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Selection and Characterization of Her2 Binding-designed Ankyrin Repeat Proteins*

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Designed ankyrin repeat proteins (DARPins) are a novel class of binding proteins that bind their target protein with high affinity and specificity and have very favorable expression and stability properties. We describe here the *in vitro* selection of DARPins against human epidermal growth factor receptor 2 (Her2), an important target for cancer therapy and diagnosis. Several DARPins bind to the same epitope as trastuzumab (Herceptin), but none were selected that bind to the epitope of pertuzumab (Omnitarg). Some of the selected DARPins bind with low nanomolar affinity (K_d = 7.3 nM) to the target. Further analysis revealed that all DARPins are highly specific and do not cross-react with epidermal growth factor receptor I (EGFR1) or any other investigated protein. The selected DARPins specifically bind to strongly Her2-overexpressing cell lines such as SKBR-3 but also recognize small amounts of Her2 on weakly expressing cell lines such as MCF-7. Furthermore, the DARPins also lead to a highly specific and strong staining of plasma membranes of paraffinated sections of human mamma-carcinoma tissue. Thus, the selected DARPins might be used for the development of diagnostic tests for the status of Her2 overexpression in different adenocarcinomas, and they may be further evaluated for their potential in targeted therapy since their favorable expression properties make the construction of fusion proteins very convenient.

Targeting of tumor cells with antibodies has been widely studied during the last decades as an alternative and a complement to conventional chemotherapy. Dozens of monoclonal antibodies targeting tumor antigens are in clinical trials, and several have been approved as drugs (1). These antibodies work by different actions, including steric hindrance of ligand binding, antibody-dependent cellular cytotoxicity, or complement activation. The high specificity of these approaches and the generally low toxicity of human antibodies reduce the severe side effects inherent in conventional chemotherapy approaches.

Human epidermal growth factor receptor 2 (Her2/c-neu, ErbB2) is a member of the EGF[5] receptor family. Its use as a target for antibody-based cancer therapy has been widely studied. The members of the EGF receptor family are able to form different homo- and heterodimers leading to altered ligand specificities. This behavior gives rise to a complex signaling network (2). In various tumors, members of the EGF receptor family are overexpressed, leading to abnormal hetero- and homodimer formation and, consequently, to altered ligand binding and aberrant signaling. Her2 is overexpressed in 20–30% of all breast cancer patients, and its overexpression has been correlated with poor prognosis (3). Several monoclonal antibodies against the extracellular domain of Her2 have been described, which show therapeutic efficacy in the treatment of Her2-positive breast cancer; Trastuzumab (Herceptin™) is a humanized monoclonal antibody (4), which binds to the membrane-adjacent cysteine-rich domain, termed domain IV (5). The mode of action is still not elucidated completely but most likely includes inhibition of shedding of the ECD from the plasma membrane by proteases (6) and induces antibody-dependent cellular cytotoxicity (4, 7). In contrast, the monoclonal antibody 2C4, also known as pertuzumab or Omnitarg™, binds to a hairpin structure on domain II, which is important for homo- and heterodimerization (8). It sterically blocks the engagement of Her2 in homo- and heterodimeric complexes and thus inhibits ligand-induced signaling (9). Some of the therapeutic antibodies are also of diagnostic relevance since the expression pattern of tumor antigens can highly vary from patient to patient and the presence of a tumor marker has to be verified in each case in diagnostic tests before applying an immune therapy.

Despite the great conceptual advantages over conventional chemotherapy, the efficacy of IgGs in tumor therapy is modest and almost never leads to a cure, as seen, *e.g.* in a phase III trial with trastuzumab (10, 11). Therefore, complementary approaches, including the fusion of targeting agents with drug-converting enzymes, cytokines, or toxins or the generation of bispecific antibodies to recruit effector cells, are continuously being developed. However, these approaches are often limited by the difficulty in manufacturing these molecules (12).

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[5] The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; DARPin, designed ankyrin repeat protein; ECD, extracellular domain; ELISA, enzyme-linked immunosorbent assay; HA, hemagglutinin; PBS, phosphate-buffered saline; BSA, bovine serum albumin.

These considerations gave rise to the development of alternative binding proteins, which retain the beneficial properties of antibodies, such as high specificity and affinity, but which overcome some of the limitations described above (13). Recently, a novel approach to generate alternative binding molecules based on repeat proteins has been described (14, 15). Repeat proteins are, next to antibodies, the most abundant binding proteins found in nature (16, 17). They are built from a single structural motif, termed repeat module, which is assembled to a repeat domain. Due to sequence homologies of the single repeat modules, a consensus sequence could be deduced from natural ankyrin repeat proteins, which consists of conserved framework residues and randomized interaction residues (14). By assembly of the randomized repeat modules, complex libraries of designed ankyrin repeat proteins (DARPins) can be generated. The number of randomized repeat modules used determines the size of the final protein, and with increasing numbers of repeat modules, the interaction interface and the library diversity can be increased. DARPins were shown to exhibit extraordinary stability and expression yield in *Escherichia coli* (18–20). Using complex DARPin libraries, binding molecules could be isolated that show great selectivity, specificity, and affinity in the recognition of their cognate antigen (20, 21). Since they encode no cysteines, they can be used for intracellular and extracellular targets.

We show here the *in vitro* selection of DARPins binding to epidermal growth factor receptor 2 (Her2) using ribosome display. We tested the selected DARPins for binding to the extracellular domain of Her2 (Her2 ECD) *in vitro*, to tumor cells in cell culture, and *in situ* on paraffinated Her2-positive breast cancer tissues. In addition, we investigated the cross-reactivity to epidermal growth factor receptor 1 and analyzed the epitopes bound by the selected DARPins using competition studies with trastuzumab and pertuzumab. The high specificity and affinity and the good expression yield of the selected DARPins in bacteria are encouraging for the development of novel diagnostic applications *in vivo* and *in vitro*. In addition, further studies can now be carried out to explore their therapeutic potential.

EXPERIMENTAL PROCEDURES

Selection Using Ribosome Display—The selection of DARPins binding to Her2 ECD was performed in parallel using two different libraries consisting of two and three randomized modules between an N- and C-terminal capping repeat, thus termed N2C and N3C, respectively. The generation of these libraries has been described previously (20). We used the extracellular domain (residues 1–621) of Her2 (Genentech) as target for the selection with ribosome display (22). For the first three rounds, Her2 ECD was immobilized directly on a Maxisorp plate (Nunc) by incubation overnight of 2 μg of Her2 ECD in PBS_{150} (20 mM Na_2HPO_4, pH 7.4, 150 mM NaCl) at 4 °C. To block unspecific binding sites, Her2 ECD-coated plates were washed three times with PBS_{150} and incubated for at least 2 h with 0.5% BSA in $TBST_{500}$ (20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.05% Tween 20).

For selection rounds 4–6, Her2 ECD was biotinylated. The biotinylation was performed by incubation of a 9.8 μM solution of Her2 ECD with a 20-fold molar excess of sulfosuccinimidyl-6-(biotinamido)-6-hexanamido hexanoate (sulfo-NHS-LC-LC-biotin, Pierce) in PBS_{150} on ice for 2 h. The biotinylated Her2 was dialyzed extensively against PBS_{150}, and the degree of biotinylation was determined as 3.1 molecules of biotin/molecule of Her2, using the 2-(4′-hydroxyazobenzene) benzoic acid (HABA) assay (Pierce).

Round four was performed on 75 nM biotinylated Her2 ECD immobilized on a NeutrAvidin-coated Maxisorp plate (Nunc). For the coating of NeutrAvidin, 100 μl of a 66 nM solution of NeutrAvidin in PBS_{150} were incubated in a Maxisorp plate at 4 °C overnight. In round five, the selection pressure was increased by applying off-rate selection. For this purpose, after the *in vitro* translation was stopped by 5-fold dilution into ice-cold WBT buffer (50 mM Tris acetate, pH 7.5, 150 mM NaCl, 50 mM $Mg(CH_3COO^-)_2$, 0.05% Tween 20), biotinylated Her2 ECD was added to a final concentration of 10 nM, and the translation was allowed to equilibrate for 2 h at 4 °C. The translation reaction was split into two aliquots, and non-biotinylated Her2 ECD was added to a final concentration of 1 μM to each aliquot, corresponding to a 100-fold excess over biotinylated antigen. The aliquots were incubated for 2 and 20 h, respectively, to increase the selection stringency for slower off rates (23). Ribosomal complexes were recovered using 30 μl of streptavidin-coated magnetic beads (Roche Applied Science). In round six, 175 nM biotinylated Her2 ECD was immobilized on a NeutrAvidin-coated Maxisorp plate. A summary of the selection process is given in Fig. 1.

In all selection rounds on solid-phase immobilized Her2 ECD, a prepanning step of 30 min on a neutravidin-coated Maxisorp plate was performed as described (20). After prepanning, the translation extracts were allowed to bind for 45 min to Her2 ECD-coated Maxisorp plates. Retained complexes were extensively washed with WBT buffer. Isolated mRNA was purified and reverse-transcribed using the oligonucleotide tolAk (5′-CCGCACACCAGTAAGGTGTGCGGTTTCAGTTGCC-GCTTTCTTTCT-3′) and PCR amplified using the oligonucleotides T7B (5′-ATACGAAATTAATACGACTCACT-ATAGGGAGACCACAACgg-3′) and tolAk (rounds 1–3) or WTC4 (5′-TTTGGGAAGCTTTTGCAGGATTTCAGC-3′) for rounds 4–6. When amplifying with primer WTC4, the tolA spacer had to be fused to the library again. Therefore, the selected pools were digested with BamHI and HindIII and ligated into pRDV (GenBank™ accession number AY327136). The ribosome display construct was PCR-amplified from the purified ligation using primers T7B and tolAk (20). All amplifications were performed using Vent DNA polymerase under stringent conditions. The mutation rate was not artificially increased during amplification.

Radioimmunoassay—To monitor the enrichment of specific DARPins in the pools, radioimmunoassays were performed after each selection round. Translation of pool mRNA was performed as described previously (23, 24) for 45 min in the presence of 0.5 mM [^{35}S]Met (1 Ci/μmol). The stopped translation mixtures were allowed to bind to surface-immobilized Her2 ECD as for the selection rounds for 30 min in the presence of 1% skimmed milk. After washing, the bound radioactivity was quantified in a scintillation counter.

Analysis of Selected Pools and Individual Clones—DNA of the selected pools after round 6 was amplified with primers SD_EWT5 (5′-AGACCACAACGGTTTCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAGATATATCCATGGGTATGAGAGGATCG-3′) and WTC4 and cloned via BamHI and HindIII into pQE30 (Qiagen). The pools were transformed into *E. coli* XL-1 Blue, and single clones were picked for plasmid preparation. Crude extracts of 1 ml of overnight protein expression cultures in LB medium containing 100 μg/ml ampicillin and 1% glucose were prepared according to previous protocols (20). Crude extracts were assayed by comparing the binding to wells containing immobilized Her2 ECD (0.5 μg/well) with the binding to wells containing NeutrAvidin alone (66 nM, 100 μl/well, Pierce). Detection was performed via an anti-RGS-His_6 antibody (Qiagen) and an anti-mouse-IgG conjugated to alkaline phosphatase (Pierce). For competition ELISA, the crude extracts were incubated with soluble Her2 ECD prior to binding to the Maxisorp plate for 1 h at 4 °C. Quantitative ELISAs were performed as described for the crude extract analysis, except that purified protein was used at 25 nM (see below).

Protein Production and Purification—For competition ELISAs, Biacore, flow cytometry, and tissue staining experiments, the DARPins were expressed on a 500-ml scale and purified on a nickel-nitrilotriacetic acid gravity flow column as described (19). For Biacore analysis, the proteins were further purified by size exclusion chromatography on a Superdex-75 column in PBS_{150} as described (19). Selected binders from each sequence group were cloned into the vector p254 with the restriction enzymes BamHI/HindIII, introducing a hemagglutinin (HA) tag (LYPYDVPDYA) to the C terminus of the ankyrin protein. p254 was constructed as follows. The two 5′-phosphorylated oligonucleotides r-cHA-Hind (5′-P-AGCTCTATTAGCGTAGTCCGGAACGTCGTACGGGTAA-3′) and f-cHA-Hind (5′-P-AGCTTTACCCGTACGACGTTCCGGACTACGCTTAATAG-3′) were annealed to each other and inserted into the HindIII site of pQE30 (Qiagen) via HindIII. HA-tagged ankyrin repeat proteins were expressed and purified as described for the RGS-His_6-tagged proteins. The single-chain Fv antibody fragment 4D5 was expressed from the plasmid pIG6 (25) in *E. coli* strain SB536 (26) and purified as described previously (27).

Surface Plasmon Resonance—Monovalent affinities were measured for selected binders using a Biacore 3000 instrument (Biacore). The measurements were performed in HBST (20 mM Hepes, pH 7.4, 150 mM NaCl and 0.05% Tween 20). Biotinylated Her2 was immobilized on a CM5 chip (Biacore), on which NeutrAvidin had previously been coated using amine coupling chemistry (according to the Biacore manual), to a final density of about 150 resonance units. The monomeric fraction of purified DARPins was injected at a flow rate of 100 μl/min at concentrations ranging from 10 nM to 1 μM, including a buffer control. The off rate was followed for at least 10 min. Data evaluation was performed by global fitting using BIAevaluation (Biacore), Scrubber (BioLogic software), and Clamp (28).

Cell Culture and Flow Cytometry—Breast cancer cell lines were obtained from the ATCC. MCF-7 and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) containing 10% fetal calf serum, 1% penicillin (10,000 units/ml), and 1% streptomycin (10 mg/ml). SKBR-3 cells were cultured in Dulbecco's modified Eagle's medium/F12 medium (Invitrogen) containing 10% fetal calf serum, 1% penicillin (10,000 units/ml), and 1% streptomycin (10 mg/ml). The cells were harvested at 80% confluence by trypsinization. Cells were washed and resuspended in PBS_{150} containing 1% BSA at 10^7 cells/ml. DARPins bearing a C-terminal HA tag were added to 1 ml of cells to a final concentration of 50 nM and incubated at 4 °C for 45 min. Cells were pelleted and resuspended in PBS_{150}/BSA containing anti-HA antibody (Roche Diagnostics, clone 12CA5, 30 min of incubation on ice according to the manufacturer's manual), pelleted again, and resuspended in PBS_{150}/BSA containing fluorescein isothiocyanate-coupled anti-mouse antibody (20 min of incubation on ice). The cells were washed with PBS_{150}/BSA and resuspended in CytoFix buffer (BD Biosciences) to a final density of 10^7 cells/ml. Measurements were performed on a Beckman Coulter Epics Altra machine at a flow rate of 500–700 s^{-1}. Fluorescence was detected at 525 nm after excitation with an argon laser at 488 nm. Data were analyzed using the software WinMDI (Version 2.8) and Microsoft Excel.

Staining of Tissue Sections—Paraffin-embedded human breast tissue specimens were cut into 5-μm sections and mounted on SuperFrost Plus microscope slides (Menzel-Gläser). Sections were deparaffinized in xylene (2 × 5 min) and sequentially hydrated by soaking first in 100% ethanol (2 × 5 min), 95% ethanol (2 × 5 min), and 75% ethanol (2 × 5 min) and finally rinsed in water for 2 min. The rehydrated tissue sections were incubated in pepsin epitope retrieval solution (25 mg of pepsin in 5 mM HCl) for 15 min at 37 °C. The sections were allowed to cool to ambient temperature for another 15 min and then transferred to the serum blocking solution (10% fetal calf serum, 1% BSA, 0.1% gelatin, 0.1% Triton X-100, 0.05% Tween 20 in PBS, pH 7.4) for 45 min. DARPins were diluted to a concentration of 200 nM in binding buffer (10 mM Tris, 155 mM NaCl, 2 mM EGTA, 2 mM $MgCl_2$ and 1% BSA, pH 7.2), added to the specimen, and incubated for 45 min. After the DARPin binding step, endogenous peroxidase was blocked for 5 min using the LSAB2 kit (Dako). Thereafter, the murine anti-HA antibody (Roche Diagnostics, clone 12CA5) was diluted 1:4000 in binding buffer and administered for 45 min. For the secondary antibody and the developing step, the LSAB2 kit (Dako) was used following the manufacturer's instructions. Finally, the specimens were counterstained for 45 s in a hematoxylin bath and thoroughly rinsed with water. The slides were mounted using the Faramount kit (Dako).

RESULTS

Selection of DARPins Using Ribosome Display—We used ribosome display to select DARPins against the extracellular domain of Her2 (Her2 ECD) from designed ankyrin-repeat protein libraries (19, 20). Ribosome display is a method to select polypeptide binders from large libraries *in vitro*. The selection is based on ternary complexes of ribosome, mRNA without a stop codon, and the newly synthesized polypeptide that form in the translation reaction and are stable after cooling (22). We used two different libraries in parallel with two or three randomized repeat modules between N-terminal and C-terminal capping repeats, termed N2C and N3C, respectively. The selec-

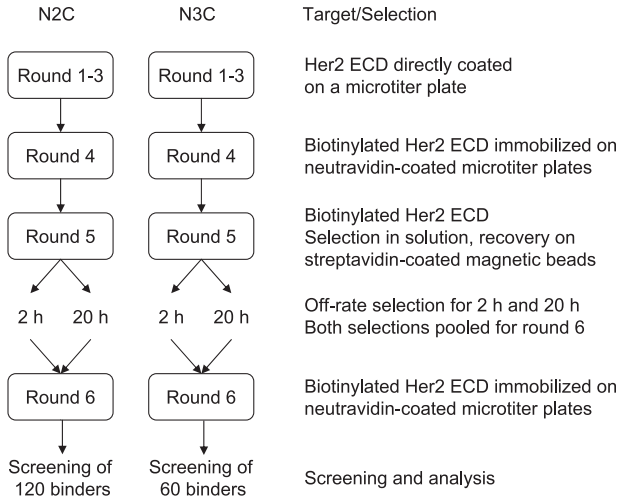

FIGURE 1. **Selection scheme.** Two DARPin libraries, termed N2C and N3C (19), were used for the selection of specific protein ligands to Her2 ECD using ribosome display. For the fist three selection rounds, Her2 ECD was directly immobilized on a Maxisorp microtiter plate. For later rounds, Her2 ECD was biotinylated, and either it was immobilized on a neutravidin-coated microtiter plate (rounds 4 and 6) or the selection was performed in solution (round 5). In round 5, the selection pressure was increased by using off-rate selection (23). The library was allowed to bind to low amounts of biotinylated Her2 ECD in solution. After equilibration, a 100-fold excess of non-biotinylated Her2 ECD was added to prevent dissociating library members from rebinding. The libraries were split into aliquots, which were incubated for 2 and 20 h, respectively, before recovering the pools with streptavidin-coated magnetic beads. Before round 6, the aliquots were pooled. From the N2C pool, 120 binders were screened for binding to Her2 ECD, and from pool N3C, 60 clones were screened for binding to Her2 ECD.

tion procedure is summarized in Fig. 1. Six panning rounds were performed to enrich for specific DARPins for Her2 ECD. The first three rounds were performed with non-biotinylated Her2 ECD. The antigen was directly coated to a microtiter plate. To be able to perform the selection rounds in solution and to increase the selection pressure by off-rate selections, Her2 ECD was biotinylated. From round 4 on, all subsequent selection rounds were performed on biotinylated antigen. The number of PCR cycles needed to recover the mRNA pools after reverse transcription decreased from round to round, which was an indication for the enrichment of specific DARPins. After the first round, 40 cycles of amplification with PCR were used. After the second round, only 35 cycles of amplification were needed to generate comparable amounts of a specific PCR band, as visualized on an agarose gel, and 30 cycles were sufficient after the third round.

In the fifth round, the pools were subjected to off-rate selection (23) to enrich for binders with slower off rates. For this purpose, the pools were equilibrated with 10 nM biotinylated Her2 ECD. Non-biotinylated Her2 ECD was then added to a final concentration of 1 μM, resulting in a 100-fold excess of competitor. After 2 and 20 h, aliquots of each pool were isolated and subjected to the standard work-up of a ribosome display round (22). A sixth panning round was performed under non-stringent conditions as it has been shown that the concentration of binders can become very low after off-rate selections when compared with unspecific background (23).

Analysis of the Pools—An indication for the specificity of the enriched DARPins in the pools for Her2 ECD was obtained by comparing the binding of the pools to immobilized Her2 ECD and to a control. Starting from the third round, selections were carried out in parallel to Her2 ECD-coated surfaces and control surfaces, coated with neutravidin or BSA. In all pools tested, only a faint band was visible for the unspecific surface when compared with the specific surface. The specific enrichment for binders during off-rate selection was further monitored by radioimmunoassays of the pools after rounds 4 and 5 (23). The binding signal of the pools to immobilized Her2 ECD increased significantly, whereas the binding signal to an unspecific surface remained about constant. For the N3C pool, 184 cpm of activity bound to a Her2 ECD-coated well after round 4 and 3345 cpm of activity bound after round 5 (18-fold more), whereas the binding activity on a neutravidin-coated surface was 180 and 206 cpm after rounds 4 and 5, respectively. The enriched pools after rounds 3 and 6 were ligated into the expression plasmid pQE30, and *E. coli* strain XL1-Blue was transformed for plasmid preparation and expression. Single clones of these pools were assayed in a crude extract ELISA in the 96-well format. After round 3, one DARPin showed significant binding over background out of 48 assayed clones from the N2C pool, and two showed significant binding over background out of 48 assayed clones for the N3C pool. After round 6, 57 out of 120 assayed clones (48%) showed specific binding in the N2C pool, and 19 out of 60 (32%) showed specific binding in the N3C pool. None of the assayed DARPin sequences showed significant binding to a BSA-coated microtiter plate. All clones showing binding signals were sequenced.

Sequence analysis of the selected clones of the N2C library revealed that most clones belonged to three sequence families (Fig. 2), representing 56, 23, and 13% of all specific binders in this pool, respectively. The members within one family only differed in a few positions. Four clones have been found several times. Only 3 out of the 57 analyzed clones did not belong to any of these major sequence families.

Analysis of the sequences in the N3C library showed that 71% of the specific binders belonged to one sequence family. The other binders had higher sequence diversity when compared with the N2C library and could not be grouped in families. No obvious sequence homology of the N3C binders could be found with selected repeat domains from the N2C pools. In both pools, binders were found that showed point mutations at positions that were not designed to be randomized. They were most likely introduced during one of the many PCR amplification cycles, despite the fact that no error-prone PCR conditions have been applied.

Competition ELISA and Affinity Determination—For a first affinity ranking, binders of all sequence families were subjected to competition ELISA with soluble Her2 ECD. The analysis was performed with cleared lysates of bacterial expression cultures. Binding of the lysates to immobilized Her2 was performed in the absence and presence of soluble Her2 ECD at 10 and 100 nM. The binding signal of more than 90% of the investigated binders could be inhibited with 100 nM free Her2 ECD by more than 70%. Even at 10 nM soluble Her2 ECD, the binding of more than half of the assayed clones could be inhibited significantly. We therefore concluded that the great majority of the DARPins present in the pools were binding with affinities better than 100 nM. For most DARPins, the binding to surface-immobilized

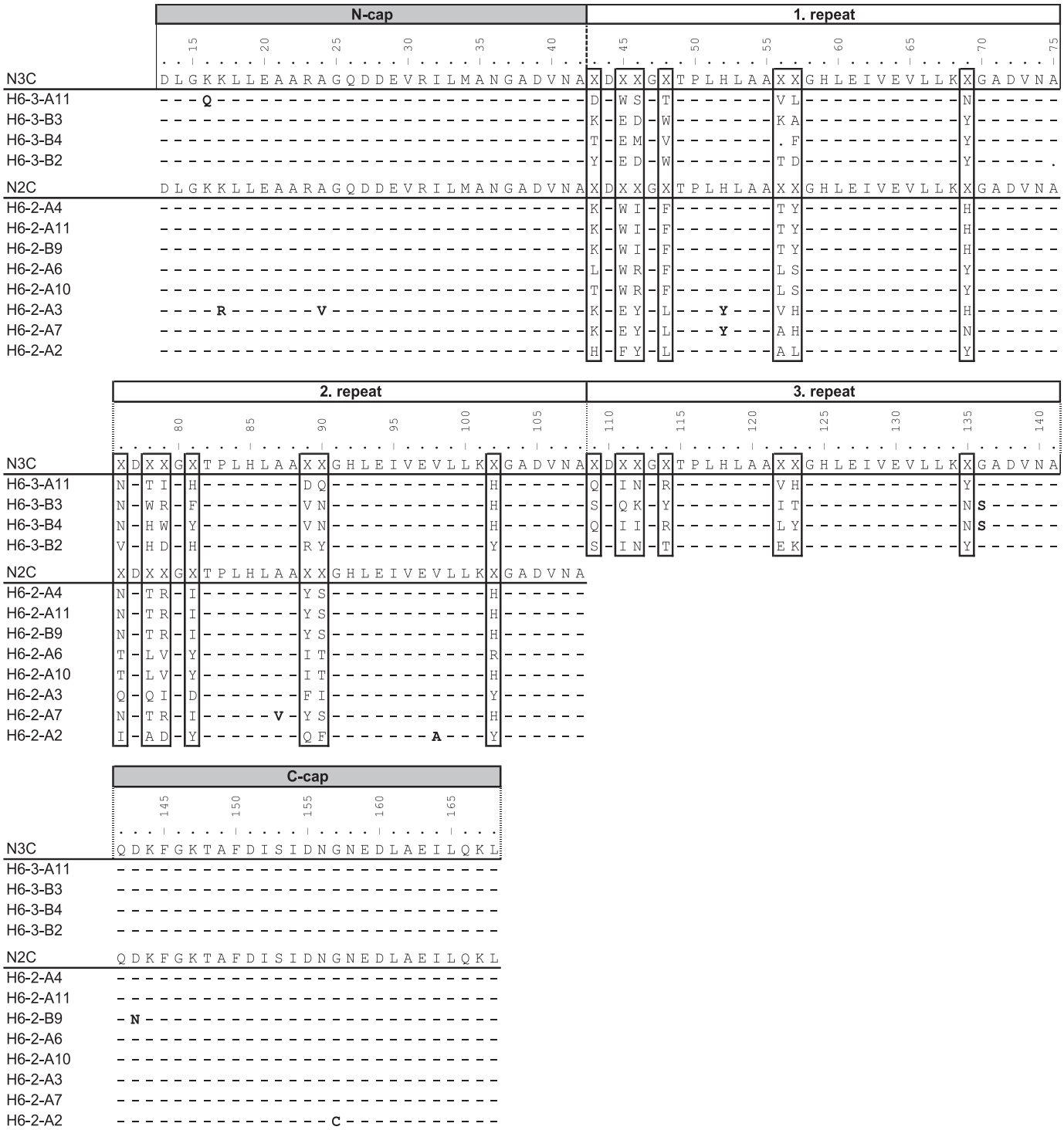

FIGURE 2. **Sequences of selected DARPins.** An assortment of protein sequences of selected DARPins from both libraries are shown in single-letter code, representing binders with promising binding signals in the ELISA (Fig. 4) and the sequence diversity. Only residues that differ from the consensus sequence (*top lines* for N3C and *lower lines* for N2C) are indicated. Clone H6-3-A11 belonged to the sequence family that had been predominantly found in the selected N3C pools. The major sequence families from the N2C pools are represented, *e.g.* by clones H6-2-A4, H6-2-A6, and H6-2-A3. Note that several N3C DARPins exhibit a deletion (positions 56 and 75, respectively), which is indicated with a *dot*. Nevertheless, these DARPins showed specific binding. Positions that had been randomized in the original design are *framed*. Framework positions that had mutated during the selection are printed in *bold*. The position of the designed cap regions and the respective repeat modules are indicated schematically.

Her2 ECD could only be inhibited by soluble Her2 ECD if the crude *E. coli* extracts containing the DARPins were diluted at least 64 times. This indicates very high levels of protein expression, as has been described earlier for both unselected and selected DARPins (19, 20). We thus expected most of the binders to be well expressed and have affinities in the low nanomolar range already before any affinity maturation. This was further verified by Biacore measurements (see below).

For further analysis, binders from each sequence family and both pools were expressed in *E. coli* XL1-Blue and purified by

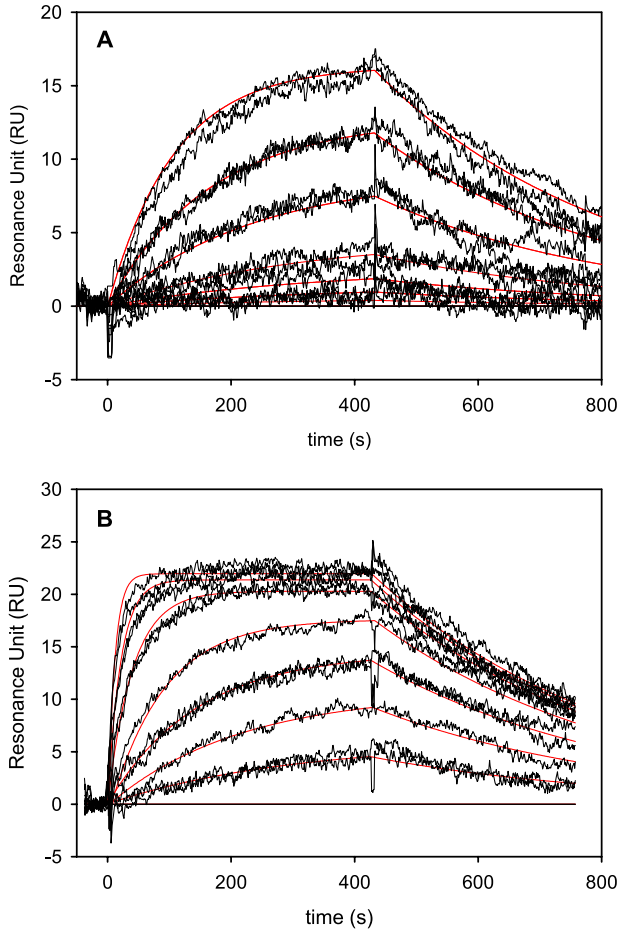

FIGURE 3. **Affinity determination of selected DARPins using surface plasmon resonance.** The binding kinetics of selected DARPins were analyzed using a Biacore 3000 instrument. Different concentrations of the corresponding binders were applied to a flow cell with immobilized Her2 ECD, and association and dissociation phases were recorded. *A*, clone H6-2-A7 had an association rate constant of $1.21 \cdot 10^5$ M^{-1} s^{-1}, a dissociation rate constant of $8.8 \cdot 10^{-4}$ s^{-1} and consequently a K_D of 7.3 nM. *B*, clone H6-3-B3 had an association rate constant of $8.9 \cdot 10^4$ M^{-1} s^{-1} and a dissociation rate constant of $2.56 \cdot 10^{-3}$ s^{-1}, resulting in a dissociation constant K_D of 28 nM. The kinetic data were evaluated by global fitting using the software Clamp (28). The curves predicted from the global fit are indicated in *red*. *RU*, resonance unit.

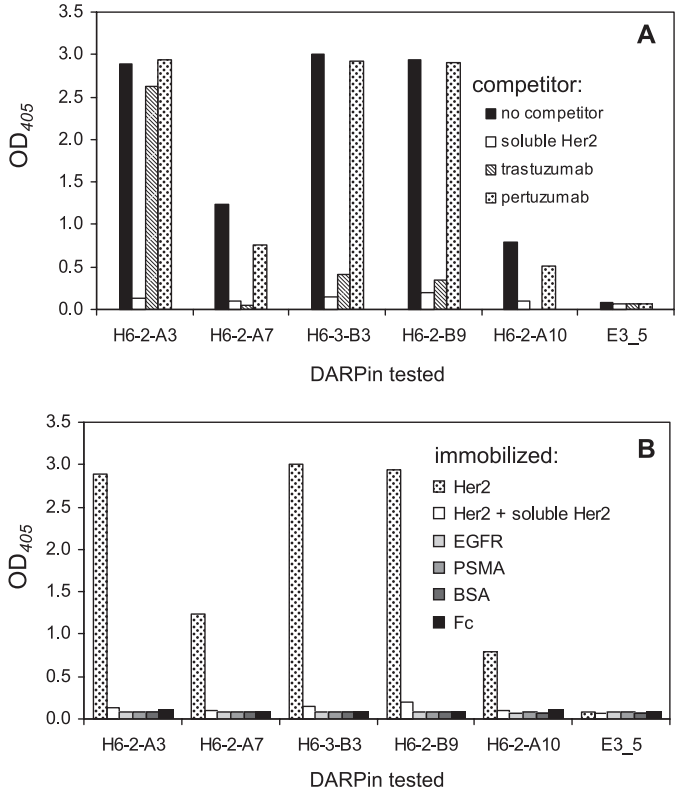

FIGURE 4. **ELISA analysis of selected DARPins.** *A*, for a closer characterization of the epitopes recognized by the selected DARPins, their binding to immobilized Her2 ECD was competed with two monoclonal antibodies, trastuzumab (Herceptin) and pertuzumab (Omnitarg) 150 nM final concentration. Several of the selected DARPins, but not all, competed for the epitope on Her2 ECD with trastuzumab. None of the DARPins was found to compete for binding of pertuzumab. The immobilized protein is always Her2, and the competitor is indicated. As a control, inhibition by soluble Her2 is shown as well. *B*, several of the selected and purified DARPins were analyzed for their specificity in binding to different immobilized target proteins. The immobilized protein is indicated. Binding could only be observed to immobilized Her2 but not to any other immobilized protein. In addition, the specific binding to immobilized Her2 ECD could be competed completely with 150 nM soluble Her2 ECD. E3_5 is a randomly picked DARPin from the synthetic unselected library (18), which was used as a control. *EGFR*, epidermal growth factor receptor 1; *PSMA*, prostate-specific membrane antigen, *Fc*, human Fc-γ1 region of an antibody.

immobilized metal ion affinity chromatography. For better detection and purification, the binders were fused to a C-terminal hemagglutinin peptide (HA tag), in addition to the N-terminal RGS-His_6 tag.

For each pool, N2C and N3C, one DARPin with a promising competition behavior in crude extract ELISA, namely H6-2-A7 and H6-3-B3, was chosen to determine the monovalent association and dissociation rates with surface plasmon resonance using a Biacore instrument (Fig. 3). Both clones were expressed and purified by immobilized metal ion affinity chromatography and by gel filtration. Analysis was performed on sensor surfaces that had been coated with low amounts of Her2 ECD. Both assayed DARPins showed specific and reversible binding to their cognate antigen. The N3C DARPin, H6-3-B3, had an association rate constant of $8.9 \cdot 10^4$ M^{-1} s^{-1} and a dissociation rate constant of $2.56 \cdot 10^{-3}$ s^{-1}, corresponding to an equilibrium dissociation constant K_D of 28 nM. Clone H6-2-A7, an N2C DARPin, displayed an association rate constant of $1.21 \cdot 10^5$ M^{-1} s^{-1} and a dissociation rate constant of $8.8 \cdot 10^{-4}$ s^{-1}, resulting in an equilibrium dissociation constant K_D of 7.3 nM (Fig. 3). The high affinity determined thus reflected the behavior observed in ELISA.

Epitope Characterization and Specificity Analysis—We aimed to further characterize the epitope of different selected DARPins. Two antibodies have been structurally described that bind to different epitopes of Her2 ECD. Trastuzumab (Herceptin) binds to the membrane-adjacent cysteine-rich domain IV (5), whereas pertuzumab (2C4) was shown to bind to domain II, which constitutes the dimerization interface to other members of the EGF receptor family, thereby preventing dimerization (8, 29, 30). To test whether some of the selected DARPins bound to the same epitope as one of these antibodies, the two antibodies were used as competitors for antigen binding in ELISA experiments. Different purified DARPins were allowed to bind to surface-immobilized Her2 ECD in the presence and absence of 100 nM free Her2 ECD, 150 nM trastuzumab, or 150 nM pertuzumab. Several groups of DARPins, including N2C and N3C DARPins, did compete with trastuzumab for the epitope, whereas none of

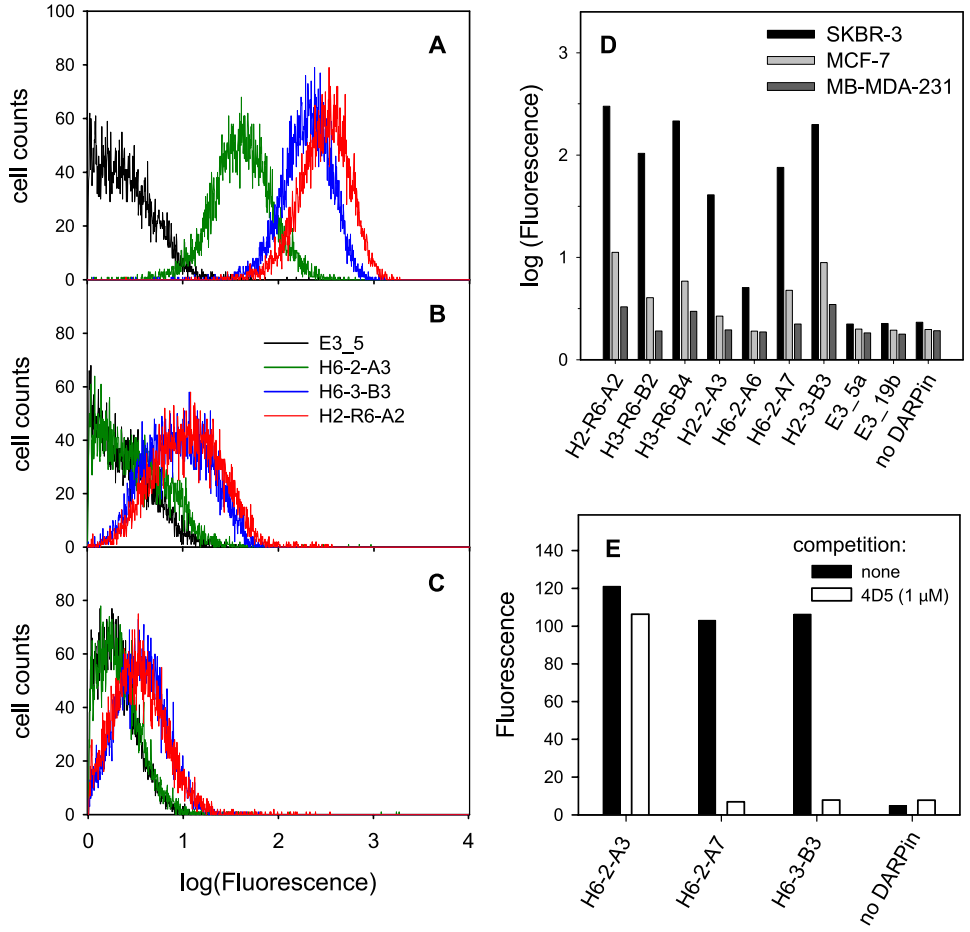

FIGURE 5. **Binding of selected DARPins to Her2 on different cell lines by analytical flow cytometry.** Selected DARPins were expressed with an N-terminal HA tag and were allowed to bind to different breast carcinoma cell lines, which had been described to express Her2 to different extent. SKBR-3 (in *A*) is known to strongly overexpress Her2, whereas MCF-7 cells (shown in *B*) only weakly express Her2. MDA-MB-231 is a cell line, which hardly expresses Her2 at all (shown in *C*). Binding was detected via HA tag-specific and fluorescein isothiocyanate-coupled antibodies. The logarithm of the fluorescence intensity of counted cells was plotted *versus* the cell count. Since all cell samples were homogeneous cell populations (*A–C*), the logarithm of the average of the fluorescence intensity was plotted as a single value for better comparison (*D*). Competition of the binding of selected DARPins with the scFv of trastuzumab, termed 4D5, is shown in *E*. Clone H6-2-A3 was shown to bind to a different epitope than the scFv 4D5, whereas clones H6-2-A7 and H6-3-B3 were shown to compete with scFv 4D5 for binding to Her2. DARPins were bound to SKBR-3 cells at 50 nM, and soluble scFv 4D5 was added to a concentration of 1 μM. Binding was detected as in *D*. The mean fluorescence intensity was plotted. The DARPins termed E3_5 and E3_19 were not selected but had been picked randomly from the naive library to be used as a non-binding control. Binding of the secondary antibody was analyzed by incubation of the cells in the absence of any DARPin (no DARPin).

the selected binders showed competition with pertuzumab (Fig. 4*A*). Domain IV, which is the membrane-adjacent domain and the epitope of trastuzumab, seems to be a preferred epitope for the binding of DARPins since binders from independent libraries and selections bound to this epitope. However, DARPins binding to other epitopes were also found, as suggested already by the presence of different sequence families. To show that the epitope was not only well accessible in soluble Her2 ECD but also in membrane-bound, endogenous Her2 ECD, binding studies on whole cells were performed (see below).

The extracellular domains of the members of the ErbB family are structurally homologous. Her2 shares 43% sequence identity with EGFR and ErbB3. The identity in domain IV between these proteins is also about 43%. We investigated the cross-reactivity of some selected DARPins against EGFR and other unrelated proteins in ELISA. Different proteins including Her2 and EGFR were coated on a polystyrene plate, and purified DARPins were allowed to bind at a concentration of 100 nM to the immobilized proteins. None of the investigated DARPins bound to any other protein than Her2 (Fig. 4*B*). The DARPin E3_5, which had been randomly selected from a naive N3C library, did not show binding to any of the immobilized proteins. This high specificity has already been observed for DARPins selected against other target proteins (20).

Flow Cytometry—In vitro selection and characterization of the binders had solely been performed with the isolated extracellular domain of Her2. To check whether the selected binders would also be able to bind to the cognate antigen on the cell surface, we used several cell lines that were known to express Her2 to a different extent (31): the strongly overexpressing cell line SKBR-3, the weakly expressing cell line MCF-7, and MDA-MB-231, which has been described not to overexpress Her2 at all. To get stringency in the binding reaction, the DARPins were allowed to bind to the cells in solution at the relatively low concentration of 50 nM. Binding was detected in a fluorescence-activated cell sorter using a HA tag-specific mouse antibody and a secondary fluorescein isothiocyanate-coupled antibody.

All analyzed DARPins showed specific binding to the Her2-positive SKBR-3 cells. Binding to MCF-7 cells was at least 30-fold reduced when compared with SKBR-3, and binding to MDA-MB-231 cells was not visible or only marginally visible. This corresponds to the previously described expression patterns of these cell lines (32). No binding to any of the cell lines could be seen for the non-binding DARPins E3_5 and E3_19 or if no DARPin was used at all (Fig. 5, *A–D*). Differences between the fluorescence intensities of the selected DARPins (Fig. 5*D*) could be due to the different affinities observed. Since the selected binders bind to several different epitopes, the differences might also be due, at least in some cases, to different accessibilities of the corresponding epitopes on the cell.

To further exclude that the DARPins would bind specifically to soluble Her2 ECD but would non-specifically aggregate on the chosen Her2-positive cell lines, the binding of different DARPins to strongly Her2-expressing cells was measured in the

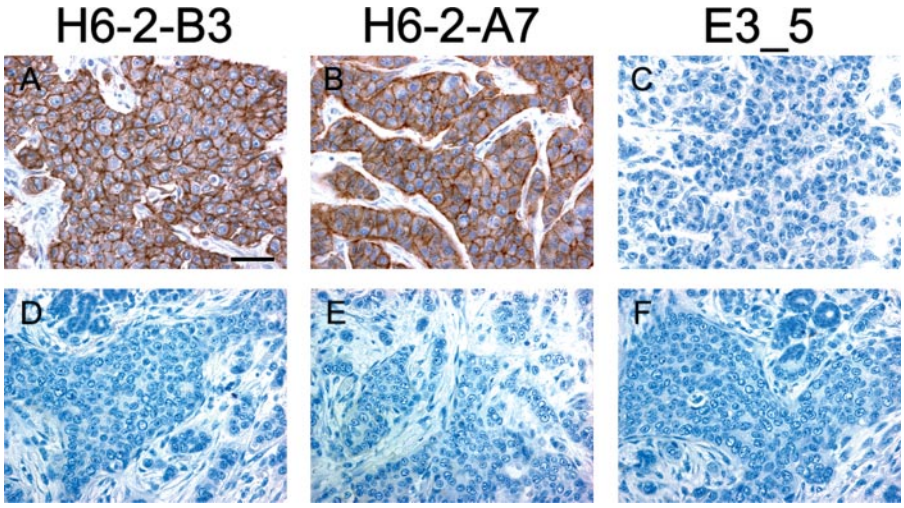

FIGURE 6. **Staining of Her2-expressing cells in human breast cancer tissue sections.** Two selected DARPins, H6-3-B3 (*A* and *D*) and H6-2-A7 (*B* and *E*) and the unselected control DARPin E3_5 (*C* and *F*), which was shown not to bind to Her2 (Fig. 4), were used to stain Her2-expressing cancer cells in paraffinated tissue sections of intraductal breast carcinoma. Two different tissues were used: one that was shown by the HercepTest (Dako) to strongly overexpress Her2 (stage 3+, *A–C*) and one that was shown not to overexpress Her2 (stage 0, *D–F*). Prior to the binding of the DARPins, the tissues were treated with proteases to unmask the epitope. Both selected DARPins show strong staining of the plasma membranes of the carcinoma cells in the Her2-positive tissue sections, whereas no staining could be observed by staining either with E3_5 or on the Her2-negative human breast tissues. The *bar* represents 50 μm.

absence and presence of the single-chain antibody Fv fragment of trastuzumab, termed 4D5 (27). The binding to SKBR-3 cells could be inhibited completely for those binders, which have been shown to bind to the same epitope as trastuzumab, whereas the binding of those, which have been shown to bind to different epitopes, could not be competed (Fig. 5*E*). This shows that the selected DARPins recognize the epitope on domain IV of Her2 ECD in its native context. The strong signals obtained for some of the DARPins and the fact that even low concentrations of Her2 on MCF-7 cells could be detected suggest that these DARPins can be used for the quantification of Her2 within a large dynamic range of detection.

Tissue Staining—For clinical diagnostics, the detection of Her2 in human mamma-carcinoma tissue is of great importance. We therefore analyzed the selected DARPins, for which the affinities had been determined and which were shown to bind well to Her2-expressing cells, in immunohistochemistry on paraffinated tissue-sections. Immunohistochemistry was also an important test to analyze the specificities of the DARPins for Her2 ECD in a complex environment. Paraffinated intraductal breast tumors, which were described to be strongly overexpressing either Her2 (type 3+) or Her2-negative (type 0) (33), were cut into 5-μm sections. Since fixation of the tissue with paraformaldehyde cross-links proteins in the tissue and therefore reduces the accessibility of the antigen, several protocols were compared to retrieve the plasma membrane-bound epitopes. We tested the retrieval by non-denaturing methods, such as limited proteolysis of the tissue by different proteases and also denaturing treatments, including strong acids, bases, and denaturing agents. We adopted a non-denaturing protocol (see "Experimental Procedures") as these resulted in much stronger plasma membrane staining than the denaturing protocols. Both assayed DARPins showed strong and highly specific plasma membrane staining of the Her2-overexpressing tumor cells, whereas no staining could be seen in the Her2-negative tissue sections and in the Her2-positive tissue sections, when using the non-selected control DARPin E3_5 (Fig. 6). We therefore concluded that the assayed DARPins bind to conformational, non-linear epitopes. This is in accordance with the structure of the DARPins, which had been designed to bind to protein surfaces rather than to peptides (19) and which exhibit a relatively flat binding surface rather than a binding cleft.

DISCUSSION

We selected high affinity consensus ankyrin repeat proteins (DARPins) against the extracellular domain of Her2 from synthetic libraries *in vitro* using ribosome display. The selected DARPins bind to Her2 with high affinity and specificity but not to any other investigated protein, including EGFR, human Fc-γ1, and prostate-specific membrane antigen. We found several sequence families in the selected pools that cover different epitopes on Her2 ECD. Several of the selected DARPins were shown to bind to the same epitope as the therapeutic antibody trastuzumab (Herceptin).

The selection experiments were performed on the isolated extracellular domain of Her2. Nonetheless, the investigated DARPins also bound to Her2 *in vivo* as shown by binding to different cell lines by fluorescence-activated cell sorter analysis and by competition of the binding to Her2-expressing cells by conformational antibodies. Furthermore, the binders could be used *in situ* for the staining of cancer cells in paraffinated tissue sections of Her2-positive mamma-carcinoma. The best results were obtained if the tissue was previously treated with proteases instead of denaturing agents, indicating that the selected DARPins bind to structured epitopes rather than to denatured protein.

Due to the short time needed for the selection and the high specificity, affinity, and stability, the selected DARPins could prove to be optimal for novel approaches in the diagnostics of cancer tissue, such as protein chip-based assays against several cancer markers in parallel. In addition, small proteins, such as DARPins, are expected to penetrate more easily into tumor tissue than the larger antibodies, whereas being cleared relatively fast from the circulation. In combination with the slow off rates observed, this could make the selected DARPins optimal candidates for *in vivo* diagnostic approaches, where these characteristics are generally linked to higher specificity, less background, and less toxicity.

The therapeutic potential of DARPins needs to be further evaluated. The fact that some DARPins bind to the same

epitope as Herceptin makes these binders interesting candidates for further studies. The high stability and the exceptional expression yields make DARPins ideal targeting modules for fusion constructs, including the fusion to toxins and other effector molecules.

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