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Receptor of activated C kinase 1 (RACK1) is necessary for the 20-hydroxyecdysone-induced expression of the transcription factor CHR3 in the spruce budworm *Choristoneura fumiferana*

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Abstract

To initiate moulting and metamorphosis, 20-hydroxyecdysone (20E) binds to its nuclear receptors and the ligand–receptor complex then mediates changes in gene expression. Phosphorylation of the receptors is required for their function. The intracellular signal transduction pathway that is involved in receptor phosphorylation remains elusive. This study provides evidence that the receptor of activated C kinase 1 (RACK1) and protein kinase C (PKC) signal transduction cascade is involved in the 20E-induced expression of the moult-associated transcription factor CHR3. A cDNA clone encoding a receptor of activated C kinase 1 was isolated from *Choristoneura fumiferana* (*Cf* RACK1). This single copy gene coded a 36 kDa protein and was expressed ubiquitously in all of the developmental stages and the tissues tested, including the midgut, epidermis, fat body, head, Malpighian tubules, ovary and testis of larvae. High levels of the transcripts were also detected in a midgut-derived CF-203 cell line. We noticed that the green fluorescence protein-fused *Cf* RACK1 protein was distributed in the cytosol surrounding the nuclei in stably transformed cells. Interference of *Cf* RACK1 mRNA suppressed the 20E-induced expression of the transcription factor CHR3. Dequalinium-14; 1,1′-decamethylenebis-4-aminoquinaldinium diiodide (DECA), an inhibitor of RACK1 binding to protein kinase C, blocked the 20E-induced expression of CHR3 and accumulation of the ecdysone receptor (EcR) in the nuclei. All of these data together suggest that 20E-induced expression of CHR3 may involve phosphorylation of the ecdysone receptor component through the PKC/RACK1 signal transduction cascade, which facilitates the import of the receptor into the nuclei of cells.

Keywords: protein kinase C, ecdysone receptor, transcription factor, signal transduction, moult, metamorphosis.

Introduction

Insect moulting and metamorphosis are triggered by 20-hydroxyecdysone (20E) through the heterodimer complex of the ecdysone receptor (EcR) and ultraspiracle protein (USP) (Riddiford *et al*., 2000). Before moulting and metamorphosis, insects secrete 20E into the haemolymph and transport it to the target tissues where 20E binds to the nuclear EcR/USP receptors, forming a ligand–EcR/USP complex that interacts with the ecdysone response elements of some moulting-triggering genes, such as transcription factors, consequently switching on expression of these genes and initiating the moulting process.

As is the case for most, if not all, vertebrate steroid hormone receptors, insect EcR and USP are post-translationally modified by phosphorylation (Rauch *et al*., 1998; Song & Gilbert, 1998; Nicolaï *et al*., 2000; Song *et al*., 2003). Phosphorylation/dephosphorylation is a mechanism to fine regulate hormone receptors for their functions in processes such as hormone binding, DNA binding and recognition of hormone response elements (Kuiper & Brinkman, 1994). Phosphorylation of the ecdysone receptor components is regulated by the ligand 20E. In *Chironomus tentans*, the phosphorylation of USP, but not EcR, was enhanced by 20E (Rauch *et al*., 1998). In *Tenebrio molitor*, 20E treatment considerably and rapidly increased the levels of the phosphorylated USP (Nicolaï *et al*., 2000). Similar results were found for the phosphorylation of *Drosophila* USP (Song *et al*., 2003). There are five to ten putative protein kinase C (PKC) binding sites in USP or EcR (Rauch *et al*.,

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1998; Song *et al*., 2003). However, it remains unknown what kinase signal transduction cascades are involved in the 20E-regulated phosphorylation of the ecdysone receptors.

The receptor of activated C kinase 1 (RACK1) (originally called receptor for protein kinase C) is a homologue of the G protein β subunit and typically contains seven internal Trp-Asp 40 (WD40) repeats (Ron *et al*., 1994). This protein appears to be involved in at least three signal transduction pathways: the PKC pathway (Ron *et al*., 1995; Ron *et al*., 1999), the cAMP-specific phosphodiesterase PDE4D5 pathway (Yarwood *et al*., 1999) and the tyrosine kinase–phosphatase pathway (Chang *et al*., 1998) (for review, see McCahill *et al*., 2002). In the PKC/RACK1 signal transduction cascade, RACK1 binds to the activated PKC and functions as a shuttling protein to translocate the activated PKC to appropriate subcellular sites for action, for example, phosphorylation of the target proteins (Ron *et al*., 1999; Rigas *et al*., 2003). Because RACK1 interacts with several different cellular proteins and may associate with several signal transduction pathways, it may play a critical role in cross-talk among different signal transduction cascades (McCahill *et al*., 2002; Rigas *et al*., 2003).

In this study, we cloned a RACK1 cDNA from the spruce budworm *Choristoneura fumiferana* (*Cf*RACK1) and demonstrated that the PKC/RACK1 signal transduction cascade is involved in the 20E-induced expression of the *Choristoneura* hormone receptor 3 (CHR3), which is a gene involved in the initiation of moulting (Palli *et al*., 1996, 1997). We found that when this pathway was blocked, either by *Cf*RACK1 RNA interference or by inhibition of PKC binding to RACK1, 20E-induced CHR3 expression was repressed.

Results

Cloning and sequence characterization of CfRACK1 cDNA

To study signal transduction of ecdysone, we identified four (out of 1500) expressed sequence tags (ESTs) in the spruce budworm cell line CF-203 that were identical and homologous to the receptor of activated C kinase 1 (RACK1) (Ron *et al*., 1994). The longest clone was completely sequenced. This complete cDNA clone was 1427 nucleotides in length and the longest open reading frame (ORF) encoded a 319-amino acid protein with a predicted molecular mass of 36 kDa and pI of 8.53 (GenBank accession number: DQ073455). The deduced amino acid sequence of this cDNA was highly similar to RACK1 from many organisms including yeast, nematodes, insects and mammals (from 62 to 86% identity at the amino acid level). Therefore, we refer to this cDNA clone as the *Choristoneura fumiferana* receptor of activated C kinase 1 (*Cf*RACK1).

Sequence analysis indicated that the amino acid sequence of *Cf*RACK1 contained seven repeating elements (Fig. 1A). Repeats II, IV, V and VI had Try-Asp (WD) dipeptides at the ends of elements, while repeats I, III and VII had Try-Lys (WK), Try-Asn (WN) and Try-Gln (WQ), respectively. There were Gly-His (GH) dipeptides at the 5′ ends of repeats I, II, IV and V, while repeats III and VI had Asp-His (DH) dipeptides. Thus, *Cf*RACK1 belongs to the super-family of proteins that includes RACK1, guanine nucleotide-binding protein beta subunit (GP-β), and others that have five to eight internally repeating elements termed WD40 motif (Fong *et al*., 1986; Simon *et al*., 1991; Ron *et al*., 1994; McCahill *et al*., 2002). Bacterial expression of the cloned cDNA resulted in a protein with an apparent molecular mass of 36 kDa (Fig. 1B), the same as the predicted size from the deduced amino acid sequence, demonstrating that the longest ORF of the cDNA does encode a 36 kDa RACK1 protein.

Cloning and sequence characterization of the Cf*RACK1 gene*

Southern blotting analysis on genomic DNA that was digested with *Bam*H I, *Xba* I and *Xho* I each gave a single hybridization band, indicating that the *Cf*RACK1 gene is present in a single copy in the spruce budworm genome (Fig. 2A).

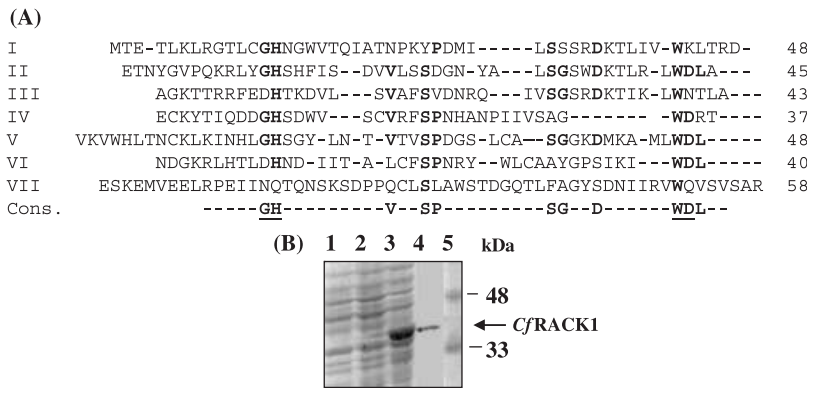

Figure 1. (A) Alignment of the seven Trp-Asp (WD) repeating sequences of the *Choristoneura fumiferana* receptor of activated C kinase 1 (*Cf*RACK1). The sequence alignment was performed using the Clustal alignment program (Higgins & Sharp, 1988). The residues that are conserved are in bold and the consensus residues are given in the cons. line of the alignment panel when at least four of seven residues match, otherwise '-' is shown. The seven repeat regions are marked with Roman numerals. The GenBank accession number for this nucleotide sequence is DQ073455. (B) Protein expression of *Cf*RACK1 in *Escherichia coli*. The open reading frame of *Cf*RACK1 was cloned into the pProEX HT vector and expressed in DH5α cells. Lane 1: control without the recombinant vector; lane 2: proteins from the *Cf*RACK1-transformed cells without isopropyl-β-D-thiogalactopyranoside (IPTG) induction; lane 3: proteins from the *Cf*RACK1-transformed cells with IPTG treatment; lane 4: His-tag purified *Cf*RACK1 protein; lane 5: protein molecular markers. The apparent molecular mass of the recombinant protein was 36 kDa.

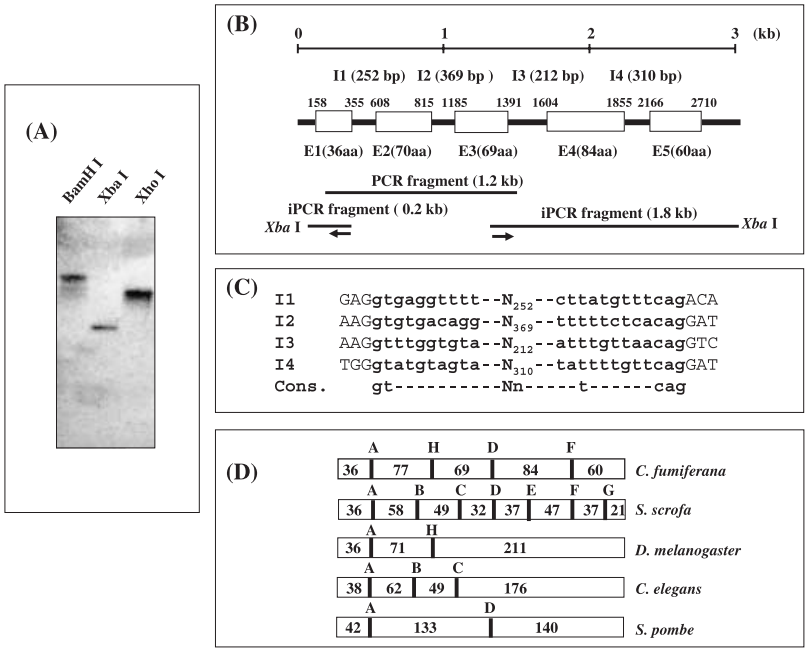

Figure 2. (A) Southern blotting analysis showing a single copy of the *Choristoneura fumiferana* receptor of activated C kinase 1 (*Cf*RACK1) gene in the genome of *C. fumiferana*. Five μg genomic DNA was digested with *Bam*H I, *Xba* I and *Xho* I, respectively. The membrane was probed with the ^{32}P-labelled full-length *Cf*RACK1 cDNA. (B) Structure of the *Cf*RACK1 gene (GENBANK accession number for this genomic DNA is DQ073456). Exons 1–3 were cloned using PCR and exons 4–5 and the core promoter region were cloned using inverse-PCR. Length of exons (E1–E5) and introns (I1–I4) are indicated by amino acid residues and nucleotides, respectively. The arrows indicate the positions and directions of the primers used for inverse-PCR. (C) Sequence alignment of the four introns showing the exon/intron boundaries. The exon sequences are indicated using capital letters, while the intron sequences are indicated using lower case letters. Consensus nucleotides are indicated on the 'cons.' line. (D) Comparison of gene structure of *Cf*RACK1 and RACK1 of *Drosophila melanogaster* (GENBANK accession number: AE003691), *Sus scrofa* (AF146043), *Caenorhabditis elegans* (Z69664), and *Schizosaccharomyces pombe* (Z98531).

To study the structure of the *Cf*RACK1 gene, the whole gene was cloned using PCR and inverse-PCR (Fig. 2B). We first amplified a 1.2 kb genomic fragment that covered exons 1–3 using the primers designed according to the cDNA sequence. We then cloned the 5′ (0.2 kb) and 3′ ends (1.8 kb) of the PCR fragments by using inverse-PCR. Comparison of the sequences of the genomic DNA (GENBANK accession number: DQ073456) and cDNA clones revealed that the *Cf*RACK1 gene had five exons (E1–E5) and four introns (I1–I4), extending approximately 3 kb nucleotides in length. The exon/intron boundaries were consistent with the typical GT/AG rule (Breathnach & Chambon, 1981) (Fig. 2C). Structural comparison of *Cf*RACK1 gene with RACK1 homologues from other species revealed that they differ mainly in the numbers of exons (Fig. 2D). For example, the *Cf*RACK1 gene has five exons whereas the *Drosophila melanogaster* RACK1 gene has three exons. The intron sites are well conserved among various RACK1 genes (Fig. 2D). Intron A in particular is preserved from yeast to mammals. Intron H may be insect-specific because it is thus far found only in genes cloned from insects.

Cellular localization of CfRACK1

Prediction analysis using the *k*-nearest neighbour (*k*-NN) algorithm method (Horton & Nakai, 1997) and Reinhardt's method (Reinhardt & Hubbard, 1998) for assessing the probability of cellular localization of a protein indicated that *Cf*RACK1 was most likely cytoplasmic. To determine the localization of *Cf*RACK1 in CF-203 cells, an expression vector containing the *Cf*RACK ORF fused with green fluorescence protein (GFP) at the C-terminus was constructed and used to stably transform CF-203 cells (Fig. 3A). The transformed cells revealed that the GFP signal was present dominantly in the cytoplasm surrounding the nuclei of the cells (Fig. 3B-a), while the GFP signal was present in both of the nuclei and cytoplasm if it was not fused with *Cf*RACK1 (Fig. 3B-c), indirectly implying the cytoplasmic localization of *Cf*RACK1. This result was consistent with the observation of mammalian RACK1 in Chinese hamster ovary (CHO) cells (Cox *et al*., 2003).

Temporal and spatial expression of Cf*RACK1*

The developmental expression profile of *Cf*RACK1 was analysed using Northern blotting. A 1.4 kb mRNA transcript was detected using Northern blotting and the size was the same as the cloned cDNA, indicating that the cDNA cloned was a full-length sequence. The *Cf*RACK1 gene was constitutively expressed in the fifth and sixth instar larvae and pupae, as well as in adults (Fig. 4A). Similar expression patterns were also observed for other larval stages (data not shown). There was no significant difference in the levels of *Cf*RACK1 expression between moulting and intermoult animals, whereas the transcription factor CHR3 was expressed periodically with moulting (Fig. 4A). CHR3 was expressed prior to headcap slippage and during the early phase of the moults as demonstrated by Palli *et al*. (1996, 1997). This result indicates that the *Cf*RACK1 mRNA does not fluctuate with moulting cycles.

*Cf*RACK1 transcripts were detected in all of the tissues tested from 5-day-old sixth instar larvae, including the midgut, epidermis, fat body, head, Malpighian tubules, ovary and testis (Fig. 4B). Higher levels were detected in the reproductive organs such as the ovaries and testes. This

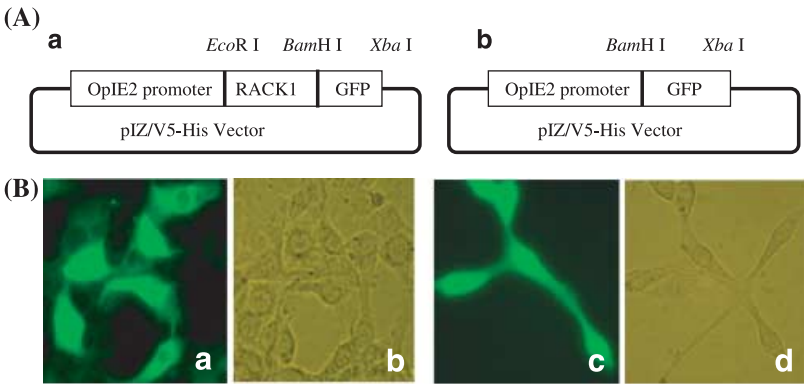

Figure 3. (A) Constructs of pIZ/*Cf*RACK1-GFP vector (A-a), in which GFP was fused with the *Choristoneura fumiferana* receptor of activated C kinase 1 (*Cf*RACK1) at its 3′ end, and pIZ/V5-His-GFP vector (A-b), in which only GFP was contained. The expression of the fused protein or GFP was driven by OpIE2 promoter. (B) Localization of *Cf*RACK1 fused with GFP in CF-203 cells. The cells were transformed with the plasmid vector pIZ/V5-His-*Cf*RACK1-GFP with GFP-fused *Cf*RACK1 under the control of the IE1 promoter (A-a, B-a, B-b) or with the plasmid vector pIZ/V5-His-GFP (A-b, B-c, B-d). The stably transformed cells were obtained after eight passages under selective medium. The green fluorescence indicates the presence of *Cf*RACK-GFP or GFP. B-a shows dominant presence of GFP in the cytosol surrounding the nuclei of the transformed cells; B-c shows presence of GFP in both of the cytoplasm and nuclei. B-a and B-c: cells under UV light by fluorescence microscopy; B-b and B-d: cells under visible light.

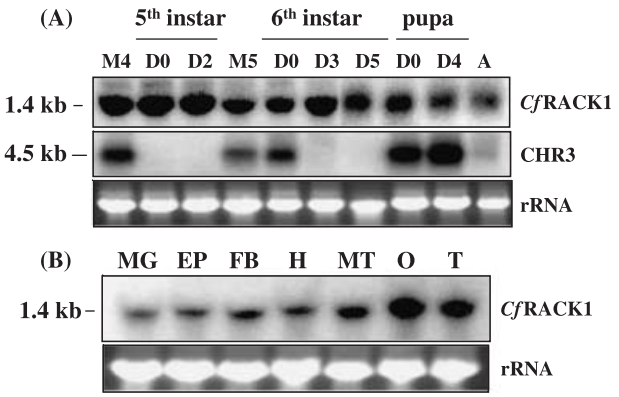

Figure 4. (A) Developmental expression of the *Choristoneura fumiferana* receptor of activated C kinase 1 (*Cf*RACK1) in fifth and sixth instar larvae, pupae and adults. M4: moulting from fourth to fifth instar; M5: moulting from the fifth to sixth instar; Dn, *n* days in the relevant larval or pupal stages; A, adults. Ten μg total RNA from whole animals was blotted and hybridized with a *Cf*RACK1 cDNA probe. The same membrane was also probed with a CHR3 cDNA probe after stripping off the *Cf*RACK1 cDNA probe. The size of the *Cf*RACK1 transcript was approximately 1.4 kb. Ribosomal RNA stained with ethidium bromide indicates equal loading of RNA. (B) Northern blot showing spatial expression of *Cf*RACK1 in different tissues of the sixth instar larvae. MG, midgut; EP, epidermis; FB, fat body; H, head; MT, Malpighian tubules; O, ovary; T, testis.

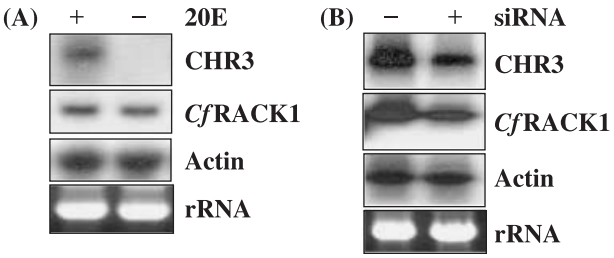

Figure 5. (A) Northern blots showing the effects of 20-hydroxyecdysone (20E) on expression of the *Choristoneura fumiferana* receptor of activated C kinase 1 (*Cf*RACK1), CHR3 and actin in CF-203 cells. The cells were treated with 2×10^{-6} M 20E for 3 h and the total RNA was extracted for analysis. 20E had no effect on the expression of *Cf*RACK1 and actin (as a control), but induced CHR3 expression. (B) Effect of *Cf*RACK1 RNA interference on expression of CHR3 in CF-203 cells. CF-203 cells were soaked with small interfering RNA (siRNA) for 40 h and 20E at 2×10^{-6} M for 3 h and the total RNA was isolated for Northern blot analysis. Green fluorescence protein (GFP) siRNA was used as a negative control. Ribosomal RNA stained with ethidium bromide indicates equal loading of RNA. Two independent replicates of the RNAi assay were conducted.

result is consistent with the observation made for human ovarian follicular cells, in which the highest levels of RACK1 transcripts were also found when these cells proliferate (Berns *et al.*, 2000). From the data on the spatial and temporal expression, it is clear that *Cf*RACK1 is a constitutively and ubiquitously expressing gene.

As mentioned above, the *Cf*RACK1 cDNA was first cloned as an EST from a CF-203 cell line, which was developed from the midgut tissues (Sohi *et al.*, 1993), therefore the transcript must be also present in these *in vitro* cultured cells. This was confirmed by Northern blot analysis (see Figs. 5 & 6). Consequently, most of the experiments in this study were carried out with CF-203 cells.

Effect of 20E on CfRACK1 and CHR3 expression

RACK1 is a scaffold protein that is involved in signal transduction by forming a complex with PKC, which then phosphorylates many proteins, initiating signal transduction cascades (McCahill *et al.*, 2002). Like most vertebrate steroid nuclear receptors, EcR and its partner USP appear to have to be phosphorylated to function during moulting and metamorphosis (Rauch *et al.*, 1998; Song & Gilbert, 1998; Nicolaï *et al.*, 2000; Song *et al.*, 2003). Therefore, we examined if *Cf*RACK1 is involved in the 20E-initiated moulting process. We used CHR3 as an indicator to study the role of *Cf*RACK1 in the 20E signal transduction process because CHR3 is a transcription factor and its expression is induced by 20E through the ecdysone receptor EcR/USP complex during the early stage of moulting (Palli *et al.*, 1996). The experimental results with CF-203 cells treated with 20E revealed that it had no effect on the expression of *Cf*RACK1, but did induce CHR3 expression (Fig. 5A).

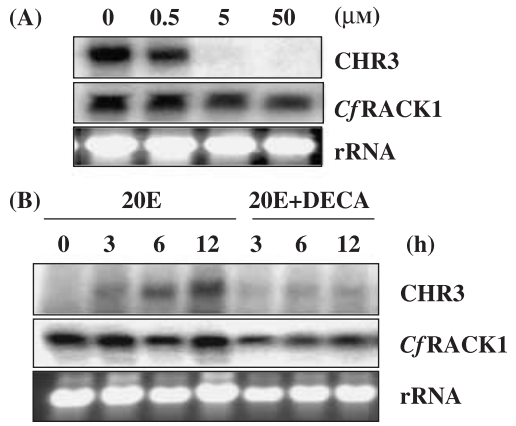

Figure 6. (A) Northern blots showing the effect of dequalinium-14; 1,1′-decamethylenebis-4-aminoquinaldinium diiodide (DECA) at different concentrations on expression of CHR3 and the *Choristoneura fumiferana* receptor of activated C kinase 1 (*Cf*RACK1) in CF-203 cells treated with 2×10^{-6} M 20-hydroxyecdysone (20E) for 3 h. Expression of CHR3, but not *Cf*RACK1, was suppressed by DECA at 5 and 50 μM. (B) Northern blots showing the time course of inducing effects of 20E (2×10^{-6} M) and the suppressive effect of DECA (2.5 μM) on the expression of *Cf*RACK1 and CHR3 in CF-203 cells. The cells were treated with the compounds for 12 h and the total RNA was extracted at 3, 6 and 12 h post treatment for analysis. The same membrane was probed, with *Cf*RACK1 and CHR3, respectively, after stripping. Ribosomal RNA stained with ethidium bromide indicates equal loading of RNA. Two independent replicates were conducted for each Northern blot.

Expression levels of *Cf*RACK1 were constant during moulting and intermoults, whereas CHR3 was expressed only before and during the early phase of the moults (Fig. 4A). These results together suggest that expression of *Cf*RACK1 might not be affected by 20E.

Effect of CfRACK1 RNA interference on CHR3 expression

We hypothesize although 20E has no direct effect on the expression of the *Cf*RACK1 gene, there is a possibility that induction by 20E of CHR3 expression may be through the action of existing *Cf*RACK1. We hypothesize that the effect of 20E on CHR3 expression may require phosphorylation of its receptors and that the phosphorylation process is the result of RACK1/PKC activity. To investigate the role of *Cf*RACK1 in 20E signal transduction and induced expression of CHR3, we knocked down *Cf*RACK1 transcripts using small interfering RNA (siRNA) in CF-203 cells treated with 20E (Fig. 5B). When the level of *Cf*RACK1 transcripts was specifically knocked down with *Cf*RACK1 siRNA, the expression of CHR3 transcripts was correspondingly decreased. Actin expression was used as a negative control to show that interference of *Cf*RACK1 had no universal effects on the expression of other genes. This result suggested that 20E-induced expression of CHR3 could be suppressed by removal of *Cf*RACK1, implying the involvement of the RACK1/PKC signal transduction cascade in the action of 20E on the expression of the transcription factor CHR3.

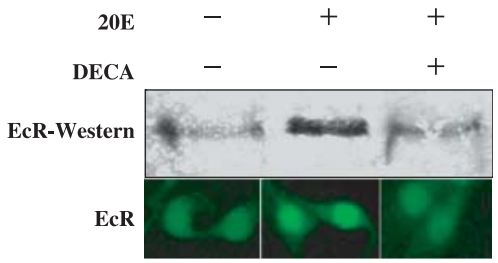

Figure 7. Western blotting and immunocytochemical staining show the accumulation of ecdysone receptor (EcR) protein in the nuclei of CF-203 cells. The cells were treated with 20-hydroxyecdysone (20E) at 2×10^{-6} M and 25 μM dequalinium-14; 1,1′-decamethylenebis-4-aminoquinaldinium diiodide (DECA) for 3 h. The nuclear proteins were extracted for EcR monoclonal antibody staining (1 : 500). The secondary antibody was goat-rabbit IgG conjugated with alkaline phosphatase (1 : 1000). Accumulation of EcR protein in the nuclei was increased by 20E treatment and suppressed by the receptor of activated C kinase 1/protein kinase C (RACK1/PKC) binding inhibitor DECA. The green fluorescence in the immunohistochemistry staining indicates the location of the EcR protein. The secondary antibody was goat anti-IgG Alexa Fluor 488 F(ab′)2 (1 : 200). Two independent replicates were conducted for each Western blot and immunocytochemical staining.

Effect of inhibition of RACK1-PKC binding on CHR3 expression

If the RACK1/PKC signal transduction cascade is involved in the 20E-induced expression of the transcription factor CHR3, specific inhibition of binding and activity of RACK1 and PKC should result in the suppression of 20E-induced expression of CHR3. We examined the effects of dequalinium-14; 1,1′-decamethylenebis-4-aminoquinaldinium diiodide (DECA), a PKC inhibitor that interacts with the RACK1-binding site of PKC preventing the formation and movement of the PKC/RACK1 complex (Rotenberg & Sun, 1998; Ron *et al*., 1999), on CHR3 expression (Fig. 6). When different concentrations of DECA were used to treat CF-203 cells, expression of CHR3 was suppressed with increases in the concentrations from 0.5 to 50 μM (Fig. 6A). 20E induced CHR3 expression in CF-203 cells 3–12 h post treatment, but DECA can suppress the effect of 20E during this time (Fig. 6B), which implies that binding of PKC to RACK1 is a requisite for 20E-induced expression of CHR3.

CfRACK1-mediated phosphorylation and transport of EcR from cytosol into nuclei

As seen in the above experiments with RNA interference and the RACK1/PKC binding inhibitor, it appears that 20E-induced CHR3 expression requires *Cf*RACK1/PKC activity. Much of the evidence has suggested that phosphorylation plays a vital role in nuclear import of steroid receptors (Rigas *et al*., 2003; Lange, 2004). We investigated the effects of 20E on EcR accumulation in the nuclei of CF-203 cells using Western blotting (Fig. 7). More EcR was detected in the nuclei of the 20E-treated cells than the

untreated cells. If the cells were simultaneously treated with both 20E and DECA, the accumulation of EcR in the nuclei was reduced. Immunocytochemical staining showed that the EcR signals in the nuclei of the 20E-treated cells were stronger than in the untreated cells or DECA-treated cells. This result indicated that the accumulation of EcR in the nuclei was suppressed by the inhibitor (DECA) of RACK1/PCK binding.

Discussion

This study suggests that the RACK1/PKC signal transduction system is involved in the 20E-induced expression of the transcription factor CHR3, which is a gene involved in the initiation of the moulting process in the spruce budworm.

Insect moulting and metamorphosis are triggered by 20E through the EcR/USP complex, which binds to the ecdysone response element of some early genes such as the ligand-inducible transcription factors (Riddiford *et al*., 2000), hormone receptor 3 in this case. While EcR and USP are post-translationally modified by phosphorylation (Rauch *et al*., 1998; Song & Gilbert, 1998; Nicolaï *et al*., 2000; Song *et al*., 2003), it is not known what signal transduction cascade(s) are involved in the phosphorylation of these ecdysone receptor components. The relationship between steroid hormone receptors and intracellular signalling pathways has been explored in vertebrate nuclear receptors such as human progesterone receptor (Lange, 2004). PKC is an intracellular signalling protein in the signal transduction cascades for several cellular responses. Inactive and activated PKC isozymes are localized in different sites in cells. Once the cells are stimulated by developmental, environmental or stress signals, the activated PKC isozymes are translocated to the target sites by the shuttling protein RACK1 (Ron *et al*., 1999). RACK1 binds to the PKC that is activated by activators, such as phorbol ester, dopamine D2 receptor agonist, and functions as a scaffold to shuttle PKC from one intracellular site to another (Ron *et al*., 1999). In other words, compartmentalization of the activated PKC to the cellular sites, where it catalyses phosphorylation of specific proteins, is a colocalization process with RACK1 movement.

In this study, we found that when siRNA knocked down transcripts of *Cf*RACK1, 20E-induced expression of CHR3 was suppressed. When RACK1 binding to PKC was inhibited by the PKC binding inhibitor DECA, which competes with RACK1 for the RACK1-binding site on PKC (Rotenberg & Sun, 1998), the inducing effect of 20E on CHR3 expression was also suppressed. Together these results are consistent with the idea that 20E-induced expression of CHR3 needs the involvement of the PKC/RACK1 signal transduction cascade. Because 20E-dependent expression of CHR3 requires the participation of the EcR/USP receptor complex (Lezzi *et al*., 2002; Kozlova & Thummel, 2002), which is phosphorylated to function in initiating gene expression (Rauch *et al*., 1998; Song & Gilbert, 1998; Nicolaï *et al*., 2000; Song *et al*., 2003), the 20E signal for triggering CHR3 expression may need phosphorylation of EcR/USP through the PKC/RACK1 signal transduction cascade.

One possible role of the *Cf*RACK1/PKC-mediated phosphorylation of ecdysone receptors is to increase binding of the receptor complex to the response elements of the transcription factor gene. Another possibility is that this facilitates transport of the phosphorylated receptors from the cytosol, where they are synthesized, to the nuclei, where they act on CHR3 gene expression. While some studies reveal phosphorylation of USP or EcR taking place in the nuclei (Song & Gilbert, 1998), Rauch *et al*. (1998) found that phosphorylated and dephosphorylated components of the ecdysone receptor are present in both the nuclei and the cytosol. More experimental evidence is needed to demonstrate where phosphorylation of EcR/USP occurs. The sequence analysis and experimental data from this study show that RACK1 is present in the cytosol. If the 20E-induced phosphorylation of EcR or USP is through the PKC/RACK1 signal transduction cascade, it probably occurs outside the nuclei. After phosphorylation, the EcR/USP components could be translocated into the nuclei. It has been suggested that phosphorylation plays an important role in nucleocytoplasmic shuttling of many proteins (Jans & Hubner, 1996; Lange, 2004). For example, it has been found that RACK1 functions as a scaffold to allow PKC to modify the androgen receptor, resulting in the translocation of the androgen receptor into the nucleus from the cytosol (Rigas *et al*., 2003). We found in this study that the inhibitor DECA of RACK1/PCK binding suppressed the accumulation of EcR in the nuclei, indicating that the import of EcR from the cytosol to the nuclei may need RACK1/PCK-mediated phosphorylation of the receptor.

On other hand, we have not demonstrated that the PKC/RACK1 system is directly involved in the phosphorylation of EcR and/or USP. The effect of 20E on induction of CHR3 expression might not involve phosphorylation of EcR and/or USP through the PKC/RACK1 system, or the PKC/RACK1 system may phosphorylate other proteins that are involved in 20E-induced CHR3 expression, but do not phosphorylate EcR/USP. It did not escape our attention that 20E did not induce expression of the *Cf*RACK1 gene. This gene is ubiquitously expressed in many tissues at all developmental stages. McCahill *et al*. (2002) have suggested that the PKC/RACK1 signal pathway is involved in many signal transduction cascades and may play a role in cross-talk of different signal transduction systems. We cannot rule out the possibility that PKC/RACK1 may regulate phosphorylation of proteins, other than EcR/USP, and indirectly affect 20E-induced CHR3 expression by signal transduction cross-talks. These possibilities are under investigation.

Based on this study, our hypothesis is that 20E stimulation activates the RACK1/PKC signal transduction cascade, resulting in phosphorylation of ecdysone receptor components. This phosphorylation of the receptor facilitates the import of the receptor proteins into the nuclei, where the phosphorylated ligand-EcR/USP receptor complex then turns on the expression of CHR3.

Experimental procedures

Experimental insect and cell line

Spruce budworm (*Choristoneura fumiferana* Clem., Lepidoptera: Tortricidae) larvae were reared on an artificial diet (McMorran, 1965) at 22 °C, 70% relative humidity and a photoperiod of 12 h light and 12 h darkness until they reached the adult stage. The spruce budworm midgut cell line FPMI-CF-203 (CF-203 Sohi *et al*., 1993) was grown at 28 °C in 25 ml flasks with SF900 medium supplemented with 10% fetal borine serum (FBS) (Life Technologies, Inc., Rockville, MD, USA).

Chemicals

20E, purchased from Sigma (St Louis, MO, USA), was dissolved in ethanol and stored at –20 °C until use. The protein kinase C inhibitor DECA (Rotenberg & Sun, 1998), also purchased from Sigma, was dissolved first in dimethyl sulfoxide (DMSO) and then diluted to different concentrations with SF900 medium (Invitrogen, Carlsbad, CA, USA).

Polymerase chain reaction (PCR) and inverse-PCR (iPCR)

Fragments of CHR3-A and the cytoplasmic actin gene for Northern blotting were synthesized using PCR with primers 5′-TGCACAGTCAGATGGGAATG-3′ and 5′-CGAAATGCTGAGAAAACCTG-3′ for CHR3-A and primers 5′-CACAATGTGCGACGAGGAAG-3′ and 5′-GAGTCCAGCACGATACCGG-3′ for actin.

PCR and iPCR were performed to amplify the *Cf*RACK1 gene. PCR was conducted with the primers 5′-CTTCCGTGGCGTTGCTGAGTG-3′ and 5′-CCAGCCAGCAGACACAATGA-3′ corresponding to the first and third exon of the gene. For iPCR, *C. fumiferana* genomic DNA was digested with *Xba*I and ligated with T4 DNA ligase. IPCR was conducted with the ligated DNA and primers 5′-CGACACTCAGCAACGCCACGGAAA-3′ and 5′-GATGTCCTATCCGTGGCCTTCTC-3′ corresponding to the first and third exon of the RACK1 gene. In all PCR and iPCR reactions, LA-Taq DNA polymerase (Takara Mirus Bio, Madison, WI, USA) was used. The PCR and iPCR products were subcloned into pGEMT for sequencing.

Fusion of the target and GFPs

The coding regions of RACK1 and GFP were amplified from RACK1 cDNA or pigA3GFPlacZ (Tamura *et al*., 2000) using PCR with primers 5′-GAATTCATGACTGAAACATTGAAGCTAAGA-3′ (*Eco*R I underlined) and 5′-GGATCCTCGCGCTGAGACTGACA-3′ (*Bam*H I underlined) for RACK1 and primers 5′-GGATCCATGGTGAGCAAGGGCGAGGA-3′ (*Bam*H I underlined) and 5′-TCTAGATCATAATCAGCCATAC-3′ (*Xba*I underlined) for GFP. The amplified products were subcloned into a pGEMT vector for sequence confirmation. The GFP ORF fragment was inserted at the 3′ end of the RACK1 ORF at the *Bam*H I site. The *Eco*R I/*Xba*I fragment containing RACK1 and GFP was cloned into a pIZ/V5-His vector (Invitrogen) (Fig. 3A-a). The same vector (pIZ/V5-His) but containing only the GFP ORF was used as a control (Fig. 3A-b).

Cell culture and transfection

CF-203 cells were seeded at 1.5×10^5 cells/ml medium in 25 ml flasks and cultured at 28 °C. For DNA transfection, the procedure was the same as for small interfering RNA transfection (see below), except that 2 μg pIZ/V5-His-RACK1/GFP or pIZ/V5-His-GFP plasmid DNA and Lipofectin Reagent (Invitrogen) were used.

Protein expression and SDS-PAGE

The ORF of the target gene was cloned into the bacterial pProEX HT expression vector (Invitrogen). The recombinant plasmid was used to transform DH5α cells. Protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM.

Proteins were extracted from the cells using homogenization buffer (20 mM Tris, 50 mM KCl, 300 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, pH 7.5). The recombinant protein was purified using the MagneHis protein purification system (Promega, Madison, WI, USA) according to the manufacturer's instruction. The protein samples were denatured at 100 °C for 5 min in 0.1 M Tris buffer, pH 6.8, containing 2% SDS, 0.5% β-mercaptoethanol, 12% glycerol, and 0.002% bromphenol blue. SDS-PAGE was performed on 12% acrylamide gels in Tris-glycine-SDS buffer (10 mM Tris, 50 mM glycine, 0.1% SDS, pH 8.0). Gels were stained with Coomassie Blue R-250.

Northern blotting and Southern blotting analysis

The guanidine isothiocyanate-phenol-chloroform extraction method described by Chomczynski & Sacchi (1987) was used for extracting total RNA from staged insects, dissected tissues or cell cultures. The NorthernMAx kit (Ambion Inc., Austin, TX, USA) was used for Northern blotting. For Northern blot hybridization, 10 μg total RNA from tissues and cells was separated on an agarose gel (1%), The RNA was transferred to Hybond N nylon membrane (Amersham Biosciences, Piscataway, NJ, USA) and fixed by UV cross-linking. The membranes were hybridized with ^{32}P-dCTP random–primer labelled DNA probes at 45 °C overnight. Prehybridization, hybridization and washes were performed according to the manufacturer's instruction for the NorthernMAx kit. Two independent replicates of the experiments were conducted for each Northern blotting.

For Southern blots, 5 μg genomic DNA from larvae was digested with different restriction enzymes followed by separation on a formaldehyde-agarose (1%) gel and transferred to Hybond N nylon membranes. Hybridization and washes were the same as for Northern blots.

Western blots and immunocytochemistry

Nuclear proteins were extracted from the nuclei of CF-203 cells according to Kethidi *et al.* (2004). Protein concentrations were determined using Bio-Rad's protein assay reagent (Bio-Rad, Hercules, CA, USA). Bovine serum albumin was used as a protein standard. The proteins were separated as above and transferred to nitrocellulose membranes, which were then probed with 1 : 500 EcR

monoclonal antibody. The monoclonal antibody was generated from mice immunized with the DNA vaccine vector pVAC1-mcs (Invitrogen) containing the EcR ORF. Goat anti rabbit IgG conjugated with alkaline phosphatase was used as the secondary antibody (1 : 1000). The substrates Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used for colour development.

For cellular immune staining, CF-203 cells were plated on glass slides. The cells were washed with 1× phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min. After being washed with PBS, the cells were treated with proteinase K (5 μg/ml) for 5 min, then washed with PBS. The cells were incubated for 2 h with 5% skimmed milk in PBS containing 0.1% tween-20 (PBST), then washed with PBST three times. The cells were incubated with monoclonal anti-ECR antibody (1 : 500) at 4 °C overnight. The cells were washed with PBST and incubated with goat anti-IgG Alexa Fluor 488 F(ab′)2 antibody (1 : 200) (Molecular Probes, Eugene, OR, USA). The cells were washed an additional three times with PBST. The signals were viewed under UV light with a Nikon fluorescence microscope (Nikon, Mississauga, ON, Canada). The experiments for Western blotting of nuclear proteins and immunocytochemical staining analysis were independently repeated two times.

siRNA

Double strand RNA (dsRNA) was prepared by *in vitro* transcription reaction with T3 and SP6 RNA polymerase using the full-length cDNAs of *Cf*RACK1 and GFP and MEGAScript SP6/T3 kits (Ambion Inc.) according to the manufacturer's instructions (Quan *et al.*, 2002). siRNA was prepared using the dsRNAs by digestion into 18–25 nucleotide RNA fragments with the Short Cut RNaseIII kit (Bio-Laboratory, New England, USA).

For siRNA transfection, CF-203 cells were set up at 2.0×10^5 cells/25 ml flask. Two μg siRNA was mixed with 100 μl neat SF900 medium. In a separate tube 15 μl CodeBreakersiRNA Transfection Reagent (Promega) was mixed with 85 μl neat SF900 medium and left for 30 min at room temperature. The two solutions (100 μl siRNA mix and 100 μl CodeBreakersiRNA Transfection Reagent mix) were combined and incubated for 30 min at room temperature. The cells were treated with trypsin for 10 s and washed three times with neat SF900 medium. The 200 μl transfection mix was mixed with 800 μl neat SF900 and then added to the cells in each flask. After rocking the cells for 40 h, the transfection mixture was removed. Five ml fresh growth medium (SF900 medium with 10% FBS) with or without 20E was added and incubated for another 3 h. The siRNA assay was independently repeated twice.

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