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Research paper

Identification and evolution of two insulin receptor genes involved in *Tribolium castaneum* development and reproduction

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ABSTRACT

The insulin and insulin-like signaling (IIS) pathway exists in a wide range of organisms from mammals to invertebrates and regulates several vital physiological functions. A phylogenetic analysis have indicated that insulin receptors have been duplicated at least twice among vertebrates, whereas only one duplication occurred in insects before the differentiation of Coleoptera, Hymenoptera, and Hemiptera. Thus, we cloned two putative insulin receptor genes, *T.cas-ir1* and *T.cas-ir2*, from *T. castaneum* and determined that *T.cas-ir1* is most strongly expressed during the late adult and early pupal stages, whereas *T.cas-ir2* is most strongly expressed during the late larval stage. We found that larval RNAi against *T.cas-ir1* and *T.cas-ir2* causes 100% and 42.0% insect death, respectively, and that parental RNAi against *T.cas-ir1* and *T.cas-ir2* leads to 100% and 33.3% reductions in beetle fecundity, respectively. The hatching rate of ds-*ir2* insects was 66.2%. Moreover, RNAi against these two genes increased the expression of the *pkc*, *foxo*, *jnk*, *cdc42*, *ikk*, and *mekk* genes but decreased *erk* gene expression. Despite these similarities, these two genes act via distinct regulatory pathways. These results indicate that these two receptors have functionally diverged with respect to the development and reproduction of *T. castaneum*, even though they retain some common regulatory signaling pathways.

1. Introduction

The insulin and insulin-like growth factor signaling (IIS) pathway is evolutionarily conserved between vertebrates and invertebrates at both the structural and functional levels (Hernandez-Sanchez et al., 2008; Guirao-Rico and Aguade, 2009). The IIS pathway is the primary signaling pathway that couples growth with nutritional status in all animals, thereby influencing animal development, metabolism, longevity, and reproduction (Tatar et al., 2003; Wu and Brown, 2006). The first component of the IIS pathway is the insulin receptor, which is a transmembrane receptor that triggers the IIS signal transduction cascade upon insulin binding.

In vertebrates, three distinct classes of receptors that bind to insulin and insulin-like growth factor (IGF) with high affinity have been described based on differences in their primary structure and function: the insulin receptor (IR), the insulin-like growth factor receptor (IGFR), and the insulin receptor-related receptor (IRR) (Hernandez-Sanchez et al., 2008). Unlike other receptor tyrosine kinases, these ligand-activated receptor kinases (Nakae et al., 2001) are expressed at the cell surface as homodimers composed of two identical α/β-monomers or as heterodimers composed of two different receptor monomers (Wei et al., 1995; Hubbard, 1997). Although the IR and IGFR share similar molecular structures, most experimental and clinical evidence is consistent with the notion that IR activation (predominantly by insulin) primarily induces glucose and lipid metabolism (Saltiel and Kahn, 2001), embryonic growth (predominantly by IGF2 and insulin) (Kitamura et al., 2003), and growth retardation (Nakae et al., 2001), whereas IGFR activation (predominantly by IGF1 or IGF2) promotes embryonic, muscle, and bone growth (Sarfstein and Werner, 2013) and increases birth weight and lifespan (Liu et al., 1993). Although no ligand associated with the IRR has yet been identified, studies in mice have shown that the IRR functions as an auxiliary member of the insulin receptor family by playing a role that may extend to other co-expressed recognition molecules, such as the *TrkA* receptor (Renteria et al., 2008).

In contrast, only one insulin receptor has been described in the model insects *D. melanogaster*, *A. aegypti*, and *B. mori* (Fernandez et al., 1995; Fullbright et al., 1997; Graf et al., 1997; Lu and Pietrantonio,

Abbreviations: IIS, Insulin and insulin-like growth factor signaling; IGF, Insulin-like growth factor; IR, Insulin receptor; IGFR, Insulin-like growth factor receptor; IRR, Insulin receptor-related receptor; RNAi, RNA interference; ORF, Open reading frame; RTK, Receptor tyrosine kinase; ERK, Extracellular-signal-regulated kinase; PI3K, Phosphatidylinositol-3-OH kinase; Vg, Vitellogenin; FOXO, Fork head transcription factor; qRT-PCR, Quantitative real-time PCR; MNCs, Median neurosecretory cells; PDK1/2, Protein kinase 1/2; AKT/PKB, Protein kinase B; JNK, c-Jun N-terminal kinase; PKC, Protein kinase C; PKA, Protein kinase A; PI3K, Phosphatidylinositol-3-kinase; PLC, Phospholipase C; ERK, Extracellular signal-regulated kinase; IP3, Inositol triphosphate; CDC42, Cell division cycle 42; IKK, IκB kinases; MEKK, Mitogen-activated protein kinase kinase kinase; ASK1, Apoptosis signal-regulating kinase 1; p38, Mitogen-activated protein kinase; ROCK, Rho-associated kinase; AC, Adenylate cyclase; FOXO, Forkhead transcription factor.

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2011). In *D. melanogaster* (*D.mel*), the insulin receptor plays a key role in growth, body size, reproduction, and lifespan (Chen et al., 1996; Brogiolo et al., 2001; Wu and Brown, 2006). Alterations in the expression of genes corresponding to the *D.mel*-IR produce phenotypic growth and organ defects in *D. melanogaster*.

The study of flies that are homozygous for a partial loss-of-function mutation in *dinr*, $dinr^{E19}$ (hereafter, *dinr* is referred to as *D.mel-ir* to maintain consistency with the representation of other genes in this manuscript) have shown that *D.mel-ir* regulates organ size during development by regulating the cell size and number in a cell-autonomous manner. In addition, this mutation causes severe growth retardation in *D. melanogaster* (Brogiolo et al., 2001). These phenotypes are similar to those observed in flies in which insulin-producing cells (IPCs) are genetically ablated (Rulifson et al., 2002). $D.mel\text{-}ir^{EC34}$ mutants exhibit recessive embryonic or early larval lethality (Chen et al., 1996). Moreover, *D. melanogaster* females carrying the $D.mel\text{-}ir^{E19}$ mutation contain ovaries that are arrested at the previtellogenic stage. The treatment of these insects with the juvenile hormone (JH) analog methoprene restores vitellogenesis (Tatar et al., 2001). This phenotype of the $D.mel\text{-}ir^{E19}$ mutant is similar to the status of diapause, suggesting that reduced *D.mel-ir* function may impact the JH levels; indeed, JH has been found to be reduced by 80% as a result of this mutation (Garofalo, 2002). In addition, the $D.mel\text{-}ir^{E19}$ mutation in *D. melanogaster* results in an 85% increase in the adult lifespan (Tatar et al., 2001). The application of an exogenous JH analog to long-lived *D.mel-ir* dwarfs restores the lifespan to the wild-type duration, indicating that the decrease in the JH level is sufficient to observe the effect of *D.mel-ir* mutations on lifespan (Garofalo, 2002).

Two insulin receptor homologs were recently discovered in the social insects honeybees and ants (de Azevedo and Hartfelder, 2008; Lu and Pietrantonio, 2011), which have endocrine mechanisms that differ from those of fruit flies and mosquitoes. Although the function of these two insulin receptors is similar to that of the insulin receptors of other insects, the direct relationship between cell size and growth and the activation of the IIS pathway that is detected in *D. melanogaster* is more complex in honey bee (Lu and Pietrantonio, 2011). In honey bee, it has been demonstrated that the IIS pathway is also a potential candidate for elucidating the relationship between diet and the downstream signals involved in caste determination and differentiation (Wheeler et al., 2006; de Azevedo and Hartfelder, 2008). Caste differentiation is regulated by nutrition and the target of rapamycin pathway, and different castes differ in not only size (as expected from IIS regulation in dipterans) but also the development of specific body structures (Lu and Pietrantonio, 2011). Similarly, in fire ant, there are also two insulin receptors. The differential expression of *S.inv-ir* genes indicates a caste-specific regulation among workers and reproductive females and males, highlighting the physiological significance of the IIS pathway in the regulation of queen physiology and behavior (Lu and Pietrantonio, 2011). Thus, in the social insects honeybees and fire ants, the insulin receptor plays certain roles in caste differentiation. In addition to the above-mentioned social insects, there are two insulin receptors in the migratory brown planthopper, *Nilaparvata lugens*, and these play opposing roles in controlling long wing versus short wing development (Xu et al., 2015).

Interestingly, we have determined that *T. castaneum*, a recently developed coleopteran insect model, also harbors two insulin receptors, which is in accordance with previous annotations (Parthasarathy and Palli, 2011; Yevgeniya Antonova et al., 2012). Although the knockdown of one of the receptors (TC010784) has been described (Parthasarathy and Palli, 2011; Donitz et al., 2015), the function of the other receptor (TC007370) and the similarities between the two receptors have not been studied. Thus, to further understand the evolution and function of insulin receptors, we cloned the full-length cDNA sequences of these two insulin receptors from beetles and investigated the transcriptional expression of both receptors at different developmental stages. Furthermore, through gene knockdown analyses, we investigated the functions of these two proteins in beetles and some of the signaling molecules downstream of each receptor.

2. Materials and methods

2.1. Insect rearing

T. castaneum strain GA-1 was used in this study. The insects were reared in whole wheat flour containing 5% yeast at 30 °C (Beeman et al., 1993; Li et al., 2014).

2.2. Identification and cloning of two putative insulin receptors in T. castaneum

Homology searches for the *D. melanogaster* insulin receptor (AAF55903.2) were conducted using the *T. castaneum* genome databases (http://beetlebase.org) (Kim et al., 2010). The initial search results were further analyzed using the gene prediction software FGENESH (http://linux1.softberry.com/berry.phtml). Based on the predicted sequences, we designed primers to amplify full-length *T.cas-ir1* and *T.cas-ir2* cDNA via PCR and 3′-RACE (3′-full RACE core set, TAKARA). The primers used for cloning are shown in Table 1. The total RNA from *T. castaneum* adults was isolated using the Trizol reagent (TAKARA). First-strand cDNA was synthesized using M-MLV Reverse Transcriptase (RNase H^-) (TAKARA) and oligo$(dT)_{18}$ as the primer, and the PCR products were cloned into the pEASY-T3 Cloning Vector (TransGene) and sequenced by MajorBio (Shanghai, China).

2.3. Sequencing and phylogenetic analyses

The candidate insulin receptors were identified from 26 sequenced metazoan genomes through BLASTP and TBLASTN searches against the genome databases using the known *D. melanogaster* insulin receptor (AAF55903.2) and human insulin receptor sequences (IR: NP_001073285.1, IGFR: NP_000866.1 and IRR: NP_055030.1) as the query sequences. The databases used for these searches included NCBI (http://www.ncbi.nlm.nih.gov/), BeetleBase (http://beetlebase.org/), FlyBase (http://flybase.org/), SilkwormBase (http://silkworm.swu.edu.cn/silkdb/), BeeBase (http://hymenopteragenome.org/beebase/), AphidBase (http://www.aphidbase.com/aphidbase/), VectorBase (http://www.vectorbase.org/index.php), and ant genomes (http://hymenopteragenome.org/acromyrmex/). The E value for evaluating all sequences in the homology searches was 10^{-6}. These candidate genes were further examined via reciprocal BLAST using the NCBI database to ensure that these sequences corresponded to insulin receptors. A total of 65 insulin receptor homologs were found in 35 species from invertebrates to vertebrates that contained complete genomic data for subsequent analysis (Table S1). Multiple sequence alignments of the amino acid sequences were performed using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The alignments were viewed and edited using BOXSHADE software (http://www.ch.embnet.org/software/BOX_form.html). The phylogenetic trees were constructed using MEGA 5.0 software according to the maximum likelihood (ML) method (Tamura et al., 2011). One thousand bootstrap tests were performed, and values lower than 50% were not considered.

2.4. Determination of expression patterns of two putative insulin receptors in T. castaneum

Using the Trizol reagent (TAKARA), the total RNA from a pool of three individuals at each of the following developmental stages was extracted: early embryo (EE, 1 day old), late embryo (LE, 3 days old), early larva (EL, 1 day old), late larva (LL, last instar larvae), early pupa (EP, 1 day old), late pupa (LP, 5 days old), early adult (EA, 1 day old), and late adult (LA, 7 days old). Reverse transcription was performed using 1 μg of total RNA. The specificity of the target amplicon was