HSP90 is a ubiquitously expressed molecular chaperone that controls the folding, assembly, intracellular disposition, and proteolytic turnover of many proteins, most of which are involved in signal transduction processes. Recently, a surface form of HSP90 has been identified and associated with cell migration events. In this paper, we explore the interaction of surface HSP90 with HER-2, a receptor-like glycoprotein and member of the ErbB family of receptor tyrosine kinases that play central roles in cellular proliferation, differentiation, and migration as well as in cancer progress. The involvement of HSP90 in the regulation of HER-2 has been attributed so far to receptor stabilization via interaction with its cytoplasmic kinase domain. Here we present evidence, using glutathione S-transferase pull-down and transfection assays, for a novel interaction between surface HSP90 and the extracellular domain of HER-2. Specific disruption of this interaction using mAb 4C5, a function-blocking monoclonal antibody against HSP90, inhibits cell invasion accompanied by altered actin dynamics in human breast cancer cells under ligand stimulation conditions with heregulin. Additionally, disruption of surface HSP90/HER-2 interaction leads to inhibition of herregulin-induced HER-2-HER-3 heterodimer formation, reduced HER-2 phosphorylation, and impaired downstream kinase signaling. Interestingly, this disruption does not affect HER-2 internalization. Our data suggest that surface HSP90 is involved in heregulin-induced HER-2 activation and signaling, leading to cytoskeletal rearrangement, essential for cell invasion.

The 90-kDa heat shock protein (HSP90) is a highly conserved and essential molecular chaperone throughout the eukaryotic lineage (1–3). HSP90 is an ATP-dependent molecular chaperone that in higher eukaryotes exists in two isoforms (HSP90α and HSP90β). It is one of the most abundant cytoplasmic proteins in unstressed cells, where it performs housekeeping functions, controlling the activity, intracellular disposition, and proteolytic turnover of a variety of proteins (4, 5). So far, at least 100 proteins are known to be regulated by HSP90, including key mediators of signal transduction and cell cycle control, steroid hormone receptors, and tyrosine/threonine kinases (6–9). HSP90 keeps these unstable proteins poised for activation through low affinity interactions, characterized by repeated ATP-dependent cycles of binding and release, until they are stabilized by conformational changes associated with signal transduction (2, 10). Over the past years, there has been increasing evidence that HSP90 interacts with a great number of molecules that are involved in the development and/or survival of cancer cells (11, 12), allowing mutant proteins to retain or gain function while permitting cancer cells to tolerate the imbalanced signaling that such oncoproteins create

The receptor tyrosine kinase HER-2 (also known as ErbB-2) is a protein whose stability depends on HSP90 function (13). HER-2 is a 185-kDa transmembrane glycoprotein and a member of the ErbB family of epidermal growth factor receptors (14–16). Binding of ligands to the extracellular domain (ECD) of ErbB receptors induces the formation of receptor homo- and heterodimers and the activation of their intrinsic kinase domain, resulting in phosphorylation of specific tyrosine residues within the cytoplasmic tail. The latter serve as docking sites for various adaptor proteins or enzymes, which simultaneously initiate many signaling cascades to produce a physiological outcome (17). HER-2 is a ligandless receptor and the preferred heterodimerization partner for ligand-bound epidermal growth factor receptor family members HER-1, HER-3, and HER-4 (17–19). By functioning as co-receptor, HER-2 mediates signal transduction, resulting in cell motility, mitogenesis, apoptosis, angiogenesis, or cell differentiation. Any alteration of the tightly regulated ErbB receptor signaling pathways results in major cellular abnormalities and tumorigenesis. For example, abnormalities in the expression, structure, or activity of HER-2 contribute to the development and maintenance of the malignant phenotype (20). Moreover, overexpression or amplification of the HER-2 receptor is associated with increased progression and metastasis in human breast cancer (21, 22).

The role of HSP90 in the regulation of HER-2 has been attributed so far to stabilization of the receptor at the cell surface, via interaction with its cytoplasmic kinase domain, such that disruption of the HER-2/HSP90 association induced by HSP90 inhibitors (e.g. geldanamycin) leads to proteosomal degradation of the receptor (23–25).

Recently, we and others have shown that HSP90 is localized not only in the cytoplasm but also on the cell surface (26–28).
addition, we have shown that surface HSP90 contributes to cell migration processes via cytoskeletal rearrangement during normal embryonic development of the nervous tissue, by exploiting a function-blocking monoclonal antibody, mAb 4C5, that recognizes both the α and, to a lesser extent, the β isoform of the HSP90 protein (26, 29, 30). Finally, we have obtained results showing that mAb 4C5 inhibits B16 F10 melanoma cell invasion and metastasis by binding selectively to surface HSP90 (31).

Taking into consideration the above, together with the fact that the migration mechanisms occurring in normal nonneoplastic cells during embryonic development are similar, if not identical, to the invasion processes of tumor cells during metastasis, in this study we examine the molecular mechanisms underlying the involvement of surface HSP90 in cancer cell invasion processes. For the first time, we show here that surface HSP90 interacts directly with the ECD of HER-2 and participates in heregulin (HRG)-induced cancer cell invasion. Moreover, specific disruption of the surface HSP90/HER-2 interaction under ligand stimulation conditions leads to inhibition of HER-2/HER-3 heterodimer formation, reduced HER-2 phosphorylation, and impaired downstream kinase signaling, without affecting HER-2 endosomal trafficking.

Our combined data strongly suggest that surface HSP90 is involved in HRG-induced HER-2 activation and signaling, which in turn will activate cytoplasmic signal transduction cascades leading to cytoskeletal rearrangement, essential for cell motility.

**EXPERIMENTAL PROCEDURES**

*Antibodies and Reagents—*mAb 4C5 was produced in our laboratory, as previously described (32). In the present study, mAb 4C5 was used as concentrated serum-free supernatant in all experiments performed. Polyclonal antibodies against HER-2 or tyrosine-phosphorylated HER-2 were obtained from Cell Signaling. Polyclonal antibodies against HSP70 as well as the phosphorylated and total forms of Akt, mitogen-activated protein kinase, and mitogen-activated protein kinase/extracellular signal-regulated kinase kinases were a kind gift from Dr. N. Grammatikakis. Polyclonal antibody against HER-3 was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). RPMI and fetal bovine serum (FBS), were from Invitrogen. All other materials were from sources previously described (26).

*Cell Cultures and Immunofluorescence—*Two different human breast cancer cell lines were used, MDAMB453, which is a HER-2-overexpressing cell line, and MDAMB435, which expresses very low levels of endogenous HER-2 (33). Both cell lines were maintained in RPMI supplemented with 10% FBS. For immunofluorescence studies, MDAMB453 cells were plated on poly-L-lysine-coated coverslips in a 48-well plate at a density of 10^5 cells/well and were left for 24 h in serum-containing medium, without further treatment. The medium was then changed to serum-free RPMI, and 16 h later, a cell-free area was generated by gently scratching the cell monolayer with a sterile yellow Gilson-pipette tip, thus resulting in the formation of an ~1-mm-wide cell-free area. Immediately after scratching, the medium was replaced with fresh medium, containing mAb 4C5 (200 μg/ml) and/or HRG (100 ng/ml) and/or Herceptin (1 μg/ml). All agents were maintained in the culture for the duration of the assay. Control cultures were grown either in culture medium alone (RPMI) or in culture medium containing a 200 μg/ml concentration of an IgG2a monoclonal antibody (concentrated serum-free hybridoma supernatant) against the unrelated protein BM88 (34). Migration of breast cancer cells within the gap was monitored microscopically at given time intervals, using a Leica DM IL inverted microscope, equipped with a LEICA DM300 video camera connected to a computer. Migration distance was estimated by acquiring and analyzing digital images, using the Image Pro Plus analysis software (35, 36) and expressed as the percentage of distance covered by cells in control cultures. Statistical analysis was performed by using Student’s t test.

*Bromodeoxyuridine Incorporation Assay—*Serum-starved MDAMB453 cells cultured in the presence of mAb 4C5 and/or HRG were exposed to bromodeoxyuridine at a concentration of previously described (32). Live MDAMB453 cells and transiently transfected MDAMB435 cells were labeled by indirect immunofluorescence as previously reported (26). Alexa488-labeled phalloidin and Alexa546-conjugated transferrin (Molecular Probes, Inc., Eugene, OR) were used to visualize F-actin and transferrin receptor, respectively, and the far red wavelength, DNA-intercalating dye To-pro-3 (Molecular Probes) was used for nuclear staining. For all experiments, controls were performed by omitting the primary antibodies and/or by using an IgG2a monoclonal antibody against the unrelated neuronal protein BM88 (34). Additional negative controls were performed using a polyclonal antibody against β-tubulin (Santa Cruz Biotechnology). Immunofluorescence was analyzed by confocal microscopy using a Leica TSC confocal microscope.

*Antibody Internalization Assay—*MDAMB453 cells were incubated while in culture with 200 μg/ml mAb 4C5 for 2, 8, and 24 h. The cells were then washed in RPMI and fixed in cold acetone for 3 min. For detection of possible internalization of the antibody, cells were permeabilized with 0.1% Triton X-100 in PBS and subsequently incubated with Alexa488-conjugated secondary antibody (Molecular Probes). For all experiments, controls were performed as mentioned above.

*Cell Fractionation—*Cellular fractionation was performed in cell cultures prepared and treated as above, using the Compartmental protein extraction kit (Chemicon), according to the manufacturer’s instructions. Protein lysates were quantified, and equal amounts of total protein were subjected to SDS-PAGE and Western blot. Protein estimations were performed with the DC protein assay (Bio-Rad), according to the manufacturer’s instructions.

*Wound Healing Migration Assay—*The assay was performed as previously described (35). Briefly, MDAMB453 and MDAMB435 cells were plated on poly-L-lysine-coated coverslips in a 48-well plate at a density of 10^5 cells/well and were left for 24 h in serum-containing medium, without further treatment. The medium was then changed to serum-free RPMI, and 16 h later, a cell-free area was generated by gently scratching the cell monolayer with a sterile yellow Gilson-pipette tip, thus resulting in the formation of an ~1-mm-wide cell-free area. Immediately after scratching, the medium was replaced with fresh medium, containing mAb 4C5 (200 μg/ml) and/or HRG (100 ng/ml) and/or Herceptin (1 μg/ml). All agents were maintained in the culture for the duration of the assay. Control cultures were grown either in culture medium alone (RPMI) or in culture medium containing a 200 μg/ml concentration of an IgG2a monoclonal antibody (concentrated serum-free hybridoma supernatant) against the unrelated protein BM88 (34). Migration of breast cancer cells within the gap was monitored microscopically at given time intervals, using a Leica DM IL inverted microscope, equipped with a LEICA DM300 video camera connected to a computer. Migration distance was estimated by acquiring and analyzing digital images, using the Image Pro Plus analysis software (35, 36) and expressed as the percentage of distance covered by cells in control cultures. Statistical analysis was performed by using Student’s t test.
10^{-5} \text{ M} \text{ for } 4 \text{ h}. \text{ Cells were subsequently rinsed in PBS and fixed in 4\% paraformaldehyde for 15 min. After washing, the cells were incubated in 2\% HCl containing 0.1\% Triton X-100 for 10 min, followed by a 20-min incubation in 0.1 \text{ M sodium borate and then processed for indirect immunofluorescence using an anti-bromodeoxyuridine antibody (DAKO) followed by an appropriate Alexa488-conjugated goat secondary antibody. Plasmid Construction—The rat HER-2 full-length cDNA (4478 bp cloned into the pcMVSPORT6.1 vector) was obtained from the I.M.A.G.E. Consortium Library, Medical Research Council Geneservice (clone MGC: 72502; IMAGE: 5597835). For construction of the GST-HER-2 plasmids, PCR was performed using Phusion polymerase (Finzymes). For production of the ECD of the rat HER-2, the following forward (F) and reverse (R) primers were used: FCD1, 5’-CCCGAATTCAATGATCATATCAGGAGCTG-3’; RCD1, 5’-ATAAGAATGGCGCCCGCTCGTCTG-3’. The resulting PCR product (1996 bp), encoding amino acids 1–650 (see Fig. 3A) was subcloned into the pGEX-4T1 vector (Amersham Biosciences) and NotI cloning sites. The intracellular domain (ICD) of rat HER-2 was also produced by PCR using the primers FICD1 (5’-CCGGAATTCGCCACCATGATCATCATGGACTGG-3’), RICD1 (5’-ATAAGAATGGCGCCCGCTCGTCTG-3’), and the resulting PCR product (1783 bp) encoding amino acids 681–1259 (see Fig. 3A) was subcloned into pGEX-4T1 between EcoRI and NotI cloning sites. Both plasmids were subjected to sequence analysis and were then used to transform BL21 cells for production of GST-HER-2 chimeric proteins.

In order to perform transient transfection experiments, the ECD of HER-2, corresponding to amino acids 1–683, including the transmembrane domain (TM) and four intracellular amino acids (KRRR), as well as the ICD of HER-2, corresponding to amino acids 684–1259 were generated by PCR (see Fig. 4D), using the following primers, respectively: FEC1 (5’-CCCGAATTCGCCACCATGATCATATCAGGAGCTG-3’), REC1 (5’-ATAAGAATGGCGCCCGCTCGTCTG-3’), and the resulting PCR product (1996 bp), encoding amino acids 1–650 (see Fig. 3A) was subcloned into pGEX-4T1 between EcoRI and NotI cloning sites. Both plasmids were subjected to sequence analysis and were then used to transform BL21 cells for production of GST-HER-2 chimeric proteins.

Surface HSP90 Interacts with the ECD of HER-2

Preparation of Cell Lysates, Co-immunoprecipitation, and Western Blot Analysis—MDAMB453 cells grown in 6-well plates in RPMI medium supplemented with 10\% FBS until subconfluence were shifted in serum-free medium for 24 h and then exposed or not to 200 \mu{g}/ml mAb 4C5 for 2 h. Excess nonbound antibody was then removed by washing with fresh medium, and cells were stimulated (or not) with HRG (100 ng/ml) for 15 min. The cultures were immediately washed twice with ice-cold PBS and lysed in 50 \mu{l} of lysis buffer (137 mM NaCl, 20 mM Tris/HCl, pH 7.4, 50 mM HEPES, 5 mM EDTA, 1 mM dithiothreitol, 1\% Triton, 10\% glycerol, 200 mM Na_{2}VO_{4}, 0.5 mM phenylmethylsulfonyl fluoride, 5 \mu{g}/ml aprotinin, 5 \mu{g}/ml leupeptin). Protein lysates were quantified, and equal amounts of total protein were subjected to SDS-PAGE and transferred to nitrocellulose. The membranes were blotted for 40 min at room temperature with nonfat dry milk (5\%) in TBS containing 0.05\% Tween 20 to block nonspecific binding sites and were then incubated with specific primary antibodies overnight at 4\°C. The membranes were washed with 0.3\% bovine serum albumin in Tris-buffered saline and incubated with horseradish peroxidase-labeled secondary antibodies for 2 h at room temperature. After washing with Tris-buffered saline, the bound antibody complex was detected using an ECL chemiluminescence reagent (Amersham Biosciences) and X-Omat AR film (Eastman Kodak Co.) as described by the manufacturers.

Co-immunoprecipitation was performed as previously described (37). In brief, equal amounts of precleared cellular lysates or brain fractions were incubated with antibodies overnight at 4\°C. The immunocomplexes were then incubated for 2 h at room temperature with protein A-Sepharose or protein G-Sepharose and washed three times with lysis buffer. Bound proteins were analyzed by gel electrophoresis and Western blot as described above. For all immunoprecipitation experiments, negative controls were performed using irrelevant IgGs.

Chemical Cross-linking—MDAMB453 cells were incubated with mAb 4C5 (200 \mu{g}/ml) and then stimulated with 100 ng/ml HRG as described above. Cross-linking was performed as pre-
Surface HSP90 Interacts with the ECD of HER-2

Previously described (38). Briefly, the cells were incubated on ice for 30 min with PBS containing a 3 mM concentration of the nonpermeable cross-linking reagent bis(sulfo-succinimidyl) suberate (Pierce). To stop the cross-linking reaction, glycine (20 mM final concentration) was added, and the cells were further incubated for 15 min at room temperature. The cells were then washed three times in ice-cold PBS and lysed. Protein complexes were analyzed using a gradient 3–8% Tris acetate NuPAGE gel (Invitrogen).

Receptor Internalization—In order to determine whether the internalization of HER-2 is affected by mAb 4C5, we examined the receptor’s endocytic trafficking using a fluorescence microscope. Briefly, serum-starved MDAMB453 cells grown in glass coverslips were treated or not with 200 μg/ml mAb 4C5 for 2 h, subsequently exposed or not to HRG (100 ng/ml) for 15 min, and surface-labeled for 60 min on ice with 100 μg/ml Alexa546-conjugated transferrin. Cells were then washed three times with cold RPMI and incubated at 37 °C for the indicated time intervals. Cells were then fixed in 4% paraformaldehyde and labeled with anti-HER-2 antibody by indirect immunofluorescence.

RESULTS

mAb 4C5 Binds to the Cell Surface and Is Not Internalized by MDAMB453 Cells—In order to investigate whether HSP90 is present on the surface of HER-2-overexpressing MDAMB453 cells, unfixed cultures were incubated with mAb 4C5. The cells were then carefully washed, fixed, and labeled with fluorescence-conjugated secondary antibody. Thus, the primary antibody had access only to the external surface of the cells. The observed typical punctuate immunostaining confirmed the cell surface localization of HSP90 (Fig. 1A). It should be noted that similar results were obtained when using commercial anti-HSP90α and anti-HSP90β antibodies (Fig. 1A). Control experiments using an antibody to the intracellular protein β-tubulin (Fig. 1A) and a mouse monoclonal antibody against BM88, an intracellular unrelated protein (data not shown), did not produce any detectable surface staining. At this point, it should be noted that mAb 4C5 also recognizes the intracellular HSP90 as demonstrated by immunofluorescence after fixation and permeabilization of MDAMB453 cells (Fig. 1B). Apparent β-tubulin staining is also observed under fixed conditions (Fig. 1B). The above results were confirmed by Western blot of MDAMB453 cell fractions, using mAb 4C5 as well as the commercial anti-HSP90α and anti-HSP90β antibodies (Fig. 1C). As positive controls, anti-HER-2, anti-HSP70, and anti-phosphohistone H3 (Upstate Biotechnology, Inc.) antibodies were used for the membrane, cytoplasmic, and nuclear fractions, respectively. Negative controls were performed using β-tubulin, which is absent in all of the above fractions but present in a separate cytoskeletal fraction (Fig. 1C).

The binding of mAb 4C5 to living MDAMB453 cells was examined at various time intervals by incubating the cells with mAb 4C5 at 37 °C for 2, 8, and 24 h. The cells were then carefully washed, and binding of the antibody was analyzed after fixation and cell permeabilization, with a fluorescence-conjugated secondary antibody. Our results showed that at all time intervals studied, mAb 4C5 was not internalized and remained bound on the cell surface (Fig. 1D).

HSP90 Is Involved in HRG-induced MDAMB453 Cell Invasion, Formation of Motile Cell Phenotypes, and Actin Rearrangement—Having established that mAb 4C5 is not internalized when incubated with living MDAMB453 cells but instead remains specifically bound to the cell-surface HSP90, we proceeded to examine the possible interaction of this molecule with HER-2 during cancer cell invasion by exploiting the function-blocking properties of mAb 4C5, in a wound healing assay. This was done either in the absence or in the presence of ligand stimulation with HRG. Furthermore, and in order to investigate the effect of HER-2 inhibition on mAb 4C5 activity, we tested the effect of Herceptin, a monoclonal antibody targeted against the ECD of HER-2, which is widely used as an inhibitor of HER-2 dimerization, alone and in combination with mAb 4C5. Control cultures were grown either in culture

![Figure 1](image-url)
Surface HSP90 Interacts with the ECD of HER-2

FIGURE 2. Surface HSP90 is involved in cancer cell invasion, formation of motile cell phenotypes, and actin rearrangement in vitro. A, wound healing assay. Photographs represent phase-contrast images obtained at zero time (left) and at 24 h after scratch formation (right), showing MDAMB453 cell migration in the presence of the following media: (a) control containing antibody against BM88; (b) containing mAb 4C5; (c) containing HRG (d) containing both mAb 4C5 and HRG; (e) containing Herceptin; or (f) containing both mAb 4C5 and Herceptin. Scale bar, 200 μm. B, quantitation of mAb 4C5, HRG, and/or Herceptin on the closure of the wound. The addition of 200 μg/ml mAb 4C5 in the culture medium resulted in a 74.4% inhibition of wound closure when compared with control cultures that were considered as resulting in 100% wound closure. HRG (100 ng/ml) enhanced cell migration to a rate of 89.7% when compared with controls, whereas the addition of both mAb 4C5 and HRG resulted to a migration rate similar to the controls (89.7% wound closure). The addition of 1 μg/ml Herceptin in the culture medium resulted in a 73.4% inhibition of wound closure, whereas the addition of both mAb 4C5 and Herceptin resulted in a 78.3% inhibition. Bars, average of three independent experiments ± S.E. Within a single experiment, each condition was tested in triplicate. Statistical significance of differences was tested by Student’s t test. The presence of 200 μg/ml of mAb 4C5, 100 ng/ml HRG, 1 μg/ml Herceptin, and both mAb 4C5 and Herceptin had a statistically significant effect on the wound closure (p < 0.0005 for each). The presence of both mAb 4C5 and HRG had no statistically significant effect when compared with control cultures (p > 0.5), whereas the presence of both inhibitors (mAb 4C5 and Herceptin) had the same significant difference from the control as when each agent alone was included in the culture medium (p > 0.005 for each). *, different from control cultures at p < 0.005; †, different from control cultures at p < 0.01; ‡, different from cultures stimulated with 100 ng/ml HRG at p < 0.01; C, cell fractionation followed by Western blot with mAb 4C5 and anti-HER-2 antibody, in various cell fractions. The status of both proteins appears with no visible differences between untreated cells or cells treated with mAb 4C5. Negative controls were performed using an antibody against the cytoskeletal protein β-tubulin. M, membrane fraction; C, cytoplasmic fraction; N, nuclear fraction; CS, cytoskeletal fraction. D, phalloidin staining. MDAMB453 breast cancer cells were treated or not with 200 μg/ml mAb 4C5 for 2 h, stimulated with 100 ng/ml HRG, and then immunofluorescently labeled with phalloidin. Control (C) (nontreated) cultures are also shown. Scale bar, 40 μm. E, higher magnification showing phalloidin staining (F-actin). mAb 4C5 effectively blocks spreading of migrating cells, cultured in the presence of 200 μg/ml mAb 4C5. Since the process of cell invasion requires, among other events, changes in cell morphology and cytoskeletal reorganization, we next examined the role of surface HSP90 in HRG-mediated invasive cellular phenotypes, using confocal microscopy. In particular, we studied the localization of F-actin in HRG-stimulated migrating MDAMB453 cells, either in the presence or absence of mAb 4C5. When these cells were immunostained with fluorescently labeled phalloidin, a rearrangement in the actin filaments was observed in the migrating cells, cultured in the presence of 200 μg/ml mAb 4C5.
In particular, when cells were exposed to mAb 4C5, we observed less cell spreading as compared with control cultures and a morphology indicative of nonmotile cells (Fig. 2D). Furthermore, when these cultures were visualized at a higher magnification, lamellipodia were less developed and spread out as compared with lamellipodia of MDAMB453 in control cultures, maintained in the absence of mAb 4C5 (Fig. 2E).

Cell Surface HSP90 Specifically Interacts with the Extracellular Domain of HER-2 both in Vitro and in Vivo—In order to investigate the possible interaction of the surface HSP90 protein with the ECD of HER-2, we performed GST pull-down assays using the GST-ECD fusion protein. As positive and negative controls, we used the GST-ICD protein and the GST protein, respectively. GST-ECD, GST-ICD, and GST proteins were incubated with Triton X-100-solubilized postnatal day 5 rat brain fractions, previously shown to contain HSP90 (26), and precipitated proteins were analyzed by Western blotting using mAb 4C5. It was thus revealed that both GST-ECD and GST-ICD interacted specifically with HSP90, whereas GST control protein gave negative results (Fig. 3B). These results demonstrate that in addition to the ICD, the ECD of HER-2 also interacts specifically with HSP90 in vitro.

In order to investigate whether surface HSP90 interacts with HER-2 in vivo, we performed transient transfection experiments in MDAMB435 cells with Myc-tagged ECD, ICD, and a fragment of HER-2 that does not interact with HSP90 (negative control). Additional control experiments were performed using the empty vector pcDNA3A (data not shown). MDAMB435 cells, in contrast to the MDAMB453 cell line, express extremely low levels of endogenous HER-2. Immunofluorescence experiments in live MDAMB435 cells confirmed the very low expression levels of HER-2 (Fig. 4A). Interestingly, expression of surface HSP90 was also very weak in these cells (Fig. 4A). As shown in Fig. 4B, when MDAMB435 cells were stimulated with HRG in the presence or absence of mAb 4C5, a weak interaction between the two endogenous proteins was observed. Since this cell line expresses very low levels of endogenous HER-2 as well as low levels of surface HSP90, the immunoreactivity obtained corresponds mainly to the intracellular interaction of these molecules. The slight reduction of the immunoreactivity observed in the immunoprecipitants derived from the mAb 4C5-treated lysates, when compared with the control lysates, most probably reflects the mAb 4C5 disruption of the extracellular interaction of HER-2 with HSP90. Moreover, it is important to note that when this cell line was used in the wound healing assay, no statistically significant difference was observed between the control cultures and the cultures performed in the presence of mAb 4C5 (Fig. 4C).

Expression of Myc-tagged ECD, ICD, and the negative control fragment of HER-2 in MDAMB435-transfected cells was confirmed by immunocytochemistry with a monoclonal anti-Myc antibody (Fig. 4F). Immunoprecipitation with the anti-Myc antibody to bring down selectively the exogenous pool of the HER-2 constructs, followed by Western blot with mAb 4C5, revealed that both the ECD and ICD of HER-2 interacted with HSP90 (Fig. 4F), whereas the intracellular fragment of HER-2 missing the kinase domain did not interact with HSP90 (negative control). Additional negative controls were performed using nontransfected cell lysates (Fig. 4F) and lysates transfected with the empty vector pcDNA3A (data not shown). Taken together, our results indicate that surface HSP90 interacts with the ECD of HER-2 both in vitro and in vivo.

Surface HSP90 Interaction with HER-2 Is Disrupted by mAb 4C5—In order to explore mAb 4C5 function in relation to the above demonstrated interaction of HSP90 with the ECD of HER-2, we performed co-immunoprecipitation experiments in MDAMB453 cell cultures pretreated or not with 200 μg/ml mAb 4C5 and then stimulated or not with HRG. HER-2 immunoprecipitants were obtained from control and treated cultures and were analyzed by Western blot using mAb 4C5. As shown in Fig. 5A, a lesser amount of HSP90 co-immunoprecipitated with HER-2, in lysates from mAb 4C5-treated cells (HRG-stimulated or not), when compared with lysates from untreated cells. This suggests that the extracellular interaction of surface HSP90 with HER-2 is specifically disrupted by the presence of mAb 4C5.

Surface HSP90 Interaction with HER-2 Is Necessary for the Receptor's Activation—To explore the possible mode of action of surface HSP90 in vivo, we examined by Western blot the expression levels of total HER-2 protein and its active phosphorylated form in nonstimulated and HRG-stimulated MDAMB453 cultures, using two different antibodies: (a) an anti-HER-2 antibody recognizing both the phosphorylated and nonphosphorylated form of the molecule and (b) an antibody recognizing only the phosphorylated form of HER-2. Living MDAMB453 cultures were treated (or not) for 2 h with mAb 4C5 to specifically disrupt (or not) the extracellular interaction of surface HSP90 with HER-2 and were then exposed (or not) for 15 min to HRG. After the removal of nonbound antibody, cell lysates were prepared and analyzed by immunoblotting. We observed that the mAb 4C5-mediated disruption of the extracellular interaction between surface HSP90 and HER-2 in HRG-stimulated cells (see Fig. 5A) resulted in substantial loss of HER-2 tyrosine phosphorylation without affecting the total protein expression levels of HER-2 (Fig. 5B). Densitometry quantification revealed that the phosphorylation levels of HER-2 after treatment with mAb 4C5 decreased by 43% when compared with control lysates.
In order to further confirm that the mAb 4C5 effects are directed toward receptor activation and not proteolytic turnover, we next explored the effect of mAb 4C5 on HER-2 internalization and endosomal trafficking, using fluorescence microscopy. Living MDA-MB453 cells were treated or not with mAb 4C5 for 2 h and surface-labeled with Alexa546-conjugated transferrin, which was used as a positive control for monitoring the internalization process. Subsequently, the cells were fixed at various time intervals and immunostained with anti-HER-2 antibody in order to evaluate the receptor trafficking. Our results showed that mAb 4C5 had no visible effect on HER-2 internalization for all time intervals studied, whereas the process of internalization preceded normally (Fig. 5C). The receptor remained bound mainly on the cell surface even after 18 h (data not shown), exhibiting extremely limited internalization in all experimental groups studied and even in the presence of HRG (data non shown).

Surface HSP90 Interaction with HER-2 Is Necessary for the HRG-induced Formation of HER-2-HER-3 Heterodimers and the Activation of Downstream Signaling Pathways—

To further elucidate the molecular interactions underlying surface HSP90 activity, we examined the effect of mAb 4C5 on HRG-induced interactions between HER-2 and HER-3 receptors in MDA-MB453 cultures by performing immunoprecipitation and cross-linking experiments in membrane fraction lysates derived from MDA-MB453 cells. The results shown in Fig. 6A, demonstrate that treatment of cultures with mAb 4C5 for 2 h, as described above, followed by stimulation with HRG, inhibited the association of HER-2 with HER-3. More precisely, the anti-HER-3 antibody co-immunoprecipitated significantly lower levels of HER-2 in response to HRG stimulation in mAb 4C5-treated cells, in which the surface interaction of HSP90 and the kinase domain of HER-2. The slight reduction observed in the mAb 4C5-treated lysates probably reflects the mAb 4C5 disruption of the extracellular interaction. C, quantitative effect of mAb 4C5 on the closure of the wound. The addition of 200 μg/ml mAb 4C5 in the culture medium resulted in a 10% inhibition of wound closure when compared with control cultures that were considered as resulting in 100% wound closure. Bars, average of three independent experiments ± S.E. Within a single experiment, each condition was tested in triplicate. Statistical significance of differences was tested by Student’s t test. The presence of mAb 4C5 had no statistically significant effect when compared with control cultures (p > 0.1). D, schematic representation of the rat HER-2 protein (1259 amino acids) and the resulting ECD and ICD fusion proteins after cloning into the pcDNA3A (His/Myc) vector. The negative fragment of HER-2 lacking the extracellular and the kinase domains (HER-2neg) is also included. E, MDA-MB453 cell cultures transiently transfected as described under “Experimental Procedures” were fluorescently labeled with mouse anti-Myc antibody 48 h after transfection. Cell surface and intracellular localization of the ECD, ICD, and the negative fragment of HER-2, respectively, was observed. Scale bar, 20 μm. F, cell lysates derived from transiently transfected MDA-MB453 cultures, as well as lysates derived from nontransfected cells (NTC), were co-immunoprecipitated with anti-Myc antibody, and immunoprecipitants were analyzed by Western blot with mAb 4C5. The immunoreactivity obtained indicates that both the ECD and the ICD (positive control) of HER-2 interact with HSP90. Nontransfected lysates and the negative fragment of HER-2 immunoprecipitants did not produce any detectable result. IP, immunoprecipitation; WB, Western blot; NTC, nontransfected cells.
Cells were treated or not with mAb 4C5, followed by stimulation with HRG, and cell lysates were assayed for the activation of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase, Akt, and Erk. As shown in Fig. 6C, treatment of cells with mAb 4C5 resulted in reduced phosphorylation levels of all of the kinases examined. At this point, it is important to note that it is not surprising that the differences observed between the controls and the cultures treated with mAb 4C5 are not dramatic, since these kinases, especially Akt, are central effectors involved in multiple signaling pathways and are subsequently phosphorylated by several molecules.

**DISCUSSION**

Our present findings demonstrate a novel role for cell surface HSP90 in the context of HRG-induced activation of HER-2 and signal transduction cascades leading to cytoskeletal rearrangement and invasion of cancer cells. Our previously reported data have demonstrated that HSP90 is localized not only in the cytoplasm but also on the cell surface of neural cells in the developing nervous system (26). Moreover, we have shown that HSP90 participates in cell migration processes and is associated with actin cytoskeletal dynamics during development of the nervous system (29, 30, 43).

In this work, we examined using mAb 4C5, an antibody targeted against HSP90, and two breast cancer cell lines, the molecular mechanisms underlying the involvement of surface HSP90 in cancer cell invasion processes. Cell surface localization of HSP90 was demonstrated both by immunocytochemistry on live MDAMB453 breast cancer cells and by Western blot using membrane fraction lysates derived from the same cell line. Inhibition of MDAMB453 cell invasion by mAb 4C5 was shown using the wound healing assay. In particular, the presence of mAb 4C5 in the culture medium strongly reduced MDAMB453 cell invasion, thus indicating the involvement of HSP90 in this event. The effects of mAb 4C5 were comparable with those of Herceptin, but inclusion of both molecules did not yield an additive effect. This is not surprising, since mAb 4C5 and Herceptin are targeted against two different molecules involved in the invasion process.

Eustace et al. (28) have recently reported that secretion of HSP90α into the extracellular matrix surrounding tumor cells seems to assist in the activation of matrix metalloproteinase-2, contributing thus to tumor cell invasiveness. To assess the participation of cell surface and not intracellular HSP90 in the above described invasion process of MDAMB453 cells, internalization of mAb 4C5 was examined at various time intervals. Interestingly, mAb 4C5 remained bound to the cell surface even after 24 h in culture. This result strongly suggests that the observed inhibitory effect of mAb 4C5 on MDAMB453 cell invasion is due to binding of the antibody to the cell surface pool of HSP90.

In order to investigate the molecular interactions of surface HSP90 during cell invasion and taking into consideration previous data showing that HRG is a growth factor implicated in the invasion of breast cancer cells (44), we examined the effect of mAb 4C5 on the invasive capacity of MDAMB453 cells in the presence of HRG. When the above cells were incubated with HRG alone and in accordance with previously reported results (35, 45), a significant enhancement of cell invasion was obtained. Moreover, an increased expression of F-actin accompanied by cell spreading and development of motile structures, such as lamellipodia, was observed. Interestingly, the addition of mAb 4C5 in the culture medium inhibited the biological effects of HRG, including invasion, reorganization of actin cytoskeleton, and lamellipodia formation. These results indicate that cell surface HSP90 may be necessary for the activation of the HER-2/HRG signaling pathway that leads to actin rearrangement and cell invasion (44, 46).

We have previously argued that since surface HSP90 is a peripheral protein loosely attached to the cell membrane, it is highly unlikely that it acts on its own in order to transmit signals within the cell (26, 30). Therefore, the inhibitory effect of mAb 4C5 on HRG-induced cell invasion prompted us to examine the
Surface HSP90 Interacts with the ECD of HER-2

A possible association of HSP90 with the ECD of HER-2. GST pull-down assays revealed that this molecule indeed interacts specifically with the ECD of HER-2 in vitro. HSP90 interacted with both the GST-ECD and the GST-ICD constructs used as baits in the GST pull-down assays but not with the GST protein alone. Evidence for interaction with the ECD of HER-2 in vivo was also obtained by performing transient transfection experiments in MDA-MB-453 cells, which express very low levels of endogenous HER-2. At this point, it is interesting to note that in this cell line, expression of surface HSP90 was also very low. Thus, the weak interaction between HER-2 and HSP90 obtained by immunoprecipitation experiments on total MDA-MB-453 lysates corresponded mainly to the intracellular interaction of HSP90 with HER-2 in vivo. However, as expected, mAb 4C5 did not significantly inhibit MDA-MB-453 cell invasion when used in the wound healing assay. When cell lysates derived from MDA-MB-453 cells transfected with the ECD and the ICD (positive control), were subjected to immunoprecipitation with anti-Myc antibody and probed with mAb 4C5, a significant immunoreactivity was obtained indicating the interaction of HSP90 with the ECD and, as expected, with the ICD of HER-2. No effect on the expression of the total pool of HER-2, but it significantly reduced the presence of its active phosphorylated form compared with control cultures (nontreated with mAb 4C5). Furthermore, data obtained from HER-2 internalization studies revealed that mAb 4C5 has no effect on receptor endosomal trafficking, and as in all experimental groups studied, HER-2 internalization remained very limited. Our combined data indicate that the extracellular interaction of HSP90 with HER-2 is necessary for maintaining the receptor in an active state and is not related to the stability of the receptor on the cell surface.

Multiple signaling pathways are initiated when ErbB receptors form dimers and are activated through trans-phosphorylation at specific tyrosine residues after ligand binding (17). To shed light on the molecular events underlying the surface HER-2/HSP90 interaction, we further investigated HER-2-HER-3 heterodimer formation and activation of downstream kinases in MDA-MB-453 cells cultured in the presence or absence of mAb 4C5. Immunoprecipitation as well as cross-linking experiments demonstrated that disruption of this extracellular interaction of HSP90 with HER-2 significantly reduced ligand-dependent HER-2-HER-3 complex formation and signaling via mitogen-activating protein kinase (MAPK), indicating that the extracellular interaction of HSP90 with HER-2 is necessary for HER-2-HER-3 complex formation. This finding is consistent with the observation that in the presence of mAb 4C5, HER-3 was no longer detected in HSP90 complexes, suggesting that the interaction of HSP90 with HER-2 is necessary for the formation of the HER-2-HER-3 complex.

**FIGURE 6.** Surface HSP90 interaction with the ECD of HER-2 is necessary for the HRG-induced receptor heterodimerization with HER-3 and activation of downstream signaling kinases. A, protein lysates derived from MDA-MB-453 cells treated as above, were immunoprecipitated (IP) with anti-HER-2 antibody, and bound proteins were analyzed by Western blot (WB) using anti-HER-3 and anti-HER-2 antibodies. Additionally, immunoprecipitation with anti-HER-3 followed by Western blot with anti-HER-3 was performed in order to detect total precipitable levels of HER-3. mAb 4C5 inhibited the association of HER-2 with HER-3 under HRG stimulation conditions, since anti-HER-3 antibody co-immunoprecipitated a lower level of HER-2 in mAb 4C5-treated cells when compared with control cultures. In the absence of HRG, there were no detectable levels of association between HER-2 and HER-3. Total precipitable levels of HER-2 and HER-3 remained the same in all cases. B, MDA-MB-453 cell lysates treated as above were chemically cross-linked with bis(sulfosuccinimidyl) suberate, and membrane proteins were analyzed using a 3–8% NuPage gradient gel. Western blot, using specific anti-HER-2, anti-HER-3 antibodies, and mAb 4C5, revealed that these proteins co-exist in an ~540-kDa protein complex. Furthermore, the immunoreactivity obtained after Western blot with anti-HER-2 corresponds to both the HER-2 homodimer-HSP90 and the HER-2-HER-3 heterodimer-HSP90 complex, since the two complexes have approximately the same $M_{r}$. The 180-kDa band corresponds to HER-2, HER-3, and the HSP90 dimer, respectively. As shown, mAb 4C5 inhibited the association of these proteins, resulting in limited complex formation. C, protein lysates derived from MDA-MB-453 cultures treated as above were analyzed by Western blot using antibodies against the total and phosphorylated forms of mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase, Akt, and Erk kinases. Western blot with anti-$\beta$-tubulin antibody is provided as a control for protein loading. Treatment of cells with mAb 4C5 reduced phosphorylation levels of all of the downstream signaling kinases examined, whereas total kinase levels remained the same. IP, immunoprecipitation; WB, Western blot.
Surface HSP90 Interacts with the ECD of HER-2

tivated protein kinase and PI3K-Akt, which are implicated in cytoskeleton dynamics necessary for cell motility (35, 44, 45, 47–49). These observations are consistent with recent reports demonstrating that inhibition of the formation of HER-2-containing heterodimers with antibodies against the ECD of HER-2 reduces ligand-activated HER-2 signaling, including HER-2 phosphorilation and activation of downstream targets, such as mitogen-activated protein kinase and Akt (50, 51).

Our combined data, in accordance with previously reported results (26), show that cell surface HSP90 participates in cancer cell invasion processes, through interaction with the ECD of HER-2, necessary for the receptor’s activation and heterodimerization with HER-3, which in turn will mediate signal transduction pathways via mitogen-activated protein kinase and phosphatidylinositol 3-kinase-Akt, leading to actin rearrangement necessary for cell motility. Taking into consideration that the physiological receptors for all epidermal growth factor-related growth factors are HER-2-containing heterodimers (52), we speculate that mAb 4C5 should inhibit migration induced by other growth factors of the EGF superfamily. What is the significance underlying the regulation of HER-2 signaling via the extracellular interaction with HSP90? We can only speculate why this receptor needs a second level of regulation by HSP90. HER-2 has a high level of constitutive (ligand-independent) activity due to its extracellular domain conformation, which is unique among the other ErbB family members. The ECD of HER-2 constitutively adopts an extended configuration, with its dimerization arm exposed, thus suggesting that it is always poised to form heterodimers with other ErbB receptors (19, 53, 54). Our present data prompt us to speculate that surface HSP90 interacts with the HER-2 ectodomain, in order to maintain this unusual active conformation, which is responsible for the receptor’s unique properties. Furthermore, the present findings reinforce the notion of a widely occurring phenomenon of extracellular molecular chaperoning and are in agreement with emerging data reporting the presence of several components of the HSP90 chaperone machinery, including HSP70, Hop, and p23, extracellularly (55–57). Indeed, it is possible that HER-2 is not the only “client” of HSP90 on the cell surface. The elucidation of the HSP90 clientele on the cell surface as well as the understanding of how this molecule and its chaperone machinery function extracellularly comprises a very ambitious goal.

In conclusion, our study suggests that specifically inhibiting surface HSP90 with mAb 4C5 may have clinical benefit in limiting cancer cell invasion and metastasis without affecting the many intracellular roles of HSP90. Indeed, we have recently shown that mAb 4C5 inhibits B16 F10 melanoma cell invasion and metastasis by binding selectively to surface HSP90 (31). On the other hand, very recent data report that high HSP90 expression is associated with elevated levels of HER-2 expression in human specimens of breast cancer (58). These results, in combination with the present findings, are very promising for a potential use of mAb 4C5 in cancer therapeutics.

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