Characterization of subclones of the epithelial cell line from *Chironomus tentans* resistant to the insecticide RH 5992, a non-steroidal moulting hormone agonist

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Abstract

Selection of hormone resistant subclones in the continuous presence of the insecticide and ecdysteroid mimick RH 5992 (tefubenozide) resulted preferentially in clones with defects in ecdysteroid receptor function. RH 5992 is already degraded to polar products in wild-type cells; no increase in metabolism of tefubenozide is observed in resistant clones. According to Western blots, ecdysteroid receptor (EcR) and its heterodimerization partner ultraspireacle (USP) are present in all resistant clones. The concentrations are comparable to wild-type cells, but in three clones the extent of phosphorylation of USP is diminished. With regard to hormone binding several types of hormone resistance are distinguished: (1) The same two high-affinity hormone recognition sites are present as in wild-type cells (K<sub>D1</sub>=0.31±0.28 nM, K<sub>D2</sub>=6.5±2.4 nM) but the number of binding sites is reduced. (2) The binding site with the lower affinity (K<sub>D2</sub>) is missing. (3) The binding site with the higher affinity (K<sub>D1</sub>) is missing. (4) No specific binding is observed. Ponasterone A binding can be rescued by addition of EcR but not by USP. (5) Ligand specificity is altered. RH 5992 can not compete [3H]-ponasterone A as efficient as in wild-type cells. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Ecdysteroid; Receptor; Tefubenozide

1. Introduction

Resistant cell lines with defects in the hormonal signaling pathway are suited tools to study the molecular mechanism of hormone action (Feyereisen, 1998). In addition, interference with the insect’s endocrine system is considered as target for the development of new insecticides with presumably low vertebrate toxicity. This approach was first used with juvenile hormone analogues and since a few years also with moulting hormone (ecdysteroid) mimicks, especially diacylhydrazines. Since these compounds are used as pesticides (for review see Dhadiilla et al., 1998) resistant cell lines may also help to anticipate the mechanisms for resistance against these compounds, which may arise under field conditions after prolonged treatment. Meanwhile, loss of sensitivity to RH 5992 is already reported in a field population of *Cydia pomonella* (Sauphanor and Bouvier, 1995).

In previous reports we have demonstrated that enhanced metabolism of 20-OH-ecdysone is associated with hormone resistance in clones of the epithelial cell line from *Chironomus tentans*, selected under the continuous presence of 20-OH-ecdysone (Spindler-Barth and Spindler, 1998; Kayser et al., 1997). In this paper we show that selection in the presence of RH 5992 results mainly in clones with target site resistance; that means defects in the hormone receptor itself. The functional ecdysteroid receptor (Yao et al., 1993) is a heterodimer of ecdysteroid receptor (EcR) and ultraspireacle (USP). Therefore we characterized these two transcription factors in resistant subclones.

2. Experimental procedures

2.1. Cell culture and selection of resistant subclones

The epithelial cell line from *Chironomus tentans* grows in multicellular spheroids. Cells were cultured in...
medium (Wyss, 1982) at 25°C and propagated by dis-
sociation of the multicellular vesicles by pipetting and
dilution with fresh medium 1:10 every 10–14 days.
According to the method described for Drosophila Kc-
cells (Stevens and O'Connor, 1982) growth inhibition
was used to select resistant subclones starting from 0.1
mM RH 5992 up to a final concentration of 0.1 μM.
Gradually increasing concentrations of the hormone mimick were added over a period of about two years.
After this time individual multicellular spheroids, which
originate from single cells by cell division were picked
under microscopical control, propagated in microtiter
plates and recloned (Spindler-Barth and Spindler, 1998).

2.2. Enzyme assay

Acetylcholinesterase activity was performed as described (Spindler-Barth et al., 1998) using a fluori-
metric assay (Parvari et al., 1983).

2.3. Metabolism of RH 5992

Two milliliters cell culture (7–10 days after dilution)
were harvested by centrifugation (20 s, 10,000 g, 4°C).
The pellets were washed once with PBS (137 mM NaCl,
2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄,
pH=6.7) and resuspended in 1 ml fresh culture medium.
After addition of [¹³C]-RH 5992 (spec. activity 329
kBq/mmol, kindly provided by Dr Carlson and Dr
Dhadialla from Rohm and Haas Company, Spring
House, PA, USA) in a final concentration of 2.5 μM the
cells were incubated for 24 h at 25°C. According to a
modified procedure of Sundaram et al. (1998) 9 ml cold
methanol containing 1% acetic acid was added and
stored overnight at −20°C. After short treatment with
ultrasonic power the samples were centrifuged (10 min,
10,000 g, 4°C). The supernatant was evaporated to 1 ml,
filtered through a 0.22 μm filter, evaporated to dryness
(Speed-Vac concentrator, Bachofer, Reutlingen,
Germany) and finally dissolved in 1 ml methanol. An
aliquot was taken for determination of radioactivity with
a 1600 TR Packard liquid scintillation counter (Packard
Canberra, Frankfurt, Germany). About 15,000 c.p.m.
were separated on a reversed phase column (Radial-Pak,
type 8NVC186, Waters, Milford, MA, USA) using a
Waters 600™ HPLC (Waters, Eschborn, Germany)
coupled to a detector (Radiomatic 500 TR, Canberra
Packard, Frankfurt, Germany). The separation was per-
formed with an acetonitrile: water gradient (each with
0.1% acetic acid) and a flow rate of 1 ml/min.

2.4. Hormone binding studies

Cells were harvested by low speed centrifugation and
washed once with PBS. The pellet was resuspended in
buffer (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1
mM 2-mercaptoethanol, 20% glycerol, pH=7.9, sup-
plemented with aprozin, leupeptin, pepstatin; final con-
centration 1 μg/ml each) and disrupted thoroughly with
an all-glass homogenizer and centrifuged (1 h, 4°C,
100,000 g). Since NaCl impedes hormone binding,
supernatants were desalted immediately with a Sephadex
G-25 column (Pharmacia, Uppsala, Sweden) using the
same buffer supplemented only with 20 mM NaCl.

Ligand binding was determined with [³H]-ponasterone
A (specific activity 7.9 Tbq/mmol; a kind gift of Prof.
H. Kayser, Novartis, Switzerland) using a filter assay as
already described in detail (Turberg and Spindler, 1992)
with slight modifications (Rauch et al., 1998). Kᵦ-values
and Bₘₐₓ were calculated according to Scatchard (1949).
Non-specific binding was determined by addition of 0.1
mM 20-OH-ecdysone or according to Chamness and
McGuire (1975) and subtracted for calculation of spe-
cific binding. For competition experiments receptor
preparations were incubated with 2 to 5 nM [³H]-ponas-
terone A for 1 h at room temperature without or with
unlabelled hormone or agonist in various concentrations.

2.5. Expression of nuclear receptors in E. coli

DNA fragments encoding EcR were cloned into the
BamH1 site of E. coli expression plasmid pGEX-KT
(Hakes and Dixon, 1992) and those for USP were cloned
into the Hind III site of pGEX-KG (Guan and Dixon,
1991) as already described (Elke et al., 1997). Overnight
cultures of E. coli strain BL21 (DE3) (Studier et al.,
1990) transformed with the respective expression plas-
mid were diluted 1:50 with fresh medium and grown at
37°C to an OD₆₀₀=0.6. Expression was induced by the
addition of IPTG (final concentration=0.2 mM) and the
culture was incubated further for 3 h at 28°C. Bacteria
were collected by centrifugation, washed once with PBS
(137 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM
KH₂PO₄, pH=7.3) and suspended in buffer (20 mM
HEPES/NaOH, pH=7.9, 20 mM NaCl, 20% glycerol, 1
mM EDTA, 1 mM 2-mercaptoethanol, 1 μg/ml of each
aprotinin, leupeptin and pepstatin). Cells were disrupted
at 0°C by sonification (Branson Sonifier B-12, Branson,
Danbury, CT, USA) using a microtip for 3×3 s with 90
W. Homogenates were centrifuged (30 min, 10,000 g,
4°C) and the supernatants used for rescue experiments.
280 ng EcR or 770 ng USP, quantified by silver stained
gels, were added to 350 μl cell extract from the resistant
clon rh-r.3

2.6. Electrophoresis and Western blots

Cell pellets were washed and dissolved in sample
buffer (100 mM Tris, 3% SDS, 1 mM 2-mercapto-
ethanol, pH=8.8), cell extracts were diluted with the same
volume of double concentrated loading buffer (sample
buffer containing 0.1% Bromophenol Blue) and boiled
for 5 min (Lämmli, 1970). 20 µg protein/lane (BCA-reagent, Pierce, Rockford, Illinois, USA) were loaded on a 1% SDS-gel (0.6xMDE gel solution, Boehringer, Ingelheim, Germany; AT Biochem., Minigel Twin, 8.6x7.2x0.1 cm, Biometra, Göttingen, Germany). Gels were electroblotted on nitrocellulose membranes (BA 85, 45 µm pore size, Schleicher and Schuell, Dassel, Germany). The membranes were soaked in blocking buffer (5% milk powder, 1% fat, in PBS, 0.05% Tween 20, pH=6.8) and probed with either an affinity purified polyclonal antibody directed against the D-domain of the cEcR, designated pABcE/D (Wegmann et al., 1995) (kind gift of Prof. M. Lezzi, ETH Zürich, Switzerland) or with a monoclonal antibody (AB 11) directed against the conserved 5’ end of the DNA binding domain common to all USPs (Khoury-Christianson et al., 1992) (kindly provided by Prof. F.C. Kafatos, EMBL, Heidelberg, Germany). The membranes were electroblotted on nitrocellulose membranes (BA 85, 45 µm pore size, Schleicher and Schuell, Dassel, Germany). The membranes were soaked in blocking buffer (5% milk powder, 1% fat, in PBS, 0.05% Tween 20, pH=6.8) and probed with either an affinity purified polyclonal antibody directed against the D-domain of the cEcR, designated pABcE/D (Wegmann et al., 1995) (kind gift of Prof. M. Lezzi, ETH Zürich, Switzerland) or with a monoclonal antibody (AB 11) directed against the conserved 5’ end of the DNA binding domain common to all USPs (Khoury-Christianson et al., 1992) (kindly provided by Prof. F.C. Kafatos, EMBL, Heidelberg, Germany) (Khyse-Andersen, 1984) as described in detail (Rauch et al., 1998). Protein bands were visualised with an ECL detection kit (Amersham-Pharmacia, Uppsala, Sweden) on X-ray films (Kodak Biomax) according to the instructions of the supplier. Quantitative evaluation of receptor concentration was done as described (Rauch et al., 1998).

3. Results

Clones resistant to RH 5992 were obtained by the same selection procedure (Stevens and O’Connor, 1982) as applied previously to select ecdysteroid resistant clones (Spindler-Barth and Spindler, 1998). A subclone was considered to be resistant, if no morphological response (Spindler-Barth et al., 1992) and no increase in acetylcholinesterase activity (Spindler-Barth and Spindler, 1998) was observed after treatment with 0.1 µM RH 5992 or 1 µM 20-OH-ecdysone (Fig. 1). Subclasses selected after about two years in the continuous presence of tefubenzoxide were kept for 20 days in hormone free medium to rule out transient receptor down-regulation. Once acquired, resistance was stable, since cultivation of e.g., rh-r.3 in the absence of the diacylhydrazine for about one year did not restore hormone sensitivity (data not shown).

Hormone resistance is frequently accompanied by increased hormone metabolism in those clones, which were selected under the continuous presence of 20-OH-ecdysone (Kayser et al., 1997); therefore we checked metabolism of RH 5992. The hormone mimick was degraded even in wild-type cells to polar compounds (Fig. 2). Since products with the same retention time under identical separation conditions were biologically inactive (Dhadialla, personal communication) these compounds were not analysed further. The pattern of degradation products in all resistant subclones tested so far was the same as for sensitive cells (data not shown). The extent of metabolism of tefubenzoxide varies about five-fold between different resistant clones, but the determination of the metabolic rate in the same clone is highly reproducible (Fig. 3A, B). RH 5992 metabolism did never exceed the rate found in wild-type cells and was sometimes considerably lower (Fig. 3B).

According to Western blots in all resistant clones EcR and USP were expressed in comparable concentrations as in wild-type cells (Rauch et al., 1998), but in three cases (rh-r.4, rh-r.5 and rh-r.6) the intensity of the phosphorylated band with the lowest electrophoretic mobility was reduced in USP (Fig. 4B, Table 1). Enhanced expression of EcR and hyperphosphorylation of USP after treatment with 20-OH-ecdysone (Rauch et al., 1998), as observed in wild-type cells was not found in resistant clones (Fig. 4).

Binding of [3H]-ponasterone A was affected in different ways. Reduced binding capacities, but normal affinity constants were observed (Table 1, Fig. 5). The selective loss of one high-affinity binding site (either K_D1 or K_D2) as well as complete loss of hormone binding were also found. In clone rh-r.3 [3H]-ponasterone A binding was rescued by addition of bacterially expressed EcR as shown in Fig. 6. Addition of in vitro translated EcR (data not shown) gave identical results. In contrast addition of USP has no effect on ligand binding.

In one resistant clone (rh-r.2) RH 5992 competed [3H]-ponasterone A less efficiently compared to wild-type cells. In addition to the considerably reduced number of binding sites this clone had an altered ligand binding specificity compared to wild-type cells. (Fig. 7, Table 2).

4. Discussion

Although RH 5992 is degraded within a few days in wild-type cells, the stability of tefubenzoxide is sufficient to allow selection of resistant clones. As outlined pre-
Fig. 2. Metabolism of RH 5992. Wild-type cells (7–10 days after dilution) were incubated with about 2.5 \textmu M \[^{14}C\]-RH 5992 (spec. activity=329 KBq/mmol) for 24 h at 25°C. (A) Degradation products were separated on a RP18 column with acetonitrile: water gradient, flow rate 1 ml/min. Radioactivity was monitored with an in line detector. (B) Control with incubation time 0 h. (C) Incubation time 24 h, retention time of non-degraded RH 5992 49.30 min, retention time of the main metabolite 5.00 min.

Usually (Spindler-Barth and Spindler, 1998), monitoring of acetylcholinesterase activity is well suited to determine resistance against ecdysteroids and hormone mimics, since hormonal stimulation of this enzyme up to about 30-fold (Spindler-Barth et al., 1988) allows the detection of very weak residual hormone responses. Monitoring of an early gene (Sundaram et al., 1998) lets expect preferentially the detection of defects in the hormone signalling pathway itself. Increase in acetylcholine-

sterase activity is a late hormone effect. Nevertheless, with this selection procedure using tefubenozide, clones with receptor defects were obtained. Selection in the continuous presence of 20-OH-ecdysone (Spindler-Barth and Spindler, 1998) resulted first in resistant clones with enhanced ecdysteroid metabolism (Kayser et al., 1997) and only at a later stage additional receptor defects were observed (own unpublished results). This means that the two most important mechanisms for insecticide resistance, metabolism and target site resistance (Mullin and Scott, 1992) against ecdysteroids and agonists arise spontaneously in the Chironomus cell line.

The ability to handle excessive concentrations of moulting hormones varies considerably among insect species and is mainly due to different efficiencies for inactivation and excretion (Blackford and Dinan, 1997a,b). Induction of inactivating enzymes after application of ecdysteroids or RH 5849 seems to be a naturally occurring mechanism as observed in Manduca (Williams et al., 1997). Increased susceptibility to tefubenozide in the presence of a monoxygenase inhibitor demonstrates also the importance of metabolism (Smagghe et al., 1998). Meanwhile in the codling moth Cydia pomonella cross-resistance with tefubenozide was observed in a field population resistant to benzoylphenylureas (Sauphanor and Bouvier, 1995) which is most likely explained by an increase in inactivating enzymes involved in the degradation of both insecticides. Altered RH metabolism does not play a role in the resistant subclones of the Chironomus cell line, since the degradation of tefubenozide never exceeds RH 5992 metabolism in wild-type cells. This is in contrast to clones selected in the presence of 20-OH-ecdysone which show enhanced ecdysteroid metabolism but normal ligand binding.

Variation in the efficiency of metabolising enzymes doesn’t seem to be the cause for the selective action of diacylhydrazines (Smagghe and Degheele, 1993; Smagghe et al., 1998) but is caused mainly by active export of these compounds (Sundaram et al., 1998) or altered ligand binding specificity to the ecdysteroid receptor (Smagghe et al., 1996).

Although target site resistance due to receptor mutations seems to be a widespread phenomenon in pest control (Mullin and Scott, 1992) and is also a considerable medical problem (Chrousos et al., 1986), this type of resistance against insect hormone receptors has not yet arisen. For example, juvenile hormone analogues were used as insecticides for more than two decades and no receptor defects were observed under field conditions.

Resistance due to insect hormone receptors was only observed after chemical induced mutagenesis in the laboratory (Wilson and Fabian, 1986; Bender et al., 1997). All ecdysteroid receptor mutants led to mortality in definite stages of development. This confirms the essential role of ecdysteroid action in arthropod development and may indicate that mutations with impaired receptor
function may be hardly compatible with life. In contrast to knock out mutations of RXR in mice (Kastner et al., 1995) which revealed that the abundance of a second isoform prevents defects, in Drosophila even one isoform of the ecdysteroid receptor can not replace the other (Bender et al., 1997).

Selection of stable hormone resistant subclones of the epithelial cell line in the continuous presence of tefubenozide took a period of about two years. Problems of reduced viability occurring in animals subjected to chemical mutagenesis were avoided in the cell line. This indicates that at least target site resistance at the hormone receptor level will not readily arise spontaneously and is an additional advantage of this environmentally friendly and safe class of insecticides. This is confirmed by treatment of Spodoptera larvae for over 12 generations with tefubenozide without loss of susceptibility for up to five generations (Smagghe et al., 1998).

For Chironomus cells cultivation in the continuous presence of hormone or agonist for about two years was necessary to obtain a number of stable hormone resistant subclones. In these clones receptor function but not expression is changed. Hormone resistance in these clones is not caused by down-regulation of receptor protein or receptor specific mRNA as described previously for Drosophila cells (Stevens and O’Connor, 1982; Koelle et al., 1991).

Addition of bacterially expressed receptor, which is still functional, clearly demonstrate that rescue of ligand binding is only possible with EcR but not with USP. This identifies EcR as cause for resistance. Not yet identified factors may play an additional role. This is reported for resistant clones, which arise spontaneously in Drosophila cells, which can not be completely rescued by ecdysteroid receptor in contrast to mutants, which were created by parahomologeous targeting with EcR fragments (Cherbas and Cherbas, 1996, 1997). In this case transfection studies were used, since bacterially expressed ecdysteroid receptor from Drosophila is still not available in a functional state.

The defects in hormone binding point to mutations mainly in the ligand binding domain (LBD). Preliminary
Fig. 4. Western blot of 0.4 M NaCl extracts from wild-type and resistant subclones of Chironomus tentans cell line. Detection of cEcR with antiserum pABcE/D. (A) Resistant subclones show the same phosphorylation pattern as in wild-type cells. (B) Detection of cUSP with monoclonal antibody AB11. In contrast to wild-type cells the phosphorylated band (Rauch et al., 1998) with the lowest electrophoretic mobility is diminished in rh-r.4, rh-r.5 and rh-r.6. (C) In wild-type cells incubation with 20-OH-ecdysone enhances the intensity of the phosphorylated band with the lowest electrophoretic mobility (77 kD), whereas in resistant subclones no hormone effect is observed. -=control; +=hormone treated (1 µM 20-OH-ecdysone, four days). Two wild-type EcR (A) and four wild-type USP (B and C) were shown to demonstrate the reproducibility of the Western blots.

data obtained with gel mobility shift assays (Elke et al., unpublished results) let assume mutations in the DNA-binding domain (DBD) also. In Drosophila, chemically induced mutagenesis with EMS (Bender et al., 1997) produced missence mutations mainly in the LBD and DBD. These regions seem to be most susceptible for mutational variation of nuclear receptors in general, since natural occurring mutations in other steroid hormone receptors e.g., the androgen receptor of patients with partial or complete insensitivity syndrome revealed that these two domains are also preferentially affected (McPhaul et al., 1993; Spindler et al., 1998).

However, it must be considered that the functional domains of steroid hormone receptors are not independent and ligand or DNA binding may be modified allosterically by changes in other functional domains or interactions with other proteins. Heterodimerization of EcR with USP is an absolute requirement for ligand binding in Chironomus, as demonstrated with purified EcR/USP preparations (Grebe and Spindler-Barth, in preparation) and is also important for DNA binding (Voegtli et al., 1998; Elke et al., 1999). Therefore defects in these functions can also be caused by mutations in the dimerization interface. In this context it is interesting that in three resistant mutants phosphorylation of USP, supposed to be essential for heterodimerization of
Table 1
Characterization of resistant subclones from the epithelial cell line from *Chironomus tentans*<sup>a</sup>

<table>
<thead>
<tr>
<th>Clone</th>
<th>$K_{D1}$ [nM]</th>
<th>$B_{max1}$ [fmol/mg protein]</th>
<th>$K_{D2}$ [nM]</th>
<th>$B_{max2}$ [fmol/mg protein]</th>
<th>“n” for $K_D$ and $B_{max}$</th>
<th>Complementation with GST-cEcR [spec. bdg. of ponA in % of pure extract]</th>
<th>cEcR hyperphosphorylation* [% of total receptor]</th>
<th>cUSP hyperphosphorylation* [% of total receptor]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.31±0.28</td>
<td>9.7±6.5</td>
<td>6.5±2.4</td>
<td>142±88</td>
<td>10</td>
<td>n.d.</td>
<td>55±5 ($n=7$)</td>
<td>23±4 ($n=5$)</td>
</tr>
<tr>
<td>rh-r.1</td>
<td>0.4±0.2</td>
<td>5.3±3.7</td>
<td>–</td>
<td>–</td>
<td>3</td>
<td>395</td>
<td>50±8 ($n=7$)</td>
<td>18±3 ($n=4$)</td>
</tr>
<tr>
<td>rh-r.2</td>
<td>0.06</td>
<td>1.2</td>
<td>3.3</td>
<td>15</td>
<td>1</td>
<td>358</td>
<td>50±5 ($n=7$)</td>
<td>17±3 ($n=4$)</td>
</tr>
<tr>
<td>rh-r.3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>6</td>
<td>see Fig. 6B</td>
<td>46±6 ($n=6$)</td>
<td>17±2 ($n=4$)</td>
</tr>
<tr>
<td>rh-r.4</td>
<td>0.08±0.02</td>
<td>1.6±0.6</td>
<td>3.6±2.6</td>
<td>9.3±5.0</td>
<td>3</td>
<td>145</td>
<td>60±6 ($n=7$)</td>
<td>8±2 ($n=4$)</td>
</tr>
<tr>
<td>rh-r.5</td>
<td>–</td>
<td>–</td>
<td>3.8±2.9</td>
<td>26±12</td>
<td>6</td>
<td>393</td>
<td>58±6 ($n=7$)</td>
<td>12±5 ($n=6$)</td>
</tr>
<tr>
<td>rh-r.6</td>
<td>0.96±0.57</td>
<td>7.0±1.3</td>
<td>–</td>
<td>–</td>
<td>5</td>
<td>736</td>
<td>60±12 ($n=7$)</td>
<td>9±3 ($n=6$)</td>
</tr>
</tbody>
</table>

<sup>a</sup> In all clones acetylcholinesterase is no longer induced. RH 5992 metabolism in no case exceeds tefubenozone metabolism in wild-type cells. In contrast to EcR, USP never restores ligand binding. *Band with the lowest electrophoretic mobility.
Fig. 5. [³H]-ponasterone A binding of 0.4 M NaCl extracts from resistant cell clones were calculated according to Scatchard (1949). Non-specific binding was determined and subtracted according to Chamness and McGuire (1975). (A) The same two high-affinity hormone recognition sites ($K_D^{1}=0.1$ nM, $K_D^{2}=7.1$ nM) are present as in wild-type cells (Rauch et al., 1998). The number of binding sites is reduced. (B) Only the binding site with the higher affinity ($K_D^{1}=0.1$ nM) is observed. (C) Only the binding site with the lower affinity ($K_D^{2}=2.5$ nM) is present. (D) No specific binding is detectable.

EcR/USP in Manduca (Song and Gilbert, 1998) is diminished.

From vertebrate receptors it is known that interactions of nuclear receptor domains either intramolecularly or intermolecularly can modify receptor function (Scheller et al., 1998). In contrast to Drosophila, where only one USP isoform has been found, in some insects two isoforms are expressed (Kapitskaya et al., 1996; Song and Gilbert, 1998) and this seems also the case in Chironomus (Rauch et al., 1998; Voegtli et al., 1999). Although both isoforms confer hormone binding to EcR it is not known, whether these isoforms are associated with different functions and whether they can replace each other in vivo. The fact that both isoforms (Song and Gilbert, 1998) or specific mRNAs (Jindra et al., 1996) are expressed differently during development in Manduca indicates, that there may be also functional differences.

Rescue experiments with either in vitro translated (data not shown) or bacterially expressed nuclear receptors (Fig. 6) identified defects in EcR as cause for resistance and demonstrates that EcR can be expressed in E. coli in a functional state. In order to correlate the observed functional defects with changes in the receptor structure we are now engaged in sequencing the ecdysteroid receptor and ultraspiracle from resistant subclones.

Acknowledgements

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Fig. 6. Reconstitution of specific [3H]-ponasterone A binding ($K_D$ = 2.5 nM) calculated according to Scatchard (1949). (○) Incubation of 0.4 M NaCl extract from the resistant subclone rh-r.3 with bacterially expressed GST-cEcR. (Δ) Same incubation conditions as before with the exception, that GST-cEcR was replaced by GST-cUSP.

Fig. 7. Altered specificity of hormone binding in the resistant subclone rh-r.2 (○) in comparison to the wild-type of Chironomus tentans (Δ). 0.4 M NaCl extracts were incubated with 2–5 nM [3H]-ponasterone A and increasing amounts of non-labelled RH 5992 for 1 h at room temperature (Means±S.D., wild-type, n=3; rh-r.2, n=8).

Table 2
Change in specificity of hormone binding in resistant clone rh-r.2 in comparison to wild-type cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_D$-values [µM]</th>
<th>$K_D$-values [µM]</th>
<th>$K_D$ rh-r.2/K_D wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muristerone A</td>
<td>0.003</td>
<td>0.08±0.02</td>
<td>27</td>
</tr>
<tr>
<td>20-OH-ecdysone</td>
<td>0.4±0.2</td>
<td>19±12</td>
<td>48</td>
</tr>
<tr>
<td>RH 5992</td>
<td>0.02±0.01</td>
<td>193±269</td>
<td>9,700</td>
</tr>
</tbody>
</table>

Kayser, Novartis, Basel, Switzerland). Antiserum pAbEc/E/D, pGBX-kG-cEcR and pGBX-kG-ctMSP were kind gifts from Prof M. Lezzi (ETH Zürich, Switzerland) and antibody AB11 was obtained from Prof F.C. Kafatos (EMBL, Heidelberg, Germany).

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