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Ligand-regulated Binding of FAP68 to the Hepatocyte Growth Factor Receptor*

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We have used the yeast two-hybrid system to identify proteins that interact with the intracellular portion of the hepatocyte growth factor (HGF) receptor (Met). We isolated a human cDNA encoding a novel protein of 68 kDa, which we termed FAP68. This protein is homologous to a previously described FK506-binding protein-associated protein, FAP48, which derives from an alternative spliced form of the same cDNA, lacking an 85-nucleotide exon and leading to an early stop codon. Here we show that epithelial cells, in which the HGF receptor is naturally expressed, contain FAP68 and not FAP48 proteins. FAP68 binding to Met requires the last 30 amino acids of the C-terminal tail, which are unique to the HGF receptor. Indeed, FAP68 does not interact with related tyrosine kinases of the Met and insulin receptor families. FAP68 interacts specifically with the inactive form of HGF receptor, such as a kinase-defective receptor or a dephosphorylated wild type receptor. *In vivo*, endogenous FAP68 can be coimmunoprecipitated with the HGF receptor in the absence of stimuli and not upon HGF stimulation. Thus, FAP68 represents a novel type of effector that interacts with the inactive HGF receptor and is released upon receptor phosphorylation. Free FAP68 exerts a specific stimulatory activity toward the downstream target p70 S6 protein kinase (p70S6K). Significantly, nonphosphorylated HGF receptor prevents FAP68 from stimulating p70S6K. These data suggest a role for FAP68 in coupling HGF receptor signaling to the p70S6K pathway.

Hepatocyte growth factor (HGF)[1] is a cytokine controlling proliferation and cell-cell dissociation ("scattering") in a broad spectrum of cells. It also induces formation of tubular structures in epithelial and endothelial cells and axon sprouting in neurons (for a review, see Ref. 1 and references within). The HGF receptor is the transmembrane tyrosine kinase encoded by the c-*MET* proto-oncogene, which has been genetically linked with human cancer (2). The downstream signaling cascades linking the ligand-activated receptor to the control of cell proliferation, scattering, and differentiation have been carefully analyzed. The three biological responses are triggered by phosphorylation of a unique multifunctional docking site containing two tyrosines (Tyr^{1349} and Tyr^{1356}), located in the receptor C-terminal tail (3, 4). The two phosphotyrosines interact with multiple cytoplasmic signal transducers either directly, or indirectly, via molecular adaptors such as Grb2 (5), Shc (6), and Gab-1 (7). After HGF stimulation, the receptor binds and activates phosphatidylinositol 3-kinase (PI3K; Refs. 8 and 9) and Src (3), recruits the Grb2/Sos complex stimulating Ras (10), and directly phosphorylates the transcriptional factor Stat-3 (11). The Gab-1 adaptor recruits additional signal transducers, such as PI3K, phospholipase C-γ, and the SHP2 phosphatase, and activates the Rac-c-Jun N-terminal kinase signaling pathway through the bound Crk adaptor (for a review, see Ref. 12 and references within). The use of dominant negative proteins and selective inhibitors indicates that PI3K, Rac, and Src activations are responsible for scattering (13–15); the Ras pathway is responsible for cell proliferation (5); and Stat-3, phospholipase C-γ, and SHP2 pathways are required for induction of branching morphogenesis (11, 16, 17). The complex, pleiotropic effects of HGF suggest that there may be additional signal transducers and effectors still to be discovered.

In this study we performed a genetic screen with the yeast two-hybrid system to identify novel proteins capable of associating with the unstimulated HGF receptor. We cloned a cDNA coding for a 68-kDa cytoplasmic protein, FAP68, which is specifically bound by the nonphosphorylated form of the HGF receptor and is released upon HGF stimulation and receptor phosphorylation. We provide evidence that forced expression of FAP68 in epithelial cells induces phosphorylation of p70S6K, without affecting the MAPK pathway. Co-transfection of nonphosphorylated HGF receptor prevents FAP68 from stimulating p70S6K. These findings suggest a potential role for FAP68 in linking HGF signaling to the regulation of protein synthesis.

EXPERIMENTAL PROCEDURES

Interaction Trap Screening of HeLa cDNA Library—To perform a yeast interaction trap screening (18), the cDNA encoding for the HGF receptor intracellular domain carrying a mutation in the ATP-binding site ($Lys^{1110} \rightarrow Ala$) was generated by PCR from the corresponding full-length construct in pMT2 vector (19). The PCR product was sequenced and inserted in pEG202 vector, fused to the LexA DNA-binding (IC-KD bait). The *Saccharomyces cerevisiae* strain EGY48 was co-transformed with the bait and the *lacZ* reporter pSH18–34. Yeast cells were then transformed with a HeLa cDNA library (from Roger Brent) in

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[1] The abbreviations used are: HGF, hepatocyte growth factor; FAP, FK506-binding protein-associated protein; FKBP, FK506-binding protein; p70S6K, p70 S6 protein kinase; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase; IGF-1R, insulin growth factor 1 receptor; GST, glutathione *S*-transferase; VSVG, vesicular stomatitis virus glycoprotein G; HA, hemagglutinin; GFP, green fluorescent protein; TOR, target of rapamycin; aa, amino acid(s); RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; bp, base pair(s).

vector pJG4–5 using the lithium acetate method (20). Over 2 million transformants were obtained and screened. Among the 700 yeast colonies isolated as Leu-positive, 35 were found to display both leucine prototrophy and *lacZ* expression only upon specific induction of the library expression with galactose. Plasmid DNA was rescued from these colonies and used for transformation of the reporter strain in combination with the IC-KD bait or with unrelated bait, pEG202-bicoid. Fourteen clones remained specifically positive. From restriction mapping it appeared that four groups of cDNAs, encoding distinct proteins, had been isolated (to be described elsewhere). The cDNA inserts from specific clones were sequenced using the ABI Prism™ 301 Genetic Analyzer (PerkinElmer Life Sciences). Clone *T291*, which is redundant with three other independent clones, was selected for the present study.

RACE Analysis—To obtain the 5′ sequence of clone *T291*, 5′-RACE-PCR on HeLa Marathon-Ready™ cDNA (CLONTECH) was performed, using as gene-specific primer an oligonucleotide annealing inside the 85-bp sequence present in FAP68 but absent from FAP48 (5′-GCCTC-CACACCTGAGTGATTACTTG-3′). Of the 17 5′ cDNAs that were retrieved, the 4 longest started at nucleotide 12 of the published *FAP48* sequence (GenBank™ accession no. U73704), and were identical to it. We performed a 3′-RACE-PCR using as gene-specific primer an oligonucleotide annealing within the 5′-untranslated region (5′-GAGGGT-TCTGGCCGATTTTAGCAT-3′). All 20 3′ cDNAs that were retrieved had the same size and contained the 85-bp sequence.

Expression Vectors and Constructs—The various HGF receptor regions were generated by PCR from a human full-length *Met* (21) and were fused to the LexA DNA binding domain in pEG202 vector (see the list in Fig. 2). The intracellular constructs of Ron (aa 983–1400 of either wild type or carrying the ATP binding site mutation) were generated by PCR from a human full-length *Ron* (22) and were fused to the LexA DNA binding domain in pEG202 vector. pEG202 and pEG202 containing the bicoid and pJG4–5 plasmids were provided by Dr. W. Kolanus. pEG202 containing the IGF-1R and insulin receptor (wild type or carrying the ATP binding site mutation) were a gift from Dr. T. A. Gustafson. The GST-fused intracellular domain of wild type HGF receptor cDNA cloned into baculovirus vector has been described previously (23). For transient transfection experiments, the *FAP68* 3′-RACE cDNA was inserted in pCR2.1-TOPO vector (Invitrogen) and sequenced. The *FAP68-VSVG* and the *FAP68-GST* were PCR-amplified from *FAP68* 3′-RACE cDNA, sequenced, and cloned in frame in *Sal*I-*Not*I of pMT2SM-VSVG-tag or pMT2SM-GST (from Dr. D. Shaap). *FAP68-VSVG* cDNA was subcloned in pcDNA3 vector with a modified polylinker (Invitrogen) for *in vitro* transcription and translation. Wild type *Met* and *TprMet* constructs in pMT2 have been described previously (3). The full-length sequence of human *p70S6Kα* was PCR-amplified with two specific primers in frame with the N-terminal Glu tag epitope (MEFMPME). Amplified cDNA fragment was cloned into the pcDNA3 vector via *Eco*RI and *Bam*HI restriction sites. Expression vectors of hamster p44 MAPK tagged with an HA peptide epitope, human ezrin protein tagged with a VSVG peptide epitope, and green fluorescent protein (GFP) were kindly provided by Dr. G. Scita, Dr. M. Arpin, and Dr. L. Naldini, respectively.

Cell Culture, Transient Transfection, and Cell Lysis—Sf9, COS-7, A549, HT29, HEPG2, SKBR3, and HeLa cells were from ATCC. HEK-293 cells used in this study express the SV40 large T antigen (kindly provided by Dr. L. Naldini). Cultures of mammalian cells were maintained in Dulbecco's modified Eagle's medium or RPMI (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.) in a humidified atmosphere of 5% CO_2 air. Insect Sf9 cells were maintained in serum-free medium (SF900; Life Technologies, Inc.) and baculovirus-infected according to Invitrogen instructions. For transient transfection experiments, subconfluent COS-7 and HEK-293 cells were transfected by DNA-calcium phosphate precipitation method. 5 μg of each cDNA were used, unless indicated. Cells from 10-cm plates were lysed after 36–48 h from transfection. HBD lysis buffer (20 mM Hepes, KOH, pH 7.8, 75 mM KCl, 0.1 mM EDTA, 2 mM $MgCl_2$, 10% glycerol, 0.5% Triton X-100, 1 mM dithiothreitol, 1 mM sodium orthovanadate, and protease inhibitors) was used for association studies. Modified EB buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 20 mM NaF, 20 mM β-glycerophosphate, 1 mM EDTA, 6 mM EGTA, 1% Triton X-100, 1 mM dithiothreitol, 1 mM sodium orthovanadate, and protease inhibitors) was used for phosphorylation studies.

Production of Fusion Proteins and in Vitro Association Assay—The control GST protein was obtained by pGEX vector transformation of BL-21 cells and induction with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside for 1 h at 37 °C. Bacteria were then lysed with B-PER (Pierce). The GST-fused intracellular domain of wild type HGF receptor was expressed in Sf9 cells using the baculovirus system and purified. Briefly, infected cells were lysed with EB buffer (20 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol, and protease inhibitors) in the presence (GST-phospho-IC) or absence (GST-dephospho-IC) of 1 mM sodium orthovanadate. Cell extracts were incubated with glutathione-Sepharose (Amersham Pharmacia Biotech) for 1 h at 4 °C and washed three times with EB buffer. The GST-phospho-IC was directly eluted with glutathione, whereas the GST-dephospho-IC was dephosphorylated with 20 units of calf intestinal alkaline phosphatase (Promega) in buffer for dephosphorylation (100 mM Tris, pH 8.0, 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 100 mM NaCl, 0.2 mg/ml bovine serum albumin), at 15 °C for 16 h. After two washings with EB buffer, the protein was eluted with 50 mM glutathione and 100 mM HEPES (pH 8).

Labeled proteins were prepared by transcription and translation of *FAP68-VSVG* in pcDNA3 vector, in the presence of [^{35}S]methionine/cysteine, using the TNT T7 coupled rabbit reticulocyte lysate system according to manufacturer's instructions (Promega). The translated lysates were diluted in HBD with 0.1% Triton X-100 and used directly in binding assay. GST alone or GST fusion proteins were immobilized on glutathione-Sepharose beads at 1 μM and incubated with diluted lysates overnight at 4 °C. The beads were washed four times with HBD with 0.1% Triton X-100, and then bound proteins were eluted in Laemmli buffer, run in SDS-PAGE, and detected by fluorography.

In Vivo Association Assay—HEK-293 cells were grown to 80% confluence and serum-starved for 24 h. Before cell lysis, cells were stimulated with 30 ng/ml HGF or not stimulated. Cells from two 10-cm plates were lysed with HBD, 0.5% Triton X-100, immunoprecipitated with 1 μl of α-HGF receptor DO24 ascites or mouse nonimmune serum as control, and immunoblotted with appropriate antibody.

Reagents, Antibodies, Precipitation, and Immunoblot—All reagents used were from Fluka and Sigma. Reagents for SDS-PAGE were from Bio-Rad. Recombinant HGF from the baculovirus expression system and α-HGF receptor monoclonal antibodies (DQ13 and DO24) have been described previously (24, 25). α-VSVG antibodies were purchased from Sigma, α-LexA from Santa Cruz, and α-HA from Roche Molecular Biochemicals. The α-Glu monoclonal antibody directed against the Glu epitope and the α-p70α rabbit antiserum were obtained from the Imperial Cancer Research Fund (London, United Kingdom). α-FAP polyclonal antibodies were produced in rabbits using aa 194–212 as immunogen. α-Phosphopeptide antibody against Thr^{412} of p70S6K (α-T412P; New England Biolabs), α-phosphotyrosine antibody (α-PY; Upstate Biotechnology), and α-active-MAPK antibody (Promega) were used.

For co-precipitation studies, cell extracts were rotated for 2 h at 4 °C with antibodies coupled to Sepharose-protein A or with glutathione-Sepharose. Beads were washed three times with cell lysis buffer and samples eluted by boiling in Laemmli buffer containing 100 mM dithiothreitol. Eluted proteins were separated on SDS-PAGE, transferred to nitrocellulose (Hybond C, Amersham Pharmacia Biotech), and probed with specific primary and secondary antibodies. Immunoblots were developed with an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech), according to the manufacturer's instructions and visualized on Amersham Hyperfilm.

RESULTS

Interaction Trap Cloning of FAP68—To identify target proteins that interact with the intracellular portion of the HGF receptor, we used the yeast Interaction Trap system (18). Because there are already many known Src homology 2 or phosphotyrosine B transducers that bind the tyrosine-phosphorylated HGF receptor, we screened for proteins that recognize the nonphosphorylated intracellular domain. To this aim a bait was produced by fusing the LexA DNA-binding domain with a cDNA encoding the cytoplasmic portion of the receptor, carrying the Lys^{1110} → Ala inactivating mutation in the ATP-binding site (IC-KD bait; see Fig. 2*A*). This mutation is sufficient to completely switch off the tyrosine kinase activity of the receptor.

Over 2 million cDNA clones from a HeLa cDNA expression library were screened; of the 700 yeast colonies selected, 14 were positive after two rounds of transformation. From restriction maps it appeared that four groups of cDNA encoding distinct proteins had been isolated. The complete sequencing of clone *T291* and comparison with the GenBank nucleotide data base using BLAST showed that *T291* is homologous to a pre-

A

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  1  MAVEELQSII KRCQILEEQD FKEEDFGLFQ LAGQRCIEEG HTDQLLEIIQ
 51  NEKNKVIIKN MGWNLVGPVV RCLLCKDKED SKRKVYFLIF DLLVKLCNPK
101  ELLLGLLELI EEPSGKQISQ SILLLLQPLQ TVIQKLHNKA YSIGLALSTL
151  WNQLSLLPVP YSKEQIQMDD YGLCQCCKAL IEFTKPFVEE VIDNKENSLE
201  NEKLKDELLK FCFKSLKCPL LTAQFFEQSE EGGNDPFRYF ASEIIGFLSA
251  IGHPFPKMIF NHGRKKRTWN YLEFEEEENK QLADSMASLA YLVFVQGIHI
301  DQLPMVLSPL YLLQFNMGHI EVFLQRTEES VISKGLELLE NSLLRIEDNS
351  LLYQYLEIKS FLTVPQGLVK VMTLCPIETL RKKSLAMLQL YINKLDSQGK
401  YTLFRCLLNT SNHSGVEAFI IQNIKNQIDM SLKRTRNNKW FTGPQLISLL
451  DLVLFLPEGA ETDLLQNSDR IMASLNLLRY LVIKDNENDN QTGLWTELGN
501  IENNFLKPLH IGLNMSKAHY EAEIKNSQEA QKSKDLCSIT VSGEEIPNMP
551  PEMQLKVLHS ALFTFDLIES VLARVEELIE IKTKSTSEEN IGIK
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B

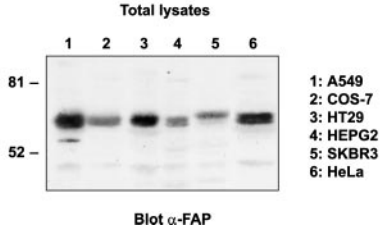

FIG. 1. **Primary sequence and biochemical structure of FAP68 protein.** *A*, the amino acid sequence was predicted from the sequence of a full-length cDNA obtained by RACE-PCR from HeLa mRNA. The cDNA includes an open reading frame of 1785 nucleotides and encodes a protein of 594 amino acid residues, with a predicted molecular mass of 68,204 kDa. *B*, a polyclonal antibody (*α-FAP*), directed against a peptide sequence encompassing aa 194–212, common to FAP68 and FAP48, recognizes a major endogenous protein resolved by 10% SDS-PAGE as a doublet of ~65 kDa in Western blot analysis.

viously identified human cDNA sequence called *FAP48* (for FKBP-associated protein 48; GenBank™ accession no. U73704, Ref. 26). *T291* starts at nucleotide 165 of the *FAP48* sequence. The two sequences are identical up to nucleotide 1307. After 85 nucleotides, absent from the *FAP48* sequence, *T291* resumes its identity with *FAP48* (1308–1834), except for the lack of nucleotide 1573. The full-size cDNA, obtained by RACE-PCR from HeLa cells, includes an open reading frame of 1785 nucleotides, which encodes a protein of 594 amino acid residues, with a predicted molecular mass of 68,204 kDa (Fig. 1*A*). It was therefore named *FAP68*. Several human expressed sequence tags contain the 85-bp sequence and the single base pair deletion present in *FAP68*. A cDNA clone from a placental library (GenBank™ accession no. AAH01257), recently deposited in GenBank™, is identical to *FAP68* cDNA. The genomic fragment of chromosome 1 (GenBank™ accession no. AC024010), deposited at the High Throughput Genomic Sequences division of GenBank™, contains the *FAP* gene. In the genomic sequence, canonical consensus splice sites (AG at the 3′ splice site and GT at the 5′ splice site) flank the 85-bp segment. Therefore, the previously published *FAP48* sequence derives from an alternatively spliced transcript that removes the 85-nucleotide exon and leads to a premature stop codon. 3′-RACE-PCR analysis of HeLa transcripts indicated the presence of a homogeneous population of *FAP68* mRNA. No transcript corresponding to *FAP48* was found.

To determine whether the two protein products were expressed in cells, a polyclonal antibody was raised against a peptide encompassing aa 194–212, common to FAP68 and FAP48. In an immunoblot of a panel of epithelial cell lines, the immune serum recognized a major band migrating in SDS-PAGE as a closely spaced doublet of ~65 kDa (Fig. 1*B*).

Interaction between FAP68 and HGF Receptor—The interaction between FAP68 protein and HGF receptor was further studied by co-transforming yeast with clone *T291* (aa 25–594) and various receptor mutants (Fig. 2*A*). A bait containing the intracellular portion of wild type HGF receptor constitutively phosphorylated on tyrosine (IC bait, Fig. 2*B*) failed to interact with clone *T291* (Fig. 2*C*). This indicates that binding is prevented by tyrosine phosphorylation of the receptor. Two deletion mutants were derived from the kinase-defective receptor used in the original screening (IC-KD bait): TKC lacking the juxtamembrane domain, and JTK lacking the C-terminal tail (Fig. 2*A*). Of these, only TKC interacted with clone *T291* (Fig. 2*C*), thus indicating that binding requires the C-terminal tail. Indeed, the last 57 amino acids of the receptor (C57 bait) showed a positive interaction, although weaker than that detected in the presence of the catalytic domain. The C-terminal tail containing the two tyrosines Tyr^{1349} and Tyr^{1356} of the multifunctional docking site but devoid of last 30 amino acids (C27 bait) failed to interact with clone *T291*. This indicates that the last 30 amino acids of the C-terminal tail are required for binding.

On the FAP68 side, we generated various deletion mutants and found that the intact molecule is required for specific binding to the receptor, except for the first 25 amino acids, which are dispensable (data not shown). The specificity of the interaction was assessed by testing baits containing the cytoplasmic domain of Ron, a Met subfamily member, and two related tyrosine kinases, the IGF-1R and the insulin receptor. Neither wild type nor kinase-defective receptors (carrying a mutated ATP binding site) did bind FAP68 (data not shown).

The interaction between FAP68 and HGF receptor was investigated in mammalian cells *in vivo* (Fig. 3). As observed in yeast, in co-transfected COS-7 cells, GST-tagged FAP68 interacted with the kinase-defective but not with the kinase-active wild type HGF receptor (Fig. 3*A*), which in COS-7 cells is autophosphorylated as consequence of overexpression (27). Identical results were obtained when TprMet was used (Fig. 3*B*). TprMet is a hybrid molecule, encoded by the *TPR-MET* transforming gene (28); the extracellular domain of Met receptor is replaced by Tpr sequences, which provide two strong dimerization motifs (29), followed by the Met kinase domain and C-terminal tail. In the $TprMet^{D}$ mutant, the two critical tyrosines of the multifunctional docking site (corresponding to the Tyr^{1349}/Tyr^{1356} in the full-size receptor) were converted into phenylalanine (3). This Tyr → Phe conversion abrogates signaling, but does not affect the $TprMet^{D}$ catalytic activity and constitutive phosphorylation of other sites. Neither $TprMet^{D}$ nor $TprMet^{WT}$ were able to bind FAP68-GST (Fig. 3*B*). This result suggests that the phosphorylated HGF receptor cannot bind FAP68 because of structural changes in the C-terminal tail rather than steric blockade by phosphotyrosine residues of the multifunctional docking site.

The interaction between FAP68 and nonphosphorylated wild type HGF receptor was confirmed *in vitro*, using purified molecules. The intracellular domain of wild type HGF receptor, purified from baculovirus-infected cell lysates and constitutively phosphorylated (GST-phospho-IC), did not interact with *in vitro* translated and radiolabeled FAP68 (Fig. 4). However, following dephosphorylation by phosphatase treatment, GST-dephospho-IC acquired the ability of binding FAP68. This confirms that FAP68 binding is specific for the nonphosphorylated HGF receptor and that the interaction of the kinase-defective

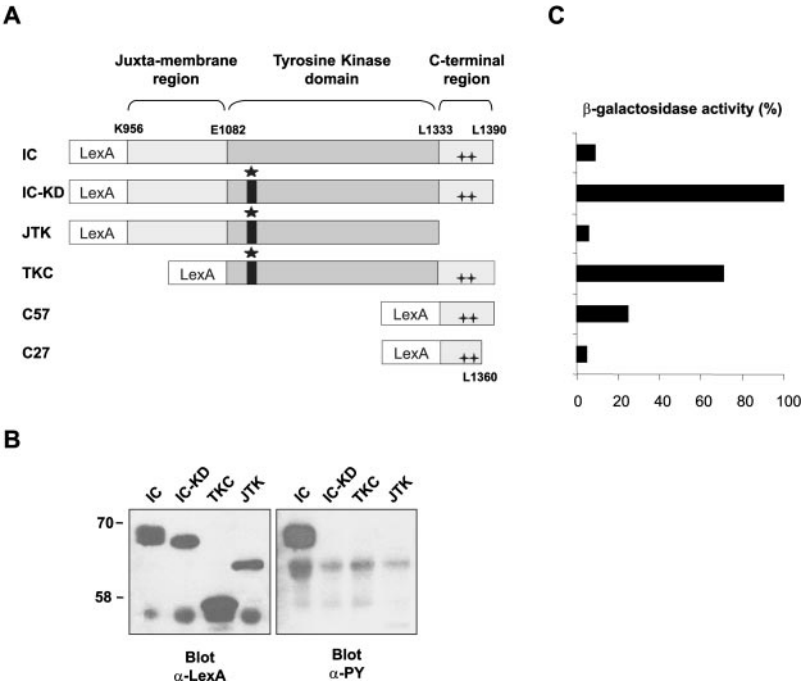

FIG. 2. **Mapping of HGF receptor sequences required for FAP68 interaction.** Yeast was co-transformed with *T291* prey in combination with different HGF receptor baits, to perform an interaction trap assay. *A*, schematic representation of the regions of the human HGF receptor intracellular portions used as baits. *Numbers* indicate the amino acid residues included in the various fusion proteins. The *star* indicates the mutation in the ATP binding site ($Lys^{1110} \rightarrow Ala$), leading to a kinase-defective mutant. The ++ indicates the multifunctional docking site (Tyr^{1349}/Tyr^{1356}). *B*, transformed yeast cells were lysed and equal amounts of total cell extracts were separated by SDS-PAGE and immunoblotted with either α-LexA or α-phosphotyrosine antibodies (*α-PY*). *C*, measurement of β-galactosidase levels was done in duplicate from two independent colonies in three separate experiments. The average values of β-galactosidase units are normalized to the maximal activity obtained with IC-KD bait.

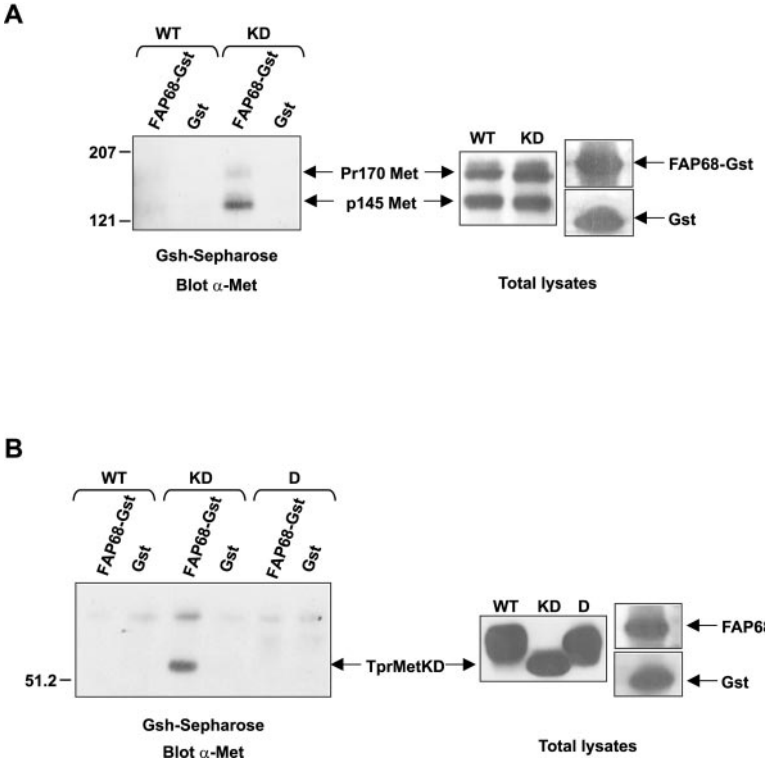

FIG. 3. **FAP68 binds the nonphosphorylated HGF receptor in mammalian cells.** COS-7 cells were co-transfected with GST-tagged FAP68 and either full-size Met (*A*) or TprMet (*B*) cDNA constructs. The various Met constructs encoded the wild type (*WT*), kinase-defective (*KD*), and $Tyr^{1349}/Tyr^{1356} \rightarrow$ Phe double (*D*) mutant receptors. Cells were lysed, precipitated with glutathione-Sepharose (*Gsh-Sepharose*), and immunoblotted with DQ13 α-Met antibody (*A* and *B*, *left panels*). Negative controls included precipitates from cells transfected with pMT2SM-GST empty vector. The expression level of transfected proteins was assayed by immunoblotting of total lysates with α-Met and α-GST antibodies (*A* and *B*, *three right panels*). *Pr170 Met* and *p145 Met* in *A* indicate, respectively, the uncleaved precursor and the mature/transmembrane β chain of the receptor, corresponding to the reported molecular weight. *Gst*, GST.

mutant is not the result of the introduced mutation in the ATP-binding site.

HGF Stimulation Causes Release of Tyrosine-phosphorylated FAP68 in Vivo—To determine, under physiological conditions, whether the association between FAP68 and the HGF receptor depends on receptor phosphorylation, we examined the interaction between the two endogenous proteins in serum-starved and HGF-stimulated HEK-293 cells. The HGF receptor was immunoprecipitated with DO24 monoclonal antibody directed against the receptor extracellular domain. This antibody preferentially reacts with the phosphorylated Met receptor (25). The immune complex was analyzed by immunoblotting with α-FAP polyclonal antiserum. Coimmunoprecipitation of FAP68 was observed with the nonphosphorylated HGF receptor but was no longer detected in the immune complex after HGF stimulation (Fig. 5*A*). We then investigated whether FAP68 was phosphorylated upon HGF receptor activation. FAP68 was immunoprecipitated with α-FAP antiserum from serum-starved and HGF-stimulated HEK-293 cells. The immune complex was analyzed by immunoblotting with α-FAP and α-phosphotyrosine antibodies. We found that following HGF stimulation a fraction of FAP68 protein underwent a gel migration shift and became tyrosine-phosphorylated (Fig. 5*B*). This indicates that *in vivo* FAP68 released from the receptor upon HGF receptor activation is tyrosine-phosphorylated.

FAP68 Increases the Phosphorylation of p70S6K—We then

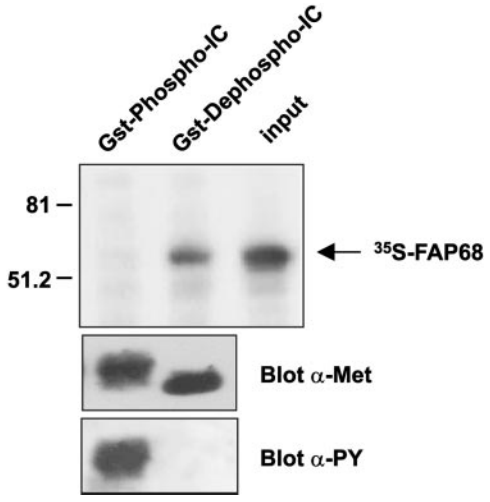

FIG. 4. **FAP68 binding to HGF receptor is negatively regulated by tyrosine phosphorylation *in vitro*.** The FAP68 cDNA was transcribed and translated *in vitro*, with rabbit reticulocyte lysate, in the presence of [^{35}S]methionine/cysteine. The radiolabeled lysate was incubated with the purified intracellular portion of wild type HGF receptor either constitutively phosphorylated (*GST-Phospho-IC*) or treated with calf intestinal alkaline phosphatase (*GST-Dephospho-IC*). After washing, the bound proteins were eluted, resolved by SDS-PAGE, and visualized by fluorography (*upper panel*). The tyrosine phosphorylation of purified wild type HGF receptor was assayed by immunoblotting with DQ13 α-Met and α-phosphotyrosine antibodies (*lower panels*).

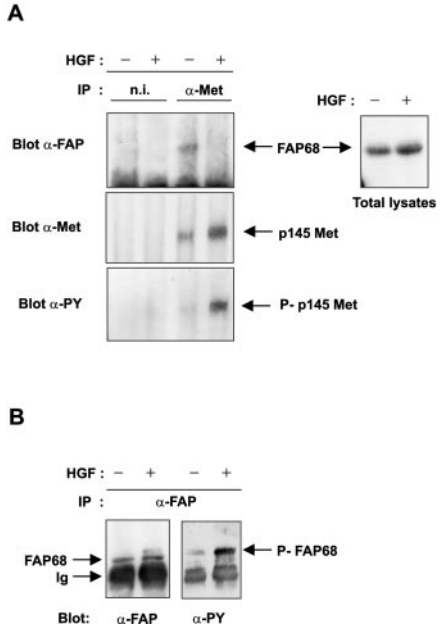

FIG. 5. **Endogenous FAP68 is released and tyrosine-phosphorylated after HGF stimulation of intact cells.** Serum-starved HEK-293 cells were either unstimulated or stimulated with HGF (30 ng/ml) for 10 min. *A*, cells were lysed in HBD buffer and HGF receptor was immunoprecipitated (*IP*) using 1 μl of DO24 monoclonal antibody directed against the extracellular portion of the receptor. The immune complex was probed by immunoblotting with α-FAP polyclonal antibody (*upper left panel*), DQ13 α-Met (*middle left panel*), and α-phosphotyrosine antibodies (*lower left panel*). Expression of endogenous FAP68 is shown in total Western blotting using α-FAP antibody (*right panel*). Nonimmune (*n.i.*) serum (1 μl) was also tested as a negative control. *B*, cells were lysed in modified EB buffer and FAP68 was immunoprecipitated using 1 μl of α-FAP antiserum. The immune complex was immunoblotted with α-FAP and α-phosphotyrosine antibodies (*left* and *right panels*, respectively).

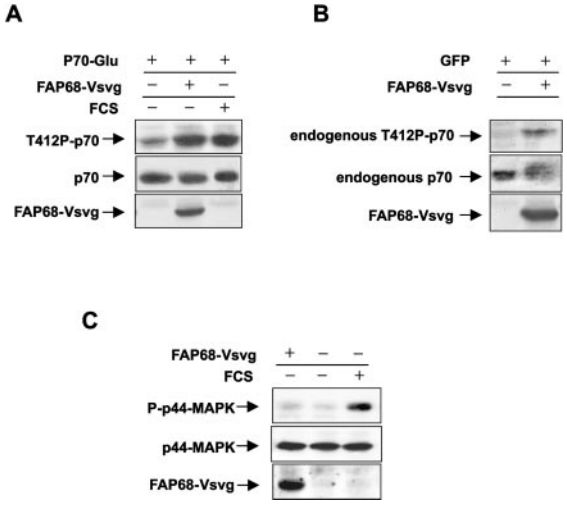

FIG. 6. **FAP68 increases p70S6K phosphorylation in mammalian cells.** *A*, HEK-293 cells were co-transfected with p70S6K-Glu (2.5 μg) and FAP68-VSVG or p70S6K-Glu and empty vector. After 36 h cells were starved for 16 h in serum-free medium, then serum-stimulated for 10 min. Lysates were normalized for amount of p70S6K (10–20 μg) and were immunoblotted with the α-p70 (T412P) phosphopeptide-specific antibody, α-Glu antibody, and α-VSVG antibody. *B*, HEK-293 cells were co-transfected with FAP68-VSVG and GFP or GFP vector alone. After 36 h, cells (80–90% GFP-positive) were lysed. 100 μg of lysates were immunoblotted with the α-p70 (T412P) phosphopeptide-specific antibody or α-p70α and α-VSVG antibodies. *C*, HEK-293 cells were co-transfected with p44 MAPK-HA and FAP68-VSVG or p44 MAPK-HA and empty vector and treated as in *A*. Lysates were normalized for amount of p44 MAPK-HA and immunoblotted with α-active-MAPK phosphopeptide-specific antibody, or α-HA and α-VSVG antibodies. *Vsvg*, VSVG.

investigated whether free FAP68 might have a role in the signal transduction pathways stimulated by the HGF receptor. We analyzed the effect of FAP68 on p70S6K, a downstream target of PI3K, and on MAPK, a downstream target of Ras. HEK-293 cells were co-transfected with p70S6K and FAP68, or p70S6K and empty vector as a control, serum-starved for 16 h, and analyzed for basal p70S6K activity. *In vivo*, p70S6K activity is closely related to the state of phosphorylation of Thr^{412} (30, 31). Using specific antibodies against the phosphorylated form of Thr^{412}, we observed that co-expression of FAP68 with p70S6K stimulated phosphorylation of Thr^{412} (Fig. 6*A*). The stimulation was comparable with that induced by addition of fetal calf serum to control cells. The effect of FAP68 was specific, as co-expression of ezrin did not result in increased p70S6K phosphorylation (data not shown). The same type of analysis was performed on endogenous p70S6K in HEK-293 cells overexpressing FAP68 and GFP. In experiments where the transfection efficiency was close to 90%, Thr^{412} phosphorylation and a shift of p70S6K were clearly detectable with the appropriate antibodies (Fig. 6*B*). We then examined the effect of FAP68 on p44 MAPK. Co-transfection with FAP68 failed to activate MAPK, as assessed by immunoblot with specific antibodies against the phosphorylated form of MAPK (Fig. 6*C*). These results indicate that FAP68 does not have a general activating effect on cellular kinases but rather specifically stimulates p70S6K phosphorylation.

A Mechanism Coupling HGF Receptor and p70S6K Activity—We consequently asked whether the stimulatory activity of FAP68 toward p70S6K could be regulated by the interaction between FAP68 and the HGF receptor. FAP68 (or empty vector as a control), p70S6K, and the kinase-defective mutant TprMet^{KD} were co-expressed in HEK-293 cells, and p70S6K phosphorylation was detected by immunoblotting with specific antibodies against the phosphorylated form of Thr^{412}. The ability of FAP68 to increase the level of the p70S6K phosphorylation was abrogated in the presence of the kinase-defective TprMet mutant (Fig. 7). Wild type TprMet is inappropriate as

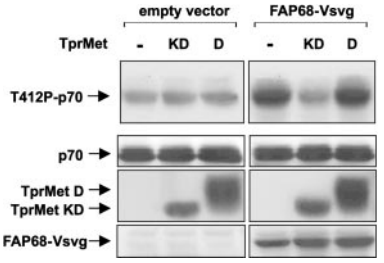

FIG. 7. **Nonphosphorylated HGF receptor prevents FAP68 stimulatory activity toward p70S6K.** *A*, HEK-293 cells were co-transfected with p70S6K-Glu (2.5 μg) and FAP68-VSVG or p70S6K-Glu and empty vector as a control, in combination with either $TprMet^{KD}$ (kinase-defective) or $TprMet^{D}$ ($Tyr^{1349}/Tyr^{1356} \rightarrow$ Phe double mutant). Cells were starved for 16 h in serum-free medium and lysed. Stimulation of p70S6K was detected by immunoblotting of normalized total lysates with the α-p70 (T412P) phosphopeptide-specific antibody (*upper two panels*). The expression of transfected proteins was assayed by immunoblotting of total lysates with α-Glu, α-Met, and α-VSVG antibodies. *Vsvg*, VSVG.

a control for this experiment, because it can enhance the p70S6K by stimulation of the PI3K pathway. We thus used the $TprMet^{D}$ mutant, unable to signal through the tyrosines Tyr^{1349}/Tyr^{1356} of the multifunctional docking site, mutated into phenylalanines. $TprMet^{D}$ did not bind FAP68 (see Fig. 3*B*) and did not interfere with the up-regulation of p70S6K activation induced by FAP68.

DISCUSSION

In this paper we have identified by interaction screening a novel protein of 68 kDa (FAP68), which specifically interacts with the HGF receptor. A splice variant, FAP48, was previously found by two-hybrid screening using FKBP52 protein as a bait (26). Our data indicate that this lower molecular mass form results from an off-frame truncation of the FAP68 transcript. Using antibodies against common peptides, we have shown that FAP68 is widely distributed in human epithelial cells, whereas FAP48 is undetectable. The high level of expression of FAP68 in epithelial cells, where HGF receptor is predominantly found, supports the idea that it may function in HGF signaling.

The interaction between FAP68 and the HGF receptor is direct and occurs with the nonphosphorylated receptor. More than one region of FAP68 is likely to contribute to formation of the receptor-binding site, because the cleavage of the molecule into two halves abrogates binding. The last 57 amino acids of the HGF receptor C-terminal tail contain a minimal binding site for FAP68, and the last 30 amino acids are an essential component of this site. This region of 30 residues in the C-terminal tail diverges even among members of the Met receptor family, suggesting that FAP68 binding may be a unique property of the HGF receptor. The sequence recognized by FAP68 is next to the region of the Met multifunctional docking site, involved in binding the Src homology 2- and phosphotyrosine B-containing effectors (3). Receptor phosphorylation inhibits FAP68 binding. This is not caused by steric hindrance of the phosphorylated tail residues Tyr^{1349} and Tyr^{1356}, as a mutant lacking these two tyrosines but still kinase-active ($TprMet^{D}$) does not bind FAP68. Rather, conformational changes in the C-terminal tail of the receptor following activation are likely to inhibit binding and to induce release of FAP68. Interestingly, upon ligand-induced receptor activation, FAP68 becomes tyrosine-phosphorylated. This modification, in addition to changes in Met structure, may contribute to the release of FAP68.

Release of FAP68 may expose a site on the receptor to which another effector may bind. In this case FAP68 may serve as an inhibitor of signaling prior to receptor activation. This is an attractive hypothesis, considering that the HGF receptor C-terminal tail is the organizing center for the assembly of large signaling complexes proximal to the receptor. Alternatively, the regulated release may serve to allow FAP68 to move to its site of action. A number of signaling mechanisms dependent on phosphorylation-induced release have been described. An example is the activation of the Stat pathway. Here, the phosphorylated receptor recruits the Src homology 2-containing transcription factor to the membrane and phosphorylates it, inducing dimerization and release into the cytoplasm. Then, the dimer migrates to the nucleus and activates transcription (32). Another example is activation of Smad transcription factors, which are released from the transforming growth factor-β family receptors upon phosphorylation on serine residues and translocated into the nucleus where they induce transcription (33). Some cytoplasmic molecules have been shown to specifically interact with nonphosphorylated tyrosine kinase receptors. For instance, a cell cycle checkpoint regulatory protein, MAD2, is released from the insulin receptor after activation (34) and a zinc finger protein selectively binds the unstimulated epidermal growth factor receptor and translocates to the nucleus upon release from the activated receptor (35).

The important question that remains open is the real function of FAP68 and its role in HGF signaling. Very little is known of this protein. It has no substantial similarities with already known sequences, except for the FAP48 isoform. It shares with FAP48 (26) the ability to associate with the FKBP proteins, the targets of immunosuppressant drugs FK506 and rapamycin, using a putative rapamycin/FK506 mimetic structure (data not shown). This raises the possibility that FAP48 and/or FAP68 act in the signal transduction pathways targeted by FK506 and/or rapamycin.

Here we show that FAP68 exerts a specific effect on p70S6K. The detailed biochemical mechanisms by which FAP68 up-regulates p70S6K are presently unclear. Regardless of the mechanism, FAP68 binding with the nonphosphorylated HGF receptor prevents its stimulatory activity toward p70S6K.

Activation of p70S6K is essential for HGF-induced hepatocyte proliferation (40). Met can activate p70S6K via PI3K (8). Our data suggest that the release of FAP68 from the activated receptor may synergize with PI3K to reinforce HGF-induced p70S6K signaling (13). This occurs via phosphorylation of Thr^{412}, which is controlled by upstream kinase(s) (37) and by serine/threonine phosphatase(s) inhibited by the mammalian target of rapamycin TOR (38, 39). An intriguing idea could be that FAP68 may participate in the cellular regulation of mammalian TOR. Work is in progress to investigate whether FAP68 is functionally linked with TOR or is involved in different aspects of HGF signaling.

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Ligand-regulated Binding of FAP68 to the Hepatocyte Growth Factor Receptor

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