Accelerated degradation of 160 kDa epidermal growth factor (EGF) receptor precursor by the tyrosine kinase inhibitor herbimycin A in the endoplasmic reticulum of A431 human epidermoid carcinoma cells

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The effect of herbimycin A on the biosynthesis of epidermal growth factor (EGF) receptor was examined in human epidermoid carcinoma A431 cells. Cells were pulse-labelled with [^{35}S]methionine, and EGF receptor biosynthesis was quantified by immunoprecipitation using a monoclonal anti-(EGF receptor) antibody. In the presence of herbimycin A, an immature 160 kDa EGF receptor precursor accumulated in 1 h and disappeared completely in 4 h. Pulse-labelled 160 kDa receptor precursor in the absence of herbimycin A, however, was converted normally into a 170 kDa one by chase with herbimycin A. Herbimycin A affected neither the synthesis of the secreted form of EGF receptor devoid of cytoplasmic domain, nor that of the transferrin receptor in A431 cells. The herbimycin A-induced degradation of 160 kDa EGF receptor precursor was not inhibited by an inhibitor of lysosomal enzymes, NH_4Cl. Endoglycosidase H digestion of the 160 kDa precursor converted it into the deglycosylated 130 kDa precursor peptide. These results suggested that herbimycin A selectively acted on the EGF receptor precursor during the synthesis of the 160 kDa form, probably on the cytoplasmic domain, to form an aberrant molecule which was subjected to rapid degradation in the endoplasmic reticulum.

INTRODUCTION

Epidermal growth factor (EGF) stimulates growth of various types of cells through its specific receptor. The EGF receptor is a tyrosine kinase, and ligand binding brings about its autophosphorylation, which triggers the signal transduction for cell proliferation [1]. The subsequent pathway of signal transduction is not yet clear, although it has recently been revealed that recognition of the phosphorylated tyrosine residue of the EGF receptor by a protein(s) with a SH2 (*src*-homologous) domain may mediate the signal to the next step [2,3].

The EGF receptor is known to play an oncogenic role in certain tumour cells, where it is over-expressed [4–6] as a result of gene amplification, enhanced transcription or change in metabolic turnover [7–9]. This abnormal expression is assumed to be closely related to the cells' abnormal proliferation, that is, their malignancy [10–12], as is true for other oncogenes. A decrease in the expression of the receptor in these tumour cells by antisense oligonucleotides [13], monoclonal antibodies [14,15], or pharmacological agents may therefore be an effective therapy for these tumours.

Herbimycin A is an inhibitor which inactivates non-receptor-type tyrosine kinase, $p60^{v\text{-}src}$ [16], and reverses transformation by various tyrosine kinase oncogenes [17]. This effect seems to be the result of direct interaction with the reactive thiol group(s) of the kinases [18,19]. Thus, this action of herbimycin A is unique compared with other tyrosine kinase inhibitors [20].

In studying of the effect of this inhibitor on EGF receptor kinase, we recently found that it decreased the number of EGF receptors in the tumour cell line A431: herbimycin A altered the cell growth behaviour stimulated by EGF so that it was normal by decreasing the EGF-receptor phosphorylation level. This decrease in phosphorylation was not due to direct inactivation of the kinase activity, but to a decrease in the amount of receptor protein [20a]. The results suggested that the mechanism of inhibition of receptor-type tyrosine kinases by herbimycin A may be different from that of non-receptor-type tyrosine kinases, and also suggested the inhibitor's potential usefulness for cancer chemotherapy. We therefore decided to study further the mechanism of herbimycin A's unique action in this tumour cell line. The previous study [20a] had suggested that the target of this inhibitor was in the biosynthetic pathway or during the turnover of the receptor. In the present study, we found that herbimycin A selectively promoted degradation of the EGF receptor precursor in A431 cells.

MATERIALS AND METHODS

Materials

Herbimycin A was isolated as described previously [21] and was used from a 1.0 mg/ml stock solution dissolved in dimethyl sulphoxide. Monensin was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and tunicamycin was from Wako Pure Chemical Industries (Osaka, Japan). EGF (1.00 mg/ml) was purchased from Takara Shuzop Co. (Kyoto, Japan). Mouse anti-(EGF receptor) monoclonal antibody Ab-1 was purchased from Oncogene Science Inc. (O.S.I., Uniondale, NY, U.S.A.). The anti-(EGF receptor) antibody B4G7 was kindly provided by Dr. N. Shimizu (Department of Molecular Biology, Keio University School of Medicine, Japan). Mouse anti-(transferrin receptor) monoclonal antibody was purchased from Amersham International (Amersham, Bucks., U.K.). The second antibody, rabbit anti-mouse IgG, was from Organon Teknika Corp.–Cappel Products (West Chester, PA, U.S.A.). Endoglycosidase H was purchased from Seikagaku Corp. (Tokyo, Japan).

Cell culture and cell labelling

A431 human epidermoid carcinoma cells (obtained from Japanese Cancer Research Resources Bank, Tokyo, Japan) were

Abbreviations used: EGF, epidermal growth factor; ER, endoplasmic reticulum.

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grown in Dulbecco's modified Eagle's medium (DME) (GIBCO, Grand Island, NY, U.S.A.) and contained 10% fetal-calf serum (FCS). To examine the biosynthesis of the EGF receptor, A431 cells were labelled with [^{35}S]methionine (50 μCi/ml; American Radiolabeled Chemicals, St. Louis, MO, U.S.A.) in methionine-free minimal essential medium (MEM) (for up to 1 h) or methionine-free MEM supplemented with 5% dialysed FCS (for up to 4 h) in the absence or presence of herbimycin A at the indicated concentrations. To examine the maturation of EGF receptor precursors, cells were incubated in methionine-free MEM supplemented with 5% dialysed FCS and labelled with [^{35}S]methionine (50 or 100 μCi/ml) for 10 min, and then chased in DME containing 10% FCS and non-radioactive methionine (150 μg/ml) with or without herbimycin A for the indicated times.

Cell lysis and immunoprecipitation

[^{35}S]Methionine-labelled cells were solubilized with RIPA buffer (20 mM Tris/HCl, pH 7.2, 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 100 kallikrein-inactivator units/ml aprotinin, 100 μM Na_3VO_4, 1 mM phenylmethanesulphonyl fluoride), and the lysates were used for measurement of protein amount and for immunoprecipitation. For analysis of secreted EGF receptor, the culture media of cells labelled with [^{35}S]methionine were used for immunoprecipitation of the secreted EGF receptor after removing cell debris by centrifugation. The cell lysates containing 20–50 μg of protein, or culture media containing corresponding amounts of protein, were treated with anti-(EGF receptor) antibody (O.S.I. Ab-1) for more than 1 h at 0 °C, followed by treatment with a second antibody for 30 min. The immune complexes were collected with Pansorbin (Calbiochem–Behring, La Jolla, CA, U.S.A.), washed with RIPA buffer and separated by SDS/PAGE (7.5% gels). Separated protein bands were revealed by fluorography, and radioactivities in these bands were quantified by liquid-scintillation counting after solubilizing the gel.

Endoglycosidase H treatment

Cells were pulse-labelled with [^{35}S]methionine for 10 min, chased for 1 h, solubilized and immunoprecipitated with anti-EGF receptor (O.S.I. Ab-1) as described above. The immunoprecipitates were boiled in 1% SDS for 2 min to solubilize the precipitates, and the mixtures were centrifuged to remove insoluble materials. The supernatants were diluted with an equal volume of digestion buffer (0.1 M sodium acetate, 0.1% Triton X-100, 1 mM phenylmethanesulphonyl fluoride, pH 5.5) and incubated with endoglycosidase H (10 m-units) for 20 h at 37 °C. Digestion was terminated by the addition of 4-fold-concentrated SDS/PAGE sample buffer, and samples were applied to SDS/PAGE (7.5% gel) as described by Gamou et al. [22].

RESULTS

Effect of herbimycin A on biosynthesis of the EGF receptor

The biosynthetic pathway of the EGF receptor in A431 cells has been extensively studied [23–25]. The receptor precursor, a 130 kDa polypeptide, is glycosylated co-translationally to give the 160 kDa precursor, and the 160 kDa glycoprotein precursor is further glycosylated to give the 170 kDa mature EGF receptor. We examined the biosynthesis of the EGF receptor in A431 cells by monitoring the time course of [^{35}S]methionine uptake into EGF receptor and receptor precursors for up to 60 min in the absence or presence of herbimycin A. The 160 kDa receptor precursor became increasingly labelled in both control and herbimycin A-treated cells (Figure 1a). After 60 min of labelling, 170 kDa mature EGF receptor became detectable in control cells, but it was labelled less in herbimycin A-treated cells (Figure 1a, lanes *e* versus *j*). In another experiment with longer labelling times, 2 and 4 h, it was demonstrated that mature 170 kDa EGF receptor accumulated after 2 h in control cells (Figure 1b, lanes *b* and *c*). However, in herbimycin A-treated cells, mature receptor accumulation was not detected (lanes *e* and *f*), and the 160 kDa receptor precursor disappeared after 4 h (lane *f*).

Effect of herbimycin A on the maturation process of the EGF receptor

To examine the effect of herbimycin A on the maturing process from 160 kDa precursor to 170 kDa receptor, cells were pulse-

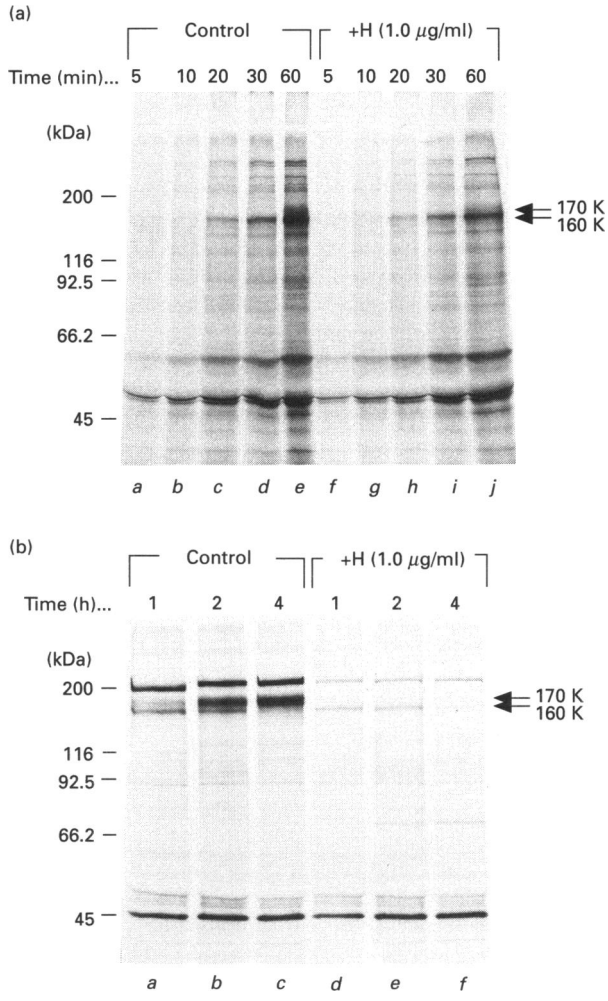

Figure 1 Effect of herbimycin A on synthesis of the EGF receptor in A431 cells

(**a**) Cells were labelled with [^{35}S]methionine for 5 min (lanes *a* and *f*), 10 min (lanes *b* and *g*), 20 min (lanes *c* and *h*), 30 min (lanes *d* and *i*) and 60 min (lanes *e* and *j*) in the absence (lanes *a–e*) or presence (+H; lanes *f–j*) of 1.0 μg/ml herbimycin A. (**b**) For longer labelling and treatment, the cells were labelled for 1 h (lanes *a* and *d*), 2 h (lanes *b* and *e*) and 4 h (lanes *c* and *f*) in the absence (lanes *a–c*) or presence (+H; lanes *d–f*) of herbimycin A.

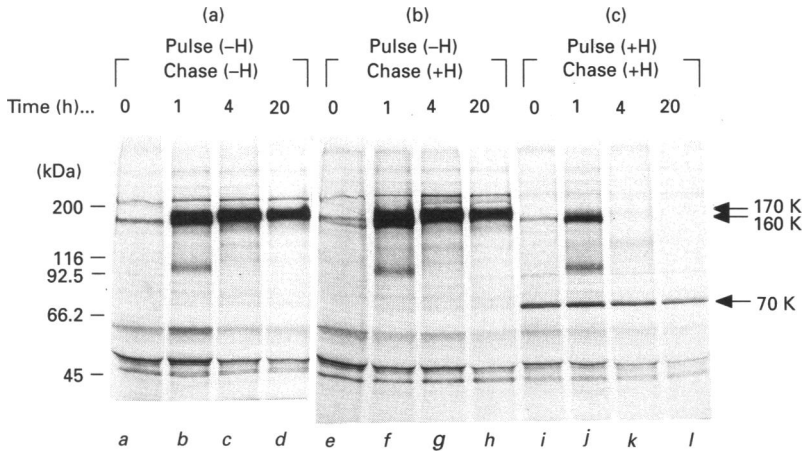

Figure 2 Effect of herbimycin A on processing of the EGF receptor in A431 cells

Cells were labelled with [^{35}S]methionine for 10 min, and chased for 0 h (lanes *a*, *e* and *i*), 1 h (lanes *b*, *f* and *j*), 4 h (lanes *c*, *g* and *k*) and 20 h (lanes *d*, *h* and *l*). In the experiment shown in (**c**), cells were treated with 1.0 μg/ml herbimycin A (+H) for 4 h, and in (**b**) and (**c**), chase medium contained 1.0 μg/ml herbimycin A.

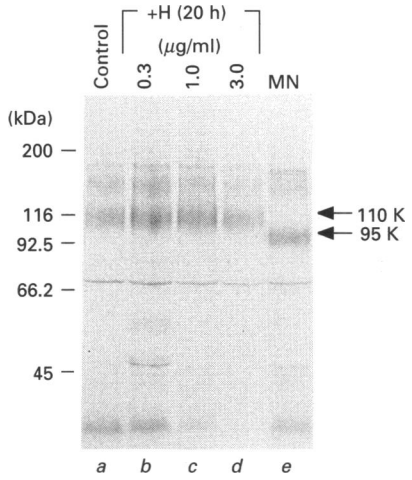

Figure 3 Effect of herbimycin A on the secreted EGF receptor in A431 cells

After treatment with 0.3 (lane *b*), 1.0 (lane *c*) and 3.0 (lane *d*) μg/ml herbimycin A (H) for 20 h, cells were pulse-labelled with [^{35}S]methionine for 10 min and chased for 4 h with the same concentration of herbimycin A. Treatment with monensin (MN) (0.5 μM) was for 1 h, and pulse (for 10 min) and chase (for 4 h) were performed in the presence of monensin. After the chase, the conditioned media were immunoprecipitated with anti-(EGF receptor) antibody.

labelled for 10 min with [^{35}S]methionine and then chased for up to 20 h in the absence or presence of herbimycin A. As shown in Figures 2(a) and 2(b), accumulation of the 160 kDa form and its conversion into the 170 kDa form were not affected by herbimycin A treatment during the chase period. However, when herbimycin A was added to the cells 4 h before and during pulse and chase periods, the 160 kDa precursor accumulated in the first 1 h of chase and disappeared after 4 h of chase (Figure 2c, lanes *j* versus *k*). Loss of the 160 kDa precursor was also demonstrated when herbimycin A was added during the pulse period without 4 h of pretreatment (results not shown). Thus the conversion of the 160 kDa precursor into the 170 kDa mature receptor did not appear to be inhibited by herbimycin A, but the 160 kDa precursor synthesized in the presence of herbimycin A was selectively subjected to degradation.

Effect of herbimycin A on secreted EGF receptor

We next investigated the effect of herbimycin A on the biosynthesis of a secreted form of EGF receptor. This secreted form is peculiar to A431 cells, and is translated from another species of mRNA different from that for the membrane-bound normal EGF receptor [26]. The structure of the secreted EGF receptor is devoid of the cytoplasmic domain of the normal EGF receptor. It is secreted extracellularly after being glycosylated in a manner nearly identical with that for the glycosylation of membrane-anchored receptor [22,27,28]. Cells were treated with herbimycin A for 20 h, before pulse-labelling with [^{35}S]methionine for 10 min, and then chased for 4 h in the presence of the inhibitor. The conditioned medium was immunoprecipitated with anti-(EGF receptor) antibody, and the precipitates were analysed. An 110 kDa protein band was detected in herbimycin A-treated cells as well as in control cell (Figure 3, lanes *a–d*). The result indicated that herbimycin A did not affect the secreted EGF receptor. Treatment with monensin, an inhibitor of glycosylation, allowed detection of a 95 kDa (lane *e*) band instead of the 110 kDa protein band (lane *e*).

Effect of herbimycin A on transferrin receptor biosynthesis

To determine its specificity, we also examined the effect of herbimycin A on transferrin receptor synthesis. The transferrin receptor is a transmembrane receptor recognized as a specific marker for rapidly growing cells, including malignant cells (for review, see May and Cuatrecasas [29]). The transferrin receptor is known to be expressed on the cell surface of A431 cell [30]. The biosynthetic pathway of the transferrin receptor, including glycosylation and translocation in the cellular organelles, is assumed to be basically similar to that of the EGF receptor [31]. Both receptors have high-mannose-type and complex-type glycosaccharides in the outer membrane domains. After A431 cells were labelled for 1, 2 or 4 h in the absence or presence of herbimycin A, immunoprecipitation with anti-transferrin receptor antibody was performed and the products were analysed.

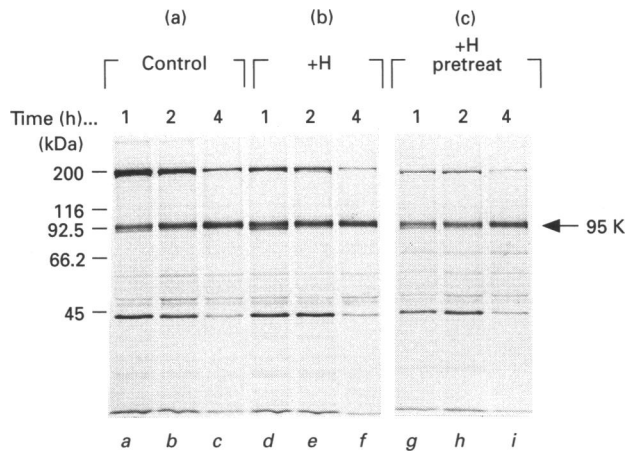

Figure 4 Effect of herbimycin A on the transferrin receptor in A431 cells

Cells were labelled for 1 h (lanes *a*, *d* and *g*), 2 h (lanes *b*, *e* and *h*) and 4 h (lanes *c*, *f* and *i*) in the absence (lanes *a–c*) or presence (lanes *d–i*) of herbimycin A (+H; 1.0 μg/ml). For lanes *g*, *h* and *i*, cells were treated with 1.0 μg/ml herbimycin A for 4 h before labelling.

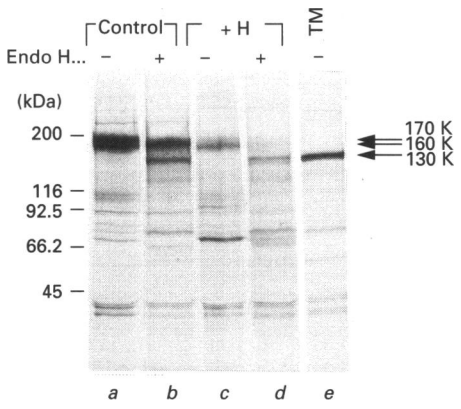

Figure 5 Subcellular localization of 160 kDa precursor receptor in herbimycin A-treated A431 cells

The cells were pulse-labelled for 10 min with [^{35}S]methionine, chased for 1 h in the absence (lanes *a* and *b*) or presence (lanes *c* and *d*) of herbimycin A (+H; 1.0 μg/ml), and then lysed and immunoprecipitated with anti-(EGF receptor) antibody. The immunoprecipitates were subjected to endoglycosidase H (Endo H) digestion. The main band in lane *e* shows the 130 K non-glycosylated precursor from tunicamycin (TM)-treated cells detected with B4G7 antibody [18].

As shown in Figure 4, under the conditions in which EGF receptor synthesis was inhibited (see Figure 1b), transferrin receptor biosynthesis was not affected by herbimycin A, indicating that the cellular functions of glycosylation and subcellular translocation of the surface receptor proteins were, in general, not affected by this inhibitor.

Subcellular localization of 160 kDa receptor precursor and its degradation by herbimycin A

Glycosylated 160 kDa receptor precursor is known to be modified to various extents by high-mannose chains in the endoplasmic reticulum (ER). The 160 kDa precursor is then subjected to removal of mannose and again modified with complex-type

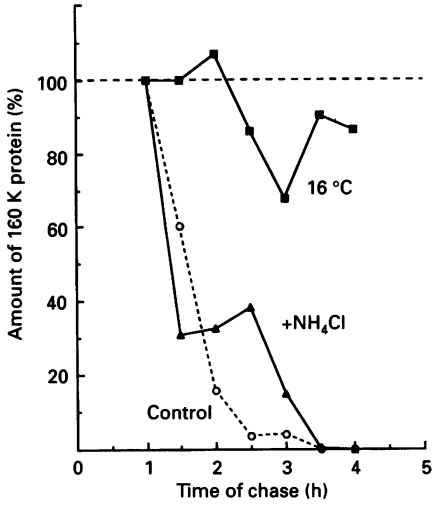

Figure 6 Effect of NH_4Cl and a low temperature on herbimycin A-induced degradation of 160 kDa precursor receptor

The pulse–chase experiment on herbimycin A-treated cells was performed by the method described for Figure 2(c), except for the chase conditions. ^{35}S radioactivity in the 160 kDa protein bands of 1 h-chased cells with herbimycin A was taken as 100%. Symbols: ○, herbimycin A-induced degradation in control cells; ▲, degradation with 50 mM NH_4Cl; ■, degradation at 16 °C.

oligosaccharide chains to become the 170 kDa mature receptor in the Golgi apparatus. Since herbimycin A seemed to act in an early stage of the 160 kDa precursor biosynthesis, we examined whether the 160 kDa receptor precursor was normally glycosylated and translocated in the subcellular organelles. Endoglycosidase H was used to distinguish proteins located in the ER from those in the distal site of the Golgi from the ER (known as *trans*-Golgi), because the glycosidase digests a high-mannose-type oligosaccharide, but not a complex-type oligosaccharide [32]. If the 160 kDa precursor is normally glycosylated in the ER, but does not translocate to the *trans*-Golgi in herbimycin A-treated cells, it will be sensitive to endoglycosidase H. Cells were pulse-labelled and chased for 1 h in the presence of herbimycin A. The cell lysate was immunoprecipitated with anti-(EGF receptor) antibody (O.S.I. Ab-1) and the immunoprecipitate was subjected to endoglycosidase H digestion. As shown in Figure 5, the 160 kDa precursor was almost completely converted into the 130 kDa non-glycosylated precursor peptide (lanes *c* versus *d*). On the other hand, in control cells the conversion into the 130 kDa form was only partial, and a major portion remained in the 170 kDa form (lanes *a* versus *b*), indicating the existence of endoglycosidase H-resistant receptor species. The 130 kDa non-glycosylated peptide which was used as a reference band is demonstrated in lane *e*. The band was revealed by tunicamycin treatment and immunoprecipitation with B4G7 antibody, which reacted with the 130 kDa EGF receptor precursor [33]. The indication was that, in herbimycin A-treated cells, the 160 kDa receptor precursor was normally synthesized and glycosylated in the ER; although it is not clear whether or not it translocated into the *cis*-Golgi, it was clear that it did not translocate up to the *trans*-Golgi. To understand the features of the herbimycin A-induced degradation of the 160 kDa receptor precursor, we examined the effect of NH_4Cl, which is known to raise intracellular pH and to inhibit activities of the lysosomal enzymes [34]. As shown in Figure 6, the more intensely labelled 160 kDa protein accumulated in a 1 h chase (see Figure

2c) was degraded by herbimycin A to about half that amount of 1 h chase in 30 min and was degraded completely in 2.5 h (Figure 6). Addition of 50 mM NH_4Cl in the chase period did not inhibit degradation of the 160 kDa precursor. This concentration of NH_4Cl was sufficient to inhibit mature EGF receptor degradation by EGF treatment of A431 cells (results not shown). In contrast, the degradation of 160 kDa precursor protein was inhibited at a low temperature (16 °C) (Figure 6), suggesting involvement of an enzyme reaction in the degradation. Leupeptin (50, 100 or 200 μg/ml), which is known to inactivate lysosomal enzymes, did not inhibit the degradation (results not shown). The data suggested that the subcellular site for the 160 kDa precursor degradation was not lysosomal, but might be ER or pre-Golgi area.

DISCUSSION

We previously reported that herbimycin A directly inactivated $p60^{v\text{-}src}$ kinase both in intact cells and in cell-free systems *in vitro*. In contrast, for EGF receptor tyrosine kinase, the protein amount of the receptor was drastically decreased in A431 cells, whereas the kinase activity itself appeared not to be affected by herbimycin A [20a]. We found that herbimycin A induced rapid degradation of the 160 kDa EGF receptor precursor in A431 cells. However, herbimycin did not affect the glycosylation process, nor the biosynthesis of either the secreted form of EGF receptor or the transferrin receptor, suggesting that it did not act on the translocation or secretion process of proteins in general in the cell. Herbimycin A therefore appeared to act selectively on the 160 kDa EGF receptor precursor, probably on its cytoplasmic domain. Although at present it is not clear whether or not its direct target site of action is the kinase domain, this is a possibility, because the corresponding sequence in the kinase domain of the receptor molecule is similar to $p60^{v\text{-}src}$. Viewed in this light, the degradation of 160 kDa EGF receptor precursor corresponds to the degradation of $p60^{v\text{-}src}$ [16]. However, the possibility cannot be ruled out that herbimycin A is acting not directly on the EGF receptor precursor, but on another protein involved in the biosynthetic pathway.

Questions remain about the difference between the effects of herbimycin A on $p60^{v\text{-}src}$ and those on the EGF receptor. Why does herbimycin A interact with and inactivate $p60^{v\text{-}src}$, but not mature EGF receptor? Why does it not degrade $p60^{v\text{-}src}$ during its synthesis and allow it to translocate to the cell membrane? One possible answer may involve the structural difference in kinase and other domains between the EGF receptor and $p60^{v\text{-}src}$. Another is the difference in translocation pathways between the two proteins. The transmembrane-protein EGF receptor is synthesized on the ER membrane-bound ribosomes and transported through the ER membrane and Golgi to the plasma membrane [35]. In contrast, $p60^{v\text{-}src}$ protein is synthesized on free polyribosomes in the cytosol, where it associates with two cellular proteins, p50 and heat-shock protein 90 (hsp 90), to form a stable complex; this complex is then transported to the plasma membrane, where it is dissociated [36]. A possible difference in the subcellular distribution of the inhibitor, as well as the subcellular site of the target proteins, might be another factor worthy of consideration.

With regard to the degradation of the receptor, we presume participation by a heat-shock protein. We previously demonstrated that herbimycin A induced the synthesis of heat-shock protein 70 (hsp70) in A431 cells and various kinds of mammalian cells [37]. Heat-shock proteins are induced by various stresses to recover damaged cellular proteins. One type of hsp70, BiP (heavy-chain-binding protein) was reported to bind to abnormal proteins [38–40]. These aberrant proteins that failed to be secreted were retained in the ER and eventually degraded [40,41]. From the similarity to these observations, we presumed that in A431 cells herbimycin A reacted with newly synthesized EGF receptor precursor to make an aberrant 160 kDa form, which was subjected to degradation with assistance of the hsp70. Consistent with the theory that hsp70 took part in the degradation of the 160 kDa EGF receptor precursor, the subcellular location of hsp70 induced by herbimycin A was in the cytoplasm [37] and the anti-EGF receptor antibody co-immunoprecipitated 70 kDa protein (Figure 2c), but it was not confirmed to be hsp70.

Among many inhibitors of tyrosine kinases, herbimycin A is unique in that it decreases the number of EGF receptors in cancer cells. We have shown that herbimycin A altered the cell growth behaviour of A431 cells to that of normal cells in response to EGF [20a]. Herbimycin A should therefore be a useful agent with which to investigate the role of over-expression of the human EGF receptor in these transformed cells. The findings also suggest the feasibility of such an inhibitor being applied in the chemotherapy for EGF-receptor-over-expressing human tumours.

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