Nuclear Import of Transcription Factor BR-C Is Mediated by Its Interaction with RACK1

Daojun Cheng1*, Wenliang Qian1*, Yonghu Wang1, Meng Meng1, Ling Wei2, Zhiqing Li3, Lixia Kang1, Jian Peng1, Qingyou Xia1*

1 State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing, China, 2 School of Life Science, Southwest University, Chongqing, China, 3 Laboratory of Silkworm Science, Kyushu University Graduate School of Bioresource and Bioenvironmental Sciences, Fukuoka, Japan

Abstract

The transcription factor Broad Complex (BR-C) is an early ecdysone response gene in insects and contains two types of domains: two zinc finger domains for the activation of gene transcription and a Bric-a-brac/Tramtrack/Broad complex (BTB) domain for protein-protein interaction. Although the mechanism of zinc finger-mediated gene transcription is well studied, the partners interacting with the BTB domain of BR-C has not been elucidated until now. Here, we performed a yeast two-hybrid screen using the BTB domain of silkworm BR-C as bait and identified the receptor for activated C-kinase 1 (RACK1), a scaffolding/anchoring protein, as the novel partner capable of interacting with BR-C. The interaction between BR-C and RACK1 was further confirmed by far-western blotting and pull-down assays. Importantly, the disruption of this interaction, via RNAi against the endogenous RACK1 gene or deletion of the BTB domain, abolished the nuclear import of BR-C in BmN4 cells. In addition, RNAi against the endogenous PKC gene as well as phosphorylation-deficient mutation of the predicted PKC phosphorylation sites at either Ser373 or Thr406 in BR-C phenocopied RACK1 RNAi and altered the nuclear localization of BR-C. However, when BTB domain was deleted, phosphorylation mimics of either Ser373 or Thr406 had no effect on the nuclear import of BR-C. Moreover, mutating the PKC phosphorylation sites at Ser373 and Thr406 or deleting the BTB domain significantly decreased the transcriptional activation of a BR-C target gene. Given that RACK1 is necessary for recruiting PKC to close and phosphorylate target proteins, we suggest that the PKC-mediated phosphorylation and nuclear import of BR-C is determined by its interaction with RACK1. This novel finding will be helpful for further deciphering the mechanism underlying the role of BR-C proteins during insect development.

Introduction

The Broad-Complex (BR-C) protein contains a pair of C2H2 zinc fingers at the C terminus and a Bric-a-brac/Tramtrack/Broad complex (BTB) domain at the N terminus [1,2]. In insects, BR-C is primarily involved in the signaling pathways of ecdysone and juvenile hormone, two key endocrine hormones that orchestrate the growth and development of insects [3]. Accumulated evidence has demonstrated the critical roles of insect BR-C in numerous developmental processes, including embryogenesis [4,5], metamorphosis [6,7], cell death [8,9], stem cell differentiation [10], wing morphogenesis [11], eye development [12], muscle attachment [13,14], metamorphic remodeling of the central nervous system [15,16], and oogenesis [17,18].

It has been reported that the transcription of insect BR-C gene is activated and suppressed by ecdysone and juvenile hormone, respectively [19]. In particular, the BR-C protein is considered an early response genes in the ecdysone signaling pathway, regulating the transcription of downstream target genes, such as cuticle protein genes [20–22], microRNA genes [23,24], caspase genes [25], glue protein genes [26], and fork head genes [27].

Although zinc fingers-mediated transcriptional regulation for BR-C protein is well understood in insects, the role of BTB domain of BR-C protein remains unknown. Given that the BTB domain is a conserved motif involved in protein-protein interaction [28], it is expected that the BTB domain would provide the BR-C protein with a facile means of interacting with some partners. In fact, several BTB domain-containing proteins have been identified to interact with partners via their BTB domain [29–34].

Silkworm (Bombyx mori) is regarded as an excellent model system for the lepidopterans [35]. The expression profile, target gene, and function during metamorphosis of the silkworm BR-C gene have all been characterized [8,20,36]. To identify potential partners interacting with BR-C protein, we performed a yeast two-hybrid screen using the BTB domain of the silkworm BR-C...
protein as bait. Ultimately, we identified a novel interacting partner for silkworm BR-C, receptor for activated C-kinase 1 (RACK1), which has been previously demonstrated to function an anchoring protein for activated protein kinase C (PKC) [37, 38] and a scaffolding protein for recruiting PKC to a number of binding partners to trigger their PKC-mediated phosphorylation [39–42]. Further examination revealed that the interaction between BR-C and RACK1 is required for the nuclear import of BR-C.

Figure 1. Identification of BR-C-interacting proteins through a yeast two-hybrid screen. (A) Yeast two-hybrid screen of BR-C-interacting proteins from a silkworm silk gland cDNA library using the BTB domain of BR-C as bait. A total of 54 positive clones were found to grow on selective medium. A negative control consisting of pGBK7-Lam with pGAD77-T and a positive control consisting of pGBK7-S3 with pGAD77-T were used to measure the efficiency of the system. (B) Yeast two-hybrid confirmation of the interaction between BR-C and its novel interacting partner RACK1. cDNA encoding full-length BR-C, the BTB domain or partial BR-C lacking the BTB domain was cloned into the pGBK7 vector. The full-length cDNA sequence of RACK1 gene was inserted into the pGADT7 vector. The yeast two-hybrid experiment was performed to test the interaction between BR-C and RACK1.

doi:10.1371/journal.pone.0109111.g001

Figure 2. Interaction of BR-C with RACK1 in vitro. (A) Interaction between BR-C and RACK1 by far-western blotting. GST, GST-tagged RACK1 (GST-RACK1), and SUMO-tagged BR-C (SUMO-BR-C) were expressed and purified from prokaryotic cells. The purified proteins were separated on 12% SDS-PAGE gels and transferred onto PVDF membranes, which were incubated with SUMO-BR-C or GST proteins at 4°C overnight, and analyzed by western blotting using anti-BR-C or anti-GST antibodies. (B-C) Interaction between BR-C and RACK1 by GST pull-down assay. purified SUMO-BR-C was incubated with purified GST-RACK1 and examined by immunoblotting using an anti-BR-C antibody (B). Precleared BmN4 cell lysates containing endogenous RACK1 were incubated with purified GST-BR-C at 4°C for 6 h, and were separated via 12% SDS-PAGE gel and visualized by immunoblotting using an anti-RACK1 antibody (C).

doi:10.1371/journal.pone.0109111.g002
Materials and Methods
Yeast two-hybrid screen
The Matchmaker Gold Yeast Two-Hybrid System (Clontech) was used to perform the yeast two-hybrid screen in this study. All experiments were carried out according to the standard protocol for this system. Briefly, the cDNA sequence encoding the BTB domain of the silkworm BR-C protein (GenBank no: NP_001104803) was amplified through a standard PCR approach and then cloned into a pGBK7T7 vector to generate the bait plasmid pGBK7T7-BTB. The primers and plasmid constructs are listed in Table S1 and Table S2. The pGBK7T7-BTB construct was transformed into Y2HGold yeast to obtain the bait strain. Given that the BR-C gene is abundantly expressed in the silkworm silk gland [36], we extracted total RNA from the silk gland using the TRIzol reagent (Invitrogen) to construct a cDNA library for preying the BTB-interacting proteins. Strains containing the pGBK7T7-BTB bait plasmid and the cDNA library were co-transfected with SUMO or SUMO-BR-C fusion proteins (50 µg per sample) were mixed with SUMO-BR-C proteins (50 µg) overnight at 4°C. Another control was prepared by the incubating the SUMO protein with GST-RACK1. Antibodies against GST (Sigma; 1:100) and silkworm BR-C (1:1,000) were used for western blotting.

Cell culture
Two silkworm ovary-derived cell lines, BmN4 cells and BmN4-SID1 cells harboring the Caenorhabditis elegans SID1 gene [24], were used in this study. The cells were cultured at 27°C in IPL-41 medium (Sigma, USA) supplemented with 10% fetal bovine serum (Life Technologies, USA).

Subcellular localization
For subcellular localization assays, full-length cDNAs encoding the silkworm BR-C and RACK1 (GenBank no: NP_001104703) genes were separately cloned into the expression vector pI2VW (a vector containing the green fluorescent protein variant Venus) to generate the Venus-BR-C and Venus-RACK1 plasmids according to the previously described procedure [43]. Meanwhile, we also generated a construct (BR-C-DsRed) containing the full-length silkworm BR-C gene fused to DsRed (a red fluorescent protein reporter) gene to examine the nuclear localization of the silkworm BR-C protein.

The plasmid constructs Venus-RACK1, Venus-BR-C and BR-C-DsRed were transfected into BmN4 cells in 12-well plates at 100 ng per well. At 48 h after transfection, the BmN4 cells were seeded onto coverslips. After washing with PBS, the cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% TritonX-100 for 15 min. BmN4 cells were then stained with DAPI (1:1,000) for 15 min, washed three times with PBS, and the coverslips were mounted on glass slides. Fluorescence signals were checked and captured via confocal microscopy (Fv1000, Olympus).

Expression and purification of recombinant proteins
The full-length silkworm BR-C gene was cloned into the vectors pET20-His and pCold-SUMO containing a His tag. The full-length silkworm RACK1 gene was cloned into the pETGST vector. After sequencing, each recombinant vector was transformed into E. coli strain BL21 and induced for 20 h by 0.2 mM IPTG at 16°C. The cells were harvested and lysed by sonication. GST and GST fusion proteins were purified using glutathione-conjugated agarose beads. The purified His-BR-C protein was used to produce a rabbit antibody against BR-C (Zoonbio Biotechnology).

Far-western blotting assay
The interaction between BR-C and RACK1 was first confirmed in vitro by far-western blotting experiments [44]. BSA, GST, and GST-RACK1 protein samples (5 µg per sample) were separately electro-transferred onto a 0.45 µm PVDF membrane. After denaturation and renaturation, the membrane was blocked with buffer containing 5% skim milk powder (Boster) and incubated with SUMO-BR-C proteins (50 µg) overnight at 4°C. Another control was prepared by the incubating the SUMO protein with GST-RACK1. Antibodies against GST (Sigma; 1:100) and silkworm BR-C (1:1,000) were used for western blotting.

GST pull-down assay
Glutathione agarose beads (25 µl) coated with either GST or the GST-RACK1 fusion protein (5 µg per sample) were mixed with SUMO or SUMO-BR-C fusion proteins (50 µg per sample) in 1 ml PBS buffer (pH 8.0). Each mixture was incubated with shaking for 8 h at 4°C. The beads were collected by centrifugation and washed three times in PBS buffer and then eluted with 80 µl 50 mM Tris-HCl (pH 8.0) containing 10 mM GSH to isolate the supernatant. The supernatants were mixed with sample loading buffer and separated on SDS-PAGE gels and immunoblotted with anti-His (Sigma; 1:100) or anti-BR-C antibodies. In addition, we also used the GST-BR-C fusion protein to pull down RACK1 that was prepared from BmN4 cells. The proteins in the supernatants were immunoblotted with anti-RACK1 antibody (Cell signaling technology; 1:100).

RNA interference
RNA interference (RNAi) was used to knockdown the expression of target genes. Double-strand RNA (dsRNA) targeting silkworm RACK1, PKC (GenBank no: NM_0011043313), or EGFP (enhanced green fluorescent protein) as a control was...
synthesized according to the RiboMAX Large Scale RNA Production System-T7 (Promega). BmN4-SID1 cells were treated with different dsRNAs as previously described [43]. Briefly, the dsRNA for each gene was added to the medium to induce RNAi. After two rounds of dsRNA treatment, the BR-C-DsRed plasmid was transfected. At 48 h after transfection, the subcellular localization of the BR-C protein was examined via a confocal microscopy.

Site-directed mutagenesis and BTB domain deletion
The full-length silkworm BR-C gene inserted into the pMD19-T vector was used as a template for mutating the PKC phosphorylation sites or deleting the BTB domain using the MutanBEST Kit (Takara). For phosphorylation-deficient mutation, each of the predicted phosphorylation sites, namely, Ser (S) or Thr (T), was mutated to Ala (A). For phosphorylation mimics, the predicted phosphorylation sites were singly mutated to Glu (E). The modified BR-C gene sequences were subcloned into a plasmid harboring the DsRed reporter to generate constructs containing the BR-C mutations or the BTB deletion fused to DsRed. Each construct was also used to check the subcellular localization as described above.

Luciferase reporter assay
Given that the silkworm wing cuticle protein gene *WCP10* is a direct target of BR-C [20], the effects of PKC phosphorylation
sites mutations or BTB deletion in BR-C on the transcription of the WCP10 gene were analyzed using the dual luciferase reporter assay system (Promega). The regulator region from −2940 to +26 in the WCP10 promoter was cloned into a pGL3 basic vector containing a dual luciferase reporter. The DsRed-BR-C, DsRed-BR-C mutants, and DsRed-BTB deletion constructs were separately co-transfected into BmN4 cells with the WCP10-lux reporter vector (1 ug per well). The cells were cultured at 27°C for 48 h. Luciferase activities were measured according to the manufacturer’s protocol for the luciferase reporter system.

Results

Identification of RACK1 as a BR-C-interacting protein by yeast two-hybrid screen

Insect BR-C transcription factors contain a conserved BTB domain, which is a protein-protein interacting motif [2]. To identify partners interact with the BR-C protein, we performed a yeast two-hybrid screen of a silkworm silk gland cDNA expression library using the BTB domain of the silkworm BR-C protein as bait. A preliminary screen identified 54 positive clones capable of growing on selective dropout medium (Figure 1A). Sequencing result revealed that 17 of these positive clones encode RACK1 (GenBank no: NP_001041703), indicating that RACK1 may be an interacting partner for the silkworm BR-C protein.

To confirm the interaction between BR-C and RACK1 in yeast cells, we obtained three silkworm BR-C gene sequences, including the full-length cDNA, the BTB domain sequence alone, and a partial cDNA sequence lacking the BTB domain, which were then fused to the GAL4 DNA binding domain (GAL4BD) to produce the bait constructs BD-BR-C, BD-BTB, and BD-BTB deletion. In addition, the complete coding sequence for the silkworm RACK1 gene was fused to GAL4 activation domain (GAL4AD) to generate the prey construct AD-RACK1. Two-hybrid analysis showed that RACK1 could efficiently interact with both full-length BR-C and the BTB domain alone, but not a partial BR-C protein lacking the BTB domain (Figure 1B), indicating the BTB domain of BR-C was necessary for the interaction between BR-C and RACK1.

BR-C interacts with RACK1 in vitro

To further confirm the interaction of BR-C with RACK1 in vitro, we first conducted a far-western blotting analysis using bacterially expressed GST-tagged RACK1 (GST-RACK1) and SUMO-tagged BR-C (SUMO-BR-C). As shown in Figure 2A, BR-C was able to interact with RACK1 but not with GST only. The interaction between BR-C and RACK 1 was also confirmed by a GST pull-down assay. The SUMO-tagged BR-C protein could be pulled down using GST-tagged RACK1 but not by GST alone, and bacterially expressed SUMO could not be pulled down by GST-tagged RACK1 (Figure 2B). Similarly, the endogenous RACK1 protein extracted from BmN4 cells could be pulled down by GST-tagged BR-C but not GST alone (Figure 2C). These data demonstrate a direct interaction between BR-C and RACK1.

BR-C and RACK1 localize to different subcellular regions in BmN4 cells

To understand how BR-C interacts with RACK1 in vivo, we surveyed the subcellular localization of BR-C and RACK1 in BmN4 cells. Venus-fused BR-C and RACK1 were individually transfected into BmN4 cells. After two days of transfection, the cells were observed via confocal fluorescence microscopy. Venus-BR-C was found to localize exclusively in the nucleus in BmN4 cells, forming multiple spots (Figure 3), which was expected due to the role of BR-C as a transcription factor. However, Venus-RACK1 was mainly localized diffusely throughout the cytoplasm of BmN4 cells (Figure 3), which was unexpected due to the role of BR-C as a transcription factor. However, Venus-RACK1 was mainly localized diffusely throughout the cytoplasm of BmN4 cells (Figure 3). Combined with the fact that RACK1 generally acts as a scaffolding/anchoring protein to coordinate the PKC-mediated phosphorylation of binding partners [45,46], we therefore hypothesized that the interaction between BR-C and RACK1 may occur transiently in the cytoplasm and further contribute to the nuclear localization of BR-C or even to its posttranslational modification.

BR-C BTB domain deletion and RACK1 RNAi both disrupt BR-C nuclear localization

To test the hypothesis that the interaction between BR-C and RACK1 may affect the nuclear localization of BR-C, we disrupted
the RACK1-BR-C interaction by either deleting the BTB domain of BR-C or performing RNAi against RACK1. For this analysis, we used the fusion protein construct BR-C-DsRed, in which full-length BR-C was inserted in the upstream of the red fluorescent protein DsRed. Consistent with the localization of Venus-BR-C, DsRed-BR-C was also localized to the nucleus (Figure 4A). Interestingly, DsRed fused to a truncated BR-C protein lacking the BTB domain was retained in the cytoplasm of BmN4 cells (Figure 4A).

Moreover, we performed RNAi-mediated expression silencing of the endogenous RACK1 gene in BmN4-SID1 cells. In contrast to control cells treated with the treatment with dsRNAs against the EGFP gene as a control, full-length BR-C fused to DsRed could not enter the nucleus and was restricted to the cytoplasm in BmN4 cells treated with dsRNAs against the RACK1 gene (Figure 4B), similar to the effects caused by deletion of the BR-C BTB domain. These results strongly demonstrated that the nuclear localization of BR-C is regulated by its interaction with RACK1.

Both PKC RNAi and PKC phosphorylation site mutations in BR-C alter BR-C nuclear localization

We next asked whether the nuclear localization of BR-C is associated with PKC-mediated phosphorylation that is regulated by RACK1. To test this hypothesis, we first performed RNAi against endogenous PKC in BmN4-SID1 cells. As shown in Figure 5, PKC RNAi resulted in a change in the cellular localization of BR-C from the nucleus to the cytoplasm, which phenocopied the effect of RACK1 RNAi.

Next, we analyzed whether mutating PKC phosphorylation sites in the BR-C protein could also affect the nuclear localization of BR-C. Based on the online NetPhosK program (http://www.cbs.dtu.dk/services/NetPhosK/), we predicted 12 PKC phosphorylation sites in the silkworm BR-C protein, including seven Ser (S) and five Thr (T) residues, of which seven sites are located in either BTB domain or zinc finger domains (Figure S1). We generated a series of BR-C mutants in which a single phosphorylation site (Ser or Thr) was specifically mutated to Ala (A), which can block phosphorylation, and then fused these to DsRed. The localization

Figure 6. Mutating the predicted PKC phosphorylation sites in BR-C disrupts the nuclear import of BR-C. Each construct expressing full-length BR-C with the mutation of a single predicted phosphorylation site to Ala (A; blocks phosphorylation) was transfected into BmN4 cells, and the nuclear import of the expressed BR-C protein was observed by confocal microscopy two days after transfection. The construct encoding wild-type BR-C without any phosphorylation site mutation was used as a control. Two predicted phosphorylation site mutations, S373A and T406A, led to the failure of BR-C nuclear import. However, mutating the predicted PKC phosphorylation site at Ser44 could not affect the nuclear localization of BR-C. doi:10.1371/journal.pone.0109111.g006
Figure 7. Mutations of phosphorylation sites to Glu in BR-C lacking BTB domain have no impact on nuclear import of BR-C. Construct expressing BR-C both lacking BTB domain and mutating the predicted phosphorylation sites of either Ser373 or Thr406 into Glu (E; mimics phosphorylation) was transfected into BmN4 cells. Confocal microscopy analysis revealed that compared to the control of BR-C only lacking BTB domain, the mutants of either S373E or T406E in BR-C without BTB domain could not change nuclear localization of BR-C.

doi:10.1371/journal.pone.0109111.g007

Figure 8. Both BTB domain deletion and PKC phosphorylation site mutants impaired the transcriptional activity of BR-C. (A) Effects of the deletion of the BR-C BTB domain on the transcriptional activity of BR-C. DsRed fused to BR-C lacking the BTB domain was co-transfected into BmN4 cells with a plasmid containing a luciferase reporter gene under the control of the promoter of WCP10 gene, which is a direct target of BR-C. Luciferase activity was measured two days after transfection. Constructs containing either DsRed fused to full-length BR-C or DsRed alone were used as controls. (B) Effects of mutating the PKC phosphorylation sites in BR-C on the transcriptional activity of BR-C. The constructs containing BR-C with mutations at the phosphorylation sites S373 or T406 or non-mutated BR-C were each co-transfected into BmN4 cells with a plasmid containing a luciferase reporter gene under the control of the WCP10 gene promoter. Luciferase activity was measured two days after transfection.

doi:10.1371/journal.pone.0109111.g008
of these mutant fusion proteins was analyzed in BmN4 cells via confocal microscopy. We found that mutations at two sites in the zinc finger motifs, Ser373 and Thr406, clearly shifted the nuclear localization of BR-C to the cytoplasm (Figure 6). By contrast, mutations at Ser44 and at other predicted PKC phosphorylation sites could not affect the nuclear localization of BR-C (Figure 6 and Figure S2). Therefore, these data suggested that PKC is involved in the nuclear import of BR-C, likely through the phosphorylation of BR-C at Ser373 and Thr406.

**Phosphorylation mimics of the PKC phosphorylation sites in BR-C lacking BTB domain have no influence on BR-C nuclear localization**

To further verify whether the interaction of BTB domain of BR-C with RACK1 is necessary for phosphorylation-mediated nuclear import of BR-C, the predicted PKC phosphorylation sites of either Ser373 or Thr406 in BR-C lacking BTB domain were mutated to Glu (E), which mimics phosphorylation, and their effects on nuclear import of BR-C were examined in BmN4 cells. As shown in Figure 7, although BTB domain was deleted, either S373E or T406E mutants did not disrupt the nuclear import of BR-C lacking BTB domain. This result, together with the observation that the phosphorylation-deficient mutations at either Ser373 or Thr406 abolish the nuclear import of BR-C, suggested that the interaction of BTB domain in BR-C with RACK1 is primarily required for PKC-mediated phosphorylation and subsequent nuclear import of BR-C.

**BTB deletion and phosphorylation site mutations in BR-C suppress the transcriptional activation of target gene**

We reasoned that the disruption of BR-C nuclear localization might affect its transcriptional activity. To confirm this possibility, we selected the silkworm *WCP10* gene, which is transcriptionally regulated by the direct binding of BR-C to the *WCP10* promoter region [20]. We generated a construct that contains a luciferase reporter gene under the control of the *WCP10* promoter. This *WCP10* luciferase reporter vector was then co-transfected into BmN4 cells with expression plasmids containing DsRed fused to either full-length BR-C or partial BR-C lacking the BTB domain. Further analysis of luciferase activity showed that overexpressing full-length BR-C could elevate the activity of the *WCP10* promoter, compared with the DsRed alone control (Figure 8A). However, deleting the BTB domain completely abolished the transcriptional activity of BR-C toward the *WCP10* promoter (Figure 8A).

To further examine the importance of BR-C phosphorylation on its transcriptional activity, we analyzed the effects of the PKC phosphorylation site mutants of BR-C on the activity of the *WCP10* promoter. As shown in Figure 8B, compared to the full-length BR-C gene, both the Ser373 and Thr406 mutants of BR-C exhibited clearly decreased activities towards the *WCP10* promoter. These data, together with above-described findings, supported that the suppression of both BTB deletion and phosphorylation site mutations in BR-C on the transcriptional activation of target gene *WCP10* is likely due to their disruption on the nuclear import of BR-C.

**Discussion**

The transcription factor BR-C is involved in controlling insect growth and development by mediating cross-talk between two endocrine hormones, ecdysone and juvenile hormone [3]. To date, the role of the DNA binding zinc finger domain of BR-C in response to endocrine hormones and transcriptional regulation of target gene expression has been well studied [20,47,48]. In addition to zinc fingers, insect BR-C proteins also features a BTB domain that is involved in protein-protein interactions [28]. However, the role of BTB domain in BR-C function remains unknown. In this study, we used yeast two-hybrid screening to
identify RACK1 as an interacting partner for the silkworm BR-C protein by using the BTB domain as bait. Further examination revealed that this BTB domain-mediated interaction between BR-C and RACK1 plays an important role in the nuclear import and signaling of BR-C.

A large number of previous studies have shown that RACK1 primarily functions as a scaffold to recruit and interact with binding partners, and this interaction endows RACK1 with critical roles in regulating the transcription, translation, and cellular localization of RACK1-interacting proteins [45,46,49]. Consistent with these observations, our present data revealed a role for RACK1 in promoting the nuclear localization of BR-C in silkworm BmN4 cells. Furthermore, this role was mediated by the interaction of RACK1 with the BTB domain of BR-C. Because BR-C and RACK1 were localized to the nucleus and the cytoplasm, respectively, in silkworm BmN4 cells, we speculated that the interaction between BR-C and RACK1 most likely occurs in the cytoplasm. These data therefore strongly supported the notion that the interaction between BR-C and RACK1 is essential for the nuclear import of BR-C.

RACK1 has been shown to regulate posttranslational modification by recruiting activated PKC to its interacting partners, which in turn leads to the activation of these interacting partners via the PKC-mediated phosphorylation [37,45]. For instance, it has been recently shown that the activity of the transcription factor ultraspireacle (USP) involved in insect ecysline signaling is regulated by PKC-mediated phosphorylation [50,51]. In addition, previous evidence has also demonstrated that the nuclear import of several proteins is modulated by their phosphorylation [52–55]. In fact, we predicted 12 PKC phosphorylation sites in BR-C and found that the mutations at only two sites, Ser373 and Thr406, could affect the nuclear import of BR-C. Taken together with the observation that the nuclear import of BR-C can be similarly disrupted by RNAi knockdown of the endogenous PKC gene in silkworm BmN4-SID1 cells, we concluded that the interaction between BR-C and RACK1 functions in a large part to recruit PKC to BR-C, such that BR-C is consequently phosphorylated by PKC and is then able to enter the nucleus.

Notably, the PKC phosphorylation sites in BR-C that are essential for the nuclear import of BR-C, namely, Ser373 and Thr406, are located in the two zinc finger domains, rather than in the BTB domain. Previous studies reported that the phosphorylation sites in several zinc finger-containing proteins are also located in the zinc finger domains [56,57]. Therefore, our findings suggested that the interaction between the BTB domain of BR-C and RACK1 primarily contribute to recruit PKC and then help phosphorylate the phosphorylation sites in the zinc finger domains of BR-C.

Our examination also revealed that deleting the BTB domain and mutating the phosphorylation sites in BR-C impaired the transcriptional activation of target genes. Taken together with the other data presented here and the functions of RACK1 reported previously [45], we propose a potential model for the roles of the novel BR-C-interacting protein RACK1 in BR-C function. As summarized in Figure 9, RACK1 recruits active PKC to BR-C in the cytoplasm of the cells through its interaction with the BTB domain of BR-C. This interaction facilitates the phosphorylation of BR-C by PKC probably at Ser373 and Thr406. Phosphorylated BR-C then translocates to the nucleus to activate the transcription of BR-C target genes. To our knowledge, this study is the first report to describe a specific protein-protein interaction with insect BR-C and will be helpful for understanding the role of the BTB domain in the functions and signaling of BR-C in insects.

Unquestionably, the mechanism underlying BR-C phosphorylation remains to be deciphered in depth. For instance, it is enticing to determine whether Ser373 or Thr406 is the authentic phosphorylation sites in BR-C using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. In addition, given that BR-C is involved in the signaling of two key endocrine hormones, ecdysone and juvenile hormone [3], it is worthy to test whether and how BR-C phosphorylation is involved in modulating endocrine hormone signaling. Undoubtedly, future studies on BR-C phosphorylation are likely to provide new insights into the functions and signaling of BR-C in insects.

Supporting Information

**Figure S1** Prediction of PKC phosphorylation sites in silkworm BR-C. PKC phosphorylation sites in silkworm BR-C were predicted using the online NetPhosK program (http://www.cbs.dtu.dk/services/NetPhosK/). Based on a threshold score of 0.6, a total of 12 sites in the BR-C protein were predicted as PKC phosphorylation sites, including Ser (S) or Thr (T). All sites are highlighted in red. The BTB domain and the two zinc finger motifs are highlighted with blue and green boxes, respectively. (TIF)

**Figure S2** Mutations at nine of the predicted PKC phosphorylation sites in silkworm BR-C have no effect on nuclear import of BR-C. Among the predicted PKC phosphorylation sites in silkworm BR-C, in addition to Ser44 described in Figure 6, mutations to Ala (A) at nine sites also have no effect on nuclear localization of BR-C in BmN4 cells. (TIF)

| Table S1 | List of primers used in this study. (TIF) |
| Table S2 | List of plasmids, strains and cell lines used in this study. (TIF) |

**Author Contributions**

Conceived and designed the experiments: QX DC WQ. Performed the experiments: WQ YW MM LW ZL LL JP. Analyzed the data: QX DC WQ YW. Contributed reagents/materials/analysis tools: WQ YW MM LW ZL LL JP. Wrote the paper: QX DC WQ.

**References**

1. DiBello PR, Withers DA, Bayer CA, Fristrom JW, Guild GM (1991) The Drosophila Broad-Complex encodes a family of related proteins containing zinc fingers. Genetics 129: 305–307.
2. Zollman S, Godt D, Prive GG, Couderc JL, Laski FA (1994) The BTB domain, found primarily in zinc finger proteins, defines an evolutionarily conserved family that includes several developmentally regulated genes in Drosophila. Proc Natl Acad Sci U S A 91: 10717–10721.
3. Dubrovsky EB (2006) Hormonal cross talk in insect development. Trends Endocrinol Metab 16: 6–11.
4. Paualachs MD, Pagone V, Belles X (2010) Key roles of the Broad-Complex gene in insect embryogenesis. Insect Biochem Mol Biol 40: 468–475.
5. Erezyilmaz DF, Rynerson MR, Truman JW, Riddiford LM (2009) The role of the pupal determinant broad during embryonic development of a direct-developing insect. Dev Genes Evol 219: 535–544.
6. Zhou X, Riddiford LM (2002) Broad specifies pupal development and mediates the 'status quo' action of juvenile hormone on the pupal-adult transformation in Drosophila and Manduca. Development 129: 2259–2269.
7. Erezyilmaz DF, Riddiford LM, Truman JW (2006) The pupal specifier broad directs progressive morphogenesis in a direct-developing insect. Proc Natl Acad Sci U S A 103: 6925–6930.
8. Ulijnrova M, Foy BD, Beatty BJ, Olson KE, Riddiford LM, et al. (2003) Use of Sindbis virus-mediated RNA interference to demonstrate a conserved role of
Broad-Complex in insect metamorphosis. Proc Natl Acad Sci U S A 100: 15607–15612.

Lee CY, Cooksey BA, Bacherere EH (2002) Steroid regulation of midgut cell death during Drosophila development. Dev Biol 250: 101–111.

Zeng XK, Hou XN (2012) Broad relays hormone signals to regulate stem cell differentiation in Drosophila midgut during metamorphosis. Development 139: 3917–3925.

Rohmu M, Belles X (2013) Subtle roles of microRNAs let-7, miR-100 and miR-125 on wing morphogenesis in hemimetabolous metamorphosis. J Insect Physiol 59: 1089–1094.

Brennan CA, Li TR, Bender M, Hsiung F, Moses K (2001) Broad-complex, but not ecdysone receptor, is required for progression of the morphogenetic furrow in the Drosophila wing discs of Bmp-22 mutant flies. Insect Biochem Mol Biol 31: 403–406.

Sandstrom DJ, Restifo LL (1999) Epidermal tendon cells require Broad Complex function for correct attachment of the indirect flight muscles in Drosophila melanogaster. J Cell Sci 112 (Pt 22): 4051–4065.

Sempere LF, Dubrovsky EB, Dubrovskaya VA, Berger EM, Ambros V (2002) Elucidation of the regulation of an adult cuticle gene Acp65A by the transcription factor Broad. Insect Mol Biol 11: 421–429.

Wang HB, Nita M, Iwanaga M, Kawasaki H (2009) betaFTZ-F1 and Broad-Complex in insect metamorphosis. Proc Natl Acad Sci U S A 106: 15607–15612.

Huang RY, Orr WC (1992) Broad-complex function during oogenesis in Drosophila melanogaster. J Cell Sci 112 (Pt 22): 4051–4065.

Daniel JM, Reynolds AB (1999) The catenin p120(ctn) interacts with Kaiso, a transcription factor that interacts with Mad and regulate transcription through the NF-E2 site. Mol Cell Biol 16: 6093–6099.

Melnick A, Carlile G, Ahmad KF, Kiung CL, Corcoran C, et al. (2002) Critical residues within the BTB domain of PLZT and bel-6 modulate interaction with corepressors. Mol Cell Biol 22: 1804–1818.

Xia Q, Li S, Feng Q (2014) Advances in silkworm studies accelerated by the genome sequencing of Bombyx mori. Anna Rev Entomol 59: 513–536.

Nishita Y, Takuya S (2004) Structure and expression of the gene encoding a Broad-Complex homolog in the silkworm, Bombyx mori. Gene 339: 161–172.

Ron D, Chen CH, Caldwell J, Jamieson L, Orr E, et al. (1994) Cloning of an intracellular receptor for protein kinase C-a: a homolog of the beta subunit of G proteins. Proc Natl Acad Sci U S A 91: 839–843.

Schechman D, Mochly-Rosen D (2001) Adapter proteins in protein kinase C-mediated signal transduction. Oncogene 20: 6339–6347.

Gahoneau AM, Hsiau R, Xu W, Shi M, Kamhudi J, et al. (2007) Myelin protein zero/F0 phosphorylation and function require an adapter protein linking it to RACK1 and PKC alpha. J Cell Biol 177: 707–716.

Nery FC, Passos DO, Garcia VS, Kobajr J (2004) Ki-1/57 interacts with RACK1 and is a substrate for the phosphorylation by phosphorylated 12-myristate 13-acetate-activated protein kinase C. J Biol Chem 279: 11444–11453.

Zhang XY, Tang LZ, Ren BG, Yu YP, Nelson J, et al. (2013) Interaction of MCM7 and RACK1 for activation of MCM7 and cell growth. Am J Pathol 182: 796–805.

Vani K, Vang G, Mohler J (1997) Isolation and cloning of a Drosophila homolog to the mammalian RACK1 gene, implicated in PKC-mediated signalling. Biochim Biophys Acta 1358: 67–71.

Li Z, Zheng D, Mon H, Tatsuke T, Zhu L, et al. (2012) Genome-wide identification of polycomb target genes reveals a functional association of Pho with Scm in Bombyx mori. PLoS One 7: e43350.

Wu Y, Li Q, Chen XZ (2007) Detecting protein–protein interactions by Far western blotting. Nat Protoc 2: 3278–3280.

Adams DR, Ron D, Kidy PA (2011) RACK1, A multifaceted scaffolding protein: Structure and function. Cell Commun Signal 9: 22.

Nilsson J, Sengupta J, Frank J, Nissen P (2004) Regulation of eukaryotic translation by the RACK1 protein: a platform for signalling molecules on the ribosome. EMBO Rep 5: 1137–1141.

Konopova B, Jindra M (2008) Broad-Complex acts downstream of Met in juvenile hormone signaling to coordinate primitive holometabolous metamorphosis. Development 135: 559–568.

Nishita Y (2014) Ecdysone response elements in the distal promoter of the Bombyx Broad-Complex gene, BmBR-C. Insect Mol Biol 23: 341–356.

Ron D, Adams DR, Baillie GS, Long A, O’Connor R, et al. (2013) RACK1 to the future - a historical perspective. Cell Commun Signal 11: 53.

Liu W, Cai MJ, Zheng CC, Wang JX, Zhao XF (2014) Phospholipase C gamma 1 connects the cell membrane pathway to the nuclear receptor pathway in insect juvenile hormone signaling, J Biol Chem 289: 13026–13041.

Wang S, Wang J, Sun Y, Song Q, Li S (2012) PKC-mediated USP acetate-activated protein kinase C. J Biol Chem 279: 11444–11453.

Li Z, Zheng D, Mon H, Tatsuke T, Zhu L, et al. (2012) Genome-wide identification of polycomb target genes reveals a functional association of Pho with Scm in Bombyx mori. PLoS One 7: e43350.

Wu Y, Li Q, Chen XZ (2007) Detecting protein–protein interactions by Far western blotting. Nat Protoc 2: 3278–3280.

Adams DR, Ron D, Kidy PA (2011) RACK1, A multifaceted scaffolding protein: Structure and function. Cell Commun Signal 9: 22.

Nilsson J, Sengupta J, Frank J, Nissen P (2004) Regulation of eukaryotic translation by the RACK1 protein: a platform for signalling molecules on the ribosome. EMBO Rep 5: 1137–1141.

Konopova B, Jindra M (2008) Broad-Complex acts downstream of Met in juvenile hormone signaling to coordinate primitive holometabolous metamorphosis. Development 135: 559–568.

Nishita Y (2014) Ecdysone response elements in the distal promoter of the Bombyx Broad-Complex gene, BmBR-C. Insect Mol Biol 23: 341–356.

Ron D, Adams DR, Baillie GS, Long A, O’Connor R, et al. (2013) RACK1 to the future - a historical perspective. Cell Commun Signal 11: 53.

Liu W, Cai MJ, Zheng CC, Wang JX, Zhao XF (2014) Phospholipase C gamma 1 connects the cell membrane pathway to the nuclear receptor pathway in insect juvenile hormone signaling, J Biol Chem 289: 13026–13041.

Wang S, Wang J, Sun Y, Song Q, Li S (2012) PKC-mediated USP acetate-activated protein kinase C. J Biol Chem 279: 11444–11453.

Li Z, Zheng D, Mon H, Tatsuke T, Zhu L, et al. (2012) Genome-wide identification of polycomb target genes reveals a functional association of Pho with Scm in Bombyx mori. PLoS One 7: e43350.

Wu Y, Li Q, Chen XZ (2007) Detecting protein–protein interactions by Far western blotting. Nat Protoc 2: 3278–3280.