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Research Article

Identification of steroid hormone signaling pathway in insect cell differentiation

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Abstract. To dissect the steroid hormone signaling pathway involved in insect cell morphological differentiation, we extended the application of the double-stranded RNA-mediated interference (dsRNAi) method to the epidermal IAL-PID2 cell line from *Plodia interpunctella* Lepidoptera. We first demonstrated that dsRNA was capable of efficiently blocking the steroid hormone 20-hydroxyecdysone (20E) inducibility of proteins that belong to the nuclear receptor superfamily, including the ecdysone receptor (EcR), its partner Ultraspiracle (USP), the insect homolog of the vertebrate retinoid X receptor and the HR3 transcription factor. We then showed that inhibiting the 20E induction of EcR, USP or HR3 proteins prevented the increased synthesis of β tubulin and consequently the morphological transformation of cells. Thanks to this functional approach, we have shown, for the first time, the participation of EcR, USP and HR3 in a 20E signaling pathway that directs morphological differentiation in insect cells by regulating β tubulin expression.

Keywords. Steroid hormone receptor superfamily, cell morphological differentiation, RNA interference, β tubulin subnit, signaling pathway.

Introduction

RNA interference (RNAi) is based on double-stranded RNA (dsRNA) that inhibits gene expression in a sequence-specific manner by triggering mRNA degradation [1]. RNAi was shown to be highly effective in *Caenorhabditis elegans* and *Drosophila melanogaster* embryos as well as other invertebrates and plants [2–4]. Subsequent studies demonstrated that RNAi could also efficiently knock down the levels of specific proteins in several *Drosophila* tissue culture cell lines [5]. The discovery of short small interfering RNAs (siRNAs) had led to the wide-scale application of this approach to a wide variety of mammalian cell lines [6]. Thus, recent experiments brought to light the *in vitro* usefulness of RNAi for the determination of gene functions in the synchronization of cellular processes [7, 8].

In higher organisms, steroid hormones control a variety of cellular functions including cell proliferation, expansion, differentiation and programmed cell death. In mammals, evidence collected during the last few years indicates that steroid hormones act by a complex interface of 'genomic' and 'non-genomic' mechanisms [9–13]. In insects, the steroid hormone 20-hydroxyecdysone (20E), which belongs to the ecdysteroids family, is known to initiate and orchestrate the morphogenetic events required for metamorphosis [14]. Most of the 20E-induced biological

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effects have been associated to genomic mechanisms that involve a hormonal binding to a nuclear heterodimeric complex composed of the ecdysone receptor (EcR) [15, 16] and Ultraspiracle (USP) [17, 18], the insect homolog of the vertebrate retinoid X receptor (RXR) [19, 20]. The 20E/EcR/USP complex then directly triggers the sequential expression of genes encoding transcription factors that ultimately regulate the activity of target genes [21–23]. In insects as in mammals, there are numerous gaps in the steroid hormone signaling pathways by which the hormonal signal is transduced to direct the biological responses. The establishment of various insect and mammalian cell lines that have retained a sensitivity to steroid hormones has provided new possibilities for specifying the mode of action of these hormones in the control of cellular processes. MCF-7, MDA-MB-231 breast cancer cells from human, MBA-15 osteoblastic cells from mouse, Kc embryonic cells from *D. melanogaster* Diptera as well as IAL-PID2 epidermal cells from *Plodia interpunctella* Lepidoptera respond to steroid hormones treatment by the arrest of cell growth and long-term spectacular modifications in the shape of cells [24–30]. In Kc and MBA-15 cells, it has also been described that morphological transformations were accompanied by a redistribution in cytoplasmic microtubules. This rearrangement was concomitant with changes in the expression level of tubulin, which is composed of non-covalently bound α- and β-chains and represents the unit of all microtubules in eukaryotic cells [31, 32]. To date, only descriptive information exits for the regulation of the tubulin gene by steroid hormones in correlation with cell morphological differentiation, and there are no functional data on the signaling transduction pathway responsible for these genetic and cellular responses. In this report, we extended the application of the double-stranded RNA interference (dsRNAi) method to a steroid hormone responsive IAL-PID2 cell line from *Plodia interpunctella* Lepidoptera. We first showed that dsRNA was capable of specifically blocking the 20E inducibility of *Plodia interpunctella* ecdysone receptor (PIEcR, [30]), Ultraspiracle (PIUSP, [33]) and transcription factor HR3 (PHR3, [34]) in IAL-PID2 cell culture. We then demonstrated that 20E-induced cell morphological transformation resulted from an increased synthesis of β tubulin. Finally, we report the consequences of PIEcR, PHR3, PIUSP proteins "knock down" on the expression level of β tubulin in correlation with the shape of IAL-PID2 cells. This functional analysis has provided evidence, for the first time, for the involvement of EcR, USP and HR3 in an ecdysteroid signaling pathway that controls the insect cell morphological differentiation by regulating β tubulin synthesis.

Materials and methods

Cell culture

The 20E-sensitive epidermal IAL-PID2 cell line was established from *Plodia interpunctella* Hübner, the Indian meal-moth [35]. Cells were grown as a loosely attached monolayer at 26°C in 25-cm^2 tissue culture flasks with 4 ml antibiotic-free Grace's medium (Gibco BRL, Life technologies, Inc) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Boehringer Mannheim, France) and 1% bovine serum albumin (BSA; Calbiochem, France). Cells were subcultured weekly to a near confluent monolayer, rinsed off the bottom of the flask in a gentle stream of culture medium and resuspended. Cell density was estimated by counting cells in an aliquot of the suspension in a Mallassez hemocytometer under the microscope. All the cultures were initiated by seeding culture flasks or 24-well cell culture dishes with 5×10^5 or 4×10^4 cells, and then cultured under normal growth conditions for 72 h at 26°C. The 20E was a gift of Dr. René Lafont (UPMC, Paris, France). For use in culture, 20E was dissolved in ethanol and diluted in appropriate volumes of sterile Grace's medium supplemented with FBS and BSA to a final concentration of 10^{-7} M. This concentration is close to physiological levels for 20E (in the order of 2×10^{-8} to 6×10^{-6} M [36]). The solutions were then directly added to cell cultures using glass capillary pipettes. Final ethanol concentration in treated and control cultures was always maintained well under 0.1% to prevent any toxic effect of the solvent.

Protein extraction and Western analysis

Cells were harvested by trituration, pelleted by centrifugation at 1000 *g*, lysed in 400 µl RIPA buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 1.0% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 0.2 mM $NaVO_4$, 10 mM NaF, 0.4 mM EDTA, 10% glycerol). Lysate (20 µl) was added to 20 µl of Laemmli loading buffer. Proteins were separated on 10% SDS-polyacrylamide resolving gel in a mini-protean vertical gel electrophoresis apparatus (Bio-Rad). Proteins were transferred to nitrocellulose membranes in a mini trans-blot electrophoretic (Bio-Rad), using 120 mA current for 1 h. Nitrocellulose membranes were blocked with 5% BSA from Sigma (St Louis, MO) in Tris-buffer saline (TBS; 10 mM Tris, 150 mM NaCl, pH 8.0) for 30 min at room temperature. Nitrocellulose membranes were then probed for 1 h at room temperature with monoclonal antibodies generated against *Plodia interpunctella* EcR, HR3, USP, RpL4 ribosomal protein and rabbit β tubulin (Sigma) at 1:1000, 1:500, 1:750, 1:800, 1:950 dilutions, respectively, in 0.2% Tween-TBS (TTBS) plus 5% BSA. The anti-EcR, anti-USP anti-HR3, anti-RpL4 antibodies were made by Covalab (France). Membranes were then washed three times with TTBS, followed by incubation with secondary antibody horseradish peroxidase (POD)-conjugated goat anti-rabbit IgG from Roche Molecular Biochemicals for 1 h at room temperature at 1:5000 dilution in TTBS plus 5% BSA. After washing with TTBS, protein bands were visualized by enhanced BM chemiluminescence blotting system from Roche Molecular Biochemicals. Total protein was quantified according to Bradford [37] using serum albumin as standard. Protein (20 µg) was loaded in each lane. After transferring proteins from gels, the nitrocellulose membranes were first stained with Ponceau-S staining to confirm the equivalent loading of protein [38]. Membranes were destained by washing with several changes of deionized water at room temperature and used in immunological probing as described above. The relative amounts of proteins were determined from the immunoblots using the Molecular Imager System, Model Bio-1D (Vilber Lourmat, Marne la Vallée, France).

Synthesis of dsRNA

The MEGAscript™ RNAi kit (Ambion) was used to generate dsRNAs corresponding to RpL4, β tubulin, the specific A/B region of PIEcR, PIUSP and specific C region of PHR3. T7 promoter sites

were added to the PCR primers RPD 5′-forward primer (5′-CCG AAC TGC AAT GTA TTG CCG-3′), RPR 5-reverse primer (5′-TTC AGG CGC GGT CCT ATG GGC-3′) for RpL4, TUD 5′-forward primer (5′-ATT AAC CGA TAC TAC AAC GAG-3′), TUR 5′-reverse primer (5′-TGG GTA TTC TTC TCG GAT CTT-3′) for β tubulin, CED 5′-forward primer (5′-CGC TGG TCC AAC AAC GGA GGG-3′), CER 5′-reverse primer (5′-TGC CGG TGA CAA CTC CTC ACG-3′) for PIEcR; USD3 5′-forward primer (5′-ATG GAG CCC TCG AGG GAA TCA-3′), USR3 5′-reverse primer (5′-AGA CGT GGC GTC GTC CCG GAG-3′) for PIUSP and A11 5′-forward primer (5′-CCG TGC AAA GTT TGC GGC G-3′), A12 5′-reverse primer (5′-GCT CAT GCC GAG TTT GAG GC-3′) for PHR3. The generated primers were T7 RPD (5′-TAA TAC GAC TCA CTA TAG GGA CCG AAC TGC AAT GTA TTG CCG), T7 RPR (5′-ATT ATG CTG AGT GAT ATC CCT TTC AGG CGC GGT CCT ATG GGC) for RpL4, T7 TUD (5′-TAA TAC GAC TCA CTA TAG GGA ATT AAC CGA TAC TAC AAC GAG), T7 TUR (5′-ATT ATG CTG AGT GAT ATC CCT TGG GTA TTC TTC TCG GAT CTT) for β tubulin, T7 CED (5′-TAA TAC GAC TCA CTA TAG GGA CGC TGG TCC AAC AAC GGA GGG-3′), T7 CER (5′-ATT ATG CTG AGT GAT ATC CCT TGC CGG TGA CAA CTC CTC ACG-3′) for PIEcR; T7 USD3 (5′-TAA TAC GAC TCA CTA TAG GGA ATG GAG CCC TCG AGG GAA TCA-3′), T7 URD3 (5′-ATT ATG CTG AGT GAT ATC CCT AGA CGT GGC GTC GTC CCG GAG-3′) for PIUSP and T7 A11 (5′-TAA TAC GAC TCA CTA TAG GGA CCG TGC AAA GTT TGC GGC G-3′) and T7 A12 (5′-ATT ATG CTG AGT GAT ATC CCT GCT CAT GCC GAG TTT GAG GC-3′) for PHR3. Separate PCR reactions were conducted to generate complementary templates with a single T7 promoter site (T7 TUD+TUR, TUD+ T7 TUR; T7 RPD+RPR, RPD+T7 RPR; T7 CED+CER, CED+T7 CER; T7 USD3+USR3; USD3+T7 USR3 and T7 A11+A12, A11+T7 A12). T7 RNA polymerase was used to transcribe ssRNA from each DNA template over 4 h at 37°C. The dsRNAs (RpL4 dsRNA, β tubulin dsRNA, PIEcR dsRNA, PIUSP dsRNA, PHR3 dsRNA) were produced by mixing solutions containing equivalent amounts of complementary ssRNA at 75°C for 5 min followed by slow cooling to room temperature. DNA and ssRNA were removed from the solution by digestion with DNase I and RNase A at 37°C for 1 h. The dsRNAs were ethanol-precipitated and resuspended in nuclease-free water. The dsRNA concentration was determined by spectrophotometry at 260 nm. Six micrograms of dsRNA were analyzed by 1% agarose electrophoresis to ensure that the majority of the dsRNA existed as a single band of expected size. The dsRNAs were stored at –20°C for several months.

DsRNAi

Preparation of dsRNA-Lipofectamine 2000 complexes. The IAL-PID2 epidermal cells were not able to naturally incorporate dsRNA molecules. We used a lipophilic transfection reagent, the Lipofectamine 2000, under standard conditions that have been established by Invitrogen. The dsRNAs and Lipofectamine 2000 were diluted separately in 500 µl FBS-free culture medium and incubated for 5 min at room temperature. The diluted dsRNA was then combined with the diluted Lipofectamine 2000 at 500 ng/ml and incubated for 2 min at room temperature to allow the formation of dsRNA-Lipofectamine 2000 complexes.

Transfection of dsRNA. For RNAi experiments, IAL-PID2 cells were seeded at 5×10^5 cells per 25-cm^2 tissue culture flask and grown under normal conditions for 72 h at 26°C with 4 ml medium supplemented with 10% FBS and 1% BSA. Then, 1 ml FBS-free medium containing dsRNA-Lipofectamine 2000 complexes was added directly to cells in the culture medium. The final concentrations of dsRNA and Lipofectamine 2000 were 2 µg/ml and 500 ng/ml, respectively. After a 10-h incubation at 26°C, cells were rinsed and then refed with a medium containing 20E at 10^{-7} M.

Isolation of RNA and RT-PCR

Total RNA from cells was isolated by guanidinium isothiocyanate-phenol-chloroform extraction method. RNA concentration was determined spectrophotometrically and the quality of RNA was checked by electrophoresis on a formaldehyde-agarose gel (1%). Using the first strand cDNA synthesis kit (Roche, Meylan, France), 1 µg total RNA was reverse transcribed into single-stranded cDNA with reverse transcriptase AMV and oligo-p$(dT)_{15}$ as primer. The resulting cDNAs were amplified by PCR a set of DNA primers (A11, A12 for PHR3; RPD, RPR for RpL4; CER, CED for PIEcR; USR3, USD3 for PIUSP) on a Techne programmable thermal cycler using recombinant Pfu DNA polymerase. Reactions were performed according to the manufacturer's instructions. Following an initial 5-min denaturation at 94°C, the thermal amplification procedure included 30 cycles of denaturation for 1 min at 94°C, annealing at 50°C for PHR3 and RpL4, or 55°C for PIUSP and PIEcR for 30 s and elongation at 68°C for 60 s.

Results

dsRNAi in IAL-PID2 epidermal cells

dsRNAi efficiency. It has been demonstrated that 20E directly initiates the transcription of PIEcR, PIUSP and PHR3 genes. To determine whether RNAi is active in IAL-PID2 cell culture, 3 µg/ml PIEcR, PIUSP, PHR3 dsRNAs combined with 500 ng/ml Lipofectamine 2000 were incorporated separately into IAL-PID2 cells. After a 24-h recovery, the effects of each transfection on the ability of 20E to induce the corresponding encoded proteins were examined by Western blotting.

In the absence of 20E, PIEcR and PIUSP proteins were constitutively expressed at a low level over time and PHR3 protein was never detectable (data not shown). Figure 1 shows that in the presence of 20E (10^{-7} M), PHR3, PIEcR and PIUSP proteins were significantly induced after 4–6 h of exposure, reached a maximum at 6, 12 and 18 h, respectively, and then declined (Fig. 1a–c). In combination with PIEcR, PHR3 and PIUSP dsRNAs, the induction level of proteins was highly reduced (Fig. 1a–c). For RNAi controls, we noticed that the incorporation of PIEcR, PHR3 and PIUSP dsRNAs had no effect on the expression of RpL4 ribosomal protein (Fig. 1a–c). In addition, the induction of PHR3, PIEcR and PIUSP proteins remained unchanged in presence of dsRNA β tubulin (Fig. 1d).

Using RT-PCR, we verified the RNAi effect on the 20E inducibility of PIEcR, PIUSP and PHR3 transcripts. In the presence of PIEcR, PIUSP and PHR3 dsRNAs, a sharp decrease in the induction level of PIEcR, PIUSP, PHR3 genes was detected after 6 h of 20E exposure, whereas the expression of RpL4 was not effected (Fig. 2).

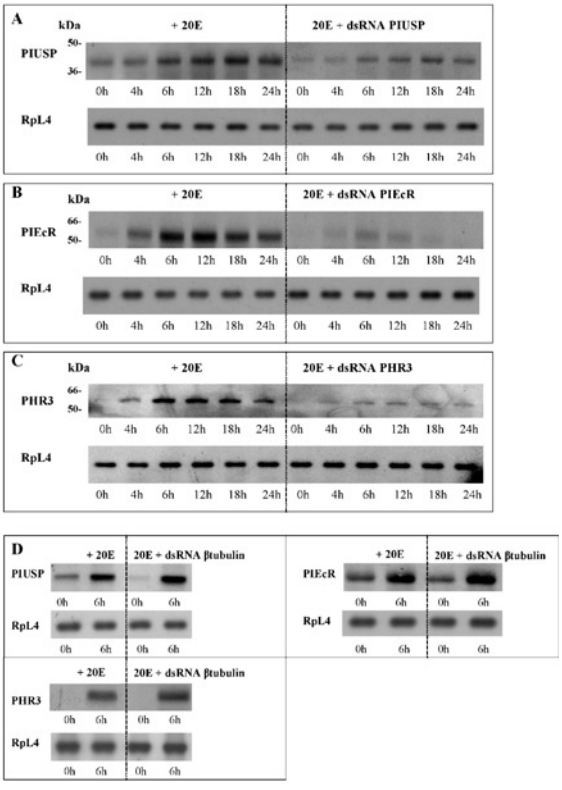


Figure 1. RNAi effect on the 20E inducibility of PIEcR, PHR3, PIUSP proteins. In combination with 0. 5 μg/ml Lipofectamine 2000, and 3 μg/ml PIEcR, PIUSP and PHR3 dsRNAs were applied separately on IAL-PID2 cells. After 24-h incubation, cells were treated with 10^{-7} M 20E. At different times of 20E exposure, 20 μg of total proteins from untransfected and transfected cells were analyzed by Western blots using antibodies directed against PIUSP (*a*), PIEcR (*b*), PHR3 (*c*). For RNAi controls, the cells were treated with 3 μg/ml β tubulin dsRNA combined with 0.5 μg/ml Lipofectamine 2000 and the induction level of PIUSP, PIEcR and PHR3 proteins was analyzed after 6 h of 20E exposure (*d*). For Western blots controls, an antibody directed against RpL4 ribosomal protein was used.

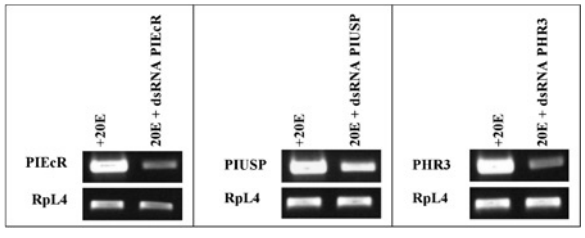


Figure 2. RNAi effects on the 20E inducibility of PIEcR, PHR3, PIUSP transcripts. In combination with 0.5 μg/ml Lipofectamine 2000, 3 μg/ml PIEcR, PIUSP and PHR3 dsRNAs were applied separately on IAL-PID2 cells for 24 h. The cells were then treated with 10^{-7} M 20E. After 6 h of 20E exposure, total RNA were isolated from untransfected and transfected cells and the induction level of PIEcR, PIUSP and PHR3 genes was determined by RT-PCR with RpL4 gene as a positive control.

dsRNAi optimization. After the discovery that the addition of dsRNA-Lipofectamine 2000 complexes to cell culture media was able to abolish the 20E inducibility of targeted proteins in IAL-PID2 cells, we determined the effect of incubation time with dsRNA. IAL-PID2 cells were treated with 3 μg/ml PIEcR, PIUSP or PHR3 dsRNA combined with 0.5 μg/ml Lipofectamine 2000 for increasing lengths of exposure time. After this incubation period, cells were refed with medium containing 10^{-7} M 20E for 12 h before examination of PHR3, PIUSP and PIEcR proteins levels. An inhibition of PHR3, PIUSP and PIEcR proteins induction was apparent when cells were exposed to dsRNA for 5 h and a maximal inhibition was reached after 10 h of continuous dsRNA exposure (Fig. 3).

We evaluated the effectiveness of RNAi by testing various dsRNA concentrations. The IAL-PID2 cells were cultured in the presence of 0.5 μg/ml Lipofectamine 2000 combined with PIEcR, PIUSP or PHR3 dsRNA at concentrations ranging from 0.2 ng/ml to 20 μg/ml for 10 h. Based on time required for the maximum induction of proteins, the amount of PHR3, PIEcR and PIUSP were then evaluated after 6, 12 and 18 h of exposure to 10^{-7} M 20E, respectively. Figures 4a–c show that the suppressive effect of PIEcR, PIUSP and PHR3 dsRNAs on the increase in the PIEcR, PIUSP and PHR3 proteins levels was concentration dependent. For the three type of dsRNA transfected into IAL-PID2 cells, a concentration of 2 μg/ml was able to maximally inhibit the induction of targeted proteins, whereas 20 μg/ml dsRNA did not result in any further inhibition (Fig. 4a–c). For PIUSP, PHR3 and PIEcR, approximate loss of protein was 71%, 82% and 92%, respectively.

dsRNAi persistence. The persistence of the RNAi effect was then examined by monitoring the induction level of PIEcR, PIUSP and PHR3 proteins over 3 days. When PIEcR, PIUSP and PHR3 dsRNAs at 2 μg/ml were present, the reduction in the expression level of PIEcR, PIUSP and PHR3 proteins were normally detected at 18 h and persisted during the whole length of their induction (Fig. 5a–c).

20E signaling pathway in cell morphological differentiation process

After defining the best conditions for RNAi use on the IAL-PID2 cell line, we dissected the 20E signaling pathway involved in the regulation of β tubulin expression during the cell morphological differentiation.

Effect of 20E on the expression of β tubulin and the shape of cells. We first examined the effects of 20E on the morphology of IAL-PID2 cells in correlation with the expression level of β tubulin, a major protein of the cytoskeleton. After different exposure times to 10^{-7} M

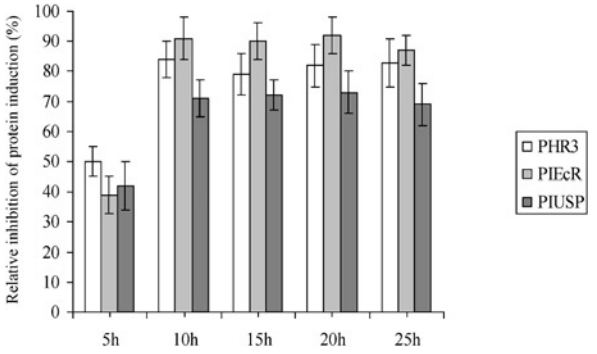


Figure 3. Effect of incubation time with dsRNA. The IAL-PID2 cells were treated with 3 μg/ml PIEcR, PIUSP or PHR3 dsRNA combined with 0.5 μg/ml Lipofectamine 2000 for different exposure times. After this incubation period, cells were rinsed and refed with medium containing 10^{-7} M 20E for 12 h before analysis of PIEcR, PIUSP and PHR3 proteins levels by Western blotting. On the y-axis, the relative inhibition of PHR3, PIEcR and PHR3 proteins induction was expressed as percentage of PHR3, PIEcR, PIUSP proteins induction after 12 h of 20E exposure in transfected cells compared to the induction of proteins measured after 12 h of 20E treatment in untransfected cells. Points are means ± SD ($n = 5$–8).

20E, the shapes of the cells were examined and total proteins then extracted. We noticed that, in the untreated cells, the expression level of β tubulin remained unchanged (Fig. 6a), whereas treatment with 20E triggered an increase in the amount of β tubulin occurring after 48 h, which reached a maximum by 72 h and then plateaued until 120 h (Fig. 6b). Moreover, we observed that the untreated cells kept a spherical shape during their growth (Fig. 7a), whereas the 20E-treated cells started elongating, emitting long pseudopodia and aggregating after 2 days treatment (Fig. 7c). By 3–4 days post treatment, cytoplasmic extensions increased in number and size, often producing long and arborized processes that formed connections between different aggregates (Fig. 7d, e).

Interaction between β tubulin and cell morphological differentiation. In combination with Lipofectamine 2000, 2 μg/ml dsRNA β tubulin was incorporated into IAL-PID2 cells. The effect of this transfection on the ability of 20E to induce the corresponding encoded protein and the cell morphological differentiation was then examined. The β tubulin protein levels were evaluated every 24 h during the 20E treatment and the morphology of the cells was observed after 4 days post treatment. The presence of β tubulin dsRNA prevented both the increased synthesis of β tubulin (Fig. 8b) and the formation of pseudo epithelial structures (Fig. 9b) as compared to untransfected cells (Figs 8a and 9a).

We noticed that the incorporation of β tubulin dsRNA had no effect on the expression of RpL4 ribosomal protein (Fig. 8b), and that the presence of dsRNA

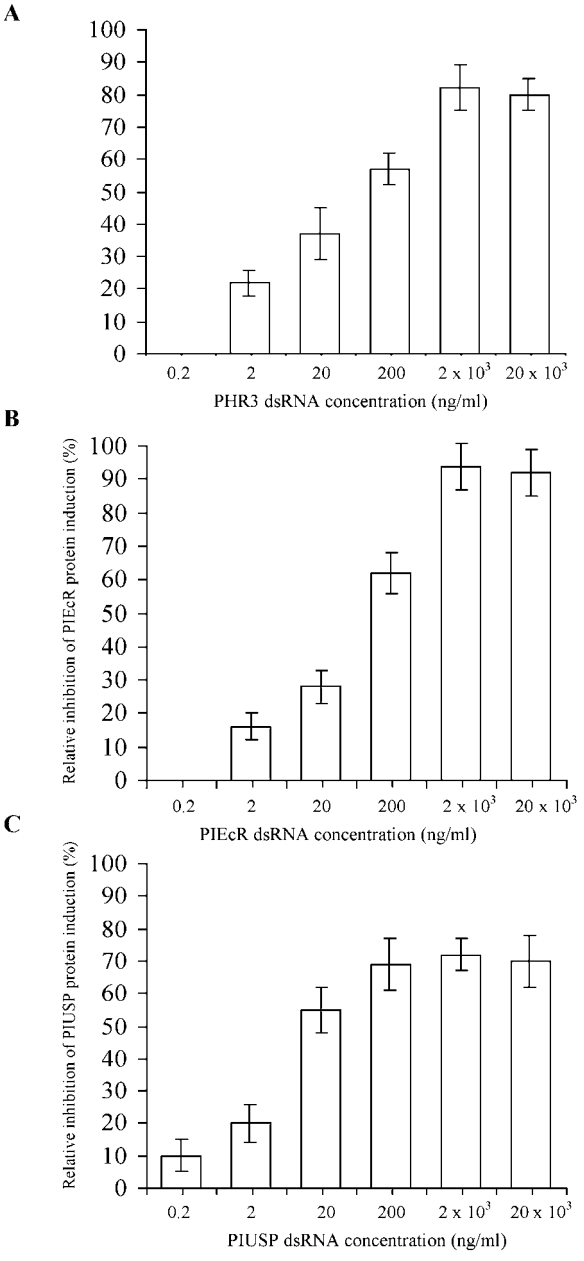


Figure 4. Effect of RNAi dose. The IAL-PID2 cells were cultured in the presence of 0.5 μg/ml Lipofectamine 2000 combined with PIEcR, PIUSP or PHR3 dsRNA at various concentrations ranging from 0.2 ng/ml to 20 μg/ml. After 10-h incubation, cells were treated with 10^{-7} M 20E and the levels of expression of PHR3 (*a*), PIEcR (*b*) and PIUSP (*c*) proteins were assessed by Western blotting after 6-, 12- and 18-h exposure to 20E, respectively. On the y-axis, the relative inhibition of PIUSP, PIEcR, PHR3 proteins induction was expressed as percentage of PHR3, PIEcR, PIUSP induction after 6-, 12- and 18-h 20E exposure in presence of dsRNA at different concentrations as compared to the induction of proteins measured after 6-, 12- and 18-h 20E treatment without applying dsRNA. Points are means ± SD ($n = 4$–8).

RpL4 did not repress the β tubulin increased synthesis (data not shown) and consequently the 20E-induced cell morphological differentiation (Fig. 9d).

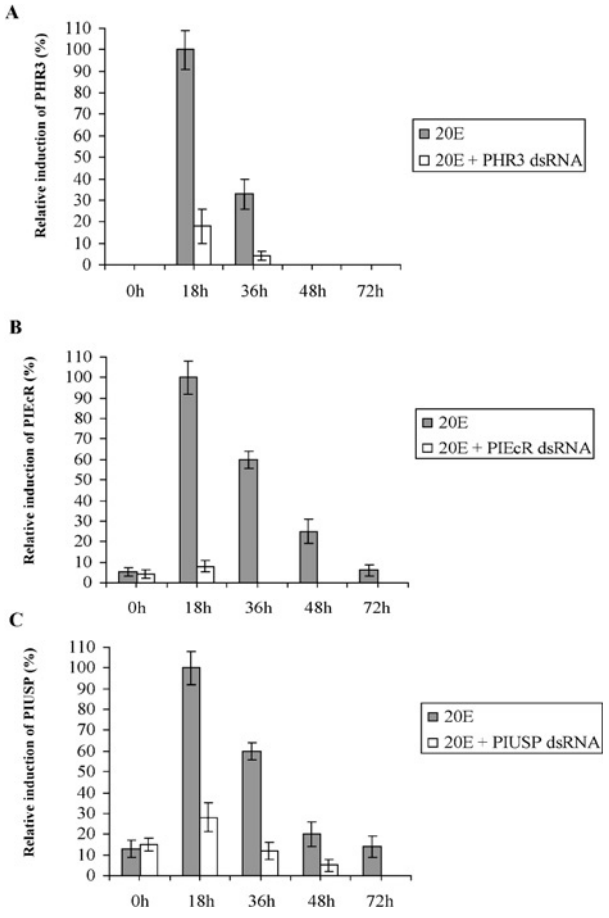


Figure 5. Persistence of RNAi. At 10 h after applying 2 µg/ml PIEcR, PIUSP, PHR3 dsRNAs combined with 0.5 µg/ml Lipofectamine 2000, cells were treated with 20E at 10^{-7} M for various continuous exposure times. At each time, 20 µg total protein was analyzed by Western blots using antibodies directed against PHR3 (*a*), PIEcR (*b*), or PIUSP (*c*). On the y-axis, the relative induction of PHR3, PIUSP, PIEcR proteins were expressed as percentages of their induction in IAL-PID2 cells cultured with 10^{-7} M 20E for 18 h. Points are means ± SD (n=2–8).

Interaction between PIUSP, PIEcR, PHR3 and β tubulin. Using RNAi, we successively blocked the induction of PIEcR, PIUSP and PHR3 proteins and then studied the effects of each disruption on both the level of β tubulin protein and the shape of IAL-PID2 cells. Applying 2 µg/ml PIEcR, PIUSP, PHR3 dsRNAs on the 20E-treated cells resulted in a decrease in the level of β tubulin protein induction (Fig. 10a–c) and in the number of pseudo epithelial structures (Fig. 11b–d) as compared to untransfected cells (Fig. 11a). We verified that β tubulin protein induction was maintained in presence of RpL4 dsRNA (data not shown).

To complete this analysis, we studied the effects of PIEcR and PIUSP dsRNAs on the 20E induction of PHR3 protein. Applying each dsRNA caused a decline in the level of PHR3 induction (Fig. 12a, b).

Discussion

In many eukaryotes, gene silencing by RNAi through the delivery of specific dsRNAs or siRNAs has become a powerful tool to knock-down specific gene expression in a wide range of cells. This RNAi technique has been reported to be effective in mammalian somatic, neuronal and embryonic cell lines, including HeLa, HEK293, and P19 [39–41]. In insect cell lines, its efficacy was mainly established in *D. melanogaster* Diptera and, under normal conditions, the *Drosophila* S2, KC and BGG2-C6 cells, originally isolated from different embryonic cell types, take up external nucleic acids with a high efficiency [5]. Using a lipophilic transfection reagent, the Lipofectamine 2000, we succeeded in extending the application of the RNAi technique to the epidermal IAL-PID2 cell line of *Plodia interpunctella* Lepidoptera.

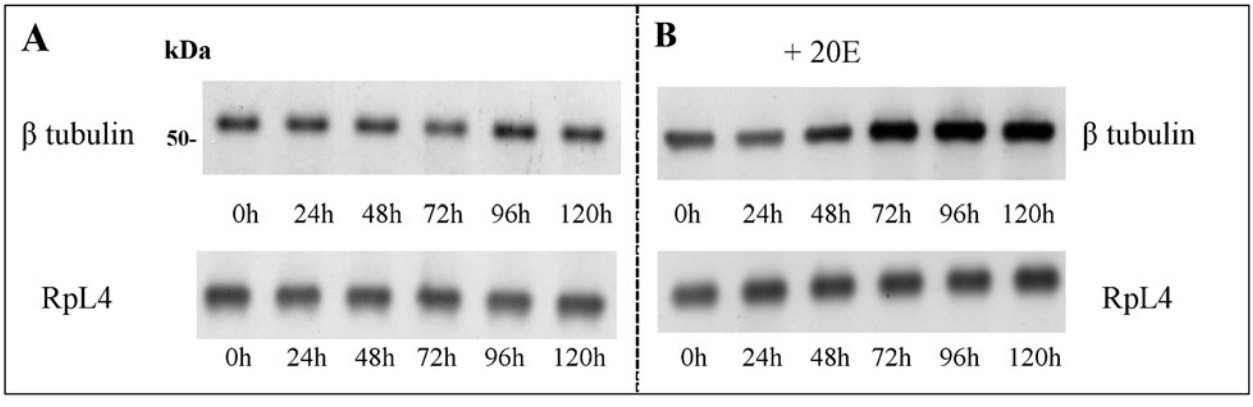


Figure 6. Effect of 20E on β tubulin protein. Twenty micrograms of total protein from IAL-PID2 cells cultured in Grace's medium with 20E at 10^{-7} M for various exposure times were analyzed by Western blot using antibody directed against β tubulin. For Western blots controls, an antibody directed against RpL4 ribosomal protein was used.

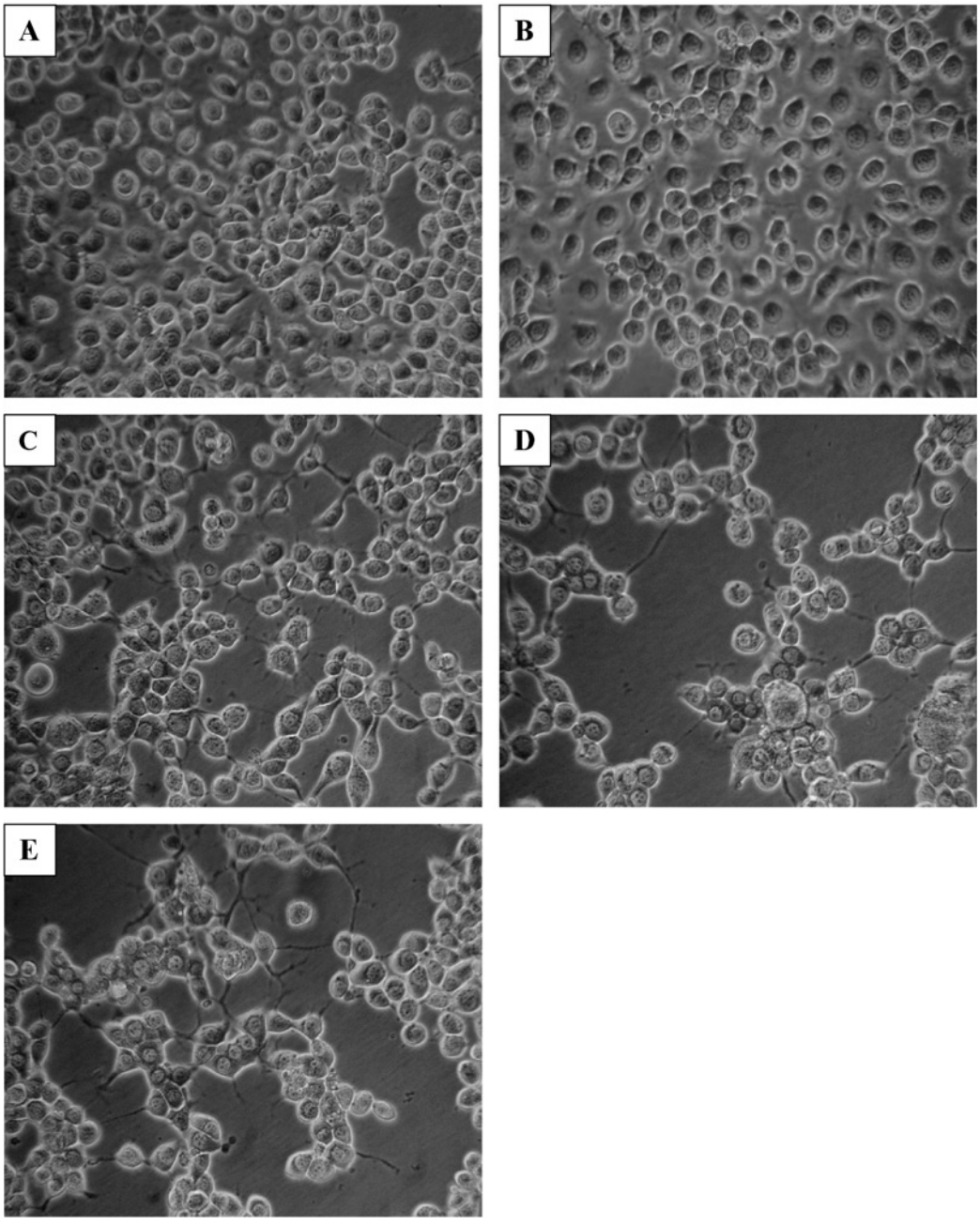


Figure 7. Effect of 20E on the shape of cells. IAL-PID2 cells were grown in Grace's medium containing 0.1% ethanol for 96 h (*a*) or 10^{-7} M 20E for 24 h (*b*), 48 h (*c*), 72 h (*d*), or 96 h (*e*). Each panel shows the representative area of three replicates.

We tested the efficacy of dsRNA-mediated interference on the steroid hormone-inducible gene expression in IAL-PID2 cells and demonstrated that dsRNA was capable of efficiently blocking the ability of the steroid hormone 20E to induce EcR, USP and HR3 genes that encode EcR, its partner USP, the insect homolog of the vertebrate retinoid X receptor (RXR) and the HR3 transcription factor. The RNAi pathway is, therefore, operative in IAL-PID2 cell culture and its use presents several advantages over methods requiring the introduction of DNA into cells. Transfection experiments require knowledge of the full sequence of the gene and of its flanking regions, whereas for RNAi, dsRNA corresponding to any gene fragments is sufficient to confer the interference effect [42]. Moreover, the use of RNAi in *Plodia interpunctella* as well as in others organisms is technically simple with the ability to produce dsRNAs with a simple PCR approach rather than purchase expensive oligonucleotides or construct complex vectors. The RNAi effects on the 20E inducibility of genes were rapid, in that the selective protein "knock-down" became

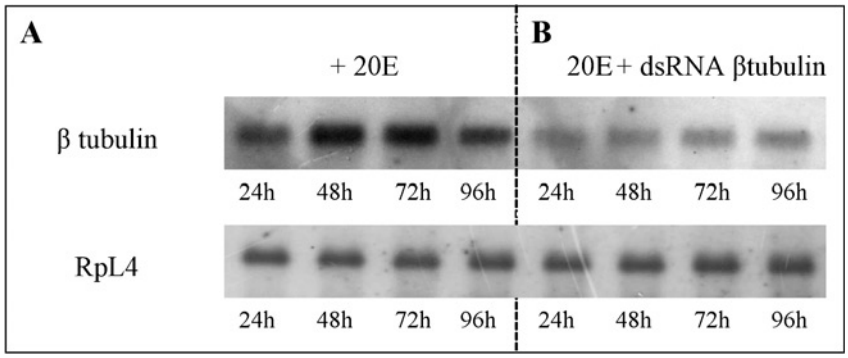


Figure 8. RNAi effect on β tubulin induction. At 10 h after applying 2 μg/ml β tubulin dsRNA combined with at 0.5 μg/ml Lipofectamine 2000, the cells were treated with 10^{-7} M 20E. The levels of β tubulin protein were analyzed at various 20E exposure times by Western blot using antibody directed against β tubulin in untransfected (*a*) and transfected cells (*b*). For Western blots controls, an antibody directed against RpL4 ribosomal protein was used.

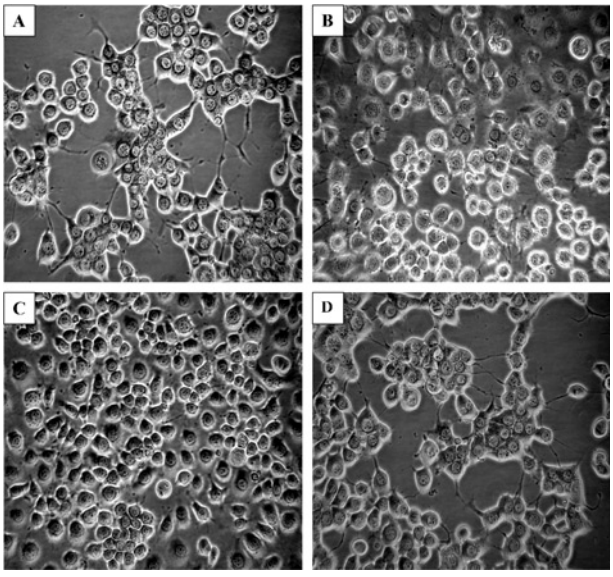


Figure 9. Interaction between β tubulin and cell morphological differentiation. At 10 h after applying 2 μg/ml β tubulin dsRNA combined with at 0.5 μg/ml Lipofectamine 2000, the cells were treated with at 10^{-7} M 20E and the shape of cells was visualized under microscope 4 days post treatment. (*a*) Cells exposed to 20E without applying β tubulin dsRNA. (*b*) Cells treated with 20E in presence of β tubulin dsRNA. (*c*) Cells cultured in Grace's medium containing 0.1% ethanol. For RNAi controls, the shapes of cells were observed after a 20E treatment in presence of RpL4 dsRNA (2 μg/ml) (*d*). Each panel is the representative area of three replicates.

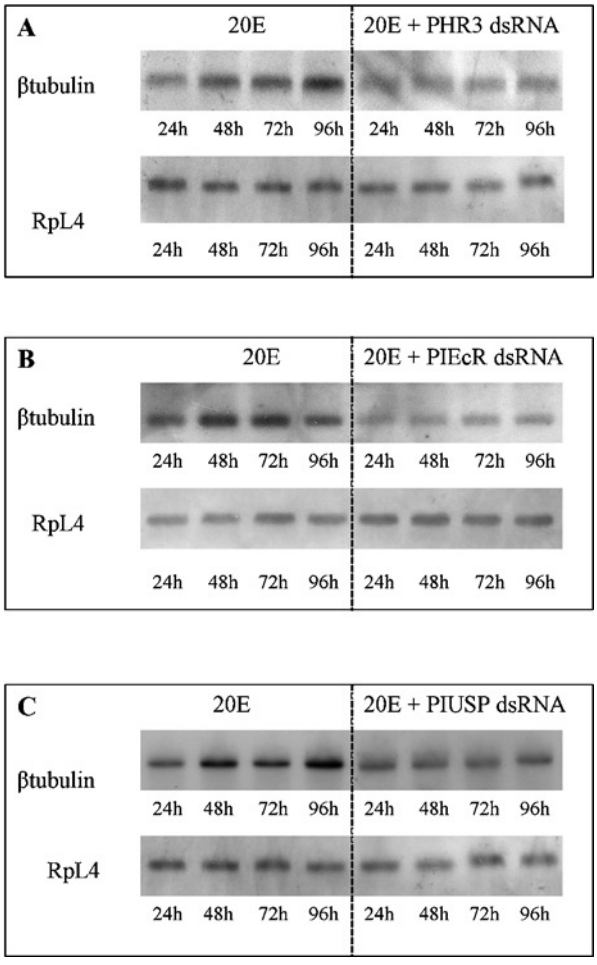


Figure 10. Interaction between PIUSP, PHR3, PIEcR and β tubulin. At 10 h after applying 2 μg/ml PIEcR, PIUSP, PHR3 dsRNAs combined with at 0.5 μg/ml Lipofectamine 2000, the cells were treated with at 10^{-7} M 20E for various exposure times. The levels of β tubulin induction in presence of PHR3 (*a*), PIEcR (*b*) or PIUSP (*c*) dsRNA were analyzed by Western blot using antibody directed against β tubulin. For Western blots controls, an antibody directed against RpL4 ribosomal protein was used.

detectable as early as after 5 h exposure of cells to dsRNAs. In addition, the RNAi was highly effective at a low concentration of dsRNAs (2 μg/ml), which caused more than 70% of inhibition in proteins induction. This suppressive effect persisted during the whole period of the induction. In other invertebrates and mammalian cell lines in which RNAi has been shown to be effective, the exposure time and the amount of dsRNAs necessary to knock-down the constitutive expression of proteins depended on their turn over and were much higher than those required for steroid-inducible proteins [5, 40, 43]. The rapidity, efficiency, specificity and longevity of the RNAi effects on steroid inducibility of gene in epidermal IAL-PID2 cells make this cell line an ideal system for identifying the role of theses genes in the steroid-regulated cellular functions. To this date, we have successfully abolished the induction of EcR, USP,

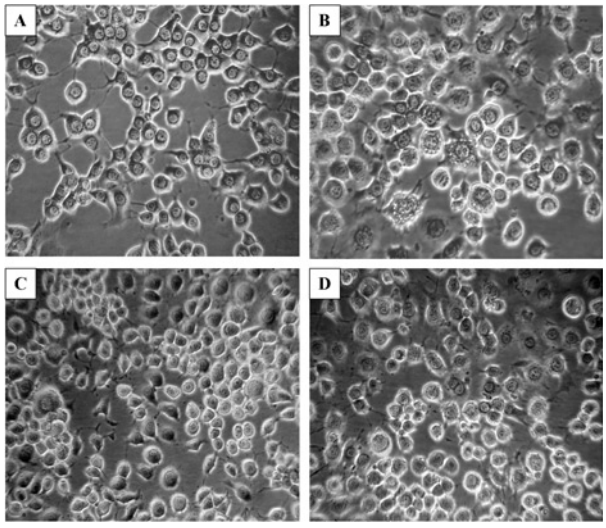
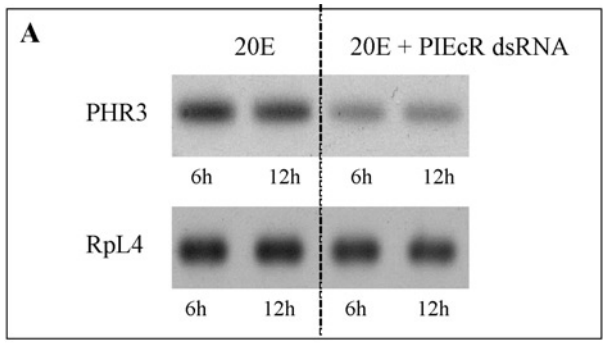


Figure 11. Correlation between PIUSP, PHR3, PIEcR and cell morphological differentiation. At 10 h after applying 2 µg/ml PIEcR, PIUSP, PHR3 dsRNAs combined with Lipofectamine 2000 (0.5 µg/ml), the cells were treated with at 10^{-7} M 20E for 4 days and the shape of cells was visualized under microscope. (*b–d*) Cells treated with 20E in presence of PHR3, PIEcR and PIUSP dsRNAs, respectively. (*a*) Cells exposed to 20E for 4 days without applying dsRNA. Each panel is the representative area of three replicates.



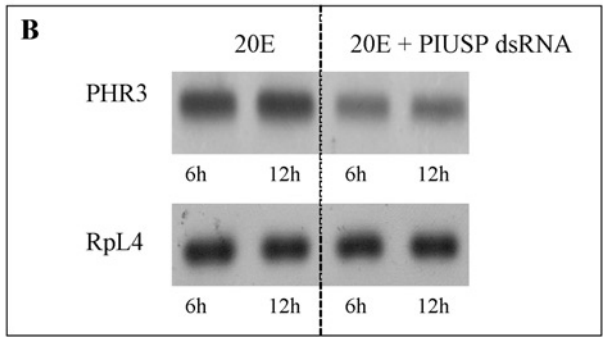


Figure 12. Interaction between PIEcR, PIUSP and PHR3. At 10 h after applying 2 µg/ml PIEcR, PIUSP dsRNAs combined with 0.5 µg/ml Lipofectamine 2000, cells were treated with at 10^{-7} M 20E for 6 and 12 h. The levels of PHR3 protein induction in presence of PIEcR (*a*) or PIUSP (*b*) dsRNA were analyzed by Western blot using antibody directed against PHR3. For Western blots controls, an antibody directed against RpL4 ribosomal protein was used.

HR3 and three other transcription factors, E75, BR-C, E74 (data not shown), thus demonstrating that the RNAi technique is efficient towards a broad range of ecdysteroid-inducible proteins belonging to the nuclear receptor superfamily. This application of RNAi methodology might be broaden to other steroid hormone responsive cell lines established from mammals and plants.

We then undertook a functional analysis of the 20E signaling cascade involved in the cell morphological differentiation process. We first examined the effects of 20E on the shape of IAL-PID2 cells in correlation with the expression level of one essential component of microtubules, the β tubulin subnit. The immunological and cytological analysis showed that 20E induced a sharp increase in the synthesis of β tubulin that preceded the morphological transformation of IAL-PID2 cells, mostly marked by the emission of long cytoplasmic extensions. These results were in agreement with those obtained on mammalian and other insect cell lines and confirmed that the tubulin cytoskeleton was a target for steroid hormone-triggered changes in cell shape [26, 31, 32, 44]. Other *in vitro* experiments performed on *Drosophila* Kc cells and mouse osteoblastic cells have revealed that steroid-induced morphological modifications were also accompanied by changes in the distribution and expression level of cytoskeleton actin [24, 26, 45–47]. It would be interesting to determine whether steroid hormones act on these two major proteins of the cytoskeleton through a common signaling pathway.

Using RNAi, we demonstrated that 20E-induced cell morphological transformation resulted from an increased synthesis of β tubulin and that inhibiting the induction of PIEcR, PIUSP or PHR3 proteins prevented the 20E effects on the β tubulin synthesis and on the morphology of IAL-PID2 cells. On the other hand, a decreased induction of PHR3 protein was observed by blocking the activation of PIEcR or PIUSP genes. Thanks to this functional approach, for the first time, we have shown that the EcR, USP and HR3 participate in a 20E signaling pathway that directs morphological differentiation in insect cells by regulating β tubulin expression.

In *Manduca*, the promoter region of the HR3 gene contains four putative ecdysone response elements (EcRE) and is activated by 20E through a binding of EcR/USP complex to EcRE [48]. In *Drosophila*, it has been demonstrated that the intronic and 5′ flanking sequences of β tubulin gene are essential to confer ecdysone responsiveness. Theses sequences are composed of motifs homologous to EcRE half sites, which appear to be involved in the level of the ecdysone response [49, 50]. To gain more precise information on the relationships between EcR, USP, HR3 and β

tubulin in the 20E signaling pathway, some experiments are in progress to characterize the presence of EcRE and a HR3 response element in the promoter regions of the *Plodia* HR3 and β tubulin genes, respectively.

Ultrastructural analysis of *Drosophila* Kc cells has shown that the addition of 20E affected the expression of β tubulin, and modified cellular architecture by inducing a redistribution of microtubules, which were thus accumulated in the pseudopodia and arranged in a parallel direction to the longitudinal axis [44]. The inhibitory effect of RNAi on the 20E-induced morphological response therefore suggested that the 20E genomic signaling pathway, described previously, could also direct the rearrangement of microtubules.

It is interesting to note that in humans, target cells and organs are regulated by a complex interplay of genomic and non-genomic signaling mechanisms of steroid hormones, and the integrated action of these machineries has important functional roles in a variety of pathophysiological processes. The steroid-regulated cytoskeletal responses are related to steroid effects on the initiation and promotion of cancer [51, 52], and it has been recently discovered that the human cancerous cell lines HT-1080 and LNCaP responded to androgen treatment by undergoing rapid cytoskeleton transformations that occurred in some minutes, a time lag incompatible with the classical scheme of a nuclear receptor action [53–56]. The cytoskeleton reorganization of these tumorous cells was mediated by a steroid extranuclear signaling pathway that involved an hormonal binding to a membrane receptor with the sequential activation of protein kinases, GTPases and the production of lipid second messengers such as phosphatidylinositol-3,4,5-triphosphate [53]. In insects, some studies have suggested the existence of extranuclear signaling for ecdysteroids by showing the involvement of nitric oxide in the control of neuroblast proliferation by 20E during *Manduca sexta* metamorphosis, and by revealing the presence of a membrane ecdysteroid receptor in the silk gland of *Bombyx mori* and the mature nervous system of *D. melanogaster* [57–59].

Finally, our experiments highlighted the IAL-PID2 cell line as an ideal system for the identification of steroid hormone genomic signaling pathways in cellular differentiation processes which could also be used to accelerate the characterization of genomic mechanisms involved in the regulation of other cellular functions (*e.g.*, proliferation and adhesion) in insects. We could thus obtain additional information on the mechanisms by which ecdysteroids control the programming of metamorphosis.

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