Benzoquinoid ansamycins (herbimycin A and geldanamycin) interfere with the maturation of growth factor receptor tyrosine kinases

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Abstract Benzoquinoid ansamycins, such as herbimycin A (HA) and geldanamycin (GA), are antibiotics that exhibit anti-tumor effects. These compounds have been shown to result in the intracellular depletion of important growth signaling molecules. Recently, GA has been shown to bind tightly to Hsp90, thereby implicating Hsp90 as a possible chaperone for those signaling molecules adversely affected by the benzoquinoid ansamycins. Here we have investigated the effects of HA and GA on the synthesis, maturation and stability of different protein tyrosine kinases. Exposing cells to either compound blocked normal maturation of the epidermal growth factor (EGF) receptor, platelet-derived growth factor (PDGF) receptor, and pp60^{v-src}. We show that only the nascent forms of the EGF and PDGF receptors are degraded under these conditions. Once the newly synthesized receptors had been translocated into the endoplasmic reticulum membrane, addition of the drugs no longer affected their stability. For the cytoplasmic tyrosine kinase, pp60^{v-src}, both the nascent as well as the mature forms of the protein were degraded in cells treated with the drugs. We discuss these observations as they pertain to the possible role of Hsp90 as a substrate-specific molecular chaperone, perhaps involved in the maturation and/or stability of proteins important for growth control.

INTRODUCTION

Many of the heat shock proteins (Hsps), most of which are expressed constitutively in all cell types, function as 'molecular chaperones', components important for protein maturation. While not conveying any direct information for the folding event, molecular chaperones appear to facilitate the folding process by reducing the possibility of misfolding and/or aggregation (reviewed by Georgopoulos and Welch 1993; Hartl 1996). Members of the Hsp70 family of Hsps, for example, bind to nascent polypeptides either being synthesized on the ribosome or translocated into the lumen of organelles. Inter-

action with the particular Hsp70 member is believed to help stabilize the maturing polypeptide, probably by preventing non-productive intra- or inter-molecular interactions that might lead to incorrect folding and subsequent aggregation. Once synthesis or translocation of the nascent polypeptide has been completed, and now with all the necessary information for protein folding being present, the Hsp70 chaperone is released from its target in an ATP-dependent fashion. Once released, folding of the newly synthesized or translocated polypeptide commences. For at least some polypeptides, subsequent interactions with yet other members of the molecular chaperone family may be required for their correct folding and/or higher ordered oligomeric assembly.

Based on recent *in vitro* studies, some investigators have suggested that Hsp90 also might be operating as a molecular chaperone (reviewed by Johnson and Craig 1997). For example Hsp90, with the help of other molecular chaperones, has been reported to play a role in both

Received 26 February 1998; Revised 30 April 1998; Accepted 30 April 1998
Available on line 10 February 1999

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preventing heat-induced protein aggregation and/or facilitating the refolding of already denatured polypeptides. Here Hsp90 was observed to 'hold' the polypeptide substrate, while the other molecular chaperones, in an ATP-dependent manner, helped facilitate the refolding event.

Other studies indicate that Hsp90 may function as a type of 'substrate-specific' molecular chaperone (reviewed by Pratt 1997; Pratt and Toft 1997). For example, Hsp90 is believed to participate in the maturation and/or stability of different steroid hormone receptors. Less well characterized is a possible role for Hsp90 in facilitating the maturation and perhaps regulation of certain protein kinases. Here Hsp90 has been identified in complexes with different members of the src family of protein kinases, Raf-1 and finally, a protein kinase specific co-factor Cdc37 (also known as p50). However, the exact role of Hsp90 as it pertains to the activity of these different protein kinases is still unclear.

Proving to be useful in helping sort out the structure and function of Hsp90 are a class of antibiotics that bind tightly to cytosolic Hsp90 and its homologue, Gsp94, present within the lumen of the endoplasmic reticulum (Whitesell et al 1994; Chavany et al 1996). These compounds, known as benzoquinoid ansamycins and represented by geldanamycin (GA) and herbimycin A (HA), have been reported to exhibit anti-tumor effects, at least in cells grown in culture. For example, both compounds elicit a morphological reversion of cells transformed by different members of the src family of oncogenic tyrosine protein kinases (Murakami et al 1988; Uehara et al 1988, 1989; Whitesell et al 1992). Mechanistically, the drugs have been shown to interfere with the interaction of Hsp90 with the newly synthesized forms of the different src-related kinases, thereby leading to the subsequent degradation of the particular kinase. (Hartson and Matts 1994; Whitesell et al 1994). As a consequence, the overall levels of active src-kinase decline over time such that the cells eventually exhibit reversion of the transformed phenotype. Others similarly have shown that HA and GA interfere with the maturation and/or stability of a number of protein kinases including Raf-1, Cdk4 and Bcr/Abl (Okabe et al 1994; Schulte et al 1995; Schneider et al 1996; Stepanova et al 1996; Stancato et al 1997).

The present study examined the effects of both HA and GA on protein maturation. In particular, we examined whether these drugs adversely affect protein maturation events in general, or whether they target only a select group of proteins involved in growth control. The results confirm and extend earlier observations showing that these compounds interfere with the maturation of viral-pp60^{src} and the epidermal growth factor receptor (EGFR) (Miller et al 1994; Murakami et al 1994; Whitesell et al

1994; Sepp-Lorenzino et al 1995; Chavany et al 1996; Minnaugh et al 1996). In addition, the maturation of yet another growth factor receptor with tyrosine kinase activity, the platelet-derived growth factor receptor (PDGFR), was blocked in cells treated with either HA or GA. Of particular interest were the observations showing that only those forms of the receptor which are in the course of synthesis appear to be affected adversely by the drugs. Once the newly synthesized receptor has been integrated into the membrane of the endoplasmic reticulum (and/or moved to the plasma membrane) addition of the drugs now had no deleterious effect on further receptor maturation and/or stability.

MATERIALS AND METHODS

Reagents

HA and GA were purchased from Gibco BRL (Gaithersburg, MD, USA). Tunicamycin and Brefeldin A were obtained from Sigma (St Louis, MO, USA). PNGase F was purchased from New England Biolabs (Beverly, MA, USA). [³⁵S]methionine was purchased from NEN (Translabel, specific activity of 1000 Ci/mMol). Proteasome inhibitor (MG132) was obtained from CalBiochem (La Jolla, CA, USA). Monoclonal antibodies (mAbs) against EGFR (B4G7 and EGFR-#1) were obtained from Takara (Kyoto, Japan) and Oncogene Research Products (Cambridge, MA, USA), respectively. Anti-Hsp90 mAb (9D2) and anti-Gsp94 (9G10) were obtained from StressGen Biotechnologies (Victoria, BC, Canada). Anti-phosphotyrosine mAb (4G10) was from Upstate Biotechnology (Lake Placid, NY, USA). Anti-pp60^{v-src} mAb (327) was the generous gift of Dr Dana Aftab and Dr S. G. Martin (UC Berkeley, CA, USA). PDGF receptor antibody was provided by L. Williams (UC San Francisco, CA, USA) (Klippel et al 1992).

Cell culture and metabolic labeling

A431 human epidermal carcinoma cells were grown on plastic dishes in bovine calf serum (CS) at 37°C. For metabolic pulse labeling, the medium was removed, the cells were washed twice with Dulbecco's modified eagle medium (DMEM) lacking methionine, and the cells then were incubated with DMEM minus methionine but supplemented with [³⁵S]methionine (100 µCi/750 µl). Cells (with or without drugs) were pulse-labeled for 30 min at 37°C, or pulse-labeled and chased for varying time periods as indicated in the Figure legends. For the latter, the culture medium containing the radiolabel was removed, the cells washed three times with 10% CS-DMEM, and the cells then incubated further in DMEM plus 10% CS for 2 or 3 h. For steady state labeling, the cells were

washed three times with DMEM lacking methionine, and incubated with [35 S]methionine (50 μ Ci/ml) in medium consisting of 97% methionine-free DMEM, 3% normal DMEM and 5% CS for 15 h at 37°C. CHO cells (2WT) expressing EGF receptor (Morrison et al 1993) and Balb/C 3T3 cells expressing the PDGF-b receptor were grown on plastic dishes in Ham's F12 containing 10% fetal bovine serum (FBS) and DMEM containing 10% CS respectively. NIH 3T3 cells expressing temperature sensitive pp60^{v-src} (NIH3T3/ts UP-1) were grown on polylysine-treated plastic dishes in DMEM containing 10% FBS and 200 μ g/ml G418 at 32°C.

Preparation of cell lysate, immunoprecipitation and gel electrophoresis

For immunoprecipitations, [35 S]methionine-labeled cells were washed with phosphate-buffered saline (PBS), lysed in PBS containing 0.1% Triton X-100, 5 mM MgCl₂, 5 units/ml aprotinase, 50 μ M Na₃VO₄, 10 μ M NaF, and 50 μ g/ml of the protease inhibitor PMSF (phenylmethylsulfonyl fluoride). The lysate was clarified at 16 000 $\times$ g for 10 min., and the supernatant used for the immunoprecipitation analysis. An aliquot from cells lysed as described above were treated with 5 $\times$ concentrated Laemmli sample buffer such that the final concentration was 0.5 $\times$ (e.g. final SDS concentration = 0.5%). The lysate was heated for 5 min at 100°C, clarified at 16 000 $\times$ g for 10 min, and then adjusted to a final 0.1 $\times$ Laemmli sample buffer concentration with RIPA buffer (1% Triton X-100, 1% sodium deoxycholate in PBS). The cells lysates were incubated with the primary antibody at 4°C. Immune complexes were captured by the addition of 40 μ l of a 50% solution of protein A sepharose (rabbit anti-mouse was used for immunoprecipitations using mouse antibody). Following four washes with RIPA buffer, the proteins present within the immunoprecipitate were released from the beads by the addition of Laemmli sample buffer and heating at 95°C for 5 min. In the case of the pp60^{v-src} studies the non-ionic detergent lysed cells were used directly to study the interaction of pp60^{v-src} with Hsp90. Here the immune complexes were washed four times with PBS supplemented with 0.1% Triton X-100 and the proteins then released from the protein A-sepharose as described above.

PNGase F digestion of the secreted EGFR

EGFR was immune-isolated and then released from the protein A sepharose by addition of 0.5% SDS plus 1% β -mercaptoethanol. After adjusting to a final 50 mM sodium phosphate (pH 7.5) and 1% NP-40, PNGase F was added (final 25 U/ μ l). After 1 h at 37°C the reaction was terminated by the addition of concentrated Laemmli sample buffer.

SDS-PAGE

Radiolabeled proteins were analysed by SDS-PAGE using gel formulations as described by Blattler et al (1972).

RESULTS

In the course of examining the effects of benzoquinoid ansamycins on protein synthesis in A431 cells, the disappearance of a polypeptide with an apparent mass of approximately 200 kDa was observed. Because these drugs previously had been reported to adversely affect the function and/or maturation of different tyrosine-specific protein kinases, along with the fact that A431 cells express high levels of the EGFR, a tyrosine kinase of approximately 170 kDa, the status of the EGFR was examined (Murakami et al 1994; Sepp-Lorenzino et al 1995). A431 cells were subjected to metabolic pulse-chase radiolabeling, in either the presence or the absence of HA or GA. Subsequently, the amount of the newly synthesized EGFR was determined by immunoprecipitation. In control cells (i.e. no drug treatment) the radiolabeled EGFR migrated in the gel with an apparent mass of 160 kDa, this representing the newly synthesized and core-glycosylated form of the receptor that is present within the endoplasmic reticulum (ER, Fig. 1B, lane 1). Upon removal of the radiolabel and further incubation of the cells (i.e. pulsed and chased) the EGFR exhibited an apparent mass of 170 kDa (Fig. 1B, lane 2), this now representing the fully glycosylated and mature form of the receptor.

In cells that were pulse-labeled for 30 min in the absence of the drugs, and then further incubated in the presence of either HA or GA (i.e. chased), maturation of the EGFR appeared to be unaffected (Fig. 1B, lanes 3 and 4). Curiously, however, the SDS-PAGE migration of the receptor in the cells chased in the presence of GA was slightly faster than that observed for the EGFR synthesized in the control cells (a point that will be returned to later). In cells pulse-labeled in the presence of either HA or GA and then immediately harvested, the 160 kDa form of the receptor again was observed, albeit at levels somewhat less than those found under control conditions (Fig. 1B, lanes 5 or 8, respectively). Finally, in cells both pulse-labeled and chased in the continued presence of the drugs, none of the newly synthesized and [35 S]methionine labeled EGFR could be detected (Fig. 1B, lanes 7 and 10). Thus, both HA and GA, if present during synthesis, acted to block the maturation of the newly synthesized EGFR.

GA has been shown to interact directly with two members of the mammalian stress protein family: Hsp90 which is present within the cytoplasm; and Gsp94, an Hsp90-related counterpart which is localized within the endoplasmic reticulum (Whitesell et al 1994; Chavany

Fig. 1 Herbimycin (H) and geldanamycin (G) interfere with the maturation of the EGFR. A431 cells, either in the presence or absence of herbimycin (1 μ g/ml) or geldanamycin (0.4 μ g/ml), were labeled with [35 S]methionine for 30 min (i.e. pulsed). Some of the cells were harvested immediately, while to the other pulse-labeled cells the medium containing the radiolabel was removed and the cells were washed with and further incubated in DMEM containing 10% CS (i.e. chased), either in the presence or absence of the drugs. Following a 3-h chase period, the cells were harvested and the EGFR immunoprecipitated as described in the methods. In parallel, immunoprecipitation was carried out using antibodies to both Hsp90 and Gsp94. Resultant immunoprecipitation products were analysed by SDS-PAGE and fluorography. Lane designations are the same in each panel. (A) Analysis of A431 cell lysates. (B) EGFR immunoprecipitates. (C) Hsp90 immunoprecipitates. (D) Gsp94 immunoprecipitates. (E) EGFR immunoprecipitates from CHO cells (2WT) expressing the human EGFR. Lane designations: 1. Pulse-label, no drug; 2. Pulse-label and chase, no drug; 3. Pulse-label in the absence of drug, chase in the presence of H; 4. Pulse-label in absence of drug, chase in presence of G; 5. Pulse-label in presence of H; 6. Pulse-label in presence of H, chase in absence of drug; 7. Pulse-label in presence of H, chase in presence of H; 8. Pulse-label in presence of G; 9. Pulse-label in presence of G, chase in absence of drug; 10. Pulse-label in presence of G, chase in presence of G. The positions of the immature (160 kDa) and mature (170 kDa) forms of the EGFR, as well as the immature secretory form of the EGFR (90 kDa) are indicated by arrowheads in (B). (E) The maturation of the EGFR in CHO cells (+/- herbimycin or geldanamycin) stably transfected with EGFR cDNA was examined by metabolic pulse-chase radiolabeling and immunoprecipitation using the EGFR antibody exactly as described above for the A431 cells. Lane designations are the same as that described above.

et al 1996). Based on our observations with the EGFR, we examined whether the maturation of either Hsp90 or Gsp94 also might be affected in cells treated with either of the two benzoquinoid ansamycins. However, unlike the EGFR the maturation of either Hsp90 (Fig. 1C) or Gsp94 (Fig. 1D) was unaffected in the drug-treated cells. Indeed this latter result is consistent with some of our earlier observations. Specifically, both Hsp90 and Gsp94, along with most of the other stress proteins, are expressed at higher levels in cells exposed to either HA or GA (Hegde et al 1995).

The effects of the drugs on the maturation of the human EGFR expressed in transfected CHO cells (2WT) were also determined. Similar to the situation with the A431 cells, maturation of the EGFR was completely inhibited in the cells treated with either of the two benzoquinoid ansamycins (Fig. 1E).

We analyzed where in the EGFR maturation pathway the two drugs were exerting their inhibitory effect. A431 cells again were analyzed by pulse-chase radiolabeling. This time, however, the drug tunicamycin (TM) was used in order to inhibit the core glycosylation of the EGFR and thereby retard the movement of the newly synthesized EGFR out of the ER. The EGFR synthesized in the presence of TM migrated with an apparent mass of 130 kDa, indicating that core glycosylation of the EGFR indeed had been blocked (Fig. 2A-pulse, TM). Note that this non-glycosylated form of the receptor was relatively stable, still being present in the tunicamycin treated cells after the 3-h chase period (Fig. 2A-chase, TM). When synthesized in the presence of both tunicamycin and the benzoquinoid ansamycins, however, very little of the radiolabeled receptor was observed (Fig. 2A-pulse, TM+HA or TM+GA and Fig. 2A-chase, TM+HA or TM+GA). From these experiments it can be concluded that HA and GA interfere with EGFR biogenesis at a very early stage in the maturation pathway, probably before the protein is transferred out of the ER.

Results of additional experiments are consistent with the idea that the benzoquinoid ansamycins interfered only with that form of the EGFR which was in the course of synthesis and translocation into the ER membrane. Specifically, both tunicamycin or another compound Brefeldin A – another drug that blocks the movement of proteins out of the endoplasmic reticulum (ER) – first were used to ‘trap’ the newly synthesized and radiolabeled EGFR in the ER. When either HA or GA was now added to the cells, no significant changes in the overall levels of the radiolabeled EGFR were observed (Fig. 2B). Thus, once its synthesis and translocation into the membrane of the ER has been completed, the drugs no longer have any obvious affect on the stability of the receptor.

Similarly, once fully matured and present at the plasma membrane the EGFR was unaffected by the addition of either of the two benzoquinoid ansamycins. For example, A431 cells were labeled to steady state (e.g. 15 h), the label removed and the cells further incubated in complete medium for 3 h (to allow for the maturation of the radiolabeled proteins). Either HA or GA was added to the cells and they were then incubated for an additional 5, 10 or 20 h. As compared to the control cells EGFR half-life appeared to be unaffected by either of the drugs (Fig. 3).

These results, when taken together, indicate that the two benzoquinoid ansamycins somehow interfered with the maturation of only that population of the EGFR

Fig. 2 Nascent, but not newly synthesized, EGFR is adversely affected in cells exposed to herbimycin or geldanamycin. (A) A431 cells were pulse-labeled with [35 S]methionine for 30 min (pulse) or pulse-labeled for 30 min and then chased in the absence of radiolabel for 3 h (chase) in the presence of either herbimycin A (HA, 1 μ g/ml), geldanamycin (GA, 0.4 μ g/ml), tunicamycin (TM, 3 μ g/ml), or a combination of the drugs as indicated in the panel. Control cells were pulsed, or pulsed-chased in the absence of any drugs. Cells were harvested and the radiolabeled EGFR analysed by immunoprecipitation. Shown are the resultant immunoprecipitates analysed by SDS-PAGE. (B) Newly synthesized EGFR present within the endoplasmic reticulum (ER) is unaffected by HA or GA. A431 cells were incubated in either the absence or presence of Brefeldin A (B, 5 μ g/ml) or Tunicamycin (T, 3 μ g/ml). After labeling the cells with [35 S]methionine for 30 min, some of the cells were harvested immediately (i.e. pulsed). From the other plates of pulse-labeled cells, the media were removed, the cells were washed with fresh culture medium and then incubated for an additional 3 h in either the absence or the presence of herbimycin A (H) or geldanamycin (G) (chase) as indicated. The EGFR then was immune-isolated from the different lysates and analysed by SDS-PAGE. Indicated at the left of the panels are the positions of the unglycosylated (130 kDa, core glycosylated (160 kDa) and mature (170 kDa) forms of the EGFR.

which was in the course of synthesis (i.e. ribosome-bound, nascent EGFR). In contrast, that population of the newly synthesized and full length EGFR, which was present in the endoplasmic reticulum, or present at the plasma membrane, appeared to be largely unaffected by the drugs.

In addition to that form of the EGFR which is integrated within the plasma membrane, A431 cells also synthesize and secrete a truncated species of the EGFR

Fig. 3 Herbimycin A and geldanamycin do not affect the stability of: mature EGFR; Hsp90; or Gsp94. A431 cells were steady state labeled with [35 S]methionine for 15 h. The culture medium containing the radiolabel was removed, and the cells washed with and further incubated in fresh culture medium. Three hours later the cells received new culture medium containing: nothing (control); herbimycin A (HA, 1 μ g/ml), or geldanamycin (GA, 0.4 μ g/ml). The cells then were incubated for an additional 5, 10, or 20 h in either the absence (lanes 2–4), or presence of herbimycin A (+HA, lanes 5–7) or geldanamycin (+GA, lanes 8–10) respectively. The amount of the radiolabeled EGFR (A), Hsp90 (B) or Gsp94 (C) remaining at each time point was determined via immunoprecipitation utilizing the relevant antibody. Resultant immunoprecipitates were analysed by SDS-PAGE and fluorography. Lane 1: cells after the 15-h labeling and 3-h chase. Lanes 2, 5 and 8: cells provided an additional 5-h chase. Lanes 3, 6 and 9: cells provided an additional 10-h chase. Lanes 4, 7 and 10: cells provided an additional 20-h chase.

(i.e. one lacking a transmembrane domain and cytoplasmic tail). As is shown in Figure 4A, neither of the two drugs had any effect on the synthesis or the maturation of the secreted form of the EGF receptor. In the presence of GA the newly synthesized and secreted form of the EGFR exhibited a slightly faster migration in the gel. A similar altered gel migration for the membrane-associated form of the receptor was observed (Fig. 1) when the cells were incubated (chased) in the presence of GA. This altered gel migration appeared to be caused by a change in carbohydrate modification. For example, upon removal of the carbohydrate moieties by the enzyme PNGase, the secreted EGFR made in the GA-treated cells now exhibited an electrophoretic mobility identical to that observed for the PNGase-treated receptor isolated from the control cells (Fig. 4B). These effects of GA on the carbohydrate processing of the secreted EGFR appeared rather specific as global changes were not observed in the relative migration of other secretory proteins synthesized in the presence of the drugs (Fig. 4C).

Use of inhibitors of different cellular proteolysis systems revealed that EGFR degradation in the benzoquinoid treated cells was mediated, at least in part, via the proteasome pathway, a result similar to that reported previously by Sepp-Lorenzino et al (1995). For example, radiolabeled EGFR synthesized in the presence of the

Fig. 4 Maturation of a secreted form of the EGFR is unaffected by treatment of cells with either herbimycin A or geldanamycin. (A) In an experiment similar to the one presented in Figure 1, A431 cells were pulse-labeled for 30 min in either the absence or presence of herbimycin A (H) or geldanamycin (G) (pulse). The medium containing the radiolabel was removed, the cells extensively washed with fresh culture medium and the cells then further incubated in the presence or the absence of the drugs as indicated above the panel (chase). The culture medium was collected and the amount of the secreted form of the EGFR present within the clarified culture medium was determined by immunoprecipitation. (B) The immunoprecipitated EGFR (from the control cells or the drug treated cells) was incubated with PNGase F, an enzyme that effectively removes the carbohydrate moieties from proteins containing N-linked sugars. Following the enzyme digestion the immobilized EGFR was released from the protein A sepharose beads via addition of Laemmli sample buffer and analysed by SDS-PAGE. (C) To examine whether herbimycin A or geldanamycin affected overall protein secretion and/or protein glycosylation patterns, the proteins present within the culture medium from those cells pulse-labeled and chased (i.e. culture medium from cells labeled in panel B) were analysed by SDS-PAGE. An equal percentage of the extracellular media from each sample was applied to the gel. Shown is a fluorograph of the gel.

benzoquinoid ansamycins could be detected, albeit at levels lower than those observed for the untreated cells, when the proteasome inhibitor MG132 was included in the culture medium (Fig. 5). Note that the EGFR synthesized in the presence of both MG132 and the benzoquinoid ansamycins exhibited a somewhat slower electrophoretic migration, perhaps as a result of modification by ubiquitin.

Other than the EGFR, overall protein maturation was not noticeably affected in the A431 cells treated with either of the two benzoquinoid ansamycins (see Fig. 1A). Consequently, we examined whether the maturation of another growth factor receptor with tyrosine kinase activity also might be adversely affected by the drugs. Balb/C 3T3 cells, expressing the PDGF- β R were analysed by metabolic pulse-chase radiolabeling in the presence

Fig. 5 Degradation of nascent EGFR in cells exposed to benzoquinoid ansamycins occurs via the proteasome pathway. A431 cells were subjected to [35 S]methionine pulse-chase radiolabeling (30 min pulse followed by a 3 h chase) in the presence of: no addition (control); the proteasome inhibitor MG132 (PI, 50 μ M); herbimycin A (HA); or HA plus MG132 (HA+PI). After either the pulse, or the pulse-chase period, the cells were harvested and the maturation of the EGFR examined by immunoprecipitation using the anti-EGFR antibody. Shown is a fluorograph of the SDS gel analyzing the immunoprecipitates. Lanes 1, 3, 5 and 7 represent the pulse-labeled cells and lanes 2, 4, 6, 8 represent the pulse labeled and chased cells. The positions of the core glycosylated (160 kDa) and fully matured (170 kDa) forms of the receptor are indicated at the left.

or absence of HA. In the absence of the drug, the newly synthesized PDGFR migrated in the gel with an apparent mass of 160 kDa. Upon further maturation, as additional carbohydrate modifications took place, the protein now exhibited an apparent mass of 180 kDa (Fig. 6A). Similar to the situation with the EGFR, maturation of the PDGFR was inhibited in the drug-treated cells. Again these adverse effects were only observed when the drug was present during the actual metabolic labeling of the receptor. Like the situation with the EGFR, if the PDGFR already had been synthesized, addition of the drugs had little or no effect on the stability of the protein (Fig. 6B).

Others have reported that benzoquinoid ansamycins can reverse the transformed phenotype in cells expressing the oncogenic form of (viral) pp60^{v-src} (Murakami et al 1988; Whitesell et al 1992). Two subsequent observations provided a possible mechanism by which this reversal was manifested: direct inhibition of pp60^{v-src} activity by the drugs (Uehara et al 1989) or inhibition of pp60^{v-src} maturation (Whitesell et al 1994). Inhibition of maturation is believed to result from a failure of the newly synthesized pp60^{v-src} to interact with Hsp90, a known chaperone involved in pp60^{v-src} maturation (Brugge 1981; Oppermann et al 1981; Courtneidge and Bishop 1982). For example, as shown in Figure 7A, newly synthesized pp60^{v-src} co-precipitated with both Hsp90 and a 50 kDa protein (this latter protein now known to represent Cdc37 (Dey et al 1996; Stepanova et al 1996) in cells pulse-labeled under control conditions. However, note that in the presence of the drugs Hsp90 was no longer observed to co-precipitate with newly synthesized pp60^{v-src}. Moreover, when the pulse-labeled cells were provided a subsequent chase period in the presence of the drugs, radiolabeled pp60^{v-src} protein could no longer be detected. Thus like the growth factor receptors,

Fig. 6 Maturation of the platelet derived growth factor receptor (PDGFR), like the EGFR, is inhibited in cells exposed to benzoquinoid ansamycins. (A) Balb/C 3T3 cells, in the presence or absence of herbimycin A (H), were subjected to metabolic pulse-chase radiolabeling with [35 S]methionine (30 min pulse, 3 h chase). Some of the cells were harvested immediately after the pulse-labeling (pulse), while to the other pulse-labeled cells the media were removed and the cells washed with and further incubated in fresh culture medium (+/- HA) for 3 h before being harvested (chase). Maturation of the PDGFR was analysed by immunoprecipitation and SDS-PAGE. Shown is a fluorograph of the gel. The positions of the core glycosylated (160 kDa) and mature forms (180 kDa) of the PDGFR are indicated at the left of the panel. (B) Mature PDGFR is unaffected by benzoquinoid ansamycins. Balb/C 3T3 cells were labeled with [35 S]methionine for 1 h, the media containing the radiolabel were removed and the cells then were washed with and further incubated in fresh culture medium for 3 h. To the culture media were added: either nothing (control) or a combination of herbimycin A and geldanamycin (H/G). After further incubation of the cells for either 2 or 4 h the cells were harvested and the relative amount of the remaining labeled PDGFR determined via immunoprecipitation and SDS-PAGE. Shown is a fluorograph of the gel.

pp60^{v-src} synthesized in the presence of the benzoquinoid ansamycins failed to undergo normal maturation.

Perhaps somewhat more interesting was the observation that the benzoquinoid ansamycins also interfered with the stability of mature pp60^{v-src}. For example, cells were subjected to metabolic pulse-chase radiolabeling to generate a population of mature pp60^{v-src}. GA plus HA then were added to the culture medium, and the cells incubated further for an additional 1, 2, 3 or 4 h. Analysis of pp60^{v-src} via immunoprecipitation revealed a significant reduction in the levels of radiolabeled mature pp60^{v-src} in the drug-treated cells as compared with the control cells (Fig. 7B). Thus, in the case of pp60^{v-src}, both the newly synthesized and mature forms of the protein were targeted for rapid degradation when the cells were incubated in the presence of the benzoquinoid ansamycins.

DISCUSSION

Herein we have confirmed and extended previous studies showing that benzoquinoid ansamycins interfere with

the normal maturation of a select number of proteins, all of which are involved in growth control related events. Initial studies using these drugs revealed their remarkable effect on Rous virus transformed fibroblasts, being effective in reversing the transformed phenotype of the cells (Murakami et al 1988; Uehara et al 1989). Subsequent work showed that a number of other macromolecules, again proteins all involved in cellular growth control, also were adversely affected in cells treated with either GA or HA. Examples include: pp60^{src} and other src-related family members (e.g. yes, fps, lck) (Uehara et al 1988; Whitesell et al 1994; Hartson et al 1996); c-Raf, a component of the MAP kinase cascade (Schulte et al 1995; Schneider et al 1996; Stancato et al 1997); the Bcr/Abl oncoprotein involved in the immortalization of hematopoietic cells (Okabe et al 1994); *erbB2* (also known as Her-2/Neu) an EGFR-related protein tyrosine kinase overexpressed in many breast, ovarian and prostate carcinomas (Miller et al 1994; Chavany et al 1996); growth factor receptors which exhibit tyrosine kinase activities such as the EGFR and insulin-like growth factor (IGF) receptors (Murakami et al 1994; Sepp-Lorenzino et al 1995) and the PDGFR (the present study); certain steroid hormone receptors (Smith et al 1995; Whitesell and Cook 1996); mutant (but not wild-type) forms of the tumor suppressor protein p53 (Blagosklonny et al 1995; Whitesell et al 1998); and finally the p50 subunit of NF- κ B, a transcription factor involved in regulating the expression of genes important for inflammatory responses (Mahon and O'Neill 1995).

Exactly how the benzoquinoid ansamycins adversely affect the aforementioned targets is still somewhat unclear. Results from initial studies examining pp60^{v-src} concluded that HA directly blocked src-kinase activities (Uehara et al 1989). For example, addition of the drug directly to immune-precipitated pp60^{v-src} resulted in a significant drop in the tyr-kinase activity of v-src. Interestingly, addition of sulfhydryl reagents such β -mercaptoethanol or dithiothreitol reversed the ability of the drug to interfere with pp60^{v-src} kinase activity. Similarly, when added to the Rous virus transformed cells in the presence of different reducing agents, HA was no longer effective in reversing the transformed phenotype. Hence it was suggested that the drugs might be interacting with a critical sulfhydryl moiety present within the kinase domain of the src-protein.

It is important to point out, however, that reversal of the transformed phenotype by HA is not immediate. Rather, only after exposure to the drug for 16–24 h do the cells regain normal contact inhibition of growth (Murakami et al 1988; Whitesell et al 1994). A similar incubation period with the drugs was required to reverse the transformed phenotype of cells expressing other pp60^{v-src}-related oncoproteins such as yes, fps, and ros

Fig. 7 Both nascent and mature pp60^{v-src} exhibit an accelerated degradation in cells treated with benzoquinoid ansamycins. (A) NIH 3T3/ts UP-1 cells expressing temperature sensitive pp60^{v-src} were pulse labeled with [³⁵S]methionine for 30 min at 32°C (i.e. permissive temperature) in either the presence or absence of herbimycin A plus geldanamycin (H/G). Some of the cells were harvested immediately after the pulse-labeling (pulse), while to the other plates the media was removed and the cells washed with and further incubated in either the absence or the presence of the drugs for an additional 2 h before being harvested (chase). The relative levels of pp60^{v-src} were determined via immunoprecipitation using an anti-src antibody and the immunoprecipitates examined by SDS-PAGE. The positions of pp60^{v-src}, Hsp90 and p50 are indicated at the left of the panels. (B) NIH 3T3/ts UP-1 cells were labeled with [³⁵S]methionine for 1 h at 32°C, the culture medium containing the radiolabel removed and the cells extensively washed with and further incubated in fresh culture medium. One plate of the cells was harvested and the amount of radiolabeled pp60^{v-src} determined via immunoprecipitation (control, 0 h). To the remaining plates was added either nothing (control), or a mixture of HA and GA (HA/GA, 1 µg/ml and 0.4 µg/ml, respectively). After further incubation of the cells for either 1, 2, 3 or 4 h, the cells were harvested and the remaining levels of radiolabeled pp60^{v-src} determined via immunoprecipitation. Shown is a fluorograph of the gel. In each case immunoprecipitation was performed with either no first antibody (Abs, -) or the anti-src antibody (Abs, +).

(Uehara et al 1988). This was also the case for the different growth factor receptors. Over time the levels of the particular growth receptor (EGFR, IGFR and the PDGFR) declined as a function of the cells' exposure to the drug (Miller et al 1994; Murakami et al 1994; Sepp-Lorenzino et al 1995). Thus, these observations as well those from the present study are consistent with the idea that the drugs, rather than directly inactivating the protein kinase target, instead interfere with its maturation and/or stability. Over time and as the levels of the particular transforming protein decline, the cells exhibit reversion of the transformed phenotype.

The decrease in growth factor receptor levels by the drugs was due primarily to a block in receptor maturation. The pulse-chase radiolabeling studies revealed that the drugs somehow interfered with the maturation of only the nascent forms of either the EGFR or PDGFR. Murakami et al (1994) similarly observed that only nascent, but not mature, EGFR was negatively affected by HA. However, Mori et al (1997) reported that addition of HA to the mature EGFR (present within a rabbit reticulocyte lysate) resulted in the receptor undergoing rapid poly-ubiquitination. Thus, all of the aforementioned studies, including our own, are in agreement that the benzoquinoid ansamycins have a negative effect on the maturation of the different tyrosine protein kinases. In particular, our studies show that the drugs adversely affect the nascent (i.e. ribosome-bound), but not the mature, forms of the growth factor receptors.

Some insights as to how the benzoquinoid ansamycins might be influencing the maturation of different signal transduction molecules have been realized. For example,

Hsp90 has been shown to interact with nascent/newly synthesized pp60^{v-src} (Brugge 1981; Oppermann et al 1981; Courtneidge and Bishop 1982). Based on these observations it was suggested that Hsp90 might play a role in facilitating the synthesis and transport of pp60^{v-src} to its final destination at the plasma membrane. Similarly, many of the other proteins whose synthesis or stability are affected by the benzoquinoid ansamycins (Raf, src and src-relatives) also have been reported to interact, at least transiently, with Hsp90 (reviewed by Pratt 1997). Hence, for these different protein kinases benzoquinoid ansamycin-mediated disruption of their interaction with Hsp90 somehow leads to the kinase being rapidly targeted for degradation.

Benzoquinoid ansamycins probably disrupt Hsp90-target protein interactions via their ability to bind tightly to Hsp90 (Whitesell et al 1994). For example, the crystal structure of the amino-terminal domain of Hsp90 bound to GA recently was reported by two groups. Here a distinct 'molecular pocket' was found to represent the binding site for the drug. While one group concluded that this 'binding pocket' might represent a potential site for the interaction of Hsp90 with polypeptide targets such as pp60^{v-src} (Stebbins et al 1997), the other study concluded that the drug was binding to a distinct nucleotide binding cleft present within Hsp90. Consistent with this latter suggestion was a recent report showing that Hsp90 does indeed interact with ATP/ADP and that such interactions appear to result in a conformational change in the protein (Grenert et al 1997). Thus, direct competition of the drugs for the nucleotide binding site within Hsp90 somehow may affect the

ability of the protein to interact with its relevant targets in vivo.

Based on this suggestion we examined the possible interaction of Hsp90 with the nascent and mature forms of the EGFR. We were unable to obtain any convincing evidence of an interaction between Hsp90 and the receptor. To our knowledge no-one has shown any direct interactions of Hsp90 with the different growth factor receptors whose maturation is adversely affected by the benzoquinoid ansamycins. However, Chavany et al (1996) did report an interaction of the *erbB2* protein with Gsp94, the aforementioned Hsp90 homologue present in the endoplasmic reticulum. Here the two proteins were reported to co-precipitate with one another, but only under conditions of very low stringency (e.g. no detergents used in the immunoprecipitation wash buffers). Indeed, other than pp60^{v-src}, we have consistently been unable to demonstrate any significant interactions of Hsp90 with other cellular proteins when detergents are present during the immune-precipitation reactions (unpublished data). In contrast, using chemical cross-linking methods we have observed a limited number of nascent/newly synthesized proteins, the identity of which are unknown, that interacted with Hsp90 in HeLa cells (Eggers et al 1997). Hence we believe it is possible that Hsp90 may be interacting with nascent growth factor receptors but that we have not identified the proper conditions by which to trap these transient interactions.

In summary, utilizing different radiolabeling protocols, the results of the present study indicate that benzoquinoid ansamycins interfere with both EGFR and PDGFR maturation, probably at a very early stage during their biosynthesis. Once the newly synthesized forms of these proteins have been inserted into the membrane of the endoplasmic reticulum addition of the drugs had little or no effect on receptor stability. Studies are currently underway to determine exactly how Hsp90 (and/or the related gnp94) participates in the early steps of growth factor receptor maturation; whether either of these two stress proteins interact directly with the maturing form of the receptors and if so, to determine exactly how the benzoquinoid ansamycins might interfere with such interactions. Considering that almost all of the known 'substrates' for Hsp90 appear to be involved in various aspects of signal transduction events, delineating how this member of the heat shock protein family participates in the maturation and/or regulation of target proteins will probably contribute to our understanding of cellular growth control.

ACKNOWLEDGMENTS

We thank L. Williams for the PDGFR antibodies, D. Aftab and S.G. Martin for providing us with the pp60^{v-src} expressing cells and the pp60^{src} antibody. Thanks to the

Welch lab and to Hisaaki Kawakatsu for helpful discussion. This work was supported by the NIH (GM33551 to WJW). Peter Morrison was supported by multi disciplinary training in Lung Disease Program, T32HL07185.

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