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The *HER-2/neu* receptor tyrosine kinase gene encodes a secreted autoinhibitor

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ABSTRACT ***HER-2/neu* (*erbB-2*) encodes an 185-kDa orphan receptor tyrosine kinase that is constitutively active as a dimer and displays potent oncogenic activity when overexpressed. Here we describe a secreted protein of ≈68 kDa, designated herstatin, as the product of an alternative *HER-2* transcript that retains intron 8. This alternative transcript specifies 340 residues identical to subdomains I and II from the extracellular domain of p185HER-2 followed by a unique C-terminal sequence of 79 aa encoded by intron 8. The recombinant product of the alternative transcript specifically binds to *HER*-2-transfected cells with a K_D of ≈14 nM and was chemically crosslinked to p185HER-2, whereas the intron encoded sequence alone also binds with high affinity to transfected cells and associates with p185 solubilized from cell extracts. The herstatin mRNA is expressed in normal human fetal kidney and liver, but is at reduced levels relative to p185HER-2 mRNA in carcinoma cells that contain an amplified *HER-2* gene. Herstatin appears to be an inhibitor of p185HER-2, because it disrupts dimers, reduces tyrosine phosphorylation of p185, and inhibits the anchorage-independent growth of transformed cells that overexpress *HER-2*.**

The *HER-2/neu* (*erbB-2*) oncogene encodes a receptor-like tyrosine kinase, p185HER-2, that has been extensively investigated because of its role in several human carcinomas and in mammalian development (1–3). The function of the *HER-2* gene has been examined mainly by the structure and biochemical properties of the 185-kDa protein product of the 4.5-kb transcript (4, 5). P185HER-2 shares a common structural organization with other epidermal growth factor receptor (EGFR) family members and consists of an extracellular domain (ECD), a single transmembrane segment, and a cytoplasmic tyrosine kinase domain. Dimerization of receptor tyrosine kinases, which typically is induced by ligand binding, is required for their activation and subsequent steps in signal transduction (6). Although p185HER-2 is highly homologous to the EGFR, no ligand that directly binds with high affinity to p185 has yet been identified (1–3). Instead of activation by direct ligand binding, p185HER-2 exhibits constitutive activity in the apparent absence of ligand, which is enhanced by *HER-2* overexpression (7–10), or p185 is recruited into heterodimers by ligand-binding members of the EGFR family (11–13). Elevated basal kinase activity and constitutive dimerization as well as the status of p185HER-2 as the preferred heterodimer partner (14, 15) may contribute to its exceptional oncogenic potency.

The most common mechanism by which *HER-2* transforms cells is by overexpression of normal p185HER-2. Overexpression, with no evidence of mutations, occurs in several human adenocarcinomas (1, 2, 16). Importantly, elevated p185HER-2 in 25–30% of breast cancers predicts significantly lower survival rates and shorter time to relapse (16, 17). Moreover, systemic administration of antibodies against the ECD of p185HER-2 can increase the time to recurrence in a subset of patients with metastatic breast cancer (18).

Here we describe a secreted protein, herstatin, that exhibits high-affinity association with p185HER-2 and is encoded by the *HER-2* gene itself. Evidence is presented that herstatin is a specific inhibitor and therefore has potential importance in the regulation of p185HER-2 in normal and malignant development.

MATERIALS AND METHODS

Cell Culture. Cell lines were from the American Type Culture Collection or the Vollum Institute Core Culture Facility. Ovarian surface epithelial cell line, IOSEVAN, was provided by Karin Rodland at the Oregon Health Sciences University. NIH 3T3 parental cells and a cell line stably transfected with *HER-2*, designated 17-3-1, were a gift from Applied Biotechnology, and the NIH 3T3 cells transfected with *src*^{527} were supplied by Brian Druker of Oregon Health Sciences University. Cells were maintained in DMEM supplemented with 10% FBS, and 0.4 mg/ml of G418 (Geneticin, GIBCO/BRL) was added to the transfected 3T3 cell cultures.

Antibodies. Anti-ECDIIIa antisera were produced by Cocalico Biologicals (Reamstown, PA) by injection of rabbits with purified polyhistidine-tagged ECDIIIa peptide. Polyclonal anti-neu(C) was made against a peptide identical to the last 15 residues of the carboxyl terminus of p185HER-2 (19). Monoclonal antiphosphotyrosine antibody, 4G10, was a gift from Brian Druker.

PCR and Primer Sets. An SKOV-3 cDNA library (Origene Technologies, Rockville, MD) was subjected to PCR using a forward primer (A) identical to nucleotides 142–161 of *HER-2* cDNA (5′-TGAGCACC$\underline{\text{ATG}}$GAGCTGGC-3′), which spans the initiation codon (underlined) and a reverse primer (B) (5′-TCCGGCAGAAATGCCAGGCTCC-3′), which is complementary to *HER-2* nucleotides 1265–1286 (4). Thirty cycles of 94°C for 30 sec, 58°C for 45 sec, and 68°C for 3 min were used for amplification.

Construction of Expression Vectors and Purification of ECDIIIa C-Terminal Peptide and Full-Length ECDIIIa Protein. The ECDIIIa sequence was amplified from a cDNA library and cloned into the pET30a vector, which encodes six histidine residues at the amino terminus of the expressed protein (Novagen). The His-tagged ECDIIIa protein was

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Abbreviations: EGFR, epidermal growth factor receptor; ECD, extracellular domain; CM, conditioned media.

Database deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF177761).

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solubilized from bacteria, absorbed onto Ni-nitrilotriacetic acid agarose (Novagen), eluted with 250 mM imidazole, and was approximately 90% pure by Coomassie blue staining of gels.

For bacterial expression of the full-length ECDIIIa protein, a cDNA expression vector was constructed by subcloning the 1.3-kb *Nco*I–*Eco*RI fragment of *HER-2* cDNA (nucleotides 149-1456) into pET30a with an N-terminal 6× His tag, and by replacing the 160-nt *Bh1*–*Bgl*II fragment (nucleotides 1075–1235) with the 435-nt *BH1*–*Bgl*II fragment containing the 274-nt ECDIIIa insert sequence cloned from a cDNA library. The BL21 strain of bacteria was doubly transformed with pET-p50ECDIIIa and with a thioredoxin expression plasmid (20), which was required to obtain a soluble form of p50ECDIIIa. Soluble extracts were applied to Ni-nitrilotriacetic acid agarose, which was washed with 20 vol of buffer containing 100 mM imidazole. The p50ECDIIIa protein was eluted with buffer containing 1.5 M imidazole, 0.5 mM NaCl, and 1% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), and then was dialyzed against 50 mM Tris, pH 9.5 with 5 mM DTT. Treatment with 5 mM DTT for ≈ 16 hr before storage under nitrogen at −80°C was required to achieve a disulfide bonded form that migrated at a faster rate in nonreducing gels. The p50ECDIIIa protein was estimated at 70% purity in Coomassie blue-stained gels.

For mammalian expression of the full-length ECDIIIa protein, the acceptor (3′) splice site was deleted by PCR using a primer spanning the initiation codon and the *Nco*I site (nucleotides 145–166 in the *HER-2* cDNA sequence) and a second primer complementary to the stop codon in the ECDIIIa sequence (nucleotides 1407–1409), which contained a *Hin*dIII site. The *Nco*I–*Hin*dIII fragment was cloned into a mammalian expression vector pJPA 5, which is driven by a cytomegalovirus promoter, (J.P.A., unpublished work).

Ligand Binding and Crosslinking Analyses. Recombinant ECDIIIa peptide, or p50ECDIIIa, purified from bacteria, were labeled with ^{125}I by using Bolton Hunter Reagent (ICN) to a specific activity of about 4×10^4 cpm/pmol. Increasing amounts of radiolabeled protein, in the presence and absence of 100-fold excess unlabeled protein, were added in binding buffer (DMEM with 1% BSA) to about 10^5 17-3-1 cells or parental NIH 3T3 cells at room temperature for 1 hr. Cells were washed and extracted, and the radioactivity was quantitated.

RESULTS

A Unique 274-nt Sequence Is in *HER-2* mRNA. PCR was used to investigate *HER-2* mRNA diversity within the ECD coding sequence. A cDNA library from SKOV-3 ovarian carcinoma cells was examined by using a forward primer specific for exon 1 (21), nucleotides 142–161, and a reverse primer complementary to nucleotides 1265–1286 in exon 9 (22). A product of ≈1,420 nt, determined to be *HER-2*-specific by Southern blotting (data not shown), was approximately 274 nt larger than expected (4). Nucleotide sequencing of the PCR product revealed the normal *HER-2* coding sequence through nucleotide 1171, followed by a 274-nt insertion that was contiguous with exon 9 sequence. Analysis of the predicted protein product shows that the first 340 aa residues, starting with the initiator methionine and signal sequence, are identical to p185 HER-2 (4) and are followed by a 79-aa extension and a termination codon encoded by the insertion (Fig. 1). Inspection of the 79-aa sequence shows a consensus N-linked glycosylation site (underlined) and a proline content of 19% (Fig. 1). The inserted sequence is designated ECDIIIa because it is located at the boundary between subdomains II and III (23) in the extracellular domain of p185HER-2.

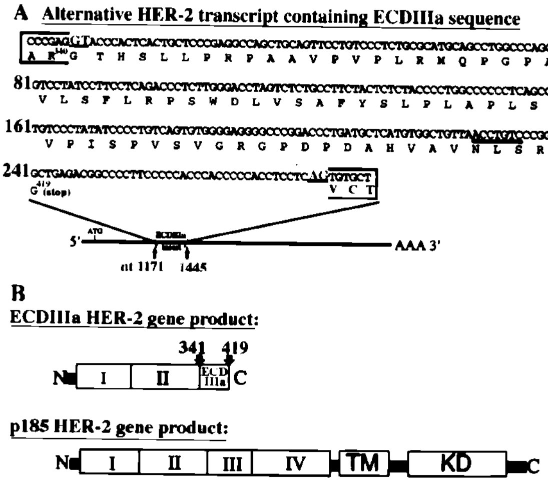

FIG. 1. Nucleotide sequence and deduced amino acid sequence encoded by the 274-nt insertion into *HER-2* mRNA. The *HER-2* ECD coding sequence from exons 1–9 was amplified by PCR from a cDNA library from SKOV-3 cells. A product of ≈1,420 bp, found to be *HER-2* specific by Southern blot analysis, was subcloned, and the nucleotide sequence was determined. (*A*) The nucleotide sequence is shown for the 274-nt insertion (outside the box) and for the adjacent 5′ and 3′ sequences, enclosed in the box. The insertion is located between nucleotide residues 1171 and 1172 and after amino acid residue 340 in p185HER-2 by using the numbering of Coussens *et al.* (4). The consensus 5′ and 3′ splice sites are underlined and shown in larger print. The inserted sequence is in-frame with 5′ *HER-2* exon sequence and is deduced to encode a 79-aa extension after Arg-340 (R^{340}). A consensus asparagine-linked glycosylation site is underlined. Comparison of the inserted nucleotides and their predicted amino acid sequence with sequences in GenBank showed no obvious homologies. (*B*) The predicted product of the alternative transcript is a truncated secreted protein that contains subdomains I and II identical to p185 and is missing the transmembrane domain and cytoplasmic domain. If fully glycosylated, the expected size is 65–70 kDa. For comparison, the schematic structure of p185 HER-2 indicates subdomains I, II, III, and IV in the ECD, the transmembrane domain (TM), and the kinase domain (KD).

Examination of the 5′ and 3′ junctions of the divergent sequence reveals consensus splice donor and acceptor sites (24) and includes a pyrimidine tract and potential branchpoint adenine residues near the 3′end of the insert sequence (Fig. 1). PCR analysis of genomic DNA indicates that the 274 nt are contiguous with *HER-2* exonic sequence (J.K.D., J.P.A., and G.M.C., unpublished work) and that the inserted sequence is intron 8 based on the location of intron 8 in the homologous EGFR and *HER-3* genes (25).

Alternative Transcripts Containing the ECDIIIa Sequence Are Expressed in Human Fetal Kidney and Liver. Poly(A)$^+$ mRNA from a variety of human fetal tissues, prepared as a Northern blot, was hybridized with a radiolabeled probe specific for the unique ECDIIIa sequence. A 4.8-kb mRNA was detected in kidney as well as in the human embryonic kidney cell line, HEK-293, whereas a 2.6-kb transcript was detected in liver (Fig. 2*A*). The 4.8-kb transcript likely corresponds to the full-length 4.5-kb transcript with the 274-nt intron sequence, and the 2.6-kb transcript may correspond to the previously described 2.3-kb alternative transcript (5, 22) with the retained intron. When the blot was stripped and hybridized with a probe specific for the 5′ *HER-2* coding sequence, a broad band representing the 4.8- and 4.5-kb mRNAs was detected in fetal kidney tissues, and the truncated 2.6-kb transcript was detected in liver, showing that these alternative transcripts contain *HER-2* ECD sequences. Because the inserted ECDIIIa sequence contains a termination codon, both of these alternative transcripts are predicted to encode the same truncated protein product.

A Secreted Protein of ≈68 kDa Contains the ECDIIIa Sequence. Antisera against the purified ECDIIIa peptide was

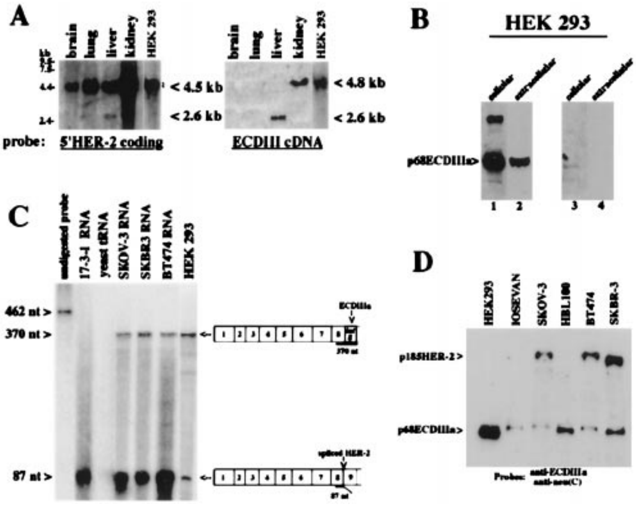

FIG. 2. Expression of the *HER-2* alternative transcript containing the ECDIIIa sequence. (*A*) Poly(A)$^+$ mRNA (2.5 μg) from different human fetal tissues (CLONTECH) or isolated TriReagent (Molecular Research Center, Cincinnati) from HEK-293 cells was analyzed as a Northern blot as described (36) by using a ^{32}P-labeled antisense RNA probe complementary to the ECDIIIa sequence. The blot was stripped and reprobed with a ^{32}P-labeled cDNA probe specific for the 5′ *HER-2* exon sequence. (*B*) Sequence-specific reactivity of anti-ECDIIIa domain antibody with a protein of ≈ 68 kDa in HEK-293 cells was detected in cell extract protein (20 μg) and 20 μl of media conditioned (CM) by HEK-293 cells. To prepare CM, 15-cm plates of confluent cells were washed 3× with PBS and then incubated for 24 hr with 12 ml of serum-free media. The CM was clarified by centrifugation at 13,000 × *g* for 20 min and was concentrated by using an Amicon filter that retains proteins of ≥30,000 daltons. Western blot analysis of cell extract and CM was conducted exactly as described (19). The blot was probed with anti-ECDIIIa diluted 1:10,000 (lanes 1 and 2) or with anti-ECDIIIA diluted 1:10,000 containing 50 μg/ml of purified His-tagged ECDIIIa peptide (lanes 3 and 4). (*C*) Ribonuclease protection assay was conducted to detect p68ECDIIIa mRNA and p185HER-2 mRNA. A template for antisense RNA probe synthesis was constructed by PCR amplification using a forward primer that is identical to *HER-2* cDNA sequence at nucleotides 1131–1152 and a reverse primer (5′-GCACGGATCCATAGCAGACTGAGGAGG-3′), which contains a 3′ *Bam*HI site and is complementary to the sequence spanning the 3′ splice site of the ECDIIIa sequence. The PCR product was digested with *Bam*HI, cloned, and sequenced, and an antisense RNA probe was transcribed by using [α-^{32}P]CTP and the T7/SP6 Riboprobe Synthesis System (Promega). RNA hybrids were prepared, digested with RNaseA (Boerhinger Mannheim) and RNase T1 (Life Technologies, Grand Island, NY), denatured, and electrophoresed in a 5% polyacrylamide/urea gel as described (37). A fragment of 370 nt was protected from RNase digestion, which is the size expected for RNA containing 5′ *HER-2* exon sequence and ECDIIIa sequence as illustrated on the right, and was in SKOV-3, SKBR-3, and BT474 RNA, but not in yeast tRNA nor in *HER-2* transfected 17-3-1 cells. A protected fragment of 87 nt, expected for RNA containing 5′ exon sequence but not ECDIIIa sequence, was detected in all cell lines but not in tRNA. (*D*) Extracts (15 μg of protein) of the indicated cell lines were resolved by SDS/PAGE in 7.5% acrylamide gels and analyzed as a Western blot using both antibodies specific for p68 (anti-ECDIIIa) and for p185 (anti-neu C).

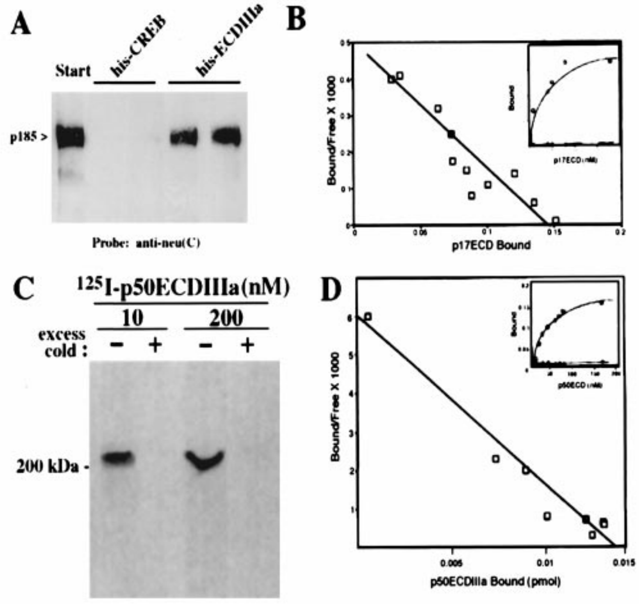

FIG. 3. The ECDIIIa protein specifically associates with p185HER-2. (A) 17-3-1 cell extract (100 μg) wase incubated in duplicate with 50 μl of packed volume of Ni-nitrilotriacetic acid agarose coupled to 20 μg of His-tagged ECDIIIa peptide containing the intron-encoded 79 residues or to 20 μg of His-tagged CREB fragment in 200 μl of wash buffer (20 mM Tris, pH 8.0/300 mM NaCl) at room temperature for 1 hr with shaking. The resin then was washed four times with 500 μl of wash buffer, and proteins were eluted by incubation with 50 μl of SDS-sample buffer at 100°C for 2 min. Eluted proteins were analyzed by Western blot analysis using antibodies against the C terminus of p185, anti-*neu*(C). (*B*) Various concentrations of radiolabeled His-tagged ECDIIIa peptide, p17, were incubated with *HER-2*-transfected 17-3-1 cells or parental 3T3 cells. Binding results were analyzed by using the Scatchard method and by plotting the saturation curve (*Inset*). (*C*) Radiolabeled p50 was bound to 17-3-1 cells and then incubated with the crosslinking reagent BS^3. The washed cells were extracted and immunoprecipitated with 5 μl of anti-*neu*(C) as described (26). The immune complex was washed and resolved by SDS/PAGE, and radiolabeled complexes were detected by autoradiography. (*D*) Various concentrations of radiolabeled p50, purified from bacteria, were incubated with 17-3-1 cells or parental 3T3 cells. Binding results were analyzed by using the Scatchard method and by plotting the saturation curve (*Inset*).

used in a Western blot of protein from HEK-293 cells, which express the ECDIIIa mRNA. A 68-kDa protein in cell extract and in extracellular media reacted with anti-ECDIIIa antibody but not with preimmune sera (data not shown), and reactivity was blocked by preincubation of the antisera with purified ECDIIIa peptide (Fig. 2*B*). The 68-kDa protein was further characterized as the product of the alternative transcript based on its reactivity with antipeptide antibody against residues 151–165 of p185HER-2 (26). The larger protein of ≈125 kDa may be an aggregate of p68. The cDNA sequence of the alternative transcript (Fig. 1) predicts a secreted protein product of 65–70 kDa if five N-linked glycosylation sites are glycosylated (27).

p68ECDIIIa Expression Is Not Elevated in Proportion to p185HER-2 in Carcinoma Cell Lines in which the *HER-2* Gene Is Amplified. The alternative transcript containing the ECDIIIa sequence could not be detected by Northern analysis of carcinoma cell lines. Therefore the more sensitive ribonuclease protection assay was used by using an antisense probe that spans the entire ECDIIIa sequence and flanking 5′ *HER-2* exon sequence. The alternative *HER-2* mRNA with the ECDIIIa insert was detected at less than 5% of the fully spliced transcripts in SKOV-3, SKBR-3, and BT474 cells, which all have their *HER-2* gene amplified about eight times (28), and was expressed at 25–30% of the p185HER-2 transcript in HEK-293 cells (Fig. 2*C*).

Fig. 2*D* shows that p185 was enhanced in the carcinoma cell lines that have their *HER-2* gene amplified. However, there was not a corresponding elevation in p68ECDIIIa. In comparison, p185 was expressed at very low levels in the HEK-293, IOSEVAN, and HBL100 nontumorigenic cells, whereas p68 was easily detected. The levels of cellular p68 were reflected in the amount secreted from these cell lines (data not shown). These results suggest that a mechanism may exist to maintain low levels of p68 when p185HER-2 is amplified in carcinoma cells.

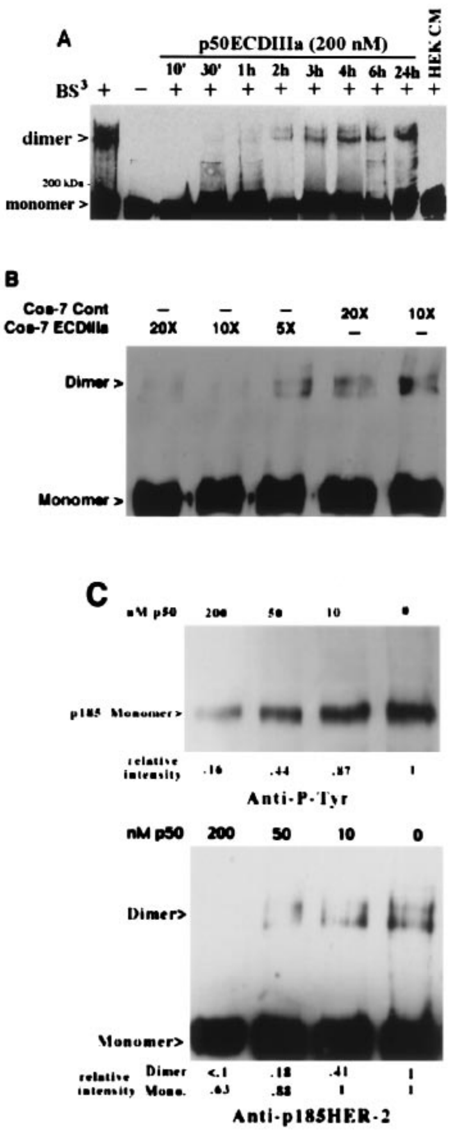

FIG. 4. The ECDIIIa protein disrupts *HER-2* dimers and inhibits tyrosine-phosphorylated p185HER-2. (*A*) Time course of the effects of purified p50ECDIIIa on *HER-2* dimers. 17-3-1 cells, in 12-well plates, were incubated at 37°C for the indicated times in binding buffer with or without 200 nM recombinant p50ECDIIIa and an equivalent concentration of p50 vehicle (50 mM Tris, pH 9.5; 5 mM DTT), or with 100 μg protein from HEK-CM prepared as described in Fig. 2*B*. The cells were crosslinked with BS^3 for 30 min at room temperature and extracted, and 20 μg of protein were resolved in 5% polyacrylamide gels. The gels were electrotransferred and analyzed as a Western blot as described (19) with anti-*neu*(C). (*B*) Effects of CM from transfected Cos-7 cells on *HER-2* dimers. Fifteen-centimeter plates were transfected with 10 μg of ECDIIIa expression vector or 10 μg of the vector without the insert by using Lipofectamine as per manufacturer's instructions (GIBCO/BRL). At ≈24 hr, the cells were washed, and 15 ml of media without serum was added for 24 hr. The CM was collected and prepared as described in Fig. 2*B* with the addition of 5 mM DTT and 1 mM PMSF as a protease inhibitor. Western blot analysis of 20 μl of 20× CM, probed with anti-ECDIIIa antibodies, demonstrated the presence of the ECDIIIa protein in the media of the ECDIIIa-transfected cells, but not in the media of cells transfected with the empty vector (data not shown). There was about 5-fold higher levels of the ECDIIIa protein in the CM of transfected Cos-7 cells compared with the CM from HEK-293 cells (see Fig. 2*B*). CM containing 1% BSA was added at the indicated concentrations to 17-3-1 cells for 1 hr. The cells were crosslinked and analyzed as a Western blot with anti-p185 antibodies. There was an approximate 5-fold reduction in dimers in cells treated with 10× or 20× CM from ECDIIIa-transfected Cos cells compared with the equivalent amount of CM from control vector-transfected Cos-7 cells. (*C*) P185HER-2 tyrosine phosphorylation, dimer, and monomer levels in response to p50ECDIIIa. 17-3-1 cells were incubated at 37°C in binding buffer for 1 hr with the indicated concentrations of purified p50 or vehicle, and then crosslinked with BS^3. Cell extract proteins (20 μg) prepared in 1 mM vanadate were examined as a Western blot using monoclonal antiphosphotyrosine antibody at 1 μg/ml. The major phosphotyrosine-containing protein was p185HER-2 based on its size, absence from parental 3T3 cells, and on previous studies demonstrating its removal by specific immunoprecipitation of p185HER-2 (data not shown). The phosphotyrosine signal of p185HER-2 was quantitated by imaging densitometry (Bio-Rad, model GS-700) of the film and standardized to the signal from the sample without p50. The tyrosine-phosphorylated p185 (*Upper*) was reduced in a dose-responsive fashion with a maximum 6-fold decrease compared with the control, untreated sample. The same blot was stripped by incubation at 55°C for 30 min in 62.5 mM Tris, pH 6.7, 100 mM mercaptoethanol, and 2% SDS, and then reacted with antibody against p185, anti-*neu*(C) (*Lower*). The relative level of dimers in each sample was quantitated by densitometry, and a lower exposure of the film (not shown) was scanned to determine the relative intensity of monomeric p185. (*Lower*) Dimers were reduced in a dose-responsive fashion with greater than a 10-fold decrease at 200 nM p50. The levels of monomeric p185 also were reduced by up to 37% at 200 nM p50, suggesting that herstatin may down-regulate p185. Taking into account the reduction in monomers, this result suggests a 3- to 4-fold reduction in the level of tyrosine phosphorylation of p185 at 1 hr of treatment with saturating concentrations of p50.

The ECDIIIa Protein Binds to *HER-2*-Transfected Cells at nM Affinity and Associates with p185HER-2. To explore the possible interaction of the intron-encoded ECDIIIa sequence with p185HER-2, the 79-aa domain, purified as a polyhistidine-tagged protein, or a control, unrelated His-tagged peptide, were used in a pull-down assay. The immobilized peptides were incubated with protein extracts prepared from 17-3-1 cells. After extensive washes, the bound proteins were eluted and prepared as a Western blot with an antibody specific for p185HER-2. Equal amounts of His-tagged ECDIIIa peptide and control peptide (His-CREB) were bound to the resin as confirmed by Coomassie staining of the eluted material in SDS gels. P185HER-2 was selectively retained by the ECDIIIa peptide, but not by resin without peptide (data not shown) nor by control peptide (Fig. 3*A*). To examine the binding properties, increasing concentrations of ^{125}I-labeled ECDIIIa peptide were added to *HER-2*-transfected 17-3-1 cells and to the parental 3T3 cells. Saturation binding to 17-3-1 cells was observed, whereas binding to 3T3 cells was not above background (Fig. 3*B*). Scatchard analysis revealed an apparent dissociation constant of about 61 nM, demonstrating specific, high-affinity binding of the intron encoded ECDIIIa sequence to cells that overexpress p185HER-2.

The binding properties of the full-length ECDIIIa protein, which contains the N-terminal subdomains I and II of p185 and the ECDIIIa C-terminal sequence, were examined next. The p68ECDIIIa cDNA was expressed as an His-tagged 50-kDa protein in bacteria, which was purified and radiolabeled with ^{125}I. Binding of p50ECDIIIa to 17-3-1 cells, but not to the parental 3T3 cells, was saturable. Scatchard analysis suggested a single, high-affinity site and predicted an apparent dissociation constant of about 14 nM (Fig. 3*D*). Fig. 3*C* shows that p50ECDIIIa was crosslinked with BS^3 to p185HER-2 on the surface of intact cells.

The Effects of the ECDIIIa Protein on p185HER-2 Dimers and Tyrosine Phosphorylation. Because the ECDIIIa protein associates with the extracellular domain of p185HER-2, we examined whether it may impact dimerization. Constitutive *HER-2* dimers in 17-3-1 cells were detected as an ≈360-kDa complex by p185HER-2-specific antibodies after treatment of cells with crosslinking reagent (Fig. 4*A*). When purified, recombinant p50ECDIIIa, at 200 nM, was added to cells before the addition of BS^3, *HER-2* crosslinked dimers were

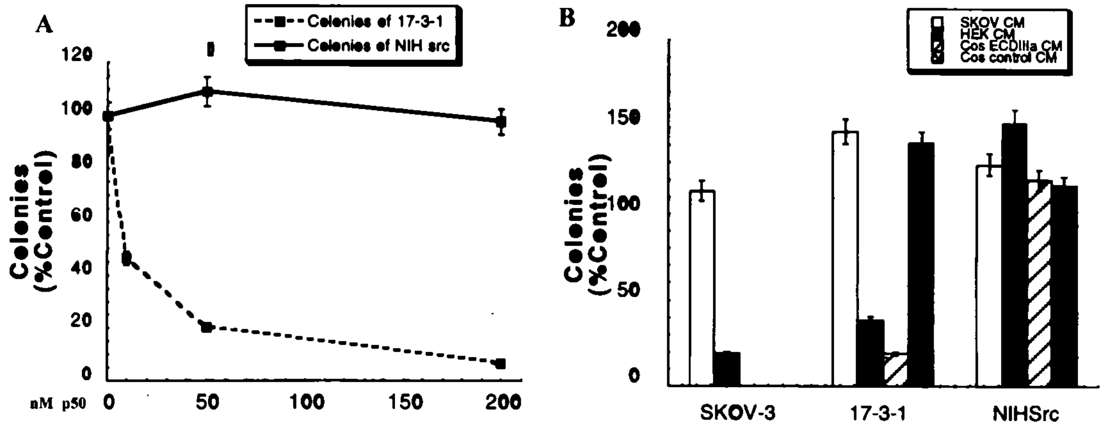

FIG. 5. Anchorage-independent growth of cells in the presence and absence of the ECDIIIa ligand. About 3,000 cells, suspended in media containing 0.3% Difco agar, were plated onto a 0.5-ml layer of media containing 0.5% agar in 12-well plates. Colonies containing at least 50 cells were counted in triplicate wells and expressed as mean percentages of untreated controls at 21 days for SKOV-3 cells and at 14 days for 17-3-1 cells or NIH-*src* transformed cells. (*A*) P50ECDIIIa, purified from bacteria, or vehicle were added at the indicated concentrations to the soft agar cultures. (*B*) Colony formation was in the absence or presence of CM (200 μg protein) prepared from SKOV-3 cells (SKOV-CM), from HEK-293 cells (HEK-CM), or in 20× CM from ECD-transfected or control, vector-transfected Cos-7 cells.

markedly reduced within 10 min of treatment, and then were partially restored by 4 hr (Fig. 4*A*). The restoration of dimers may occur because the p50ECDIIIa protein is inactivated with time in binding buffer, or p50 may be internalized. A native source of p68ECDIIIa in conditioned media (CM) from HEK-293 cells (see Fig. 2*B*) as well as the CM of p68ECDIIIa transfected Cos-7 cells compared with the CM from control, vector-transfected Cos-7 cells, also inhibited the levels of p185-containing dimers (Fig. 4 *A* and *B*). However, neither an irrelevant His-tagged protein purified from bacteria, nor the intron-encoded 79-residue peptide (used in Fig. 3*A*) inhibited p185 dimers (data not shown).

To examine the impact of p50ECDIIIa on tyrosine phosphorylation, 17-3-1 cells were treated with recombinant p50, crosslinked, and examined as a Western blot that was probed first with antiphosphotyrosine antibody then stripped and probed with anti-p185. There was a dose-dependent decrease in the phosphotyrosine signal associated with p185 that correlated with inhibition of dimers (Fig. 4*C*). Depression of the tyrosine phosphorylation signal, like dimer inhibition, was transient with restoration to nearly control levels by 6 hr (data not shown). The decreased phosphotyrosine signal was caused, in part, by suppression of the level of tyrosine phosphorylation as well as by a small decrease in the amount of p185HER-2, suggesting that both tyrosine dephosphorylation and down-regulation of p185 occurred in response to p50ECDIIIa.

The Anchorage-Independent Growth of Cells that Overexpress p185HER-2 Is Inhibited in the Presence of the ECDIIIa Protein. Overexpression of p185HER-2 causes transformation of cells, manifested by their anchorage-independent growth (8, 9). To examine the effect of the ECDIIIa protein on anchorage-independent growth, 17-3-1 cells were plated in soft agar cultures containing recombinant p50ECDIIIa. The number of colonies, consisting of at least 50 cells, was inhibited in a dose-dependent manner with about 90% inhibition at 200 nM of p50 (Fig. 5*A*). Neither the His-tagged CREB protein, nor the recombinant peptide identical to the C terminus of p50ECDIIIa (described in Fig. 3 *A* and *B*) affected the growth in soft agar (data not shown). The CM from ECDIIIa-transfected Cos-7 cells, but not from control vector-transfected Cos-7 cells, also inhibited the soft agar colony formation by 17-3-1 cells, indicating that the mammalian recombinant ECDIIIa protein displayed the growth inhibitory activity (Fig. 5*B*). In the presence of the native ligand, p68, from HEK-293 CM, growth in soft agar of 17-3-1 cells and of the SKOV-3 ovarian carcinoma cells, which also depend on p185HER-2 overexpression for anchorage-independent growth (29, 30), was inhibited several-fold compared with cultures treated with CM from the SKOV-3 cells (Fig. 5 *B* and *C*), which does not contain detectable p68 (data not shown). The growth in soft agar of the NIH 3T3 cells, transformed by the *src* oncogene (*src*^{527}), was not suppressed by any source of the ECDIIIa protein (Fig. 5*A* and *B*), indicating that its inhibitory effect was specific for cells transformed by p185HER-2.

DISCUSSION

The results presented here demonstrate tissue-specific expression of alternative *HER-2* mRNA, which contains an additional 274 nt, probably intron 8. The protein product of the alternative transcript, designated herstatin, was secreted from human cells.

Herstatin binds specifically to *HER-2*-transfected cells at nM affinity to a single class of saturable binding sites. A K_D of ≈14 nM was determined for the bacterially produced herstatin (Fig. 3*D*). Binding studies using the glycosylated version, synthesized in mammalian cells, will be necessary to determine the affinity of native herstatin. An extensive search has not yet yielded identification of a characterized, secreted factor that specifically binds with high affinity to p185HER-2 (1–3). Although radiolabeled herstatin was crosslinked to p185HER-2 at the cell surface (Fig. 3*C*), we cannot rule out the possibility that a coreceptor may be required for binding.

The significance of the C-terminal sequence of herstatin, encoded by the retained intron, was suggested by the specific, high-affinity ($K_D \approx 61$ nM) binding to *HER-2*-transfected cells and by the association with p185HER-2 displayed by the recombinant peptide (Fig. 3 *A* and *B*). One model is that the intron-encoded sequence confers binding to p185; a possibility generally supported by the *HER-2*-specific binding displayed by the recombinant peptide. Another model, which cannot yet be ruled out, is that the N terminus of herstatin, consisting of subdomains I and II from the extracellular domain of p185, is sufficient for binding to p185 and for the biological activity. The role of the retained intron, in the second model, would be to supply a termination codon to cause truncation, because subdomains I and II, in the context of the entire extracellular domain, do not bind to p185 (31, 32).

Binding of ligands for mammalian EGFR family members is tightly coupled to stimulation of receptor dimerization and tyrosine phosphorylation (1–3, 33). Although herstatin binds with high affinity, it does not activate, but rather inhibits p185

(Fig. 4). Disappearance of dimers within 10 min suggests that herstatin either disrupts existing dimers, shifts the equilibrium between dimers and monomers by stabilizing monomers, or causes their down-regulation and degradation. Herstatin may inhibit dimerization in a dominant negative fashion by occupying monomeric receptors and blocking their recruitment into dimers (32). We also observed a depression in p185-associated tyrosine phosphorylation that correlated with dimer inhibition (Fig. 4*C*). The significance of the decline in tyrosine phosphorylation to signal transduction will require determination of the specific phosphorylation sites in p185HER-2 that are affected.

Anchorage-independent growth, a property of tumorigenic cells that overexpress *HER-2* (17, 18, 29, 30), was suppressed by bacterial recombinant herstatin, CM from Cos-7 cells transfected with herstatin, and CM from human cells that secrete native herstatin, whereas cells transformed by *src*^{527} were not inhibited. There are several possible mechanisms by which herstatin could interfere with anchorage-independent growth. Transient interruption of *HER-2* signaling caused by disruption of dimers and inhibition of tyrosine phosphorylation may be sufficient to allow apoptosis to occur in suspended cells. Herstatin also may execute a signal in addition to or distinct from disruption of p185 dimers. Whatever the mechanism, growth inhibition by herstatin could provide a selective pressure for overexpression of p185 to overcome the repressive effects of herstatin in tumor cells with *HER-2* gene amplification (Fig. 2*D*).

Very few natural ligands that inhibit receptor tyrosine kinases have been identified to date. The Argos protein is an example of an extracellular inhibitor of the *Drosophila* EGFR (34). Likewise, angiopoietin-2 is a natural antagonist for the Tie 2 endothelial receptor tyrosine kinase (35). Herstatin is distinguished from these by its structure, which consists of part of the extracellular domain of the receptor to which it binds.

In summary, our results support the model that herstatin is a naturally occurring inhibitor of p185HER-2. Future studies investigating its impact on growth factor-induced recruitment of p185 into heterodimers and on second messengers that propagate *HER-2* signaling will be required to understand the extent to which herstatin interferes with p185 signaling. As a growth inhibitor (Fig. 5), herstatin could have therapeutic value against human cancers that are driven by overexpression of p185HER-2.

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