Concurrent Binding of Anti-EphA3 Antibody and Ephrin-A5 Amplifies EphA3 Signaling and Downstream Responses: Potential as EphA3-Specific Tumor-Targeting Reagents

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Abstract

The Eph receptor tyrosine kinases and their membrane-bound ephrin ligands form a unique cell-cell contact–mediated system for controlling cell localization and organization. Their high expression in a wide variety of human tumors indicates a role in tumor progression, and relatively low Eph and ephrin levels in normal tissues make these proteins potential targets for anticancer therapies. The monoclonal antibody IIIA4, previously used to isolate EphA3, binds with subnanomolar affinity to a conformation-specific epitope within the ephrin-binding domain that is closely adjacent to the “low-affinity” ephrin-A5 heterotetramerization site. We show that similar to ephrin-A5, preclustered IIIA4 effectively triggers EphA3 activation, contraction of the cytoskeleton, and cell rounding. BIACore analysis, immunoblot, and confocal microscopy of wild-type and mutant EphA3 with compromised ephrin-A5 or IIIA4 binding capacities indicate that IIIA4 binding triggers an EphA3 conformation which is permissive for the assembly of EphA3/ephrin-A5-type signaling clusters. Furthermore, unclustered IIIA4 and ephrin-A5 Fc applied in combination initiate greatly enhanced EphA3 signaling. Radiometal conjugates of ephrin-A5 and IIIA4 retain their affinity, and in mouse xenografts localize to, and are internalized rapidly into EphA3-positive, human tumors. These findings show the biological importance of EphA3/ephrin-A5 interactions and that ephrin-A5 and IIIA4 have great potential as tumor targeting reagents. (Cancer Res 2005; 65(15): 6745-54)

Introduction

Eph receptor tyrosine kinases (Ephs) and their membrane-bound ephrin ligands (ephrins) control cell positioning and tissue organization during essential embryonic development programs (1). During adulthood, many Ephs and ephrins are expressed preferentially in malignant tissue, where they are thought to participate in progressing invasive and metastatic cancers, including malignant melanoma, sarcoma, lung cancer, kidney, and brain tumors (2–4). Ephs probably do not function in mitogenesis (5) but promote cancer progression by reactivation of their embryonal functions thus facilitating neoangiogenesis and directing tumor cell motility, adhesion, and positioning (1, 2).

Many biological effects attributed to Eph function, such as segregation of mixed cell populations into Eph and ephrin expression domains, require concurrent “forward” signaling in Eph and “reverse” signaling in ephrin-expressing cells, which occurs in vivo when cell surface–bound Ephs and ephrins interact in trans. Thus, soluble Eph and ephrin exodomains bind their corresponding partner as function-blocking antagonists and have been tried as inhibitors of Eph function during tumor progression and neovascularization (6–8). However, most ephrins can bind and activate several Eph receptors within their subclass with comparable affinities (9, 10), a redundancy that provides a considerable challenge for selective targeting of Eph- or ephrin-expressing tumor cells with soluble Eph or ephrin proteins. In this regard, monoclonal antibodies can be more specific and have been described for EphA3 (11), EphB2 (12), EphB6 (13), mouse ephrin B1 (14), and EphA2 (15), whereby an apparent antiproliferative activity of the latter (15) has not been reproduced for any of the other antibodies (12). We previously used a monoclonal antibody (mAb), IIIA4, raised against LK63 human acute pre-B leukemia cells to affinity isolate EphA3 (11). We later confirmed that IIIA4 binds to the native EphA3 globular ephrin-binding domain (16, 17) with subnanomolar affinity (K_D ~ 5 × 10^-10 mol/L) reflecting a very low apparent dissociation rate k off = 3 × 10^-4/s (18).

A number of recent studies have provided insight into the contact surfaces that are involved in the assembly of Eph/ephrin signaling complexes. Crystallography (10, 19) and complementing structure-function analysis (18) revealed that high-affinity, 1:1 dimers of the isolated monomeric Eph/ephrin-binding domains (16, 20), through engagement of two additional interfaces, assemble at high local concentration (within the crystal structure) into 2:2 heterotetramers. In the resulting ring-like heterotetramer, Ephs and ephrins are oriented so that their COOH termini point in opposing directions of each other, in agreement with an orientation that facilitates signaling into each of two opposing cells. However, the assembly of active signaling complexes (21) and their internalization into the Eph-expressing cell (22) requires further oligomerization, which is routinely achieved in vitro through preclustered, tetravalent ephrin-Fc fusion proteins (23). We postulated that the heterotetrameric conformation of EphA3 harbors the propensity for Eph/Eph oligomerization via membrane-proximal Eph domains.
in tumor-targeting studies, we prepared $^{125}$I or $^{111}$In radioisotope conjugates of IIIA4 and ephrin-A5 mAb, both of which retain the number of binding sites per cell were calculated as described previously (26). Iodination with $^{125}$I was achieved by direct diethylenetriaminepentaacetic acid, allowing convenient chelation of the radioligand-binding analysis as described previously (18, 22). Labeled proteins were recovered from the reaction mixture on a Phast Desalting column (Pharmacia, Piscataway, NJ) and stored in aliquots at $-80^\circ$C.

**Cell culture.** Human acute lymphoblastic pre-B cells (LK63), AO2, and SK-Mel28 melanoma lines and human kidney epithelial 293 (HEK293, American Type Culture Collection, Manassas, VA) cells have been described previously (11, 24, 26) and were cultured in RPMI or DME (HEK 293), containing 10% FCS. Transfection was carried out using Fugene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN). Before each experiment, cells were serum starved in culture medium containing 0.5% FCS for at least 4 hours.

**Microscopy and immunocytochemistry.** Immunocytochemistry and time-lapse confocal microscopy on an Olympus FV500 microscope using 100×, 1.4 numerical aperture (NA) oil (fixed cells) and 60×, 1.0 NA water (live cells) immersion lenses, were done as described (24). Images of EGFP (EphA3) and Alexa$^{488}$ and Alexa$^{647}$ (IIIA4) fluorescence were collected sequentially to minimize “bleed-through” from spectral overlap. EPFP was excited with the 488-nm line of a 100-mW argon ion laser; Alexa$^{546}$ was excited with the 543-nm HeNe laser line; Alexa$^{647}$ with the 635 HeNe laser line.

Sections from mouse xenografts were snap frozen, or frozen in optimal cutting temperature compound (Tissue-Tek, Sakura Finetek U.S.A., Inc., Torrance, CA); Sections (5 μm) were fixed in ice-cold acetone and treated with 0.8% H$_2$O$_2$ to eliminate endogenous peroxidase and nonspecific binding sites saturated with a protein-blocking reagent. Biotin-conjugated IIIA4 antibody was used for mouse xenograft sections, and hematoin-counterstained sections developed with streptavidin HRP and 3-aminon-9-ethylcarbazole (AEC) as chromogenic substrate. Sections in Crystal/Mount (Abbott, North Chicago, IL) for microscopic viewing.

**Immunoprecipitation and Western blotting.** Phosphorylated proteins and EphA3 were immunoprecipitated (minimum 4 hours, 4°C) from Triton X-100 lysates of stimulated or control cells (24) using 4G10 anti-phosphotyrosine agarose (Transduction Laboratories) and with IIIA4 mAb agarose, respectively, the latter prepared by coupling the purified antibody to Mini-leaf agarose (Kern-En-Tec, Copenhagen, Denmark) according to the manufacturer’s instructions. Lysates and washed immunoprecipitates were analyzed by Western Blot with appropriate antibodies and visualized using an enhanced chemiluminescence substrate (Pierce, Rockford, IL).

**Labeling of IIIA4 monoclonal antibody and a control monoclonal antibody (CLB-CD19) with $^{111}$In and $^{125}$I.** The IIIA4 antibody and ephrin-A5 Fc were conjugated with the bifunctional metal ion chelator CHX-A$^\delta$-diethylenetriaminopentaacetic acid, allowing convenient chelation of the resultant conjugate with $^{111}$In. Iodination with $^{125}$I was achieved by direct coupling using Chloramine-T as described previously (26). Retention of the affinity of labeled proteins was confirmed by radioiodination and binding analysis, whereby serial dilutions of unlabeled ephrin-A5 Fc or IIIA4 mAb (from) and a constant concentration (20 ng) $^{125}$I or $^{111}$In protein were incubated with 1.5 × 10$^5$ SK-MEL-28 or EphA3/HEK 293 cells for 45 minutes at room temperature with continuous mixing. Radioactivity in washed cell pellets levels was determined on a gamma counter. The $K_a$ (affinity) and the number of binding sites per cell were calculated as described previously (26).

**In vitro properties of protein-riadiometal conjugates.** The retention of binding affinities of radiolabeled ephrin-A5 Fc and IIIA4 mAb were confirmed by the Lindmo assay as described previously (26). Briefly, cells expressing radiolabeled monoclonal antibodies were transfected with serial dilutions SK-MEL-28 (3 × 10$^7$) or LK63 (4.7 × 10$^7$) cells, incubated (45 minutes, room temperature) with 20 ng of $^{125}$I or $^{111}$In-mAbIIIA4 was determined on a gamma counter. The stability of $^{125}$I- and $^{111}$In-labeled proteins, incubated for 2 and 7 days at a constant radioactive concentration in human serum was determined by binding to SK-MEL-28 cells and expressed as percentage of radioactivity at day 0.

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**Materials and Methods**

**Expression vectors and reagents.** Expression vectors encoding full-length wild-type (wt) and mutant EphA3 proteins, containing either tyrosine-to-phenylalanine substitutions in the three major phosphorylation sites (3' YF EphA3, ref. 24) or substitutions Phe$^{189}$-to-Leu and Val$^{192}$-to-Glu within the putative EphA3 heterodimerization and heterotetramerization domains with or without addition of a COOH-terminal enhanced green fluorescent protein tag (EGFP, Clontech, Mountain View, CA) were prepared as described previously (18, 22). Expression plasmids (pGL5OS) encoding extracellular domains of ephrin-A5 or EphA3 (25) fusion proteins with the hinge and Fc region of human IgG1 (gift from A. van der Merwe, Oxford University, Oxford, United Kingdom) were prepared and Protein-A affinity purified from culture supernatants of stable Chinese hamster ovary transfectants as described (18, 22).

**Anti EphA3 mAb, IIIA4 and affinity-purified rabbit polyclonal antibodies were described (11, 24). Other antibodies and reagents were from Immunoresearch Laboratories [West Grove, PA; anti-human Fc and anti-phosphotyrosine antibody 4G10 and 4G10-agarose], Jackson ImmunoResearch Laboratories (Eugene, OR; Alexa488-phalloidin).**

**Alexa$^{488}$ and Alexa$^{647}$ protein conjugates.** Recombinant, purified ephrin-A5-Fc and the IIIA4 mAb were labeled with Alexa Fluor$^{488}$ and Alexa Fluor$^{647}$ (Molecular Probes); labeling efficiency and biological integrity of the proteins during labeling were monitored by spectral (high-performance liquid chromatography, HP LC, diode array detection) and Biacore binding analysis as described previously (18, 22). Labeled proteins were recovered from the reaction mixture on a Phast Desalting column (Pharmacia, Piscataway, NJ) and stored in aliquots at $-80^\circ$C.
A view. In images were acquired over 10 minutes and data recorded on a standard containing 10% of the injected dose was placed within the field of camera (Twinsbury, OH) equipped with a medium energy collimator. A protein.

Results

Parallel binding of monomeric ephrin-A5 Fc and IIIA4 to EphA3 triggers strong, sustained signaling without need for agonist preclustering. The structures of Eph/ephrin complexes (10, 19) suggest that the assembly of Eph signaling clusters involves the coordinated tethering of several distinct Eph contact surfaces by ephrin oligomers (27). We noted previously that the anti-EphA3 mAb IIIA4 also recognizes part of the Eph/ephrin tetramerization interface, whereby ephrin-A5 Fc and IIIA4 can bind to the same EphA3 molecule with minimal effect on their individual interaction kinetics (18).

These findings prompted us to examine if IIIA4 could act as agonist to initiate EphA3 signaling and if concurrent binding of IIIA4 and ephrin-A5 Fc would trigger assembly of EphA3 signaling complexes. We first compared the capacities of a 1:1 mixture of the monomeric proteins or of clustered ephrin-A5 Fc or IIIA4 to activate signaling in EphA3/HEK 293 cells. SE-HPLC of the proteins for use in these experiments allowed us to confirm the oligomerization of the clustered proteins, and the absence of protein aggregates from nonclustered ephrin-A5 and IIIA4 (Supplementary Fig. S1). Western blot analysis revealed that clustered IIIA4 triggers stronger EphA3 phosphorylation (Fig. 1A–B, peak D) and CrkII recruitment (Supplementary Fig. S1, C-D) than ephrin-A5 Fc, whereas neither nonclustered protein induced EphA3 activation above background levels. Interestingly, an equimolar mixture of nonclustered ephrin-A5 Fc and IIIA4 together stimulated EphA3 phosphorylation to an even stronger degree than clustered IIIA4 on its own (Fig. 1A-B, peak M). Furthermore, whereas IIIA4-induced, similar to ephrin-A5-induced phosphorylation reached a plateau after 5 to 10 minutes (Fig. 1C), we observed a continuously increasing EphA3 phosphorylation in IIIA4/ephrin-A5 stimulated samples over 30 minutes.

Figure 1. Parallel binding of ephrin-A5 Fc and IIIA4 triggers synergistic EphA3 activation without requiring agonist preclustering. A. Superose-12 SE-HPLC profile of IIIA4 and ephrinA5-Fc complexes used as EphA3 agonists. Fractionation of preclustered ephrin-A5-Fc (ephrinA5-Fc-X) yields fractions of oligomeric (O), dimeric (D), and uncomplexed ephrinA5-Fc (X), the latter containing residual cross-linking antibody; retention times of protein standards (gray vertical lines) are overlaid onto the chromatogram. An analogous profile is obtained for anti-mouse-Fc preclustered IIIA4 (Supplementary Fig. 1D). Inset, monomeric IIIA4 and ephrin-A5 Fc (M) were obtained from samples lacking anti-Fc antibodies; the gray shaded area in the chromatogram illustrates nonclustered IIIA4 (M) used for experiments. B, preclustered IIIA4 (1.5 µg/mL), or ephrinA5-Fc (1.5 µg/mL) or a combination (comb) of the nonclustered proteins (0.75 µg/mL each) were purified by SE-HPLC and protein fractions (denoted as in A) used for stimulation (10 minutes) of HEK293/EphA3 cells. Anti-phosphotyrosine (PY) immunoprecipitates from cell lysates were blotted with anti-EphA3 antibodies, a sample of nonstimulated HEK293/EphA3 cells (Nil) was analyzed in parallel. C, time course of EphA3 phosphorylation, triggered by treatment with combined IIIA4/ephrin-A5 Fc. Anti-phosphotyrosine (PY) immunoprecipitates of cell lysates, after HEK293/EphA3 activation for the indicated times with monomeric IIIA4, preclustered ephrinA5-Fc, or monomeric IIIA4, and ephrinA5-Fc in unison before probing with anti-EphA3 antibodies; combined monomeric agonists induce escalating EphA3 activation. Cell lysates (lysat) analysed in parallel in anti-EphA3 Western blot confirmed even gel loading. D, actin cytoskeletal changes following stimulation with clustered or nonclustered IIIA4 or ephrin-A5-Fc or with their combination. HEK293/EphA3 cells, incubated (10 minutes) with reagent controls (PBS or anti-mouse Fc antibody, z-mFCC), clustered ephrinA5-Fc (c ephn), nonclustered (nc IIIA4) or clustered IIIA4 (c IIIA4), and combined monomeric IIIA4 and ephrinA5-Fc (nc IIIA4 + ephn) were fixed and stained with AlexaFluor phalloidin to highlight the cytoskeleton for confocal microscopy.
We next examined the molecular surfaces of EphA3 that may be differentially affected in ephrin-binding domain mutants. In agreement with the observed EphA3 activation, exposure of EphA3/HEK293 cells to nonclustered ephrin-A5 Fc and IIIA4 resulted in dramatic cell rounding and membrane blebbing, exceeding the responses observed with the clustered ephrin-A5 Fc or IIIA4. As expected, nonclustered IIIA4 on its own (Fig. 1D) did not affect the actin cytoskeleton or cell morphology.

IIIA4-binding and IIIA4/ephrin-triggered EphA3 activation is differentially affected in ephrin-binding domain mutants.

We assessed by confocal microscopy binding of Alexa487-labeled IIIA4 to cell surface expressed mutant or wt EphA3, transiently expressed in HEK 293 cells as fusion protein with a COOH-terminal EGFP (Fig. 2A). The Alexa487 antibody bound strongly to the wt receptor, as well as to the 3×YF EphA3-EGFP control mutant lacking three functionally relevant EphA3 tyrosine residues (18, 24), as evidenced by merged images of EphA3 (green) and IIIA4 fluorescence (blue), indicating a convincing overlap of the fluorescent proteins (cyan) at the cell membrane. EphA3 [Phe152-Leu], despite low expression in these experiments, also bound Alexa487-III A4 considerably, and the merged image reveals an almost complete overlap of cell surface EGFP and Alexa487 staining at the cell membrane. By contrast, a mutation in the low-affinity binding site, EphA3 [Val133-Glu], on its own, or in combination with the Asn232-Ile substitution, affected IIIA4 binding notably and the merged images of these cells, despite robust expression of the mutant EphA3, reveal little of IIIA4-associated fluorescence on the cell membrane and thus little overlap in the merged images.

We assessed the functional relevance of impaired IIIA4 binding by studying phosphorylation levels of IIIA4-stimulated EphA3 by immunoprecipitation analysis. Comparison of anti-phosphotyrosine levels in these samples confirms that site-directed substitution Phe152-Leu within the high-affinity ephrin-A5 binding site does not affect IIIA4 induced activation of EphA3. By contrast, tyrosine phosphorylation of the Val133-Glu low affinity binding site mutant, and of EphA3 bearing a Val133-Glu/Asn232-Ile double mutation were reduced to background levels, confirming this EphA3 interface as the principle IIIA4 binding region. In agreement with the synergistic activity of ephrin-A5 and IIIA4, the phosphorylation level in wt EphA3-transfected cells was notably increased after stimulation with an equimolar mix of both agonists. Interestingly, the relative EphA3 phosphorylation level was modulated differently in the EphA3 mutants, likely reflecting the combined contributions of ephrin-A5 Fc and IIIA4, which are differently affected by these mutations (18), to the formation of these signaling complexes.

Figure 2. Compromised IIIA4 binding and IIIA4/ephrin-triggered phosphorylation in ephrin-binding domain EphA3 mutants suggests proximity of interaction sites. A, confocal microscope images of HEK293 cells transiently transfected with chimeric proteins of NH2-terminal EGFP and wt EphA3, or mutant EphA3 as indicated: 3×YF, V133E, F152L, N232I, V133F/N232I. Cells were exposed (10 minutes) to preclustered, Alexa487-labeled IIIA4, and nonbound IIIA4 removed before fixation of cells and microscopy. Images of GFP fluorescence (green), Alexa487 fluorescence (blue), and merged images (cyan) of GFP and Alexa487 fluorescence. Fluorescence signals inside the cells represent cytosolic pools of GFP-EphA3 (green). B, activation of kinase activity in mutant EphA3 receptors. Protein-G immunoprecipitates from lysates of HEK293 cells transiently transfected with a vector control, wt or mutant EphA3 (as indicated), were stimulated with preclustered IIIA4 or with combined, nonclustered ephrin-A5 Fc and IIIA4 and analyzed with anti-PY Western blots (WB). Analysis of cells expressing EphA3 mutants at the principle autophosphorylation sites (3×YF) was used as control. To assess even loading and equivalent transfection levels, the Western blot membrane was reprobed with a polyclonal anti-EphA3 antibody. Overlays of α-PY and anti-EphA3 Western blots indicate that only the prominent upper band in the anti-EphA3 blot is phosphorylated. Bar, 20 μm.
The coordinated interaction of IIIA4 and ephrin-A5 Fc triggers accelerated EphA3 activation and cell-morphologic responses. Our finding, that combined non clustered IIIA4 and ephrin-A5 trigger EphA3 signaling to a scale exceeding that of the clustered individual agonists, prompted us to examine the assembly and dynamics of IIIA4/ephrin-elicited EphA3 clusters by confocal microscopy (Fig. 3A). In agreement with earlier time-lapse experiments (22), binding of clustered Alexa546 ephrin-A5 Fc or clustered Alexa647 IIIA4 to EphA3-transfected A02 melanoma cells or HEK293 cells (data not shown) is clearly visible 1 minute after addition. Already at this early time point clustering of the fluorescent agonists, in particular of IIIA4, is apparent. As expected from previous studies (24), the cells respond within 10 minutes, with pronounced cytoskeletal contraction, cell rounding and membrane blebbing, which is notably stronger in IIIA4 rather than in ephrin-A5 Fc-stimulated cells. Nonclustered ephrin-A5 Fc or IIIA4 on their own do not induce a cellular response, but interestingly, after extended exposure assemble into small punctuate aggregates on the cell membrane (Fig. 3A, arrows). We assessed if this aggregation, or "patching," might be an intermediate step during the formation of active signaling clusters, and treated EphA3/HEK293 cells with increasing times with nonclustered ephrin-A5 Fc or IIIA4, before addition of the complementary agonist (IIIA4 or ephrin-A5, respectively) for 1 minute only. However, increasing the duration of this cluster "preassembly" on the cell membrane did not increase EphA3 phosphorylation above the level observed if both reagents were added together (Fig. 3C, 0-minute clustering time). This suggests an instantaneous formation of active signaling complexes once both agonists are available. In agreement, a dramatic contraction of the cytoskeleton, cell rounding and extensive membrane blebbing was apparent within 1 minute when nonclustered ephrin-A5 and IIIA4 were added together (Fig. 3A, nc IIIA4+ephn). The merged image suggest an almost complete overlap of red (ephrin-A5), blue (IIIA4) and green (rhodamine, polymerized actin) fluorescence, indicative of ephrin-A5 and IIIA4 coassembly into common clusters. Likewise, Western Blot analysis of parallel samples reveal coinciding, rapid and sustained EphA3 phosphorylation in EphA3/HEK293 cells or in EphA3-transfected A02 cells (Fig. 3B), confirming that cooperative

Figure 3. Synergistic binding of IIIA4 and ephrin-A5 to EphA3-expressing cells triggers accelerated clustering and cytoskeletal contraction. A, confocal microscopic images of EphA3-expressing A02 (A02/EphA3) melanoma cells, left untreated (nil), or stimulated for indicated times with clustered (c), Alexa546-labeled ephrin-A5-Fc (c ephn), clustered Alexa647-labeled IIIA4 (c IIIA4), or SE-HPLC-fractionated, nonclustered (nc) ephrin-A5-Fc (nc ephn), or IIIA4 (nc IIIA4), or their combination (nc IIIA4+ephn). Washed and paraformaldehyde-fixed A02/EphA3 cells were stained with Alexa488-phalloidin to accentuate the actin cytoskeleton (green fluorescence). Individual channels recording Alexa546 (ephrin, red) and Alexa647 fluorescence (IIIA4, blue) as well as merged images, indicating colocalization of actin with Alexa546 ephrin-A5 Fc (yellow), Alexa647 IIIA4 with actin (cyan) and colocalization of all three fluorescent labels (white). Nonstimulated cells (nil) reveal the cell morphology in the absence of EphA3 activation. Arrows indicate latent and assembled clusters. Bar, 20 μm. B, comparison of EphA3 activation in (preclustered) ephrin-A5-Fc-stimulated HEK293/EphA3 or A02/EphA3 cells. EphA3 phosphorylation levels in anti-EphA3 immunoprecipitates from A02/EphA3 or HEK293/EphA3 cell lines were monitored by anti-PY Western blots (WB). Parallel anti-EphA3 Western blots confirm equivalent loadings. C, sequential EphA3 activation with ephrin-A5 Fc or IIIA4 does not affect the net phosphorylation level. HEK293/EphA3 cells were incubated with nonaggregated EphA3 agonist (clustering molecule, IIIA4, top; or ephrin-A5-Fc, bottom) for specified times (minutes) to induce formation of latent EphA3 clusters. Receptor activation was initiated by the addition of the complementary agonist for 1 minute and analyzed on anti-Eph-A3 Western blot of anti-PY immunoprecipitates (IP).
transmembrane EphA3, we compared confocal microscopic sections (at 50% cell height) of EphA3/HEK293 cells that had been exposed to the agonists either alone or in combination (Fig. 4 A and B). We showed previously, that assembly of cell membrane ephrin-A5/EphA3 clusters precedes internalization (22). Aggregation of ephrin-A5 or of IIIA4 proceeds slowly if either of the two nonclustered proteins is applied on its own (Fig. 3 A). In agreement, cytosolic ephrin-A5 Fc-associated fluorescence becomes noticeable only after 40 minutes, or in the case of IIIA4, only after 60 minutes (Fig. 4 A). By contrast, exposure to combined, nonclustered ephrin-A5 Fc and IIIA4 significantly accelerates the internalization kinetics of both proteins. Already after 10 minutes, clusters of cytosolic ephrin-A5-associated fluorescence are abundant, whereas cytosolic IIIA4-associated fluorescent vesicles are clearly obvious after 20 minutes, suggesting a notable difference in the internalization kinetics of these two agonistic proteins (Fig. 4 B). However, after 40 minutes, the majority of the ephrin-A5 and IIIA4-associated fluorescence is cytosolic and, as suggested by the merged image (yellow pseudocolor), a large proportion seems to colocalize within the same cytosolic compartment.

Overall, our experiments show that exposure of EphA3-positive cells to IIIA4 mAb and ephrin-A5 Fc in combination dramatically accelerates and enhances EphA3-specific cellular responses and trafficking of both agonistic proteins into common cytosolic compartments. It seems that this unique property of IIIA4, not shared by two other mAbs derived from the same immunization protocol (Supplementary Fig. S2), is due to its ability to bind to EphA3/ephrin-A5 complexes in a manner that promotes the assembly of higher-order signaling clusters (Fig. 4 C). In this regard, IIIA4 binding replaces anti-Fc-mediated ephrin clustering that is normally required for the assembly of oligomeric complexes from Eph/ephrin heterotetramers.

Together, our findings lead us to explore the concept that rapid EphA3-mediated agonist uptake (22) could be exploited to develop effective targeting reagents for EphA3-positive human tumors.

Biodistribution of [111]In-ephrin-A5 and IIIA4 and γ-camera imaging in solid tumor-bearing xenografts. We analyzed the potential tumor targeting properties of ephrin-A5 Fc and IIIA4 in three different xenograft models, including SK-MEL28 cells, a malignant melanoma line that develops solid xenograft tumors in nude BALB/c mice (26). EphA3-overexpressing HEK293 cells have been extensively characterized for their EphA3 signaling and biological responses (22, 24), whereas LK63 acute pre-B leukemia cells were used as original source for the isolation of EphA3 (11). For the targeting studies we prepared radio conjugates of IIIA4 and ephrin-A5 using the bifunctional metal ion chelator, CHX-A00-diethylenetriaminepentaacetic acid, as stable chelation of radioisotopes proved to yield higher retention of biological activities compared with conventional methods, yielding proteins with 50% to 75% binding capacity to EphA3-positive cells. In agreement with...
the binding properties of nonderivatized proteins (18), the Scatchard and Lindmo analysis revealed subnanomolar affinities of iodine or indium-labeled ephrin-A5 Fc (K_D = 7.3-7.5 × 10^{-10}), and IIIA4 (K_D = 4.2-5.5 × 10^{-10}), and an estimated 20,000 to 40,000 IIIA4-binding sites on SK-MEL 28 cells, in agreement with strong IIIA4 staining of SK-MEL 28 tumor or control sections from xenograft-bearing mice. We initially assessed biodistribution and clearance of IIIA4 and ephrin-A5 Fc in SK-Mel28 xenograft-bearing BALB/c Nude mice; however, due to a rapidly decreasing blood concentration of 125I- or 111In-labeled ephrin-A5 Fc, the retention in SK-Mel28 tumor was difficult to assess (Fig. 5A).

By contrast, 111In-IIIA4 has a longer, ~48-hour blood half-life and a significant uptake and retention in EphA3/HEK293 xenografts, that was lasting for 8 to 10 days (Fig. 5B). A survey of the biodistribution of 111In-IIIA4 revealed rapid accumulation within 4 days of tumor-associated radioactivity to 27% of injected dose per gram (ID/g), whereas the 111In concentration in blood and all other tissues dropped below 10% ID/g within 24 hours (Fig. 5C). Likewise, the 125I concentration in all tissues, but also in the xenografted tumor decreased to less than 5% ID/g within 72 hours (Fig. 5C). This observation is in line with the notion of IIIA4 internalization into tumor cells and its degradation to release 125I radio-halogen, whereas the radiometal 111In is retained within the cell for much longer, as has been shown in previous studies (see ref. 26 and references within).

To visualize the deposition of 111In-labeled IIIA4 in the grafted EphA3/HEK293 tumors, we did whole body gamma camera imaging of a tumor-bearing mouse at various times after receiving the injection of the radio-metal antibody conjugate. As shown in Fig. 5D, the blood pool in the heart was clearly visible at all time points, whereas accumulation of 111In-IIIA4 within the tumor on the left dorsal flank was barely visible at 4 hours after injection. However, concentration of 111In-IIIA4 was clearly apparent at 24, 48, and 96 hours after injection, whereas the whole-animal images confirm the absence of 111In-labeled IIIA4 deposition in other organs, such as liver or spleen.

111In-ephrin-A5 Fc and IIIA4 biodistribution in leukemia-xenografted BALB/c nude mice. We were interested to assess if 125I- or 111In-labeled ephrin-A5 Fc, despite its rapid clearance from the circulation, could be used to target haematopoietic tumors. BALB/c nude mice, following i.v. injection of 2 × 10^7 LK63 leukemia cells, were injected with radiolabeled ephrin-A5 Fc or IIIA4, and relative levels of either protein in the blood and the various organs determined (Fig. 6A). We observed a rapid accumulation of ephrin-A5 in spleen, lung, and liver, reaching after 4 hours relative concentrations of 24%, 33%, and 35% ID/g, respectively. To assess if this was reflecting ephrin-A5 Fc binding to an Eph receptor or was due to anti-human Fc immunoreactivity, we carried out the targeting experiment in mice that had received saturating amounts of hu3S193, a humanized antibody against the nonrelevant Lewis Y [Le(y)] carbohydrate antigen (28) to block anti-human Fc binding sites. An unchanged biodistribution profile of 111In-ephrin-A5 Fc in these mice (Fig. 6B) indicated that Fc receptor-mediated uptake was not contributing notably to the distribution of ephrin-A5 Fc in these mice. Comparison of the relative 125I and 111In levels 4 hours after injection of ephrin-A5 Fc, or 24 hours after injection of IIIA4, revealed in all organs that had preferentially accumulated the targeting proteins, a notably lower 125I concentration (Fig. 6A, white

Figure 5. Biodistribution of 111In and 125I-labeled IIIA4 mAb in EphA3/HEK293 xenograft-bearing BALB/c nude mice. A, mice bearing SK-Mel28 or (B) HEK293 tumor xenografts were injected with a mixture of 111In (green, black graphs) and 125I (red, blue graphs)-conjugated ephrin-A5 Fc (A) or IIIA4 (B), and at indicated times after injection five animals per group were sacrificed and radioactivity in the blood (red, black) and tumor (green, blue) determined. The 111In (green, black) and 125I (red, blue) radioactivity is expressed as mean fraction of the injected dose/gram of tissue or blood (% ID/g). C, the concentration of 111In and 125I-labeled IIIA4 (as indicated) in individual tissues of tumor-bearing mice was assessed at indicated times as described above. D, whole body gamma camera images taken from EphA3/HEK293 xenograft-bearing BALB/c nude mice (dorsal left flank) at indicated times following injections of 111In-IIIA4. A standard containing 10% of the injected dose was placed into each field of view (white arrowheads). The yellow arrows indicate the approximate position of the tumor xenograft.
columns) suggesting similar to the solid tumor model, an active uptake and degradation into LK63 cells colonizing spleen, lung, and liver. The $^{111}$In-IIIA4 localized to the same organs, whereby the relative IIIA4 concentration in the spleen amounted here to some 70% ID/g 4 hours after injection. Indeed, detection of the infiltrating human cells by immunohistochemistry (data not shown) or flow cytometry using anti-human CD45 and Alexa488-labeled IIIA4 for detection revealed abundant population with CD45 and EphA3-positive tumor cells of spleen (Fig. 6D), liver and bone marrow (data not shown). In agreement with the biodistribution data, immunohistochemical analysis of EphA3 expression in sections of these mice confirmed IIIA4-specific immunoreactivity in the spleen above that observed in the liver (Fig. 6C). We noted however an $^{111}$In-IIIA4 concentration in liver and lung that was substantially lower than that of $^{111}$In-ephrin-A5, possibly suggesting that an endogenous Eph receptor other than EphA3 is targeted by ephrin-A5 Fc. Whereas there is no known expression of EphA3 in either organ, quantitative reverse transcription-PCR indicated substantial expression of EphA1 and EphA2 in normal liver samples, interestingly coincident with elevated levels of the cognate ephrin-A1 (Supplementary Fig. S3A). In agreement, extraction of endogenous Ephs from normal mouse liver with Protein-A Sepharose-bound ephrin-A5 confirmed the presence of EphA2 but not of EphA3 as candidate receptor binding ephrin-A5 Fc (Supplementary Fig. 3B), explaining the localization of ephrin-A5 Fc to these organs.

In summary, these tumor targeting studies show that in tumor xenograft-bearing mice radiometal conjugates of ephrin-A5 and IIIA4 effectively localize to, and are internalized, into EphA3-positive human tumor cells. We also observe that ephrin-A5 Fc and IIIA4 act strongly synergistic on EphA3-positive tumor cells, together providing the basis for ongoing studies to develop effective tumor targeting strategies that combine the specificity and avidity of these two EphA3 agonists.

**Discussion**

**IIIA4 monoclonal antibody is an ephrin-A5 mimetic.** One of the typical features of Eph signaling is the formation of oligomeric
Eph/ephrin signaling clusters that are necessary for biological responses (21–23), and in vivo may involve preformed aggregates of cell surface associated ephrins, achieved experimentally by cross-linking of soluble ephrins into clusters containing four Eph binding modules (Fig. 4C). In the present study, we have shown that IIIA4, a mAb which had been raised for the isolation of EphA3 (11) and which is specific for the ephrin-binding domain of EphA3 (20), mimics this ephrin-A5 property and in dimerized form but not as monomeric nonclustered antibody triggers robust EphA3 phosphorylation, CrkII recruitment, and cell rounding.

Whereas agonistic antibodies against the related EphA2 receptor have been reported, the authors of these studies suggested that the mAb epitope is distinct from the ephrin-A1 binding site, present only in EphA2-positive cancer cells, and only in these facilitates EphA2 phosphorylation, degradation, and inhibition of tumor growth in vitro and in vivo (15, 29). By contrast, a recently described anti-EphB2 mAb, which in agreement with lacking mitogenic activity of Ephs does not affect tumor growth, effectively blocks ephrin-B2 binding and EphB2 phosphorylation in colon carcinoma cells in vitro, thus suggesting overlapping ephrin and mAb binding sites (12). In the case of IIIA4, earlier structure-function studies revealed close proximity of one of the three ephrin-A5-binding sites and the IIIA4 interaction surface (Fig. 4C): specifically, amino acid substitutions within the low-affinity Eph/ephrin interface (19) affected ephrin-A5 as well as IIIA4 binding (18). It is established from detailed structure/function studies (10, 18, 19) that high-affinity Eph/ephrin complexes are held together by three distinct interfaces forming a ring-like heterotetrameric structure (Fig. 4C) and likely serve as nucleating unit for the formation of oligomeric signaling clusters (22). It is of note that the low-affinity Eph/ephrin interface (Fig. 4C) is not apparent in the crystal structure of the EphB2/ephrin-A5 complex, which, compared with the EphB2/ephrin-B2 or EphA3/ephrin-A5 complexes, is of lower-affinity and has reduced signaling capacity (10). We argue that this contact might function to stabilize the complex for effective signaling, whereas ephrin-binding is maintained in particular by the high-affinity ephrin-A5/EphA3 interface (18). This rationale would provide an explanation, why in contrast to competitive blocking by antagonistic antibodies (12, 30), high-affinity IIIA4 binding to this EphA3 surface has little effect on the overall affinity of ephrin-A5 interaction but provides a molecular tether for the formation of active signaling clusters (Fig. 4C).

**Concurrent binding of nonclustered IIIA4 and ephrin-A5 is more efficient than aggregation.** Our demonstration of significantly elevated and accelerated EphA3 activation and internalization by two distinct, nonclustered agonists which share a common binding site on EphA3, superficially seem counter-intuitive to the known, well-established Eph activation mechanism. The individual, monomeric IIIA4 and ephrin-A5-Fc proteins seem to facilitate protracted assembly of small "microclusters" of transmembrane EphA3, that remind of "capped" immune receptors (31) but have no apparent cell morphologic effect and clearly do not trigger notable EphA3 activation. It is of note that Eph activation without the need for cross-linking of either ephrin (4) or agonistic antibody (15) has been reported. However, our experiments clearly show that in the case of IIIA4, this activity is due to small amounts of antibody aggregates that are frequently present in the antibody preparation. Indeed, we observed the presence of higher molecular weight aggregates also in some of our ephrin-A5 Fc preparations. To avoid potential ambiguities arising from these aggregates, we routinely used SE-HPLC-purified protein in all our experiments.

That addition of IIIA4 to cells, already binding nonclustered ephrin (or vice versa), results in almost instantaneous EphA3 activation and cell rounding suggests that the additional tether immediately converts the latent microclusters into functional signaling complexes. It is plausible, that due to the tight binding of ephrin-A5 to the high-affinity interface but much weaker binding to the low-affinity interface (to which IIIA4 binds with very high affinity), will lead to the formation of complexes where each EphA3 is tethered by an ephrin-A5 and a IIIA4 (Fig. 4C). This allows for the assembly and propagation of clusters that is limited only by the concentration and diffusion rate of components on the cell membrane and in solution. Interestingly, whereas IIIA4 was isolated together with two other mAbs with very similar binding epitopes, but differing considerably in their affinities, only IIIA4 mAb in combination with ephrin-A5-Fc can induce receptor activation effectively, suggesting that in addition to the binding site, also the high affinity dominated by a very low dissociation rate may be essential for its agonistic properties.

**Ephrin-A5 and IIIA4 as tumor targeting reagents.** The pronounced synergistic mode of ephrin-A5 and IIIA4 interacting with cell surface EphA3, leading to almost quantitative internalization of resulting signaling complexes, suggests their potential as tumor-targeting reagent for EphA3-positive cancer cells. Whereas ephrin-A5 on its own, despite its preferential interaction with EphA3, will bind most EphA receptors, as well as EphB2 (10, 32), IIIA4 binds exclusively to EphA3 (data not shown) and in combination with ephrin-A5 does not activate EphB2 (Supplementary Data). In contrast to the reports on direct antitumor effects of anti-EphA2 mAbs (15, 29), we have not observed an effect of EphA3 activation, either with ephrin-A5, IIIA4, or their combination, on cell viability in vitro or in vivo. However, the efficient receptor mediated uptake of both agonists, and the specificity, in particular of the combined proteins, for EphA3-expressing cells advocates their use as tumor-targeting reagents suited to specifically deliver a conjugated cytotoxic cargo to EphA3-positive tumor cells. Thus, radiometal conjugates of ephrin-A5 and IIIA4 retain their binding specificities, and in mice bearing solid hematopoietic tumor xenografts are effectively taken up into EphA3-positive cancer cells. In its current form, the rapid clearance of ephrin-A5 Fc from the circulation hindered assessment of its properties as targeting reagent in solid tumors, whereas the longer-lasting retention of IIIA4-associated radioactivity in EphA3-positive, kidney endothelial tumor xenografts for 14 days form the basis for development of a specific tumor targeting strategy. Despite its short blood half-life, ephrin-A5, similar to IIIA4, effectively targets hematopoietic tumor xenografts. As expected, its targeting properties are determined by the expression pattern of endogenous binding partners; in contrast to marginal EphA3 expression we show here significant endogenous EphA2 mRNA and protein in normal mouse liver, emphasizing the conceptual difficulties of using ephrin-Fc fusion proteins as specific Eph agonists in targeting studies. Experiments, exploiting the possibility to combine ephrin-A5 and IIIA4 to increase the targeting of the combined therapeutic reagent, are ongoing.

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EphA3-Specific Tumor-Targeting Reagents
References
Concurrent Binding of Anti-EphA3 Antibody and Ephrin-A5 Amplifies EphA3 Signaling and Downstream Responses: Potential as EphA3-Specific Tumor-Targeting Reagents

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