Signal transduction by dioxin is mediated by the intracellular basic helix-loop-helix dioxin receptor which, in its ligand-activated state, binds to target DNA as a heteromeric complex with the partner factor Arnt. In contrast, the repressed form of the receptor is a complex with hsp90 which appears to maintain the receptor in an inducible conformation. In human keratinocytes dioxin receptor activation has previously been shown to depend on phosphorylation processes. To further dissect mechanisms regulating dioxin receptor function the importance of tyrosine phosphorylation was investigated by the use of specific tyrosine kinase inhibitors. Here we report that the inhibitor genistein inhibited dioxin-dependent induction of expression of the target gene cytochrome P-450IA1. This effect was rapid and reversible and did not lead to altered levels of dioxin receptor protein. Analyses of dioxin receptor or Arnt fusion proteins that function independently of one another showed that the target for genistein action was the dioxin receptor, and, more specifically, a region of the receptor harboring its ligand-binding domain. In addition, function of an unrelated transactivator, the glucocorticoid receptor, was inhibited by genistein while a truncated form lacking the ligand-binding domain was not. A common denominator between the ligand-binding domains of both receptors is their ability to interact with hsp90. Importantly, co-immunoprecipitation experiments showed that genistein inhibited ligand-induced release of hsp90 from the glucocorticoid receptor. Thus, the interaction of these transactivators with hsp90 may be regulated by a tyrosine kinase-dependent pathway.

Induction of cytochrome P-450IA1 (CYP1A1) gene transcription has been studied to dissect the mechanism of action of aromatic hydrocarbons including dioxin (for recent reviews, see Refs. 1 and 2). This environmental contaminant binds to the intracellular dioxin or aryl hydrocarbon receptor that belongs to the basic helix-loop-helix (bHLH) class of transcription factors (3, 4). The receptor is most similar to a protein termed Arnt (5) and the Drosophila melanogaster proteins Per (Period) and Sim (Single Minded). The former protein plays a role in generation of circadian rhythm patterns while Sim is necessary for embryonic development of the nervous system (reviewed in Ref. 6). The non-activated dioxin receptor (7) is similar to some steroid receptors (most notably the glucocorticoid receptor) and protein kinases (e.g. v-Src and c-Raf) complexed with heat shock protein 90 kDa (hsp90, for a recent review, see Ref. 8). Hsp90 seems to maintain the dioxin receptor in an inducible conformation (9). Moreover, studies using a yeast strain in which hsp90 expression levels can be modulated or yeast strains expressing mutant forms of hsp90 indicate that hsp90 may be required for ligand-dependent activation of the glucocorticoid receptor in vivo (10, 11). This effect of hsp90 may be related to the finding that hsp90 can chaperone protein folding of many different classes of proteins (12, 13), apparently including the bHLH factors MyoD and Elavl1 (14, 15). A possibly important role of hsp90 in dioxin receptor function was recently further indicated by the co-localization of ligand and hsp90 binding activities within a minimal region of the dioxin receptor (16).

Challenge with ligand results in nuclear accumulation of the dioxin receptor and activation to a DNA-binding form. The receptor-homologous, non-dioxin binding protein Arnt (5) has been shown to be part of the DNA binding receptor complex (17–20). The activated receptor specifically binds to DNA sequences termed xenobiotic response elements (XREs), present in several copies upstream of the CYP1A1 gene. When positioned upstream of a heterologous promoter XREs can mediate transcriptional regulation by dioxin upon transfection into permissive cells (21, 22).

The non-activated dioxin receptor is a phosphoprotein (23), and, following activation with ligand, three species with different isoelectric properties have been described (24). Moreover, the DNA binding activity of the receptor is lost upon phosphorylation treatment in vitro (25). Several recent reports suggest that dioxin receptor activation is dependent upon functional protein kinase C (23, 26–28). In order to further delineate intracellular signals that modulate receptor activity we have investigated a possible role of tyrosine kinases by using specific inhibitors. Here we report that activation of the dioxin receptor in normal human keratinocytes is dependent upon a putative tyrosine kinase that is specifically inhibited by genistein. Strikingly, activation of a distinct hsp90-associated transactivator, i.e. the glucocorticoid receptor, was also inhibited by genistein, indicating that the target for regulation was interaction of the receptor with hsp90.
MATERIALS AND METHODS

Cell Culture, Treatments, and RNA Analysis—Human keratinocytes were isolated from adult donors and cultured as described previously (29). Cells were maintained in 15% fetal calf serum, 100 pg/ml insulin, 100 pg/ml bovine pituitary extract (Pelfreeze), 100 pg/ml streptomycin, 100 IU/ml penicillin (Nordcell), and 0.25 pg/ml fungizone Nordcell. Cells at the third to fifth passage were routinely used for experiments. In order to maximize the cytochrome P-450A1 induction by TCDF when GENISTEIN INHIBITION OF LIGAND-RECEPTOR FUNCTION  

Amersham, respectively.

Cell Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)—Cells were treated with 50 nm TCDF in the absence or presence of 100 µg genistein for the indicated period of time. Control cultures received MeSO for 1 h before harvest. Nuclei were prepared and protein extracts were isolated as described previously (43). When preparing the cell extract, the cells were broken, after a washing step, by Dounce homogenization in low KC1 extraction buffer (40), KCl was added to a final concentration of 300 mm, and proteins were extracted for 30 min on ice before subsequent centrifugation at 15,000 × g at 4 °C. DNA binding reactions were assembled in a total volume of 20 µl with 10 pg of nuclear or whole cell protein and a final concentration of 25 µm HEPES (pH 7.9), 0.2 mm EDTA, 75 mm KCl, 2 mm MgCl2, 1 mm dithiorethiol, 0.1 mm phenylmethylsulfonfluoride (PMSF), 100 µm iodoacetamide (PMSF), 10 µg/ml leupeptin, 5 µg pepstatin (Boehringer Mannheim), 5% glycerol, 4% Ficoll, 100 µg/ml poly(dI-dC) (Pharmacia Biotech Inc.), and 12.5 µg/ml poly(dA-dT) (Pharmacia) using a double stranded 32P-labeled oligonucleotide as probe. The sequence of which corresponded to 968–997 of the human CYP1A1 gene (5'-CTCCGCGTCTTCTTACCCAAACGCTGGCCCA-3', sense orientation). In competition experiments a 100-fold molar excess of unlabeled oligonucleotide was used. Unspecific competitor DNA was described in Ref. 22. DNA-protein complexes were separated under nondenaturing conditions on a 4% polyacrylamide gel (29,1) run in 1 × TGE (50 mm Tris, 2.7 mm EDTA, and 380 mm glycine) at 4 °C.

Dioxin Receptor Antiserum and Immunoblot Analysis—An anti-dioxin receptor antiserum was raised against a synthetic peptide corresponding to amino acids 12–51 of the murine dioxin receptor (41). The peptide was coupled to keyhole limpet hemocyanin prior to immunization of rabbits (18, 22). Cellular protein was prepared by lysing the cells in 20 mm sodium phosphate (pH 7.2), 50 mm β-glycerophosphate, 10% (v/v) glycerol, 1 mm EDTA, 150 mm NaCl, 0.1 mm Na3VO4, 0.2 mm phenylmethylsulfonfluoride, 5 µm pepstatin, 10 µg/ml leupeptin, 100 KIU/ml aprotinin, and 0.1 mm Nondes P-40, and the lysates were cleared by centrifugation for 45 min at 15,000 × g at 4 °C. Proteins were separated on 9% SDS-polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes, and the membranes were subsequently stained by peroxidase-conjugated swine anti-rabbit immunoglobulin (Dako) using anti-dioxin receptor antiserum (20). When these antibodies were used, we did not observe any cross-reactivity observed, using as substrates luciferin from BioThema and [3H]chloramphenicol from Amersham, respectively.

RESULTS

Genistein Specifically Inhibits Cytochrome P 450A1 mRNA Induction in Human Keratinocytes—To examine a possible importance of tyrosine phosphorylation for the dioxin receptor activation response we initially investigated the effect of tyrosine kinase inhibitors on induction of cytochrome P 450A1 mRNA steady-state levels by the dioxin receptor ligand TCDF. After simultaneous treatment of keratinocyte cultures with TCDF and genistein, a marked reduction of the P 450A1 mRNA induction response was observed (Fig. 1A). Genistein is a competitive inhibitor with regard to ATP, however, it also inhibits the epidermal growth factor receptor tyrosine kinase in a noncompetitive manner. In addition, genistein has been shown to inhibit the effects of other tyrosine kinases including those of the platelet-derived growth factor and insulin receptors (42). These data were reviewed in Ref. 43). Genistein does not inhibit serine and threonine kinases such as protein kinase C (44).

Inhibition of cytochrome P 450A1 mRNA induction by genistein was dose-dependent. At a concentration of 100 µg/ml, cytochrome P 450A1 mRNA levels were reduced to ~2% of the levels obtained in the absence of genistein. In order to test the specificity of the inhibition caused by genistein an inactive isomer, daidzein (44), was used. Daidzein did not influence cytochrome P 450A1 mRNA induction by TCDF when

Genistein Inhibition of Ligand-Receptor Function
Genistein Inhibition of Ligand-induced Receptor Function

**Fig. 1. Inhibition of the cytochrome P-450IA1 mRNA induction response by tyrosine kinase inhibitors.** Representative Northern blot analyses of cytochrome P-450IA1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA steady-state levels are shown. A, human keratinocytes were simultaneously treated with 50 nM TCDF and increasing concentrations of genistein (25, 50, 100, and 200 μM, respectively), daidzein (50 and 100 μM), or herbimycin A (0.04, 0.2, 1.0, and 5 μg/ml, respectively). Cells were harvested after 24 h of treatment and RNA prepared for analysis. B, human keratinocytes were treated with 50 nM TCDF for 24 (lanes 2 and 4) and 48 h (lanes 3 and 5) in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of 100 μM genistein (GENI). These P-450IA1 mRNA levels should be compared to the levels obtained in lane 6 where the cells first received TCDF and genistein for 24 h and then TCDF only for additional 24 h. The cells represented by lane 7 had received TCDF for 24 h before genistein was added for the remaining 24 h before harvest.

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Additionally, the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA steady-state levels are shown. The results indicate that genistein and herbimycin A often inhibit the same cellular processes (reviewed in Ref. 45). As shown in Fig. 1A, 5.0 μg/ml herbimycin A decreased the cytochrome P-450IA1 induction response to a similarly low level as did genistein. The treatments did not, however, influence expression of the mRNA levels for the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase that were analyzed in control experiments (Fig. 1A).

Inhibition of cytochrome P-450IA1 mRNA induction by genistein was reversible since P-450IA1 mRNA levels could be induced by TCDF back to maximal levels 24 h following withdrawal of genistein treatment (Fig. 1B, compare lanes 2, 4, and 6). Interestingly, genistein could suppress already maximally induced P-450IA1 expression levels (Fig. 1B, compare lanes 3 and 7). Taken together, these results suggest that the inhibitory effect on tyrosine kinase activity. In addition to regulation of tyrosine kinase activity, genistein has been reported to inhibit topoisomerase II (46). However, this effect was not produced by topoisomerase II inhibitors including, for instance, VP-16 and Novobiocin (data not shown).

These results argue that the effect of genistein was not due to toxicity or an irreversible change in cellular functions. Moreover, the putative target tyrosine kinase activity seemed to be necessary not only to produce the initial induction response but also for maintaining prolonged induction. Since dioxin receptor ligands evoke a strikingly pleiotropic response in vivo (47), we compared the effect of genistein on cytochrome P-450IA1 expression in keratinocytes to that produced by genistein in human cells of another origin. These experiments demonstrated that genistein was inert as an inhibitor of the induction response in the human hepatoma cell line HepG2 (data not shown).

Genistein Inhibits Induction of Dioxin-responsive Promoters—As demonstrated in Fig. 2A, sensitivity to genistein could be conferred to a chloramphenicol acetyltransferase reporter gene under the control of the -1140 to +2435 portion of the human CYPIA1 gene (pRNH11C). Human keratinocytes were transiently transfected with this reporter gene construct, and chloramphenicol acetyltransferase activity was determined after 40 h of treatment with TCDF, genistein, or a combination of both. To test if genistein was affecting the TCDF induction response through the dioxin receptor binding sequence, a minimal reporter gene construct containing two directly repeated XREs in front of the thymidine kinase promoter (pTX.DIR) was also introduced into keratinocytes in parallel experiments. Transiently transfected cells were treated with TCDF in the absence or presence of genistein prior to determination of luciferase activity. The TCDF induction response of this minimal dioxin-responsive promoter construct was also inhibited by genistein (Fig. 2B). pTX.DIR was also cotransfected with cytomegalovirus enhancer/promoter-driven expression vectors for Arnt (pCMV-Arnt) and the dioxin receptor (pCMV-DR). Increased expression of dioxin receptor and Arnt resulted in a 20-fold TCDF induction response compared to the 3.5-fold induction response produced by the endogenous receptor-Arnt complex (Fig. 2C). This enhanced TCDF induction response was as sensitive to genistein treatment as the endogenous response (Fig. 2C). Hence, under these conditions, the activity of the putative target tyrosine kinase that was inhibited by genistein did not seem to be limiting in regulation of dioxin receptor function.

To rule out the possibility that genistein was specifically down-regulating the levels of the dioxin receptor protein in human keratinocytes, we prepared whole cell extracts from both untreated and genistein-treated cells, and receptor levels were monitored by immunoblot analysis using an anti-dioxin receptor antisera. As demonstrated in Fig. 3, genistein did not have any effect on the intracellular levels of the receptor. Moreover, cellular levels of Arnt were not altered by genistein treatment (data not shown).

Rapid Inhibition of Activation of the Dioxin Receptor to Its DNA Binding Form by Genistein—In view of the results above and the background that the DNA binding activity of the dioxin receptor appears to be modulated by phosphorylation-dependent processes (26, 23), we wanted to examine if genistein treatment could affect the DNA binding activity of the
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A, human keratinocytes (25 cm$^2$) were transfected with 2 µg of the CAT reporter vector pRNH11C that contains a portion (-1140 to +2435) of the human cytochrome P-450IA1 gene. CAT activity was analyzed by thin layer chromatography after treatment with 50 nM TCDF and/or 100 µM genistein (GENI) for 40 h. Cellular extracts were normalized according to the level of luciferase activity obtained from 0.2 µg of the co-transfected control plasmid pRSV-Luc. The mobilities of [Cl-chloramphenicol (CM) and acetylated products (AcCM) are indicated.

B, human keratinocytes (10 cm$^2$) were transfected with 2 µg of a luciferase reporter plasmid driven by an XRE containing minimal TK promoter (pTX.DIR). Luciferase activities were determined after treatment with 50 nM TCDF in the presence or absence of 100 µM genistein. The values have been normalized to the protein content of the cellular extract and to the activity of the parental construct lacking the XRE sequence (pTX1). The bars represent average values obtained from three independent experiments.

C, human keratinocytes (10 cm$^2$) were transfected with 1.5 µg of pTX.DIR and 1 µg each of dioxin receptor and Arnt expression plasmids, and luciferase activity was assayed after treatment with 50 nM TCDF in the presence or absence of 100 µM genistein (GENI). The values have been normalized to the protein content of the cellular extracts. The bars represent average values obtained from two independent experiments.

Fig. 2. Inhibition by genistein of TCDF-induced expression of reporter gene constructs containing dioxin-responsive promoters.

A, human keratinocytes that had been treated for 1 h with TCDF in the absence or presence of genistein. These extracts were analyzed for the DNA-binding form of the dioxin receptor (Fig. 4A) by EMSA using a $^{32}$P-labeled XRE oligonucleotide as a probe. Genistein treatment potently inhibited XRE binding by the dioxin receptor (compare lanes 2 and 3), whereas it did not affect the DNA binding activity of the constitutive and ubiquitous bHLH transcription factor USF (compare lanes 4-6). To establish that inhibition was not due to a change in intracellular distribution of the ligand-activated receptor, EMSA was also performed using whole cell extracts. As shown in Fig. 4B, addition of genistein almost completely inhibited formation of a TCDF-induced XRE-receptor complex. The effect of genistein was rapid since simultaneous treatment with both compounds was as efficient in inhibiting DNA binding activity by the receptor as pretreatment of cells with genistein prior to addition of TCDF. In fact, even addition of genistein to human keratinocytes during the last hour of a 2-h long TCDF treatment period was sufficient to inhibit the XRE binding activity of the dioxin receptor (Fig. 4B). This result supports the model that a genistein-sensitive tyrosine kinase activity might be important either for the receptor activation process or for maintenance of the ligand-activated receptor form.

The Ligand-binding Domain of the Dioxin Receptor Is the Target for Negative Regulation by Genistein—In order to deter-
mine if a specific dioxin receptor subunit or both were targeted by the action of genistein, we analyzed the activity of chimeric protein constructs in transiently transfected cells. In these experiments dioxin receptor or Arnt protein fragments lacking their very N-terminal bHLH domains were fused to a constitutively active glucocorticoid receptor fragment (rDBD) containing its DNA-binding (DBD) and major transactivating (τ) domains (shown schematically in Fig. 5A). Importantly, the rDBD fragment lacks the large C-terminal portion of the glucocorticoid receptor harboring its hormone-binding domain and is a constitutive transcriptional regulator. In contrast to wild-type dioxin receptor or Arnt, the resulting rDBD-dioxin receptor (rDBD/DR, Ref. 16) or rDBD/Arnt fusion proteins (Fig. 5A) regulate transcription of glucocorticoid response element (GRE)- driven reporter genes independently of their endogenous bHLH partner factor. Thus, the glucocorticoid receptor transactivation domain of rDBD/DR construct is conditionally regulated by the ligand-binding domain of the dioxin receptor in an Arnt-independent fashion in cells of hepatic origin (16).

The chimeric receptor constructs were transiently transfected into keratinocytes, and their activity was assayed as their ability to induce GRE-dependent expression of a co-transfected MMTV promoter-driven alkaline phosphatase reporter gene (pMMTV-AF) or a herpes simplex thymidine kinase promoter-driven luciferase reporter gene (p(GRE)₅T105Luc). Based on this assay, activation of the GRE-driven promoter by dioxin could be examined. Notably, the C-terminal part of Arnt (rDBD/Arnt) seemed to harbor a potent transactivating capacity that was unaffected by TCDF and genistein treatment (Fig. 5B). As expected, both rDBD/DRaa:83–805 and rDBD/DRaa:83–593 were repressed in the absence of dioxin and derepressed upon exposure to dioxin (Fig. 5B). TCDF-induced activity of the rDBD/DRaa:83–805 fusion protein was efficiently abolished by genistein treatment while the activity of the rDBD control was unaltered by this treatment (Fig. 5B). In addition, the same result was obtained using the rDBD/DRaa:83–593 construct lacking the C-terminal transactivation motif (16).

Genistein Inhibits Ligand-dependent Activation of the Glucocorticoid Receptor—Although the parental rDBD fragment did not show genistein sensitivity (Fig. 5B), the full-length glucocorticoid receptor construct did (Fig. 5C). These results argue against the notion that DNA binding per se was affected by genistein. On the contrary, both the C-terminal hormone-binding domain of the glucocorticoid receptor and a large portion of the dioxin receptor containing its ligand-binding domain appeared both to be the targets of negative regulation by genistein treatment. These domains are under noninduced conditions mediating association with hsp90.

To assess if the inhibitory action of genistein was at the level of the receptor-hsp90 interaction, keratinocytes were treated for 2 h with 5 μM dexamethasone or vehicle alone in the absence or presence of 100 μM genistein. Subsequently, cellular extracts were prepared and analyzed by an hsp90 co-immunoprecipitation assay. The glucocorticoid receptor was visualized by im-
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**Fig. 5.** Genistein inhibits transactivation by the τDBD/DR fusion protein and full-length glucocorticoid receptor. A, a schematic representation is shown of the wild-type human glucocorticoid receptor (hGR), mouse dioxin receptor (mDR), and the human Arnt protein (hArnt) and their functional domains. The transactivating and DNA-binding domains of the glucocorticoid receptor are indicated by τ1 and DBD, respectively. In the case of the dioxin receptor and Arnt, sequence motifs are indicated by bHLH, Per-Arnt-Sims (PAS) homology region containing PAS repeats A and B, and a glutamine-rich sequence (Q). The chimeric receptor constructs contain the N-terminal 500 amino acids of the hGR (termed τDBD) fused to varying C-terminal segments of the dioxin receptor and Arnt. B, human keratinocytes (10 cm²) were transfected with 1 µg of pMMTV-AF as a reporter and 1 µg of expression plasmid encoding the indicated glucocorticoid receptor derivatives. The bars represent average values of four experiments and have been normalized to the activity obtained from transfections with pMMTV-AF only. C, human keratinocytes (10 cm²) were transfected with 1 µg of pMtGlucocorticoid receptor (pmtGR) expression plasmid and 1 µg of the reporter constructs pMMTV-AF or p(GRE)₅TI05Luc. The bars show a representative experiment, and the values have been normalized according to the protein content of each extract and to the activity obtained from transfection with pMMTV-AF or p(GRE)₅TI05Luc only. The rationale for also using p(GRE)₅TI05Luc. The rationale for also using p(GRE)₅TI05Luc is that the alkaline phosphatase activity can be induced in response to glucocorticoids in some cell types. Note that the induction of p(GRE)₅TI05Luc was inhibited by genistein treatment while pMMTV-AF and pDBD/Arnt were unaffected by treatment.

**DISCUSSION**

We have shown that the tyrosine kinase inhibitor genistein caused a rapid and reversible inhibition of ligand-induced activation of the dioxin receptor. This mode of regulation has important functional consequences for XRE-mediated induction of gene expression. The target for genistein action was the dioxin receptor and not the Arnt partner factor. In addition, ligand-dependent activation of the glucocorticoid receptor was inhibited by genistein treatment. In analogy to the dioxin receptor system (16), the ligand-binding domain of the glucocorticoid receptor mediates association with the molecular chaperone hsp90 in the absence of ligand. Thus, it is plausible that maintenance of the receptor in an inducible conformation by the action of hsp90 is a regulated event requiring input from distinct intracellular signal systems.

**Fig. 6.** Co-immunoprecipitation of the glucocorticoid receptor with hsp90-specific antibodies. Human keratinocytes were treated for 2 h with vehicle alone (lanes 1 and 2), 5 µM dexamethasone (lanes 3 and 5), or 100 µM genistein (lanes 4 and 5) and cytosolic extracts were prepared. Extracts were immunoprecipitated with hsp90-specific antibodies as described under "Materials and Methods," and analyzed for the glucocorticoid receptor by SDS-PAGE and subsequent immunoblotting using a glucocorticoid receptor-specific antibody. In lane 1 the hsp90 antibody is excluded. The positions of the glucocorticoid receptor (GR) and of the molecular mass standard proteins are indicated.

Protein kinase C has previously been implicated in regulation of dioxin receptor function (23, 26–28). Consequently, an interesting question is if the genistein-sensitive putative tyrosine kinase is upstream of protein kinase C in a phosphoryla-
tion cascade, that is important for dioxin receptor function or whether these kinase activities belong to two independent signal pathways that converge at the dioxin receptor level. Although mechanistically not understood, it has recently been indicated that second messenger activation may influence glucocorticoid receptor activity (48). Genistein-sensitive tyrosine phosphorylation processes have been shown to constitute an early event in keratinocyte differentiation (45). It is an intriguing possibility that cellular differentiation processes may influence genistein-sensitive signaling pathways. In murine keratinocytes, phospholipase C activity is induced in vitro in response to a differentiation signal (elevated extracellular Ca++) and this activity is inhibited by genistein (50). Phospholipase C activity can, in turn, lead to activation of protein kinase C (recently reviewed in Ref. 31). Moreover, terminal differentiation of keratinocytes is important for high-level induction of cytochrome P-450IA1 mRNA expression by dioxin (29, 52). In this context it is interesting to note that the effect of genistein on the cytochrome P-450IA1 induction response seemed to differ between cell types. For instance, induction of cytochrome P-450IA1 mRNA levels by TCDD was not altered in human hepatoma cells by genistein treatment. A similar observation has been made using murine hepatoma cells (26). This apparent insensitivity of hepatoma cells to genistein can be interpreted as cell type-specific differences in the putative tyrosine kinase-mediated intracellular signaling pathway. However, this may rather be a reflection of the cellular state of the hepatoma cells in culture.

Since dioxin receptor function is not immediately dependent on exogenously added growth factors in keratinocytes, one could speculate that the signaling pathway that is inhibited by genistein is constitutive in keratinocytes due to, possibly, autocrine stimulation. Interestingly, keratinocytes produce cytokines such as interleukin-1, the action of which has been shown to be specifically inhibited not only by genistein but also by herbimycin A (53). Both these compounds are equally potent in inhibiting induction of cytochrome P-450IA1 mRNA levels by TCDD. Moreover, the epidermal growth factor receptor is a tyrosine kinase that is inhibited by both genistein and herbimycin A. Evidently, several possibilities exist to account for constitutive genistein-sensitive phosphorylation in human differentiated keratinocytes.

There are several phosphorylation-dependent regulatory events that may be necessary to produce a dioxin receptor form with capacity to bind to DNA. The dioxin receptor is a phosphoprotein in its non-activated state (23) and both its Arrt dimerization and DNA binding activities appear to be modulated by phosphorylation-dependent processes (23). By creating fusion proteins of the dioxin receptor and Arrt fragments (lacking their corresponding bHLH motifs) with a heterologous DNA-binding domain the independent function of these two factors can be studied in transiently transfected cells. These experiments showed that the target subunit for regulation by genistein was the dioxin receptor and not Arrt. Importantly, Arrt does not interact with hsp90 (43, 54) while the dioxin receptor in its inactive conformation forms a stable complex with hsp90 (reviewed in Ref. 1). It is noteworthy that the glucocorticoid receptor belongs to a distinct class of inducible transactivators that is stably associated with hsp90 in its latent form (for recent reviews, see Refs. 8 and 55). Although the glucocorticoid receptor is phosphorylated the functional significance of this modification remains unclear (56). Remarkably, treatment of human keratinocytes with genistein also inhibited glucocorticoid receptor-mediated induction of target genes. However, the activity of the constitutively active rDBD glucocorticoid receptor fragment was unaffected by genistein treatment. The rDBD fragment lacks the hormone-binding domain of the glucocorticoid receptor and does not bind to hsp90 (57, 58). Moreover, we observed that genistein inhibited hormone-induced dissociation of hsp90 from the glucocorticoid receptor, as assessed in a co-immunoprecipitation assay. Thus, a common functional property between the genistein-sensitive proteins described here is association with hsp90. These data suggest that tyrosine phosphorylation may be important for a very early step in receptor regulation, i.e. interaction between the receptors and hsp90. The inhibitory effect of genistein may be exerted indirectly through hsp90 or directly on the dioxin and glucocorticoid receptor proteins. In order to examine these models in closer detail and in order to identify the putative tyrosine kinase and its regulatory role it will be necessary to specify the target amino acid(s) in the receptor and/or hsp90 proteins. Against this background, regulation of receptor function by genistein may prove to be a valuable tool to further understand the role of hsp90 in modulating the function of a subclass of inducible transactivators in vivo.

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