Signaling of Hepatocyte Growth Factor/Scatter Factor (HGF) to the Small GTPase Rap1 via the Large Docking Protein Gab1 and the Adapter Protein CRKL*

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Hepatocyte growth factor (HGF; scatter factor) is a multipotent protein with mitogenic, motogenic, and developmental functions. Upon activation, the HGF-receptor c-Met binds and phosphorylates the multisite docking protein Gab1. Besides binding motifs for phosphatidylinositol 3-kinase and Grb2, Gab1 contains multiple Tyr-X-X-Pro (YXXP) motifs which, when phosphorylated, are potential binding sites for the adapter proteins c-Crk and Crk-like (CRKL). Stimulation of human embryonic kidney cells (HEK293) with HGF leads to Gab1 association with CRKL. The Gab1-CRKL interaction requires both, the SH2 domain of CRKL and the region containing the YXXP motifs in Gab1. CRKL binds via its first SH3 domain to several downstream signal transducers, including C3G an activator of the small GTPase Rap1. Indeed, Rap1 was rapidly activated after HGF stimulation of HEK293 cells. Rap1 activation through HGF was suppressed through transfection of a truncated C3G protein which only contains the SH3-binding motifs of C3G. Transfection of nonmutated Gab1 led to a strong increase of Rap1-GTP in the absence of HGF. In contrast, transfection of the Gab1YXXP mutant abolished the elevation of Rap1-GTP by HGF. A replating assay indicated that HGF decreases the adhesion of HEK293 cells. The results presented here delineate a novel signaling pathway from HGF to the GTPase Rap1 which depends on the interaction of the adapter protein CRKL with the exchange factor C3G and could be linked to cell migration.

Signal transduction events regulating the development and homeostasis of complex multicellular organisms are prominently regulated through the timely activation of different enzymes but also through the rapid and selective formation of multiprotein complexes which are often a prerequisite for the appropriate activation of intracellular enzymes (reviewed in Refs. 1 and 2).

The Crk family of adapter proteins was initially discovered in the form of an oncogene carried by two sarcoma-inducing retroviruses (CT10, ASV1; 3, 4). The normal cellular counterparts of this oncogene, c-Crk I and c-Crk II, as well as the highly homologous, but distinct gene product Crk-like (CRKL; 5) are characterized through their almost exclusive composition from building blocks that mediate selective protein-protein interactions and are known as SH2-domain and SH3-domain receptors (reviewed in Refs. 2 and 6). Crk, CRKL, and their SH2 and SH3 domain-interacting proteins have been suggested to play a role in many signaling pathways, including neuronal differentiation of PC12 rat pheochromocytoma cells (7, 8), T-cell receptor signaling (9, 10), integrin signaling (11, 12), cell migration (13, 14), and signal transduction of the CML-causing Bcr-Abl oncoprotein.2

Physiological and pathological signals mediated by Crk and CRKL affect several different downstream targets, including the Rap1-activator C3G, via DOCK180 the GTPase Rac and via HPK1 so called “stress kinases” of the JNK/SAPK family (15–21).

Since SH2 and SH3 domains are very widely present in signal transduction proteins, considerable effort was made to understand the binding preferences of these protein interaction domains. In the case of Crk, useful clues for the specificity of the SH2 domain came from phosphopeptide library studies (22), as well as the identification of intramolecular and intermolecular CrkSH2 binding motifs in c-Crk II (23), p130Cas (24, 25), and other proteins. It is now well established that the “in vivo” binding sites of the Crk and CRKL SH2 domains commonly conform to the sequence phospho-Tyr-X-X-Pro (pYXXP).

The downstream signal transduction of c-Crk and CRKL seems to be largely mediated by the first of the two SH3 domains (SH3(1)). The SH3(1) domains of c-Crk and CRKL recognize with great preference sequence motifs which conform to the consensus Pro-X-X-Pro-X-Lys (26). Multiples of these Crk(CRKLSH3(1))-binding motifs are found in Crk(CRKL-signal transducers like C3G and DOCK180 (reviewed in Ref. 2). C3G activates the Ras-like GTPases Rap1A and Rap1B (16, 17), while DOCK180 up-regulates by a not yet exactly defined mechanism the activity of Rac1 (18, 19, 27).

Rap1 can counteract Ras in some cell types and leads to T-cell anergy and neuronal differentiation (7, 9). In fibroblasts

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1 The abbreviations used are: SH2, Src homology domain 2; HGF, hepatocyte growth factor; GST, glutathione S-transferase; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; BES, 2-[bis(2-hydroxyethyl)aminio]ethanesulfonic acid; HEK, human embryonic kidney cell.

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it is activated in response to growth-stimulating factors like bombesin which do not activate Ras (28). Furthermore, injection of Rap1-overexpressing fibroblasts leads to tumor formation in nude mice (29). In platelets, Rap1 is strongly activated by thrombin (30, 31). The distinct roles of Rap1 thus appear to depend largely on the cellular context.

Hepatocyte growth factor/scatter factor (hereafter simply referred to as HGF) is a multifunctional factor produced by many cell types, including normal fibroblasts, epithelial and endothelial cells, but also different oncogenic cells (32). It has been reported to influence cell proliferation of hepatocytes and multiple other cell types, kidney cell migration, organ and tissue development, but also the invasiveness of tumor cells and other biological responses (for reviews, see Refs. 33–35). Kidney epithelial cells show three types of responses to HGF in a sequential, timely regulated and to some extent interdependent order. First, HGF leads to scattering and increased motility which requires the detachment of cell-cell contacts and cytoskeletal rearrangements. Phosphatidylinositol 3-kinase plays an important role in this process (36). Second, cells show an increased proliferation, a response which requires the activation of mitogenic kinases (mitogen-activated protein kinases/extracellular regulated kinase) (Ref. 37). The third response is the formation of branched tubules, a developmental process which occurs only in three-dimensional culture systems and appears to require signaling through STAT3 (28).

HGF binds to the receptor tyrosine kinase c-Met which transmits the HGF signal into the cytoplasm. c-Met autophosphorylation leads to the recruitment of signal transduction proteins like the c-Src kinase, phospholipase C-γ, the Grb2-SoS complex, and phosphatidylinositol 3-kinase (Ref. 38). These proteins are known to result in tyrosine phosphorylation of Gab1 (2, 6). Furthermore, the c-Met substrate Gab1 contains six putative Crk/CRKLSH2-binding sites (XXP motifs; see also Fig. 1). Therefore, we explored a possible link from the HGF-c-Met-Gab1 signaling pathway to Crk/CRKL and its downstream targets. The results document the existence of HGF-induced signals via complex formation of Gab1 and CRKL to the small GTPase Rap1.

**MATERIALS AND METHODS**

Reagents—Antibodies used in the experiments were from commercial sources: anti-c-Met (65-237 and 65-238, UBI), anti-C9G (sc-889, Santa Cruz), anti-Crk (Transduction Laboratories, C12230), anti-CRKL (sc-319, Santa Cruz), anti-cyclin A (sc-596, Santa Cruz), anti-epidermal growth factor receptor (IgG2a; GR01, Calbiochem), anti-FLAG (M2, Kodak), anti-Gab1 (sc-6292, Santa Cruz), anti-GRAP (sc-6101, Santa Cruz), anti-Nck (N15920, Transduction Laboratories), anti-phospho-tyr (PY20, Transduction Laboratories), and anti-Rap1 (R22020, Transduction Laboratories). Anti-C9G for detection of a transfected C3G-CBR fragment (details below) was generated by immunizing rabbits with a GST fusion protein of the CBR region of C3G as described previously (15). Western blot detection was done with horseradish peroxidase-coupled secondary antibodies (715-036-151 and 711-036-152, Jackson ImmunoResearch Lab., West Grove, PA; or sc-2020, Santa Cruz) and ECL (Amersham Pharmacia Biotech). The expression of GST fusion proteins and confirmation of their functionality has been previously described (Refs. 20 and 45, and references therein). The pGEX-CRKL vectors are kind gifts of Brian J. Druker, Portland, OR (CRKLASH2; Ref. 46) and John Groffen, Children’s Hospital, Los Angeles, CA (CRKL full-length; Ref. 47). The pCDNA3-C3G-CBR vector was kindly provided by Randall D. York and John Groffen, Children’s Hospital, Los Angeles, CA (CRKL full-length; Ref. 47). The pCDNA3-C3G-CBR vector was kindly provided by Randall D. York and Philip J. S. Stork, Portland, OR (7). The pMT2-ΔHA-Rap1 vector is a gift of Fried Zwaritrkus and Johannes L. Bos, Utrecht (53).

pBat-Flag-Gab1 expresses full size Gab1 (mouse) and has been described (40). pBat-Flag-Gab1-DYxxF was constructed in two steps. A polymerase chain reaction fragment encoding amino acids 411 to 695 of Gab1 was cloned into NotI and SalI sites of the pBat-Flag expression vector (40). A cDNA fragment encoding amino acids 1 to 241 of Gab1 was then inserted in-frame with the C-terminal coding sequences using the NotI site.

Cell Culture, Transfection, Stimulation, and Immunoprecipitation—HEK293, 293T, and A431 cells were cultured in Dulbecco’s modified Eagle’s medium with 10% FBS and antibiotics. Active (processed) HGF was made as described previously (48). One unit of HGF (defined by the minimal amount of HGF required to induce scattering of a highly sensitive Madin-Darby canine kidney cell clone) equals 0.5 ng of pro-
tein. Cells were stimulated with HGF as indicated in the figures. Cell lysis was done as described (20) but with a buffer containing only 1% Triton X-100 as detergent (Triton X-100 buffer). Immunoprecipitations were carried out with Triton X-100 buffer lysates overnight and precipitates were washed three times with low stringency buffer (IP buffer: 20 mM Tris 7.5, 100 mM NaCl, 1 mM Na2EDTA, 0.1% Tween 20, 5% glycerol) prior to SDS-PAGE. Calcium phosphate/BES transfection was done as described (49) with 5 μg of DNA per plasmid used. Transfected 293T cells were harvested 24–30 h after the start of the transfection as described below.

**GTPase Assays**—The Rap1 activity assay has been previously described in detail (28, 31). GST-RalGDS-RBD was a gift of B. Franke and J. Bos (31). Briefly, cells were lysed with Nonidet P-40 containing buffer as described (28) and extracted for 30 min, insoluble material was removed by centrifugation. After protein quantification, 1 mg of extracted protein was incubated for 90 min with 20 μg of GST-RalGDS RBD and GSH-Sepharose beads, precipitates were washed 3 times with lysis buffer, and analyzed for bound Rap1 GTP by 12% SDS-PAGE and Western blot with anti-Rap1.

**Replating Assay**—Cells were grown in Dulbecco’s modified Eagle’s medium with 10% FBS until approximately 80% confluent and subsequently maintained in medium with 0.1% FBS, allowing cell survival without significant proliferation. To some wells HGF was added as indicated while control wells were maintained without HGF. After 2 days the culture medium containing floating and loosely attached cells was carefully removed. These cells were washed once with phosphate-buffered saline and replated with medium containing 10% FBS but without HGF to allow viable cells to grow into clusters. Cells were then fixed with 100% methanol, stained for 1 h with Giemsa (Merck, 1.09204), rinsed with water to remove unbound dye, and air dried.

**RESULTS AND DISCUSSION**

***Gab1 Tyrosine Phosphorylation and Formation of Gab1-CRKL Complexes in Response to HGF***—To investigate a potential link between HGF signaling and Crk family adapter proteins, human epithelial kidney (HEK) 293 cells were chosen as a model system. They form epithelial layers similar to the widely used Madin-Darby canine kidney cells and are much easier to transfect. HEK293 cells express both c-Crk and CRKL (20), as well as the HGF receptor c-Met (Fig. 2A). Human epithelial A431 cells which have previously been reported to express c-Met (50, 51) and 100 μg of extracted A431 cell proteins were used as positive control. B, HGF increases the amount of tyrosine-phosphorylated proteins in HEK293 cells in a dose-dependent manner. 50 μg of total Triton X-100 buffer lysates from cells stimulated for 2 min with the amount of HGF indicated were separated by 10% SDS-PAGE and analyzed by Western blot with an anti-phosphotyrosine specific monoclonal antibody (PY20) or probed for Nck (loading control). C, maximum of protein tyrosine phosphorylation through HGF is seen after 3 min. Cells were stimulated with 200 units/ml of HGF for the time indicated and lysed with Triton X-100 buffer and 50 μg of the lysates analyzed as in B.

![Figure 2](http://www.jbc.org/01710774/)

**FIG. 2. Response of HEK293 cells to HGF.** A, HEK293 cells express the c-Met receptor. 500 μg of Triton X-100 buffer lysates from human epithelial cells were immunoprecipitated with anti-c-Met (IgG2a) or the same amount of control immunoglobulin of the same species and isotype (abbreviated as CI; here: anti-epidermal growth factor receptor). 2 μg (middle lanes) or 4 μg (right lanes) of anti-c-Met or control antibody were used. Precipitates were washed 3 times with Triton X-100 buffer, separated by 7% SDS-PAGE, and analyzed by Western blot for c-Met expression. 100 μg of total lysate from 293 cells were also analyzed. Human epithelial A431 cells have been previously described to express c-Met (50, 51) and 100 μg of extracted A431 cell proteins were used as positive control. B, HGF increases the amount of tyrosine-phosphorylated proteins in HEK293 cells in a dose-dependent manner. 50 μg of total Triton X-100 buffer lysates from cells stimulated for 2 min with the amount of HGF indicated were separated by 10% SDS-PAGE and analyzed by Western blot with an anti-phosphotyrosine specific monoclonal antibody (PY20) or probed for Nck (loading control). C, maximum of protein tyrosine phosphorylation through HGF is seen after 3 min. Cells were stimulated with 200 units/ml of HGF for the time indicated and lysed with Triton X-100 buffer and 50 μg of the lysates analyzed as in B.
Endogenous Gab1 isolated by immunoprecipitation with Gab1-antiserum co-precipitated CRKL only after stimulation of serum-starved cells with HGF. This co-precipitation of CRKL was further increased by overexpression of a FLAG-tagged Gab1 protein (Fig. 3B). Endogenous CRKL co-precipitates with endogenous Gab1 and overexpressed Gab1 in an HGF-dependent manner, 1 mg of protein of Triton X-100 buffer lysates from cells transfected with FLAG-tagged Gab1 or control vector were precipitated with anti-Gab1 (4 μg) or control Ig (Cl, anti-GRAP) and protein A-agarose as indicated, washed with Triton X-100 buffer, separated by 9% SDS-PAGE, blotted, and probed with anti-CRKL or anti-Gab1 as indicated. Left small panels show blots of total lysates transfected with FLAG-Gab1 or control vector and Western blotted as indicated.

HGF Activates the Small GTPase Rap1—To analyze the functional consequences of the Gab1-CRKL interaction induced by HGF stimulation of starved cells, downstream effector proteins of CRKL were investigated. One of the best characterized signal transducers of Crk and CRKL is the guanine nucleotide releasing protein C3G, which binds with great selectivity to the SH3(1) domains of Crk and CRKL. C3G is found in constitutive complexes with CRKL in many cell types and is known to activate the GTPase Rap1 in cells and "in vitro." C3G is also constitutively bound to CRKL in HEK293 cells (Fig. 4A). HGF-induced CRKL binding to Gab1 requires the 6-Tyr-X-X-Pro region (amino acids 242–410) of Gab1. HEK293 cells were transfected with FLAG-Gab1, FLAG-Gab1 with a deleted CRKL-SH2-binding region (FLAG-Gab1DYxxP), or control vector, then stimulated and analyzed as described in C. Effect of HGF stimulation on tyrosine phosphorylation of proteins and the expression of endogenous and transfected Gab1 is documented in the left two panels.

HGF Signals via CRKL to Rap1

Fig. 3. CRKL forms complexes with Gab1 after stimulation of HEK293 cells with HGF.

A, Gab1 from HGF-stimulated cells interacts with GST-CRKL in an SH2-dependent manner. Nonstimulated cells or cells stimulated with 200 units/ml HGF for 2 min were lysed with Triton X-100 buffer and lysates precipitated with 20 μg of GSH-Sepharose-immobilized GST, or eqimolar amounts of GST-CRKL or GST-CRKLSH2. Precipitates were washed with Triton X-100 buffer, separated by 7% SDS-PAGE, blotted, and probed with anti-Gab1. B, endogenous CRKL co-precipitates with endogenous Gab1 and overexpressed Gab1 in an HGF-dependent manner, 1 mg of protein of Triton X-100 buffer lysates from cells transfected with FLAG-tagged Gab1 or control vector were precipitated with anti-Gab1 (4 μg) or control Ig (Cl, anti-GRAP) and protein A-agarose as indicated, washed with Triton X-100 buffer, separated by 9% SDS-PAGE, blotted, and probed with anti-CRKL or anti-Gab1 as indicated. Left small panels show blots of total lysates transfected with FLAG-Gab1 or control vector and Western blotted as indicated.

C, Gab1 co-precipitates with CRKL after treatments of cells with HGF. Cells were stimulated with 200 units/ml HGF where indicated, and 100 μg of total lysates or precipitates from 1 mg of Triton X-100 buffer-extracted protein with anti-CRKL (1 μg) or control Ig (Cl, anti-cyclin A) and washed with Triton X-100 buffer separated by 9% SDS-PAGE, blotted, and probed as indicated. Increased phosphotyrosine in proteins after stimulation of cells with HGF is documented in the left panel. Total lysates of nonstimulated and HGF-stimulated cells were also probed for CRKL (bottom panel, two left lanes). D, HGF-induced CRKL binding to Gab1 requires the 6-Tyr-X-X-Pro region (amino acids 242–410) of Gab1. HEK293 cells were transfected with FLAG-Gab1, FLAG-Gab1 with a deleted CRKL-SH2-binding region (FLAG-Gab1DYxxP), or control vector, then stimulated and analyzed as described in C. Effect of HGF stimulation on tyrosine phosphorylation of proteins and the expression of endogenous and transfected Gab1 is documented in the left two panels.
protein (31). Analysis of tissue-derived cells with this assay was previously shown to lead to a 2–4-fold increase of GTP-loaded endogenous Rap1 (28, 54).

Activation of Rap1 was seen with HGF after 3 min, but Rap1 was down-regulated again after 10 min (Fig. 4B). Stimulation of 293 cells with the tyrosine phosphatase inhibitor vanadate/H$_2$O$_2$ also lead to a significant elevation of Rap1$^{z}$GTP. Stimulation of endogenous Rap1 was partly inhibited by the transient transfection of a C3G fragment which contains the Crk/CRKLSH3(1)-binding region of C3G (C3G-CBR; 7) (data not shown). A total inhibition of endogenous Rap1 activity is not expected since not all of the 293 cells are transfected. To observe the Rap1 inhibition effect by C3G-CBR more clearly, HA-tagged Rap1 was co-transfected with C3G-CBR or control vector. HA-Rap1 from transfected cells can then be selectively detected by subsequent anti-HA blotting of precipitated Rap1$^{z}$GTP. HA-Rap1 activation of transfected 293T cells in response to HGF was strongly decreased by C3G-CBR (Fig. 4C). The basal Rap1 activity in nonstimulated cells was also slightly reduced, indicating the importance of C3G for the Rap1 activity in 293 cells.

To determine whether Gab1 is crucial for the HGF-mediated Rap1 activation, nonmutated Gab1, mutant Gab1 with a deleted YXXP-region (Gab1ΔYxxP), or control vector was co-transfected together with HA-tagged Rap1 into 293T cells. As shown in Fig. 5, the empty control vector did not affect the HGF-induced activation of Rap1. Overexpression of wild-type Gab1 prominently increased the basal Rap1 activity to levels comparable to those normally seen after HGF treatment. On the other hand, expression of the Gab1ΔYxxP mutant protein completely abolished the HGF-inducible Rap1 activation. We conclude that the HGF-induced complex formation of Gab1 and CRKL is an important prerequisite for the transmission of signals from HGF to Rap1.

To understand the biological effects of HGF on 293 cells several analyses were performed. Since HGF has been reported to stimulate the proliferation of certain cells, the possibility of a dose-dependent proliferation increase by HGF was investigated. We were unable to observe a marked effect on cell numbers when cells were treated with HGF over a period of several days (data not shown). However, during these experiments it was noted that HGF appeared to reduce the adhesion of HEK293 cells.

To investigate this further, cells maintained in low serum were treated with different doses of HGF for 2 days. Floating and loosely attached cells were then carefully collected with the culture medium and replated into normal 10% serum-containing culture medium in the absence of HGF for 5 to 7 days to...
again allow proliferation and growth of the 293 cells into small clusters. The cells were then fixed and stained with Giemsa for better visibility.

In addition, observation of untreated and HGF-treated 293 cells by video microscopy indicated an increased motility upon treatment with HGF (data not shown). Further studies will be necessary to clarify whether these biological effects of HGF on HEK293 cells depend on CRKL and Rap1 signals.

Crk, CRKL, and their SH2 and SH3 domain-binding proteins (p130Cas, paxillin, C3G, and DOCK180 etc.) have been implicated in the regulation of a number of signaling events including cell proliferation and growth of the 293 cells in a dose-dependent manner. Further studies will be required to determine whether these biological effects of HGF on HEK293 cells depends on CRKL and Rap1 signals.

Crk adapters can signal at least two GTPases, namely Rap1 and Rac. The latter is closely implicated in the regulation of cell migration events (see e.g. Ref. 60 for review). By contrast, little is known about the biological functions of Rap1 and its downstream targets remain largely elusive.

The only present indication that Rap1 is involved in the regulation of cell migration events comes from Drosophila studies with deleted Rap1. These flies display, for example, defects in ventral and dorsal closure of the developing embryo. This may be explained by a failure of specific cells to migrate to their appropriate location in the absence of Rap1 (61).

It will be important to find out whether Rac1 is also activated in a CRKL-dependent manner after HGF stimulation of HEK293 cells and to determine whether Rap1 and Rac signals are somehow interconnected.

In our current study, we were only able to detect the HGF-induced complex formation of CRKL and Gab1. However, a role for the c-Crk proteins cannot be excluded at this point. While the polyclonal CRKL antiserum used in the experiments shown here works well for coimmunoprecipitations and does not cross-react with c-Crk proteins, such a reagent specific for c-Crk still needs to be generated.

Gab1 is also a substrate of other tyrosine kinases, for example, the epidermal growth factor and nerve growth factor receptors and the kinases coupled to the erythropoietin and interleukin-6 receptors (39–43). It remains to be studied whether complexes between Crk family adapters and Gab1 can also form after the activation of other receptor systems.

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Note Added in Proof—Additional results pertaining to HGF signaling via Crk family adapters have recently been published (Garcia-Guzman, M., Dolfi, F., Zeh, K., and Vuori, K. (1999) Oncogene 18, 7775–7786).



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