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Hsp90 restrains ErbB-2/HER2 signalling by limiting heterodimer formation

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ErbB-2/HER2 is an oncogenic tyrosine kinase that regulates a signalling network by forming ligand-induced heterodimers with several growth factor receptors of the ErbB family. Hsp90 and co-chaperones regulate degradation of ErbB-2 but not other ErbB members. Here, we report that the role of Hsp90 in modulating the ErbB network extends beyond regulation of protein stability. The capacity of ErbB-2 to recruit ligand-bound receptors into active heterodimers is limited by Hsp90, which is dissociated from ErbB-2 following ligand-induced heterodimerization. We show that Hsp90 binds a specific loop within the kinase domain of ErbB-2, thereby restraining heterodimer formation and catalytic function. These results define a role for Hsp90 as a molecular switch regulating the ErbB signalling network by limiting formation of ErbB-2-centred receptor complexes.

Keywords: ErbB-2/HER2; Hsp90; tyrosine kinase; EGF; signal transduction

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INTRODUCTION

ErbB receptor tyrosine kinases act in the context of a signalling network, in which the diversity of ligand growth factors, as well as the identity of homo- and heterodimeric receptors, defines the type of signalling module formed (reviewed in Yarden & Sliwkowski, 2001). Of the four ErbBs, ErbB-2 is the most associated with human malignancies, but is believed to be a ligandless receptor acting solely in the context of ErbB heterodimers. In addition, its favoured dimerization partner, in the context of which the most potent signalling dimers are formed, is ErbB-3, a kinase-defective receptor. Of the four ErbB receptors, only ErbB-2 is subject to an additional layer of regulation, mediated by molecular chaperones, primarily Hsp90 (Xu *et al*, 2001, and references therein). The role of Hsp90 in the regulation of ErbB-2 has so far been attributed to stabilization of the receptor at the cell surface, such that following prolonged inhibition of Hsp90 by ansamycins the receptor undergoes proteasomal degradation (Sepp Lorenzino *et al*, 1995).

The Hsp90 chaperone has been implicated in the stabilization of several cellular proteins, many of which have central roles in signal transduction (reviewed in Pratt & Toft, 2003). Recently, evidence has begun to accumulate for an additional role for Hsp90 in regulating the intrinsic activities of its client proteins. For example, Hsp90 both facilitates and represses the double-stranded RNA (dsRNA)-dependent kinase PKR (Donze *et al*, 2001). Likewise, Hsp90 regulates ligand-induced activation of steroid hormone receptors, which has been a principal focus of interest (Pratt & Toft, 2003). Here, we address the role of Hsp90 in the context of ligand-induced activation of receptor tyrosine kinases. The results indicate that the chaperone maintains the ErbB network in a restrained state by binding to a specific loop located within the kinase domain of the shared co-receptor, ErbB-2/HER2.

RESULTS

Hsp90 restricts dimerization and activation of ErbB-2

Assuming a role for Hsp90 in regulation of ErbB signalling, we addressed the effect of ligand-induced heterodimerization and activation of ErbB-2 on its association with Hsp90. Stimulation of SKBR3 human breast cancer cells, which express high levels of ErbB-2, with the Neu differentiation factor (NDF/NRG1) resulted in dissociation of Hsp90 from ErbB-2 (Fig 1A). A comparable effect was observed on treatment of cells with a well-characterized ansamycin antagonist of Hsp90, geldanamycin (GA). ErbB-2 is also recruited into heterodimers by stimulation of cells with ErbB-1 ligands such as epidermal growth factor (EGF) or transforming growth factor-α (TGF-α). The results shown in supplementary Fig 1A,B online indicate that both ligands dissociate Hsp90 from ErbB-2. Further, we noted an additive effect of relatively low concentrations of NDF and GA on dissociation of Hsp90 from ErbB-2 (Fig 1B), as expected from summation of the effects of compounds acting on different components.

To address the possibility that Hsp90 limits the capacity of ErbB-2 to heterodimerize and *trans*-phosphorylate, we examined the effect of blocking Hsp90 on NDF-induced receptor heterodimerization, which was determined by covalent crosslinking

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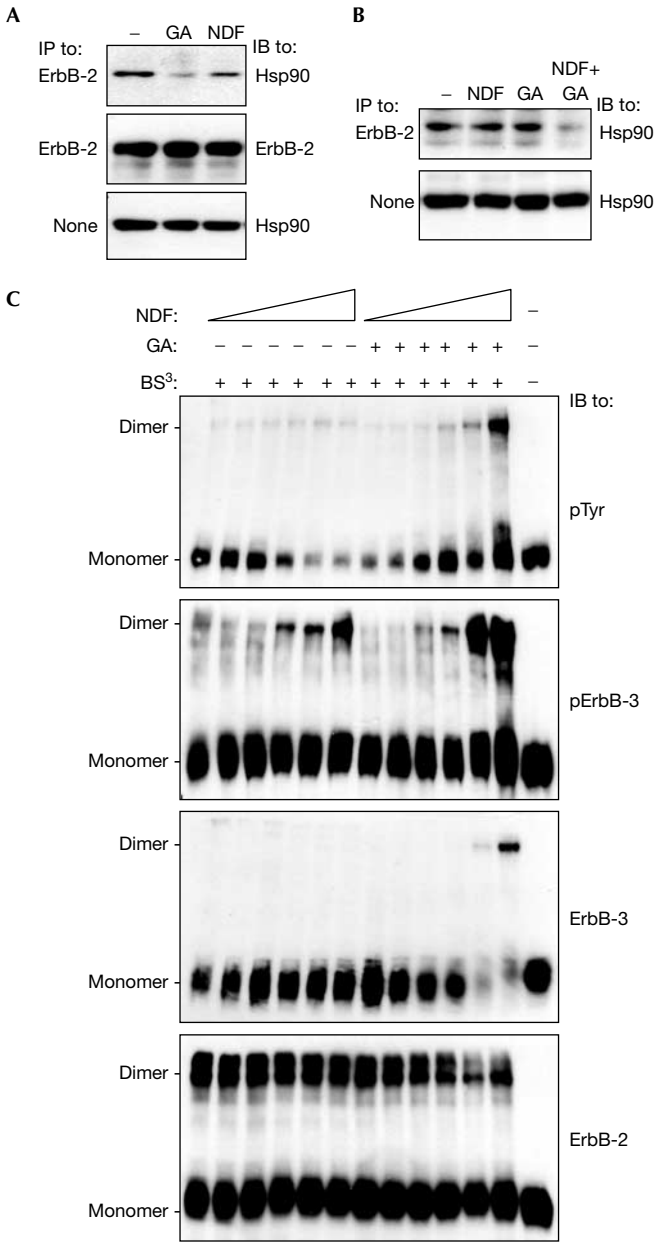


Fig 1 | Hsp90 regulates ligand-induced dimerization of ErbB proteins. (**A**) SKBR3 cells were subjected 24 h after plating to serum starvation for an additional 24 h before treatment for 5 min with GA (5 μM) or NDF (100 ng/ml). Cells were then lysed and the interactions of ErbB-2 with Hsp90 determined by immunoprecipitation (IP) of ErbB-2, followed by SDS–polyacrylamide gel electrophoresis and immunoblotting (IB). (**B**) SKBR3 cells were treated for 5 min with relatively low concentrations of NDF (5 ng/ml) or GA (0.5 μM), or a mixture of both compounds, followed by treatment as in (**A**). (**C**) SKBR3 cells were treated with increasing concentrations of NDF (0.1, 0.5, 1, 5 and 10 ng/ml; 5 min), in the absence or presence of GA (10 μM). Cells were then washed and lysed in solubilization buffer containing BS^3. Note that antibodies to the phosphorylated form of ErbB-3 detected minor intermediary species, along with monomeric and dimeric forms, but their identity remains unknown.

(Fig 1C). Clearly, ligand-induced formation of active ErbB-3 · ErbB-2 complexes is potentiated following inhibition of Hsp90 by GA. A similar phenomenon was observed when addressing EGF-induced heterodimerization of ErbB-1 and ErbB-2 (supplementary Fig 1C online). Interestingly, ErbB-2 is constitutively dimeric, especially in cells expressing high levels of ErbB-2, whereas the recruitment of ErbB-3 into dimers is associated with enhanced *trans*-phosphorylation of ErbB-3 and reduction of both monomeric ErbB-3 and dimeric ErbB-2, perhaps owing to the formation of receptor oligomers once stripped from Hsp90. Consistently, the phosphorylation of ErbB-3 was enhanced following NDF stimulation, whereas the phosphorylation of ErbB-2 was decreased (supplementary Fig 1D online). These observations imply transition from a dimer of two kinase active ErbB-2 molecules to an ErbB-2 · ErbB-3 heterodimer, in which ErbB-2 cannot be phosphorylated. In contrast, following EGF stimulation of the same cells, the levels of phosphorylation of both ErbB-1 and ErbB-2, as well as two downstream targets, were increased (supplementary Fig 1E online), consistent with formation of a dimer of two kinase active molecules.

Hsp90 limits signalling downstream to ErbB-2

In an attempt to verify a role for Hsp90 in regulating kinase activity, we found that short treatment of SKBR3 cells with GA promoted the kinase activity of ErbB-2, tested *in vitro* (Fig 2A). GA enhanced the catalytic activity of the receptor also when administered *in vitro*, and phosphorylation was inhibited by pretreatment with CI-1033 (Fig 2B), a highly selective inhibitor of ErbB kinases (Fry, 2003). Covalent crosslinking of these *in vitro* reactions further established a role for Hsp90 in limiting the potential of ErbB proteins to form active receptor dimers (Fig 2B). Taken together with the results presented in Fig 1C, these observations suggest that Hsp90 restrains the kinase activity of ErbB-2 as well as the ligand-induced formation of active ErbB-2-containing heterodimers.

Phosphorylation of the mitogen-activated protein kinase pathway (MAPK/Erk) is a pivotal outcome of ErbB activation. Hence, we used MAPK activation as readout for ErbB activity following inhibition of Hsp90 with either GA or several other Hsp90 inhibitors (17-alkylamino-17 demethoxygeldanamycin (17-AAG), Herbimycin-A and Radicicol), mechanisms of action of which differ. Activation of Erk was observed following treatment of SKBR3 cells with GA, as well as with the other Hsp90 inhibitors, and the kinetics of stimulation was reminiscent of that observed following treatment with ErbB ligands (Fig 2C). Indeed, independently of antagonist identity, Erk activation was inhibited after addition of CI-1033, confirming induction through ErbB receptors. This was further established using 32D myeloid cells singly expressing ErbB-1 (D1 cells) or ErbB-2 (D2 cells; Pinkas-Kramarski *et al*, 1996) on an ErbB-null background. In contrast with the parental 32D cells, which showed no Erk activation following treatment with either GA or EGF, Erk of D1 cells was stimulated by EGF but not by GA (Fig 2D) and only cells in which ErbB-2 was expressed (D2 cells) demonstrated an induction of Erk following treatment with GA. Interestingly, in these cells, Erk showed relatively high basal activity. As expected from the ability of Hsp90 to limit ligand-induced receptor activation, the activation of Erk and phospholipase C (PLC)-γ observed following stimulation of HeLa cells with a low dose of EGF was accelerated and

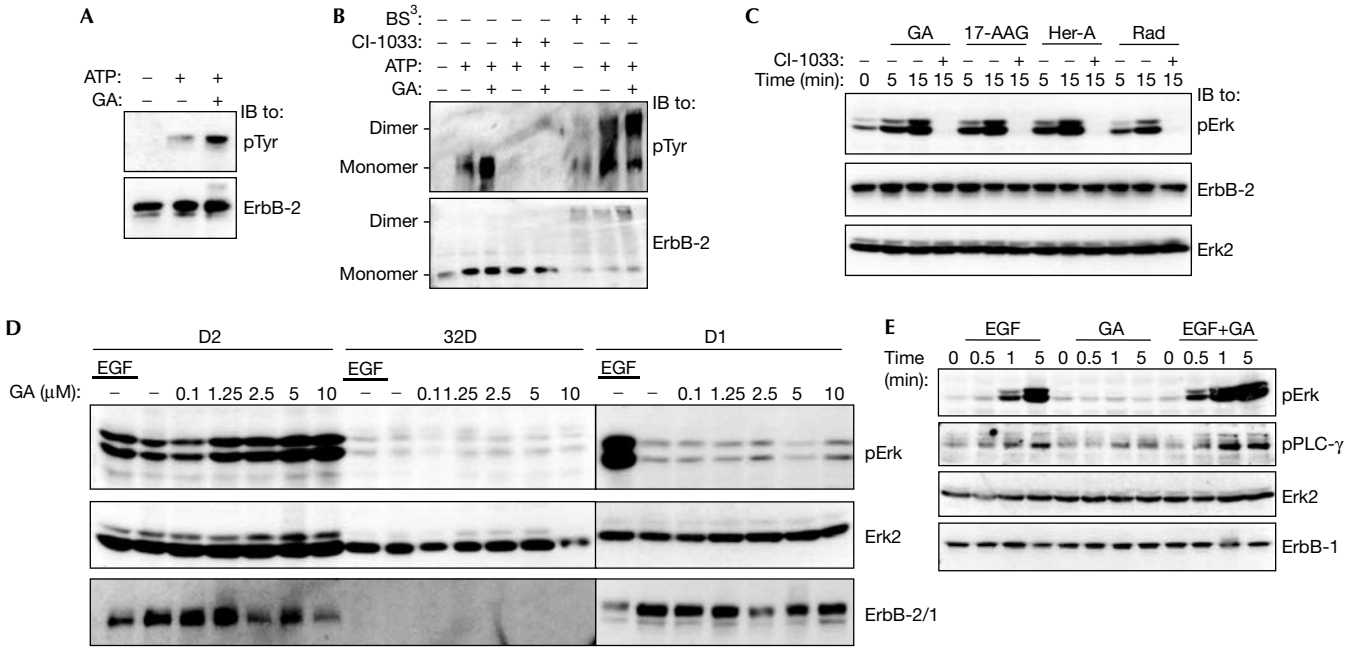


Fig 2 | Inhibition of Hsp90 enhances dimerization, activation and signalling downstream of ErbB receptors. (**A**) A solubilized membrane fraction isolated from SKBR3 cells treated with GA (5 μM; 1 min) was subjected to *in vitro* kinase reactions (30 °C; 5 min) in the absence or presence of ATP. (**B**) Solubilized membranes, purified from SKBR3 cells, were subjected to preincubation in the absence or presence of GA (0.1 μM) or CI-1033 (10 μM), followed by *in vitro* kinase reactions. Where indicated, kinase reactions were followed by crosslinking in the presence of BS^3 (2 mM). (**C**) SKBR3 cells were treated for the indicated intervals with GA (10 μM), 17-AAG (10 μM), Herbimycin-A (Her-A; 10 μM) or Radicicol (Rad; 10 μM), in the absence or presence of CI-1033 (10 μM). Whole-cell lysates were immunoblotted with the indicated antibodies, including antibodies to active, phosphorylated Erk (pErk). (**D**) 32D cells or their derivatives expressing ErbB-2 (D2) or ErbB-1 (D1) were treated with the indicated dose of GA (in μM) or with EGF (100 ng/ml) for 5 min. (**E**) HeLa cells were subjected to a short time course (0, 0.5, 1 and 5 min) of treatment with low doses of EGF (5 ng/ml), GA (0.5 μM) or a mixture of both compounds.

potentiated following co-treatment with a low dose of GA (Fig 2E). In summary of the results shown in Fig 2C–E, Hsp90 restrains signalling downstream of ErbB-2 but not downstream of ErbB-1.

The Hsp90 interaction motif restricts ErbB-2 signalling

Immunoprecipitation of ErbB-1, ErbB-2 and ErbB-3 from SKBR3 cells demonstrated a specific interaction of Hsp90 with ErbB-2 and not with the other two receptors (Fig 3A). Hsp90 has been demonstrated to interact with the kinase domain of ErbB-2 (Xu *et al*, 2001; Citri *et al*, 2002), and comparison of the highly homologous kinase domains of ErbB-1 and ErbB-2 led to the identification of a motif within the kinase domain of ErbB-2, which, following substitution with the orthologous sequence in ErbB-1, conferred refractoriness to GA-induced degradation (Tikhomirov & Carpenter, 2003). This motif lies within the amino-terminal lobe of the kinase, in close association with the ATP-binding pocket and the αC helix (Fig 3B), a region involved in regulating kinase activity (Huse & Kuriyan, 2002). Assuming that the motif confers recognition of ErbB-2 by Hsp90, we replaced the respective amino-acid sequence of ErbB-1 with the corresponding motif of ErbB-2 (protein denoted 1L2). Likewise, we established the reciprocal fusion protein, denoted 2L1 (Fig 3C). Consistent with the surveillance status of the different receptors by Hsp90, receptors that are coupled to Hsp90 (ErbB-2 and 1L2) demonstrated enhanced basal ubiquitination, and reduced phosphorylation in comparison with their Hsp90-refractory counterparts (ErbB-1 and 2L1; Figs 3D,4A). Further characterization of the ErbB-2 chimaera 2L1 demonstrated that it lost GA-sensitive recognition of Hsp90 (Fig 4A), as well as GA-dependent ubiquitination, in accordance with chaperone-mediated surveillance of proteasomal degradation (supplementary Fig 2A online). Accordingly, 2L1 showed enhanced kinase activity, both in living cells (Fig 4A) and *in vitro*, which was insensitive to GA (supplementary Fig 2B online). Consistent with these observations, coupling of 2L1 to downstream effectors (that is, PLC-γ and Erk) was enhanced relative to ErbB-2 (Fig 4A,B). In contrast to ErbB-2, which is found constitutively in homodimers of limited activity and retains responsiveness to GA, 2L1 formed receptor dimers of enhanced activity, into which ErbB-3 was more easily recruited (Fig 4C, and data not shown). As expected from its constitutive activation, stable expression of 2L1 drove enhanced proliferation of NIH-3T3 cells when compared with equivalent expression of wild-type ErbB-2 (Fig 4D). These observations reinforce the following scenario: Hsp90 binds to a specific loop within the kinase domain of ErbB-2, thereby restraining the catalytic function and the ability to heterodimerize. Replacement with the corresponding loop of ErbB-1 abolishes interaction with Hsp90, resulting in enhanced heterodimerization, signalling and mitogenic capability.

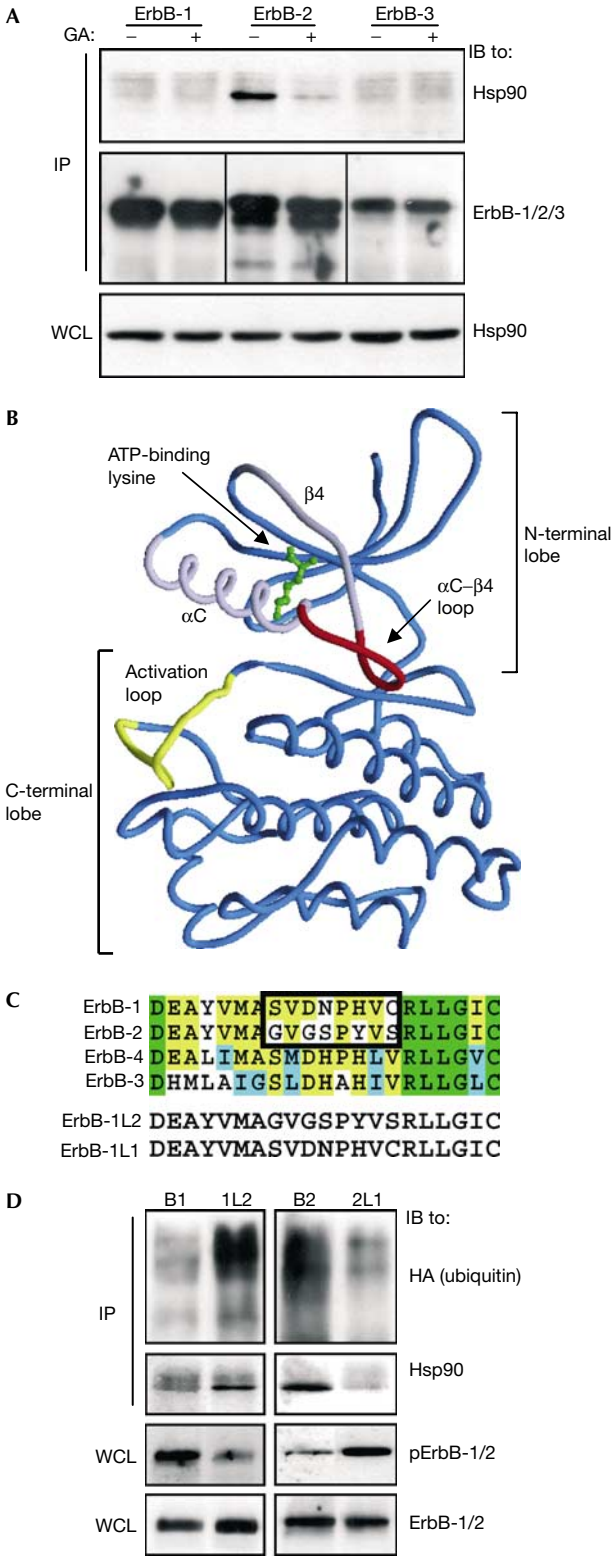
DISCUSSION

The data we obtained with a receptor tyrosine kinase add to the evidence acquired for steroid hormone receptors (Pratt & Toft,

2003) and cytosolic kinases (Donze *et al*, 2001), extending the role of the Hsp90 chaperone to regulation of client activity. The data also identify a specific loop of ErbB-2 as a recognition site for the Hsp90 complex. By binding to this loop within the kinase, Hsp90 restrains the catalytic function of ErbB-2 and limits the capacity of other ErbB proteins to engage in active heterodimers with ErbB-2 (Figs 1,2 and supplementary Fig 1 online). We propose that Hsp90 sequesters homodimers of ErbB-2 but the restrained catalytic activity is sufficient for weak *trans*-phosphorylation within homodimers (see model in Fig 4E). Conceivably, ligand-induced receptor heterodimerization brings the two intracellular kinase domains into close proximity, resulting in dissociation of Hsp90 by steric hindrance.

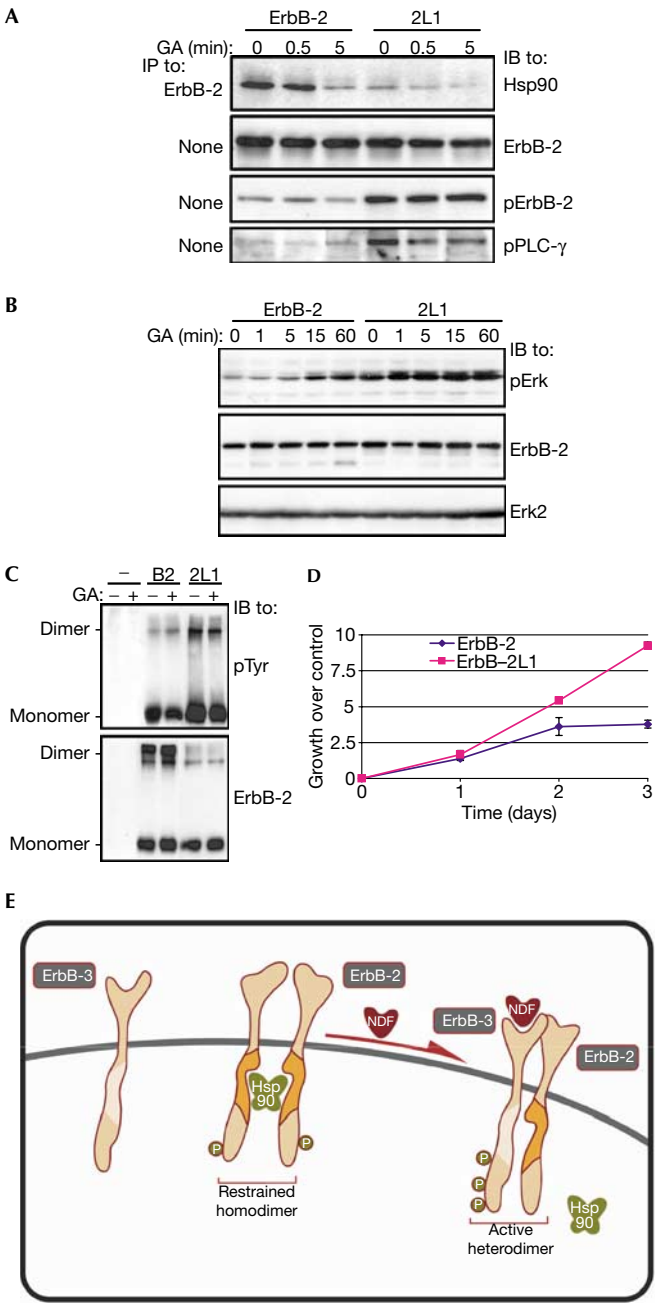
In the context of the ErbB signalling network, the extended role for Hsp90 is underscored by the selection of ErbB-2 as the only target receptor (Fig 4A). The unique function of this ligandless kinase is best exemplified by its interactions with the kinase-dead receptor ErbB-3. Heterodimers containing ErbB-2 and ErbB-3 generate extremely potent mitogenic signals, although each receptor is signalling-incompetent in isolation. By sequestering ErbB-2, Hsp90 prevents formation of ErbB-2·ErbB-3, as well as ErbB-2·ErbB-1 heterodimers (Fig 1 and supplementary Fig 1 online). Although the interaction of Hsp90 with ErbB-2 regulates both receptor heterodimerization and intrinsic kinase activity, it is unclear to what extent these two roles are distinct. Previous studies confined the binding site for Hsp90 to the kinase domain of ErbB-2 (Xu *et al*, 2001; Citri *et al*, 2002), suggesting that the chaperone represses heterodimerization by masking a dimerization motif within the kinase domain. A role for the kinase domain in receptor dimerization has previously been suggested, and receptor dimerization is enhanced after drug-induced modulation of the kinase (Arteaga *et al*, 1997). Hsp90 has previously been demonstrated to maintain an activatable conformation of steroid hormone receptors (reviewed in Pratt & Toft, 2003). Similarly, Hsp90 restricts activation of some clients by various mechanisms, including limiting the kinase activity of the PKR kinase (Donze *et al*, 2001) and stalling trimerization of the transcription factor Hsf1 (Zou *et al*, 1998).

On the basis of recognition of a region defining the sensitivity of ErbB-2 to ansamycins (Tikhomirov & Carpenter, 2003), we have established that the respective motif confers association with Hsp90 and demonstrated its function in limiting receptor activation. The motif maps to a loop connecting the αC helix and the β4 strand in the N-terminal lobe of the kinase domain (see model in Fig 3B). This location enables the motif to act as a hinge dictating the movement of the N-terminal lobe relative to the carboxy-terminal lobe, which governs the capacity of the kinase to bind both ATP and a substrate (Huse & Kuriyan, 2002). Regulatory



◀ **Fig 3** | A motif within the kinase domain of ErbB-2 confers recognition by Hsp90. (**A**) SKBR3 cells were treated with GA (5 μM; 5 min). Cells were then lysed and the interactions of the different ErbBs with Hsp90 determined by analysing co-immunoprecipitation in comparison with whole-cell lysates (WCLs). (**B**) Model of the kinase domain of ErbB-2 depicting the αC helix and β4 strand (both in silver), αC–β4 loop (red), activation loop (yellow) and the ATP-binding lysine (green). (**C**) Sequence alignment of the region corresponding to the putative Hsp90-binding loop of ErbB-2. A box marks the amino acids swapped in the 1L2 and 2L1 chimaeras. (**D**) HEK-293T cells were transfected with plasmids encoding HA-tagged ubiquitin and either ErbB-1, 1L2, ErbB-2 or 2L1. After 48 h, cells were subjected to the indicated analysis.

mechanisms often modulate kinase activity by altering the conformation of the αC helix. Examples include the interactions of cyclins with the αC helix of cyclin-dependent kinases (Jeffrey *et al*, 1995) and the inactive conformation of Src kinases, which stabilizes the αC helix in an inactive conformation (Sicheri *et al*, 1997). It is interesting to note that a region structurally similar to the putative Hsp90 binding site of ErbB-2 has been defined as the chaperone-binding motif on the glucocorticoid receptor, as well as within the kinase domain of Lck (Prince & Matts, 2004). The possibility that additional Hsp90 clients share a similar motif, and the relevance of our model to other receptor tyrosine kinases, are matters for future investigation.



MATERIALS AND METHODS

Reagents and plasmids. Unless otherwise indicated, materials were purchased from Sigma (St Louis, MO, USA). CI-1033 was received from Pfizer (Ann Arbor, MI, USA). GA and 17-AAG were received from Kosan Biosciences (Hayward, CA, USA). Anti-Hsp90 antibodies were from Stressgen (San Diego, CA, USA), anti-haemagglutinin (anti-HA) from Roche (Basel, Switzerland), anti-EGF receptor from Alexis (Montreal, Canada), phospho-specific antibodies to ErbB-1 (tyrosine 1148), ErbB-2 (tyrosine 1248) and ErbB-3 (tyrosine 1289) were from Cell Signaling (Beverly, MA, USA) and the phospho(783)-PLC-γ antibody was from Upstate Biotechnology (Charlottesville, CA, USA). All other antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Construction of mammalian expression vectors. Reciprocal replacements within the kinase domains of ErbB-1 and ErbB-2 (1L2 and 2L1) were generated by three consecutive rounds of PCR mutagenesis using the Pfu-Turbo enzyme (Stratagene, La Jolla, CA, USA). An expression vector encoding HA-tagged ubiquitin was a gift from Dirk Bohmann (EBI, Heidelberg, Germany).

Cell culture and transfection. 32D cell derivatives were cultured as described (Pinkas-Kramarski *et al*, 1996). All other cell lines were cultured in DME medium. Transfection of HEK-293T cells was performed using the calcium phosphate method. Lipofectamine2000 (Gibco BRL, Grand Island, NY, USA) was used for transfection of HeLa cells.

Lysate preparation, immunoprecipitation and immunoblot analyses. Where specified, RIPA buffer (25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40 and 0.1% SDS) was used. Lysis, immunoprecipitation and western blotting were carried out as described previously (Citri *et al*, 2002). Crosslinking experiments were carried out by adding *bis*(sulphosuccinimidyl) suberate (BS^3) (2 mM) to the solubilization buffer for 20 min on ice and terminated by addition of glycine (50 mM) for an additional 5 min.

Cell proliferation assay. Stable, low expression of 2L1 and ErbB-2 from the pcDNA3 expression vector was established in NIH-3T3 cells. Proliferation of the cells was assayed by using the MTT

Fig 4 | An ErbB-2 molecule unable to bind Hsp90 shows enhanced activity. (**A**) HEK-293T cells were transfected with plasmids encoding ErbB-2 and 2L1. After 48 h, cells were subjected to treatment for the indicated times with GA (5 μM), followed by lysis, immunoprecipitation of ErbB-2 and analysis as indicated. (**B**) HeLa cells were transfected with plasmids encoding ErbB-2 or 2L1. After 24 h, each plate was divided into six. Cells were then treated with GA (5 μM), and analysed as indicated. (**C**) HEK-293T cells were transfected with plasmids encoding ErbB-2 or 2L1. After 24 h, each plate was divided into six. The following day, the cells were treated with GA (0.1 μM; 5 min), followed by lysis in solubilization buffer containing BS^3 (2 mM). Cleared lysates were analysed as indicated. (**D**) The proliferation of NIH-3T3 cells stably expressing ErbB-2 or 2L1 (5×10^3 cells/well in 96-well plates) was followed over the course of 3 days using the MTT assay. Results presented are the average of six samples, relative to the growth of mock-transfected cells. (**E**) Model depicting the role of Hsp90 in regulating ErbB signalling. NDF binding to ErbB-3 drives heterodimerization of ErbB-2 and ErbB-3, while dissociating dimeric Hsp90 from ErbB-2 and disrupting ErbB-2 homodimers. Transphosphorylation of ErbB-3 subsequently takes place within the ErbB-2·ErbB-3 heterodimer.

(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl) assay as described (Pinkas-Kramarski *et al*, 1996).

***In vitro* kinase assays.** Assays were carried out in a final volume of 60 µl, and included a solubilized membrane fraction (30 µl; see supplementary information online) and 20 µl buffer (final concentrations of 20 mM Hepes (pH 7.4), 0.1 mM Na_3VO_4, 1 mM dithiothreitol, 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 0.1 mM ATP). The remaining volume was used for introduction of GA and CI-1033. Pretreatment with GA or CI-1033 took place for 30 min on ice, followed by addition of ATP and transition to 30 °C for 5 min, and was terminated by addition of boiling sample buffer.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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