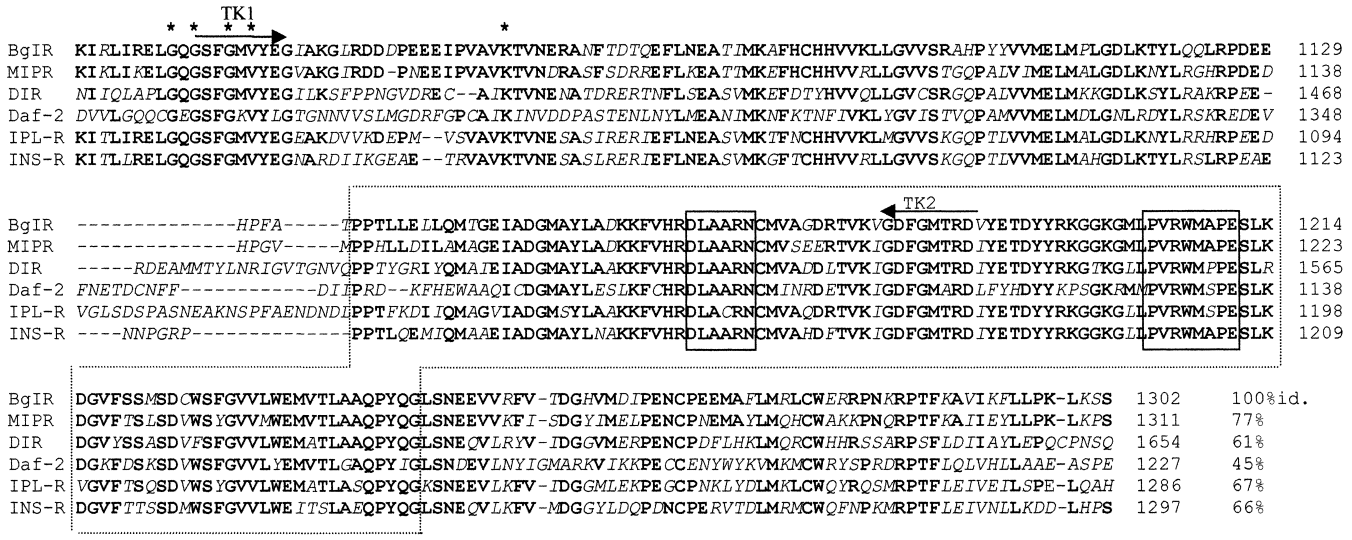


Fig. 3. Comparison of the protein kinase catalytic domain of BgIR with tyrosine kinase domains of various insulin-related peptide receptors. The BgIR amino acid sequence comprised between $K_{1026}$ and $S_{1302}$ was aligned by the Clustal method with tyrosine kinase domains of *L. stagnalis* MIPR ([12]; GenBank Q25410), *D. melanogaster* DIR ([28]; P09208), *C. elegans* daf-2 ([29]; AF012437), *Amphioxus* ILP receptor ([26]; O02466) and human INS-R ([30]; P06213). Stars indicate the conserved residues of the ATP binding site. The central core of the catalytic domain is boxed as are the two consensus sequences for tyrosine substrate specificity. Numbers indicate the percentage of identical residues (in bold letters) found at similar positions in BgIR and individual related sequences.

of smaller domains each containing eight C residues, and surrounded by two homologous domains (L1 and L2) [25]. Fig. 2 illustrates the presence of this typical structure in the BgIR molecule, that constitutes an additional argument to consider BgIR as a member of the IR family. The L1 and L2 domains which are primarily involved in insulin binding, have been found to be conserved throughout evolution. Data in Table 1 indicate that the degree of identity of BgIR varies for both the L1 and L2 domains from 47–49% with another mollusc (*L. stagnalis*) to 25–37% with a nematode (*Caenorhabditis elegans*) receptor. Such values are comparable to those obtained from the amino acid sequence comparison of various

Table 1
Identity (%) of BgIR L1 and L2 domains with other IR ligand binding domains

| | BgIR L1 | BgIR L2 |
|---|---|---|
| BgIR | 100 | 100 |
| MIPR | 47 | 49 |
| DIR | 41 | 36 |
| Daf-2 | 37 | 25 |
| IPL-R | 42 | 35 |
| INS-R | 44 | 35 |

Amino acid sequences from residues 17 to 154 (putative L1 domain) and from residues 332 to 448 (putative L2 domain) of BgIR (see Fig. 2) were compared independently to amino acid sequences of *L. stagnalis* (MIPR), *Drosophila melanogaster* (DIR), *C. elegans* (daf-2), *Amphioxus* (IPL-R) or human (INS-R) analogous domains (GenBank accession numbers are given in the legend to Fig. 3). Numbers indicate the percentage of identical residues found at identical positions following alignment by the Clustal Method (Megalign, DNASTAR program).

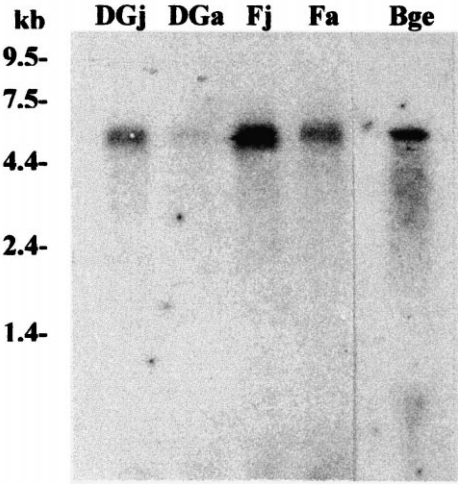


Fig. 4. Detection of BgIR transcripts in snail tissues. Northern blot results indicated the presence of a 5.6 kb transcript in total RNA extracted from Bge cells and from digestive gland (DG) or foot (F) of juvenile (j) or adult (a) snails.

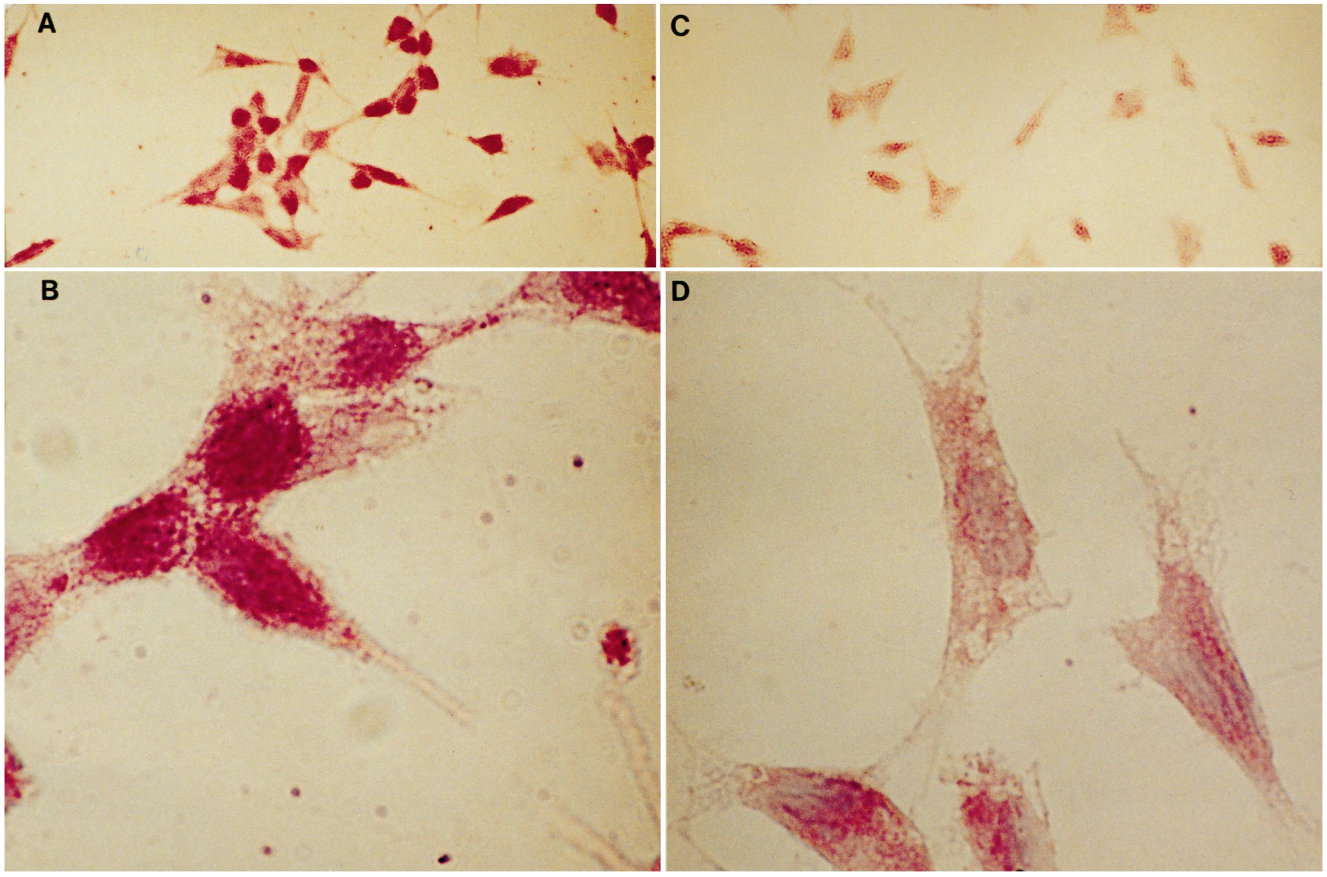


Fig. 5. Immunocytochemical staining of Bge cells with mouse anti-BgIR serum (A,B) or with preimmune serum (C,D). ×200 (A,C); ×1000 (B,D).

insulin and insulin-related peptides [26] and probably reflect coevolution of receptor and ligand molecules.

Sequence analysis thus indicates that BgIR exhibits all of the typical features of insulin receptors but it still remains to be determined that this receptor indeed is functioning as a receptor for insulin-related peptides.

### 3.4. Expression of BgIR in snail cells and tissues

Further experiments were concerned with the detection of BgIR messenger RNAs in Bge cells and mollusc tissues. Northern blot analysis (Fig. 4) confirmed that the BgIR sequence corresponded to the full-length cDNA molecule and indicated its transcription in Bge cells as well as in mollusc tissues. Experiments performed on *B. glabrata* total RNA extracted from foot or hepatopancreas showed the presence of common mRNA species in snail muscles and digestive gland, suggesting a wide distribution of BgIR in the body of juvenile and adult snails.

Immunodetection of the BgIR protein in Bge cells was performed using antibodies produced against the putative TK domain of BgIR expressed as a recombinant protein in *E. coli*. Fig. 5 illustrates the presence of the BgIR protein in Bge cells but did not indicate a particular subcellular localization of the protein. A more intense labeling was obtained in permeabilized cells that could be explained by the recognition of the intracellular TK domain of BgIR.

## 4. Conclusion

Signaling pathways mediated through tyrosine kinase receptors regulate metabolism, growth and development in metazoans. One of these pathways, ac-

tivated by insulin or insulin-like molecules plays a central role in invertebrate organisms, affecting metabolism, cell division and differentiation in arthropods and molluscs and also cnidaria, one of the most ancient metazoan phyla [27]. This work describes the characterization of an insulin-like receptor of *B. glabrata* expressed in an embryonic cell line of this mollusc. BgIR, like other TK receptors, presents a typical tripartite structure with extracellular, transmembrane and cytoplasmic regions. One of the characteristics of IR molecules is that they are composed of two subunits encoded by a common cDNA. The precursor protein is cleaved at a tetrabasic site to generate α and β chains respectively bearing the extracellular ligand binding domain and the intracellular catalytic site. The presence of this potential site for cleavage in the BgIR sequence indicated that it could also be composed of two separate chains. The identification of conserved residue patterns confirmed the tyrosine kinase specificity of the catalytic domain.

Although the BgIR extracellular domain conserves the characteristics of an insulin binding domain, it is interesting to note that a significant divergence exists between these domains in the two freshwater snails *B. glabrata* and *L. stagnalis*, that could suggest a structural divergence of MIP molecules in different gastropod families. Five members (I, II, III, V and VII) of the MIP family have been characterized from *L. stagnalis* [6–10] and we are currently attempting to identify MIP molecules in *B. glabrata* in order to confirm this hypothesis. *L. stagnalis* is also the intermediate host for a schistosome, the avian parasite *Trichobilharzia ocellata*, and the marked specificity of the trematode parasites for their hosts indicates the importance of an adaptative molecular dialogue between specific partners. For this reason, we are looking for the presence of specific insulin-like peptides in Bge cells that could be potentially implicated in interaction between Bge cells and *S. mansoni* sporocysts in vitro. We have recently demonstrated that insulin increases the metabolic activity of *S. mansoni* sporocysts and characterized an insulin-like receptor in this parasite (Vicogne et al., manuscript in preparation). All these data lead us now to analyze the role of insulin-like peptides in molecular interactions between mollusc cells and sporocysts and their effect on cell activation and parasite differentiation in order to evaluate the importance of insulin signaling pathways in schistosome-host relationships.

## Acknowledgements

The authors thank J. Trolet and J.M. Merchez for technical assistance and Dr. Raymond Pierce for helpful discussions. This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), Institut Pasteur de Lille and Ministère Education Nationale Recherche Technologie (MENRT).

## References

[1] B. Pimentel, E. de la Rosa, F. de Pablo, Insulin acts as an embryonic growth factor for Drosophila neural cells, Biochem. Biophys. Res. Commun. 226 (1996) 855–861.

[2] H. Duve, A. Thorpe, N.R. Lazarus, Isolation of material displaying insulin-like immunological biological activity from the brain of the blowfly *Calliphora vomitoria*, Biochem. J. 184 (1979) 221–227.

[3] J.-M. Lebel, W. Giard, P. Favrel, E. Boucaud-Camou, Effects of different vertebrate growth factors on primary cultures of hemocytes from the gastropod mollusc, *Haliotis tuberculata*, Biol. Cell 86 (1996) 67–72.

[4] A. Kawakami, M. Iwami, H. Nagasawa, A. Suzuki, H. Ishizaki, Structure and organization of four clustered genes that encode bombyxin, an insulin-related brain secretory peptide of the silkmoth *Bombyx mori*, Proc. Natl. Acad. Sci. USA 84 (1989) 6843–6847.

[5] M. Lagueux, L. Lwoff, M. Meister, F. Goltzené, J. Hoffmann, cDNAs from neurosecretory cells of brains of *Locusta migratoria* (Insecta, Orthoptera) encoding a novel member of the superfamily of insulins, Eur. J. Biochem. 187 (1990) 249–254.

[6] A.B. Smit, E. Vreugdenhil, R.H.M. Ebberink, W.P.M. Geraerts, J. Klootwijk, J. Joosse, Growth-controlling molluscan neurons produce the precursor of an insulin-related peptide, Nature 331 (1988) 535–538.

[7] A.B. Smit, W.P.M. Geraerts, I. Meester, H. Heerikhuizen, J. Joosse, Characterization of cDNA clone encoding molluscan insulin-related peptide II of *Lymnaea stagnalis*, Eur. J. Biochem. 199 (1991) 699–703.

[8] A.B. Smit, S.F.T. Thijsen, W.P.M. Geraerts, I. Meester, H. Heerikhuizen, J. Joosse, Characterization of cDNA clone encoding molluscan insulin-related peptide V of *Lymnaea stagnalis*, Mol. Brain Res. 14 (1992) 7–12.

[9] A.B. Smit, A. Marle, R. Elk, J. Bogerd, H. Heerikhuizen, W.P.M. Geraerts, Evolutionary conservation of the insulin

gene structure in invertebrates: cloning of the gene encoding molluscan insulin-related peptide III from *Lymnaea stagnalis*, J. Mol. Endocrinol. 11 (1993) 103–113.

[10] A.B. Smit, S. Spijker, J. Van Minnen, J.F. Burke, F. De Winter, R. Van Elck, W.P.M. Geraerts, Expression and characterization of molluscan insulin-related peptide VII from the mollusc *Lymnaea stagnalis*, Neuroscience 70 (1996) 589–596.

[11] W.P.M. Geraerts, A.B. Smit, K.W. Li, E. Vreugdenhil, H. van Heerikhuizen, Neuropeptide gene families that control reproductive behaviour and growth in molluscs, Curr. Aspects Neurosci. 3 (1991) 255–305.

[12] E. Roovers, M.E. Vincent, E. Kesteren, W.P.M. Geraerts, R.J. Planta, E. Vreugdenhil, H. Heerikhuisen, Characterization of a putative molluscan insulin-related peptide receptor, Gene 162 (1995) 181–188.

[13] E.L. Hansen, A cell line from embryos of *Biomphalaria glabrata* (Pulmonata), establishment and characteristics, in: Kurstak, Maramorosch (Eds.), Invertebrate Tissue Culture: Research Applications in Medicine and Biology and Agriculture, Academic Press, New York, 1976, pp. 75–97.

[14] T.P. Yoshino, J.R. Laursen, Production of *Schistosoma mansoni* daughter sporocysts from mother sporocysts maintained in synxenic culture with *Biomphalaria glabrata* embryonic (Bge) cells, J. Parasitol. 81 (1995) 714–722.

[15] C. Coustau, G. Ataev, J. Jourdane, T.P. Yoshino, *Schistosoma japonicum*: in vitro cultivation of miracidium to the daughter sporocyst using a *Biomphalaria glabrata* embryonic cell line, Exp. Parasitol. 87 (1997) 77–87.

[16] P. Duclermortier, V. Lardans, E. Serra, F. Trottein, C. Dissous, *Biomphalaria glabrata* embryonic cells express a protein with a domain homologous to the lectin domain of mammalian selectins, Parasitol. Res. 85 (1999) 481–486.

[17] B.J. Davids, X.-J. Wu, T.P. Yoshino, Cloning of a b integrin subunit cDNA from an embryonic cell line derived from the freshwater mollusc *Biomphalaria glabrata*, Gene 228 (1999) 213–223.

[18] C.M. Adema, R.A. Harris, E.C. Deutekom-Mulder, A comparative study of hemocytes from six different snails: morphology and functional aspects, J. Invertebr. Pathol. 59 (1992) 24–32.

[19] J.M. Chirgwin, A.E. Przybyla, R.J. MacDonald, W.J. Ruther, Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease, Biochemistry 18 (1979) 5294–5299.

[20] J. Vaitukaitis, J.B. Robbins, E. Nieschlaf, G.T. Ross, A method for producing specific antisera with small doses of immunogen, J. Clin. Endocrinol. Metab. 33 (1971) 988–991.

[21] H. Nielsen, J. Engelbrecht, S. Brunak, G. von Heijne, Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites, Protein Eng. 10 (1997) 1–6.

[22] G. von Heijne, Membrane protein structure prediction, hydrophobicity analysis and the positive-inside rule, J. Mol. Biol. 225 (1992) 487–494.

[23] S.K. Hanks, A.M. Quinn, T. Hunter, The protein kinase family: conserved features and deduced phylogeny of the catalytic domains, Science 241 (1988) 42–52.

[24] Y. Yarden, A. Ullrich, Growth factor receptor tyrosine kinases, Annu. Rev. Biochem. 57 (1988) 443–478.

[25] M. Bajaj, M.D. Waterfield, J. Schlessinger, W.R. Taylor, T. Blundell, On the tertiary structure of the extracellular domains of the epidermal growth factor and insulin receptors, Biochim. Biophys. Acta 916 (1987) 220–226.

[26] M. Pashmforoush, S.J. Chan, D.F. Steiner, Structure and expression of the insulin-like peptide receptor of amphioxus, Mol. Endocrinol. 10 (1996) 857–866.

[27] R.E. Steele, P. Lieu, N.H. Mai, M.A. Shenk, M.P. Sarras, Response to insulin and the expression of a gene encoding an insulin receptor homologue suggest a role for an insulin-like molecule in regulating growth and patterning in *Hydra*, Dev. Genes Evol. 206 (1996) 247–259.

[28] Y. Ruan, C. Chen, Y. Cao, R.S. Garofalo, The Drosophila insulin receptor contains a novel carboxy-terminal extension likely to play an important role in signal transduction, J. Biol. Chem. 270 (1995) 4236–4243.

[29] K.D. Kimura, H.A. Tissenbaum, Y. Liu, G. Rukvun, daf-2, an insulin receptor-like gene that regulates longevity and diapause in caenorhabditis elegans, Science 277 (1997) 942–945.

[30] A. Ullrich, J.R. Bell, E.Y. Chen, R. Herrera, L.M. Petruzzelli, T.J. Dull, A. Gray, L. Coussens, Y.C. Liao, M. Tsubokawa, P.H. Seeburg, C. Grundfeld, O.M. Rosen, J. Ramachandran, Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes, Nature 313 (1985) 756–761.