A Five-Amino-Acid Peptide Blocks Met- and Ron-Dependent Cell Migration

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Abstract

Various human cancers express elevated levels of the receptor tyrosine kinases Met or Ron and v6-containing isoforms of CD44. The activation of Met and Ron requires the presence of such CD44 v6-containing isoforms that act as coreceptors. Three amino acids within the v6 sequence were identified by mutational analysis to be essential for the coreceptor function: EWQ in the rat sequence and RWH in human. Peptides comprising these three amino acids (the smallest containing only five amino acids) efficiently act as competitors and block ligand-dependent activation of Met or Ron and subsequent cell migration. (Cancer Res 2005; 65(14): 6105-10)

Introduction

The receptor tyrosine kinase Met controls migration, differentiation, and proliferation in development, in normal adult, and in pathologic processes (reviewed in ref. 1). In the adult, Met is expressed in numerous epithelial tissues as well as in lymphoid cells, whereas its ligand hepatocyte growth factor (HGF) is predominantly secreted by stromal cells (reviewed in ref. 1). Met is the prototypic member of a small family of related receptor tyrosine kinases with similar domain structure but different ligands: Sea in chicken (2) and Ron in humans (3). Like Met and HGF, Ron and its ligand macrophage-stimulating protein (MSP) are involved in the regulation of proliferation, migration, and differentiation (4, 5). Both Met and Ron are overexpressed in several cancer cell lines and in tumors (6).

We and others have shown that the activation of Met profits from the presence of a neighboring coreceptor (7–9). In several cancer cell lines as well as primary cells, we have shown that Met activation depended on the presence of a specific CD44 isoform carrying the sequence of exon v6 in its extracellular domain (8). This is interesting in that v6-containing CD44 variants are not only overexpressed in many types of human tumors but also can confer metastatic and invasive properties to tumor cell lines (10–13). The coreceptor induces the formation of a ternary complex consisting of Met, CD44 v6, and HGF, which is required for Met autophosphorylation (8). Recently, we have obtained in mice *in vivo* evidence of a role of CD44 in the Met pathway as the inactivation of CD44 creates Met haploinsufficiency.[3]

We present here experiments that address the mechanism of the coreceptor function of CD44 v6. Such a study requires cell culture experiments. We report here on the minimal sequence in CD44 v6 required for Met activation: a section of three consecutive amino acids in v6 suffices for the HGF-dependent activation, whereas all other portions of v6 can be mutated without loss of this function. Excess of a peptide comprising these three amino acids added to the culture medium prevents Met activation and the Met induced migration phenotype. Ron also requires CD44 v6 for ligand-dependent activation. Sequence comparisons of the rat and human receptors provide insights into the mechanism of coreceptor action.

Materials and Methods

Cells and cell culture. The human colon adenocarcinoma cell lines HT29, a gift of A. Zweibaum (Institut National de la Sante et de la Recherche Medicale, France) and the human kidney cell line 293 (American Tissue Culture Collection, ATCC, Wesel, Germany) were grown in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (PAA, Cölbe, Germany). The human colon carcinoma cell line HCT116 (ATCC) was grown in McCoy's medium (PromoCell, Heidelberg, Germany) plus 10% FCS. The rat pancreatic carcinoma cell line BSp73AS10 (also designated AS; ref. 14) and its transfectants were grown in RPMI (PAA) plus 10% FCS. Generation and properties of AS cell clones that had been transfected with various CD44 expression constructs have been described (8, 10).

Transfection. Transfection of AS cells was done using Tfx-50 (Promega, Mannheim, Germany) according to the manufacturer's instructions.

Mass cultures expressing different linker scan mutants were obtained by cotransfection of the respective linker scan constructs with the plasmid pBabe puro (15) and selection for puromycin resistance (1 μg/mL; Sigma-Aldrich, Munich, Germany).

Plasmids and mutagenesis. All mutants were produced starting from the pGKs6 plasmid encoding the CD44 constant region plus the exon v6 (16) controlled by the phosphoglycerate kinase promoter. The linker scan mutants ls1-ls14 were obtained by replacing stepwise 9 bp in frame in the 127 bp of the v6 exon by a 9-bp *Not*I linker (GCGGCCGCT) coding for alanines. In cases where one of the triplets to be exchanged already encoded an alanine, the sequence was changed to encode a glycine. The mutagenesis was PCR-based using the Quick change site-directed mutagenesis kit (Stratagene, La Jolla, CA). The sequences of the primers can be obtained upon request.

Immunoprecipitation, Western blotting, peptide competition, and RNA interference inhibition. HGF induction and determination of Erk or Met phosphorylation have been published (8). Induction by platelet-derived growth factor (PDGF) or MSP was done similarly to that by HGF. Ron phosphorylation was determined similarly to Met phosphorylation immunoprecipitating with Ron-specific antibody C-20 (Santa Cruz, Heidelberg, Germany). For peptide competition, the cells were treated with the different peptides (50 ng/mL) for 5 minutes at 37°C before HGF induction.

To reduce gene expression of CD44 v6, cells were transfected with siRNA against CD44 v6 (AAGCAGCTACCCAGAAGGAGA; synthesized by MWG, Ebersberg, Germany) or with a control siRNA (Qiagen, Hilden, Germany) using oligofectamine (Invitrogen) according to the manufacturer's protocol. After 24 hours, the cells were starved and after additional 24 hours, the cells were treated with HGF.

Fluorescence-activated cell sorting analysis. Binding of hyaluronan to cells was measured by fluorescence-activated cell sorting analysis. Cells

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were harvested with PBS/EDTA and resuspended in 100 μL PBS, 3% FCS. Cells (1×10^6) were incubated with FITC-labeled hyaluronan (10 μg/mL; rooster comb, Sigma, Taufkirchen, Germany; labeled according to ref. 17) for 30 minutes on ice, washed twice, and resuspended in PBS. The fluorescence was analyzed by a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany).

Antibodies and other reagents. The rat CD44 exon 15 specific antibody 5G8 (18) and the rat exon v6-specific antibody 1.1ASML (19) were prepared from hybridoma supernatants. The human pan CD44 antibody J173 was obtained from Immunotech (Marseille, France); the human CD44 exon v6-specific antibody VFF18 was a gift of Bender (Vienna, Austria). The phosphotyrosine-antibody 4G10 was obtained from BIOMOL (Hamburg, Germany); the phospho-Erk antibody from Cell Signaling Technology (Beverly, England); the Erk antibody K-23, the Met antibody sc-161, and the Ron antibody C-20 from Santa Cruz. Recombinant human HGF and MSP (R&D Systems, Wiesbaden, Germany) were activated with 5% FCS overnight. PDGF was obtained from Calbiochem (Darmstadt, Germany). The growth factors were used at a concentration of 10 ng/mL. Rabbit IgG and mouse IgG were obtained from DAKO (Hamburg, Germany). The mitogen-activated protein kinase (MAPK) kinase (MEK) inhibitor UO126 was purchased from Promega and was used at a final concentration of 10 μmol/L.

The rat peptides (A, WADPNSTTEEAATQ; B, KEKWFENEWQGKNP; C, PTPSEDSHYTEGTT) were synthesized by Invitrogen; the human peptides (A, QATPSSTTEETATQ; B, KEQWFGNRWHEGYR; C, QTPREDSHSTTGTA) were a gift from the bio-company G2M Cancer Drugs AG (Frankfurt, Germany). Other rat peptides (3-mer rat, EWQ; 5-mer rat, NEWQG; 5-mer mut rat, NAAAG) and human peptides (5-mer hum, NRWHE; 10-mer hum, WFGNRWHEGY) were synthesized by NMI Technologietransfer (Reutlingen, Germany). The control peptide HNREQANLNSRTEETI was a gift from Jonathan Sleeman (Karlsruhe, Germany).

Scattering and migration assays. The assays for scattering and invasion into Matrigel (BD Bioscience, Heidelberg, Germany) have been described previously (8). For the invasion assay, 12-well plates with 8-nm pores and HT29 cells were used. Treatment with the human peptides (50 ng/mL) or with the MEK inhibitor was done 30 minutes before HGF induction.

Results and Discussion

Minimal sequence of v6 required for coreceptor function. The activation of the Met receptor depends in several cell lines on exon v6-containing CD44 isoforms. This was shown by means of CD44 v6-specific antibodies (8), by v6-specific RNA interference (Fig. 1*A*), or by transfection of CD44 v6-containing isoforms into cells that express Met but not CD44 v6 (8). In these transfection experiments, a CD44 variant containing exclusively the v6 exon sequence sufficed to support Met activation (ref. 8; Fig. 1*A*). The v6 exon comprises 42 amino acids. To identify the minimal v6 sequence required for Met activation, we generated linker scan mutations of the v6 sequence (Fig. 1*B*), replacing three amino acids at a time by alanines or glycine (in place of alanine). We transfected these CD44 mutants into BSp73AS cells that do not express CD44 splice variants. Like wild-type CD44 v6, most mutants acted as coreceptors for Met except for the ls8 mutant (examples shown for HGF-dependent Erk and Met activation; Fig. 1*C*). The sequence identified by ls8 is also part of the epitope recognized by the v6 antibody VFF18 (20) that had been used to inhibit Met activation. Thus, most sections of v6 can be mutated without affecting the coreceptor function. All mutants including ls8 were expressed to similar levels in the transfected cells (Fig. 1*C*, CD44 blot). Despite its lack of coreceptor function, the ls8 mutant still bound hyaluronan similarly to CD44 v6 wild type (Fig. 1*D*) indicating that the overall structure of CD44 v6 was not altered and suggesting that CD44 clustering that promotes

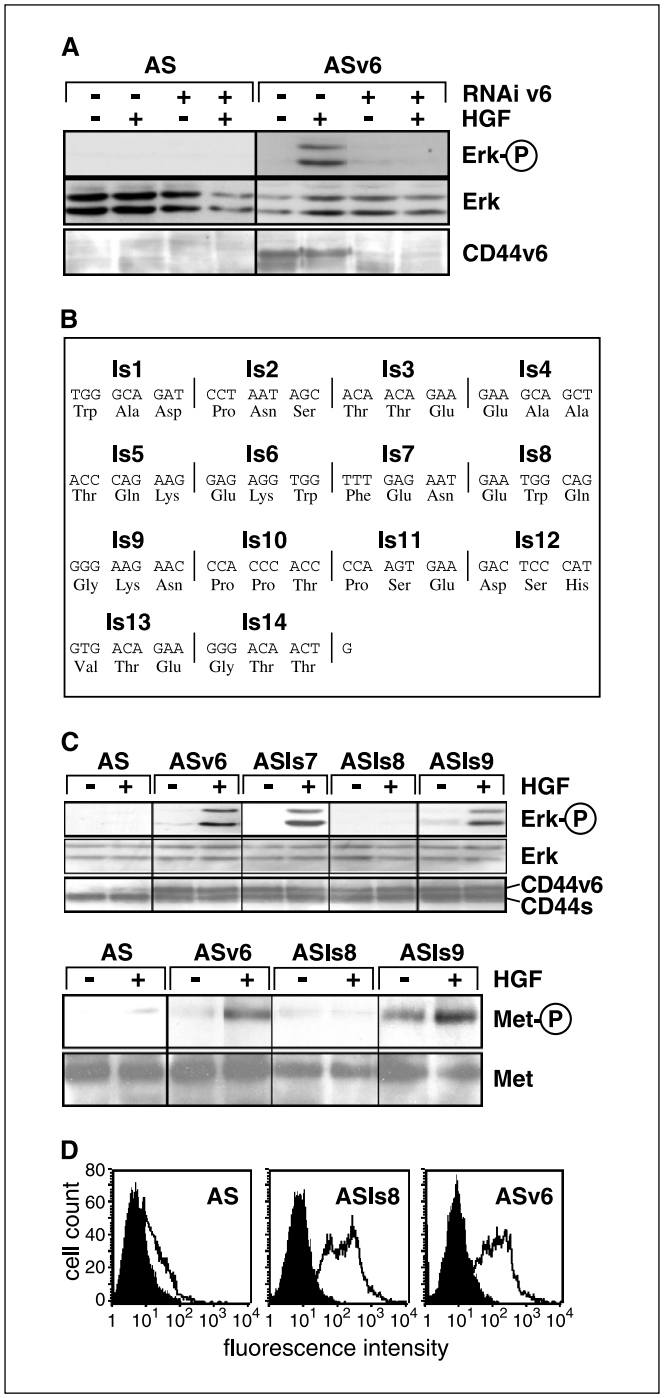

Figure 1. Minimal sequence of CD44 v6 supporting Met activation. *A,* AS and ASv6 cells were treated with RNA interference (*RNAi*) directed against v6 where indicated, starved, and induced by HGF (10 ng/mL for 5 minutes). Lysates were resolved by SDS-PAGE and immunoblotted with Erk, phospho-Erk, or rat CD44 v6-specific antibodies. The lanes indicated by − RNAi v6 refer to treatment with nonsilencing RNA interference (Materials and Methods). *B,* schematic representation of linker scan mutations in the exon v6 sequence. *C,* HGF induced Erk or Met phosphorylation in AS cells transfected with CD44v6 or a selection of linker scan mutants. Cells were maintained as mass cultures, HGF treated as in A and lysates resolved by SDS-PAGE. Erk, phospho-Erk, Met, and phospho-Met were determined as described (8). The Western blotting for CD44 was done with the 5G8 antibody that recognizes both CD44s and CD44v6 (CD44s is the smallest CD44 isoform also expressed in AS cells). *D,* fluorescence-activated cell sorting analysis of AS transfectants binding FITC-labeled hyaluronan (Materials and Methods).

hyaluronan binding (21) does not depend on the sequence mutated in ls8.

Excess of a five-amino-acid v6 peptide blocks coreceptor function. The coreceptor function of CD44 v6 requires the molecule to be membrane bound: expression of the ecto-domain of CD44 v6 but not of CD44s competed efficiently against the membrane-bound form and abolished its coreceptor capacity (Fig. 3 in ref. 8). Although the three-dimensional structure of the complete ecto-domain might be important for the blocking effect, we nevertheless investigated whether we could mimic the inhibitory effect of the ecto-domain by smaller linear peptides. First we designed 14-amino-acid peptides corresponding to three nonoverlapping regions of v6 (A, B and C; for sequences, see Materials and Methods). Exclusively, the addition of peptide B spanning the middle part of exon v6 to a culture of CD44 v6-expressing AS cells (ASv6 and ASv4-7; Fig. 2*A*) blocked HGF-dependent Erk phosphorylation. Peptide B comprises the sequence mutated in linker scan mutant ls8 that abolished Met activation.

We then reduced the size of the peptide to five and three amino acids comprising the three amino acids mutated in ls8. Indeed, excess addition of the five-amino-acid peptide sufficed to block Met activation, whereas the three-amino-acid peptide did not inhibit (Fig. 2*A*). Interestingly, flanking the three amino acids by amino acids other than the ones present in v6 (e.g., alanines) still allowed competition for Met activation (data not shown), a mirror image of the linker scan analysis data where also the amino acids outside the central ones are not decisive. A five-amino-acid peptide carrying the three alanines of ls8 (5-mer mut) did not inhibit. We conclude that a sequence of three amino acids in v6 is instrumental for the coreceptor function and in addition that these three amino acids within a linear peptide suffice to compete for the activation of Met.

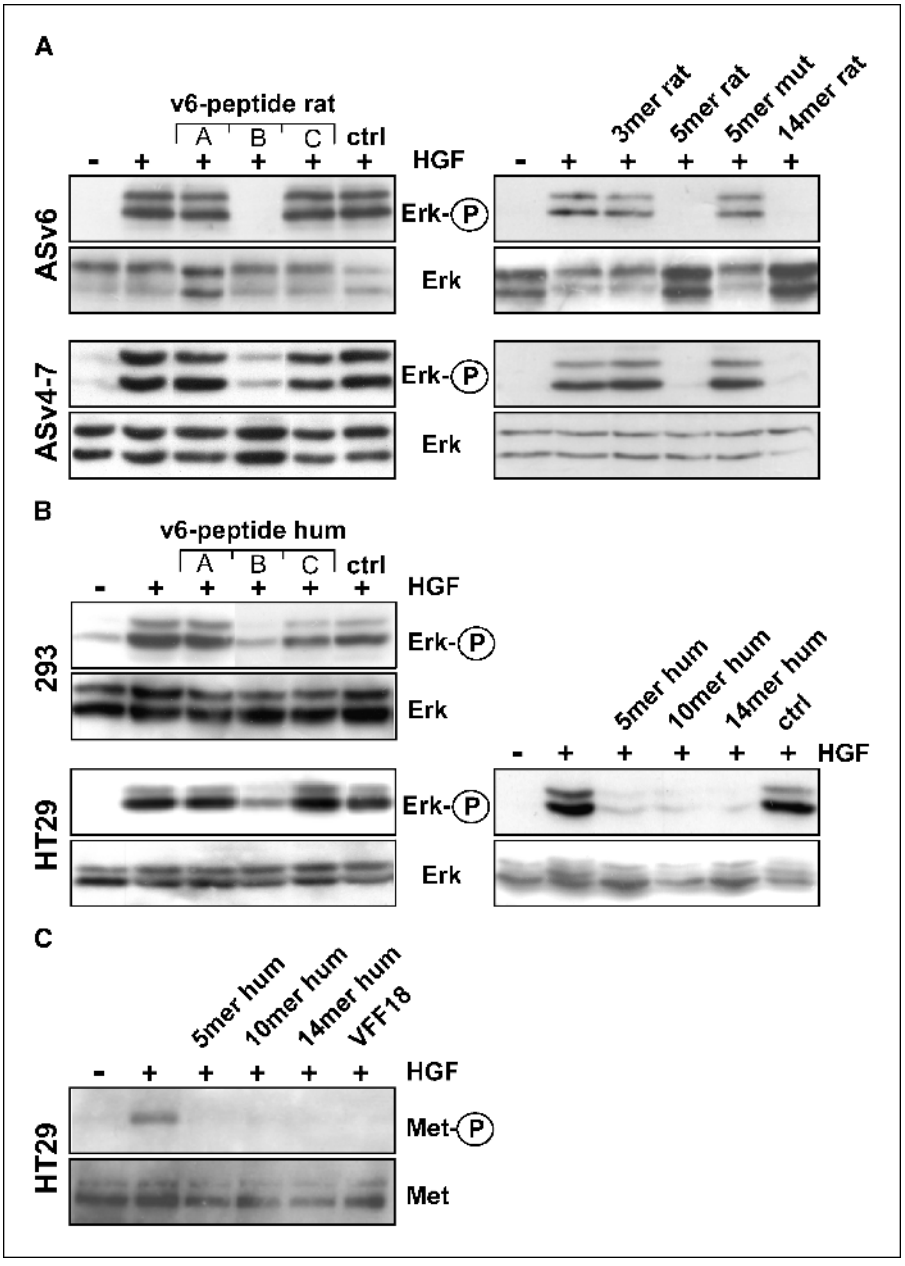

Figure 2. A linear peptide comprising three v6-specific amino acids abrogates Met activation. *A-C,* Met activation is abrogated by CD44 v6-specific peptides. Peptides corresponding to the (*A*) rat v6 exon sequence or (*B-C*) to the human v6 sequence or a control peptide (*ctrl*) were used as competitors for CD44 v6-dependent Met activation (either measured directly on Met phosphorylation, *C*; or on Erk phosphorylation, *A* and *B*) in the cell lines indicated. *ASv6,* AS cells stably expressing CD44 v6; *ASv4-7,* AS cells stably expressing CD44v4-7. For 293 and HT29 cells and the sequence of the peptides, see Materials and Methods.

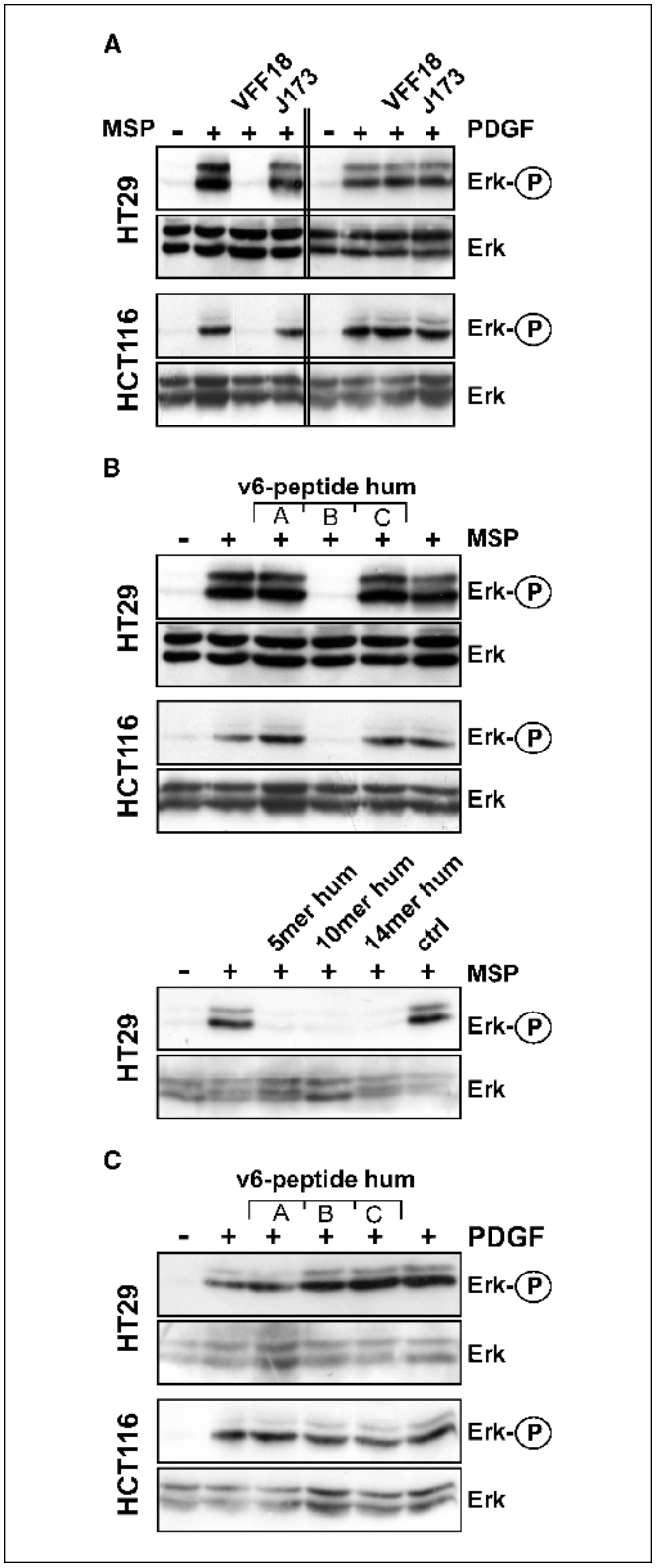

Figure 3. Ron activation is inhibited by CD44 v6-specific antibodies and peptides. *A,* HT29 or HCT116 cells were starved and induced by MSP or PDGF (10 ng/mL for 5 minutes each) as indicated and Erk activation was determined as in Fig. 1*A*. Where indicated, cells were incubated with human CD44 v6-specific VFF18 or pan CD44-specific J173 antibodies (100 μg/mL). Peptides corresponding to the sequence encoded by the human v6 exon were used as competitors for CD44 v6 in MSP-dependent Erk phosphorylation (*B*) or PDGF dependent Erk phosphorylation (*C*) in the cell lines indicated.

Rat and human v6 sequences differ but fulfill identical function. The critical sequence replaced in mutant ls8 (EWQ in the rat; Fig. 1*A*) is not entirely conserved in the human v6 sequence (RWH; ref. 22). Antibodies to the human v6 sequence, however,

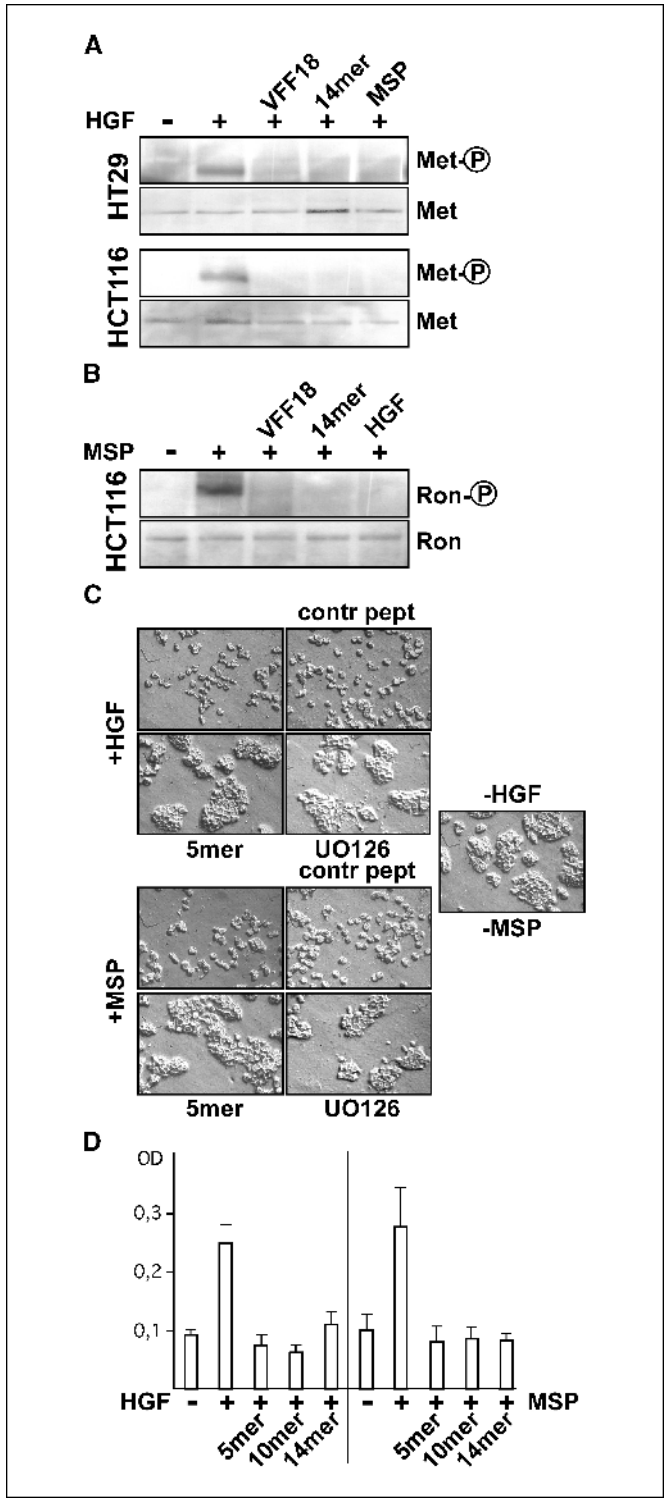

Figure 4. CD44 v6 peptide inhibits MSP- or HGF-dependent scattering and migration. *A-B,* no heterodimerization between Met and Ron. Determination of Met and Ron phosphorylation as in Fig. 1*C*. Addition of antibody, peptide, or ligands are indicated. *C-D,* cell scattering and invasion through Matrigel was determined as described in Materials and Methods. Magnification in (*C*) was 40×.

blocked Met activation in human cell lines (8). It is therefore plausible that in both human and rat the homologous sequences confer the coreceptor function. We tested whether peptides comprising the sequence RWH would also compete with endogenous CD44 v6 in the activation of Met. This was indeed the case: in the human colon carcinoma cell line HT29 and in human kidney carcinoma 293 cells, the activation of Met by HGF was strongly reduced by addition of the corresponding human 14 mer (peptide B) to the medium (Fig. 2*B*). As one might expect, the peptides are species specific and the addition of rat peptides (containing the EWQ sequence) to human cells or the human peptides (containing the RWH sequence) to rat cells had no effect on Met activation (data not shown). As for the rat peptides, human 10- and 5-amino-acid peptides sufficed to abolish Met activation (Fig. 2*B*). This inhibition was observed both by measuring activation of the Met receptor directly or of the downstream target Erk (Fig. 2*B-C*).

Ron activation by macrophage-stimulating protein requires CD44 v6 as coreceptor. Human cells express two related receptor tyrosine kinases, Met and Ron (3). We chose to also examine Ron for dependence on or independence of CD44 v6. Interestingly, Ron activation in response to its ligand MSP also required CD44 v6 in the human colorectal carcinoma cell lines HT29 and HCT116 (Fig. 3*A*; inhibition by v6-specific antibody VFF18, but not by antibody J173 directed against another CD44 epitope). The HCT116 cell line is the standard cell line for determination of MSP-induced invasiveness and migration (5). These results suggest that the receptors Met and Ron and/or their ligands HGF and MSP share surfaces responsible for their CD44 v6 dependency. CD44 v6 is, however, not a general coreceptor for receptor tyrosine kinases: activation of the PDGF receptor by its ligand could not be inhibited by the v6 antibody (Fig. 3*A*). These data prompted us to explore the effect of the v6 peptide on Ron activation in the human cell lines that express both Met and Ron. The result was identical to that obtained for Met (Fig. 3*B*). The human v6 sequences of 14, 10, or 5 amino acids efficiently blocked Ron dependent Erk activation (data on the activation of the receptors are shown in Fig. 4*A-B*). The activation of the PDGF receptor, however, was not affected by the addition of the competing peptides (Fig. 3*C*).

Because Met and Ron activation requires dimerization and heterodimerization between Ron and Met has been reported (23), one could speculate that CD44 v6 might address Ron via Met or vice versa. This is not the case. Neither did MSP induce Met phosphorylation (Fig. 4*A*) nor did HGF induce Ron phosphorylation (Fig. 4*B*). The activation of the receptors by their authentic ligands, however, could be blocked by the v6-specific antibody and v6 peptides (Fig. 4*A-B*).

Excess of a five-amino-acid v6 peptide blocks Met- or Ron-dependent scattering and migration. The receptor tyrosine kinases Met and Ron induce cell migration and scattering upon treatment with their ligands. We investigated the effect of v6-blocking peptides on scattering and migration. The 5-, 10-, and 14-amino-acid peptides prevented completely scattering (Fig. 4*C*, only the 5 mer is shown) or invasion through the extracellular matrix (Fig. 4*D*). The addition of a control peptide had no effect. HGF and MSP induced migration in the absence of extracellular matrix as measured in transwell chambers gave similar results (data not shown).

In HT29 cells, the activation of the MAPK pathway does not lead to proliferation (24) but rather to scattering (25). We could confirm this result in that the treatment of HT29 cells with the MAP kinase inhibitor UO126 prevented HGF or MSP induced scattering (Fig. 4*C*). The specificity of the inhibitor was verified by its block of Erk phosphorylation but not of Met or PI3 kinase activation (data not shown).

Conclusion

A small portion encoded by the CD44 exon v6 is critical for ligand-dependent activation of two related receptor tyrosine kinases, Met and Ron. Although this sequence of v6 differs between rat and man, in both species, it is required for the coreceptor function. The critical sequence of v6 is obviously exposed in that a specific antibody can bind and block the coreceptor function. The CD44 v6 molecule must be anchored in the plasma membrane because neither the complete extracellular domain of CD44 v6 nor the v6 peptides on their own promote ligand-dependent Met or Ron activation. The soluble peptides rather compete for an essential component and thereby inhibit receptor activation. Could this essential component be the ligand? No, certainly not directly, as the competing amino acid peptides act species specifically although the same recombinant HGF was used for Met activation in rat and human cells. Options are that the competing soluble v6 peptides interfere with homoclustering of the endogenous CD44 v6 (21), or with the interaction between Met and CD44 v6. Although we have yet no direct evidence for the v6 peptide binding to Met, we consider this interaction more likely because the elevated binding of hyaluronate generated by homoclustering of CD44 variant molecules was not inhibited by a v6 peptide (data not shown) nor abolished by the ls8 mutation in CD44 v6 (Fig. 1*D*). We propose that v6 peptides disrupt the interaction between the receptor and its coreceptor which is required to enhance ligand binding and/or possibly to eliminate a negative control factor counteracting receptor activation. A comparison of the amino acid sequence of the human Met and Ron receptors revealed several conserved stretches of more than four amino acids, which could be considered the sites of CD44 v6 interference. More precisely these sites should differ in human and rat Met to explain the species specificity of the peptides. By such a comparison, we failed thus far to identify a primary Met and Ron sequence that would fit to the basic RWH motif in the human v6 sequence, whereas the corresponding rat Met sequence would fit to the more acidic rat EWQ motif. It is of course not certain that the counterpart in the receptors must be a linear sequence.

Irrespective of the mechanism of coreceptor function, the inhibitory ability of a short peptide is surprising. There are only few examples for such effective action of a peptide, RGD interference with integrin function being the most well known case (26). The inhibitory action of these v6 peptides offers the promise for a future development of more stable and systemically usable compounds (peptidomimetics) preventing invasion and metastatic spreading of tumor cells by blocking the coreceptor action.

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