The β -subunit of the hepatocyte growth factor/scatter factor (HGF/SF) receptor phosphorylates and associates with CrkII: expression of CrkII enhances HGF/SF-induced mitogenesis

Stephen M. RIORDAN, Sukhwinderjit LIDDER, Roger WILLIAMS and George G. SKOUTERIS¹

Laboratory of Cell Biology, Institute of Hepatology, Department of Medicine, Royal Free and University College London Medical School, London WC1E 6HX, U.K.

CrkII, a 40 kDa adaptor possessing a Src homology (SH)2 domain followed by two SH3 domains, although not endowed with catalytic activity, participates in intracellular signalling, presumably by activating the Ras pathway. CrkII was found to be phosphorylated in response to hepatocyte growth factor/scatter factor (HGF/SF) and to associate with the beta-subunit of the HGF receptor (MET). CrkII associated with p^{145/MET} via its SH2 domain. Growth-factor-receptor-bound protein 2 (Grb2) co-immunoprecipitated with CrkII species. By transient transfection of A431 human epidermoid carcinoma cells with wild-type and dominant-negative Grb2 expression constructs lacking either the SH2 or SH3 domains, we have concluded that

Grb2 does not contribute to the 'presentation' of CrkII to p^{145/MET}. Overexpression of wild-type CrkII in A431 cells enhanced HGF/SF-induced proliferation, while a CrkII dominant-negative mutant lacking the SH2 domain prevented a similar proliferating response to HGF/SF. The effect of CrkII on HGF/SF-induced proliferation was also abolished in cells co-expressing CrkII and Son-of-sevenless lacking the guanine exchange domain, suggesting that CrkII is likely to induce cell proliferation partly via the Ras/mitogen-activated protein kinase route.

Key words: tyrosine phosphorylation, mitosis, MET, Grb2.

INTRODUCTION

Crk is a member of the family of adaptor proteins and is the cellular equivalent of an avian retrovirus encoding the oncogene product v-Crk. The cellular equivalents of v-Crk include CrkII, CrkI and CrkL. CrkII is a 40 kDa protein comprised of an Src homology (SH)2 domain located at its N-terminus followed by two SH3 domains [1–2]. CrkI is a 21 kDa protein lacking the second SH3 domain. Both CrkII and CrkI are alternatively spliced forms of a single gene [3–4]. CrkII and CrkL, a 36 kDa Crk-like protein possessing two SH2 domains and one SH3 domain, are substrates of the B-cell antigen receptor [4]. Crk proteins do not contain catalytic domains. However, transformation by v-crk has been reported to increase cellular phosphorylation, probably by modulating the kinase-phosphatase equilibrium in responsive cells [5]. Crk, via its SH3 domains, associates with C3G (a guanine nucleotide exchange factor for Rap-1) and mouse Son-of-sevenless (SoS), also a guanine nucleotide releasing protein, with the subsequent formation of a Crk–C3G complex leading to the activation of c-Jun N-terminal kinase (JNK). Crk-induced JNK activation is believed to play an important role in integrin-mediated signal transduction [6,7]. CrkII has been shown to be an endogenous substrate of both the insulin-like growth factor (IGF)-I and the epidermal growth factor (EGF) receptor tyrosine kinases via its SH2 domain [5,8]. In addition, phosphoinositide 3-kinase (PI-3K), CrkII and c-Cbl were reported to form a quaternary complex with growth-factor-receptor-bound protein 2 (Grb2), and to interact with the colony

stimulating factor 1 receptor tyrosine kinase via the unoccupied SH2 domain of Grb2 [9]. These data support a role for endogenous c-Crk as an adaptor contributing to the integration of signals from receptors to downstream targets.

Hepatocyte growth factor/scatter factor (HGF/SF) induces mitogenesis, scattering, invasion and morphogenesis in a number of endothelial and epithelial cells, including primary hepatocytes [10–11]. The HGF/SF signals are transduced by the HGF receptor (MET), the product of the c-MET oncogene, which is endowed with tyrosine kinase activity [12]. The beta-subunit of MET activates, via tyrosine phosphorylation, a number of transducers and adaptors including phospholipase- γ , PI-3K, Grb2, pp60^{src} and Src homology collagen (Shc) [13]. An important issue is to identify downstream effectors contributing to the integration of signals to a particular phenotype. Endogenous c-Crk is a candidate adaptor, possessing the hallmarks (SH3 domains) to interact with other effectors involved in MET signalling, potentially contributing to the amplification of the initial signal. There are reports suggesting that Crk may contribute to activation of the Ras/mitogen-activated protein kinase (MAPK) pathway in a manner similar to Grb2 [6]. The Ras/MAPK pathway is activated in cells responsive to HGF/SF either by proliferation or scattering [14].

The purpose of the present study was to investigate whether endogenous c-Crk could be a target for phosphorylation in response to HGF/SF. Our data suggest that HGF/SF stimulates tyrosine phosphorylation of CrkII, which associates via its SH2 domain with p^{145/MET}. Grb2 was detected in CrkII immuno-

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FCS, foetal calf serum; Grb2, growth-factor-receptor-bound protein 2; GST, glutathione S-transferase; HGF/SF, hepatocyte growth factor/scatter factor; IGF, insulin-like growth factor; JNK, c-Jun N-terminal kinase; LB, lysis buffer; MAPK, mitogen-activated protein kinase; MET, HGF receptor; NGF, nerve growth factor; PI-3K, phosphoinositide 3-kinase; SH, Src homology; Shc, Src homology collagen; mSoS, mouse Son-of-sevenless.

¹ To whom correspondence should be sent, at present address: Laboratory of Cellular and Molecular Biology, Centre for Hepatology, Royal Free and University College London Medical School, Department of Medicine, Upper third floor, Rowland Hill Street, London NW3 2PF, U.K. (e-mail g.skouteris@rfc.ucl.ac.uk).

precipitates, although Grb2 dominant-negative mutants did not abolish CrkII phosphorylation in response to HGF/SF. Overexpression of CrkII in A431 cells enhanced HGF/SF-induced mitogenesis, thereby establishing CrkII as a docking site for the proliferating signal of HGF/SF.

MATERIALS AND METHODS

Cells and materials

A431 epidermoid carcinoma cells were obtained from the Imperial Cancer Research Fund cell bank (London, U.K.) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) foetal calf serum (FCS; Helena Laboratories, Sunderland, U.K.) in a 5% CO₂-humidified atmosphere. Cells were serum-starved for 2 days and stimulated, where appropriate, with 50 ng/ml of HGF/SF for various times. HGF/SF was a gift from Genentech (San Francisco, CA, U.S.A.). Chemicals were purchased from Sigma Chemical Co. Anti-MET and anti-phosphotyrosine (PY99) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Peroxidase-labelled anti-mouse and anti-rabbit antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, U.S.A.). The anti-(PI-3K) antibody was a gift from the Ludwig Institute for Cancer Research (UCL, London, U.K.) and the anti-Crk antibodies were obtained either from Transduction Laboratories (raised against Crk-SH2) or kindly provided by Dr M. Matsuda (3A8). Radioactive compounds were obtained from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.) and the enhanced chemiluminescence system was purchased from Pierce & Warriner (Chester, U.K.).

Preparation of total lysate, and detergent-soluble and cytoskeletal fractions

Total cell lysate was prepared from subconfluent (90%) serum-starved cells, stimulated or not with HGF/SF, which were harvested in ice-cold PBS and lysed in lysis buffer [LB; 10 mM Tris/HCl (pH 7.4), 1% (v/v) Triton X-100, 0.5% Nonidet P40, 150 mM NaCl, 0.2 mM sodium orthovanadate, 1 mM PMSF, 40 µg/ml leupeptin and 45 µg/ml aprotinin].

Detergent-soluble and -insoluble fractions were prepared by scraping the cells into Hepes buffer containing 25 mM Hepes (pH 7.4), 2 mM MnCl₂, 0.2 mM vanadate, 1% Triton X-100, 1 mM PMSF and protease inhibitors as in LB. Cells were then incubated on ice for 10 min and mixed by inverting the tubes at approximately 30 s intervals during the incubation period. The mixture was centrifuged for 5 min at 15 000 g and the supernatant was defined as the detergent-soluble fraction. The pellets were resuspended in LB buffer and represented the detergent-insoluble or cytoskeletal fraction.

Immunoprecipitation and Western blotting analysis

Cell lysates were supplemented with the appropriate antibody and incubated at 4 °C for 2 h and for a further 30 min with Protein A-Sepharose (Roche Diagnostics, Lewes, East Sussex, U.K.). Complexes were washed three times with LB lacking protease inhibitors, and protein species were then eluted by heating in 4 × Laemmli sample buffer [200 mM Tris/HCl (pH 6.8), 8% SDS, 400 mM dithiothreitol, 0.4% Bromophenol Blue and 40% (v/v) glycerol]. Eluted proteins were immunoblotted and probed with anti-MET, anti-Crk or anti-phosphotyrosine antibodies. Reactive species were visualized using an enhanced chemiluminescence kit. Blots, for further probing, were stripped

in 62.5 mM Tris/HCl (pH 6.7) containing 2% (w/v) SDS and 100 mM 2-mercaptoethanol, for 30 min at 50 °C.

Purification of recombinant proteins and *in vitro* association

Crk cDNAs subcloned into the pGEX-2T vector were expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* and purified as previously described [15]. GST-Grb2, GST-P49LGrb2 (SH3 mutant) and GST-R36KGrb2 (SH2 mutant) fusion proteins were purified as previously described [16]. Lysate protein (1–1.5 mg) was incubated with GST-Crk fusion proteins for 2 h at 4 °C. Beads (GSH-Sepharose 4B) were collected and washed three times with LB. Bound species were released by heating in 4 × Laemmli sample buffer, followed by SDS/PAGE and immunoblotting analysis.

Transfection and establishment of A431 cell lines

Dominant negative SH2/SH3 Grb2 expression-constructs were generated by mutating the conserved arginine of the FLVRES sequence within the SH2 domain to lysine [17]. The tryptophan within the tryptophan doublet of the SH3 domain was also mutated to lysine. The capacity of mutants to eliminate ligand binding of the mutated domain without interfering with the binding capacity of the unmutated domain was assayed as previously described [17]. Wild-type or dominant-negative Grb2 constructs were transiently imported into A431 cells (see below) and after transfection the cells were serum-starved for 36 h and stimulated with 50 ng/ml of HGF/SF for 15 min prior to lysis in LB. Protein expression of the transfected dominant-negative Grb2 was examined by immunoblotting, using anti-Grb2 specific antibodies (Transduction Laboratories), and similar levels of expression of the Grb2 mutants were confirmed (results not shown). Lysates from transfected cells were then immunoprecipitated with anti-Grb2 and immunoblotted as described above.

A431 cell lines expressing exogenous wild-type or dominant-negative CrkII constructs (see Figure 6) were generated by transfecting cells using the calcium phosphate methodology, followed by selection in geneticin (500 µg/ml) [18]. A431 cells were transfected with CrkII, together with either full-length mSoS or a mutant mSoS1 lacking the guanine nucleotide exchange domain (ΔSoS). Stable transfectants were isolated as previously described [19]. Expression of exogenous CrkII and SoS was then confirmed by immunoblotting with anti-Crk and anti-SoS antibodies. Cell lines were stimulated and processed as described in the Figure legends.

Proliferation assay

A431 parental or CrkII overexpressing cells were grown in monolayers in multi-well plates and serum-starved for 48 h. The monolayers were then treated with various concentrations of HGF/SF (10–100 ng/ml) in DMEM supplemented with 2 µCi/well of [³H]thymidine, incubated for 2 h and then rinsed with ice-cold PBS/5% (w/v) trichloroacetic acid followed by 95% ethanol. Cells were then lysed and solubilized samples were subsequently counted for radioactivity.

RESULTS

Endogenous Crk is phosphorylated by HGF/SF and associates with the MET beta-subunit

A431 cells express significant levels of Crk and respond to HGF/SF by proliferation and scattering [20]. HGF/SF (50 ng/

Figure 1 HGF/SF induces tyrosine phosphorylation of endogenous Crk, which associates with the beta-subunit of MET

Cell lysates from serum-starved and HGF/SF-stimulated (50 ng/ml) A431 cells were immunoprecipitated (IP) with anti-Crk antibody and reactive species were resolved by SDS/PAGE and immunoblotted with anti-phosphotyrosine (PY99) antibody (A). The blot shown in (A) was stripped and re-probed with either anti-Crk (B) or anti-MET (C) antibodies. Immunoprecipitated MET species from A431 cells stimulated with HGF/SF for various times were collected in Protein A-Sepharose beads. Immune complex kinase assays were performed in the presence of 10 μ Ci of [γ - 32 P]ATP for 20 min at room temperature. 4 $\times$ Laemmli sample buffer was then added. Samples were heated at 95 $^{\circ}$ C and were subsequently analysed by SDS/PAGE (D). One-half of the boiled samples was immunoblotted and probed with anti-MET (E). Ab, antibody.

ml) stimulated phosphorylation of two major protein species in Crk immunoprecipitates probed with anti-phosphotyrosine antibody (Figures 1A–1C). One migrated at approximately 150 kDa and the other at 40/42 kDa, corresponding to the molecular sizes of the beta-subunit of MET and CrkII respectively (Figure 1A). Tyrosine phosphorylation of p $^{145/\text{MET}}$ peaked at 30 min, whereas Crk phosphorylation was significant up to 3 h after the addition of HGF/SF (Figure 1A). Re-probing the blot with anti-Crk antibody (Transduction Laboratories) revealed that Crk immunoprecipitates contained similar amounts of Crk (Figure 1B).

Stripping and re-probing the blot in Figure 1(A) with anti-MET antibodies revealed that the 145 kDa phosphorylated band was recognized by anti-MET and corresponded to the beta-subunit of MET. The MET beta-subunit therefore co-immunoprecipitated with Crk species (Figures 1A and 1C). Maximal association of p $^{145/\text{MET}}$ with CrkII was detected at 60 min post-stimulation (Figure 1C).

We also determined the time course of MET phosphorylation, by performing immune complex kinase assays. MET species from A431 cells treated for various times with HGF/SF were immunoprecipitated with anti-MET and the kinase activity was assayed in the presence of [γ - 32 P]ATP (Figure 1D). MET was maximally phosphorylated 30–60 min after HGF/SF addition. Equal content was confirmed by immunoblotting and probing the immune complex kinase assays with anti-MET (Figures 1D and 1E).

Crk-SH2 associates with the beta-subunit of MET

The role of the individual SH2/SH3 Crk domains in mediating the interaction with the beta-subunit of MET was then investigated (Figures 2A and 2D). CrkII proteins contain one SH2 domain located at the N-terminus and two SH3 domains (N-SH3

Figure 2 GST-SH2-Crk binds p $^{145/\text{MET}}$, and Grb2 co-immunoprecipitates with Crk

GST-Crk fusion proteins illustrated in the lower panel (D) were prepared as described in the Materials and methods section. Wild-type CrkII contains one SH2 domain and two SH3 domains. GST-CrkII proteins, namely v-Crk, c-Crk, SH2-Crk, CrkII lacking the SH2 domain (2R38K), N-SH3-Crk and CrkII lacking the N-SH3 domain (2W170K), were reacted with cell lysates from A431 cells either treated or not treated for 15 min with HGF/SF (50 ng/ml). Reacting species were eluted in 4 $\times$ Laemmli sample buffer, separated by SDS/PAGE and immunoblotted with anti-MET antibody (A). A A431 lysate (100 μ g) not brought into reaction with GST proteins is also included in the panel (A431). The blot shown in (A) was stripped and re-probed with anti-Grb2 antibody (B). To identify whether Crk associated with Grb2, the blot shown in Figure 1(A) was stripped and re-probed with anti-Grb2 antibody (C). wt, wild-type.

and C-SH3) (Figure 2D). GST-Crk fusion proteins reacted with lysates from A431 cells, stimulated or not with HGF/SF, were then probed with anti-MET antibody. GST-v-Crk, GST-c-Crk and GST-SH2-Crk, but not N-SH3-Crk, were found to bind p $^{145/\text{MET}}$ (Figure 2A). In lysates from HGF/SF-stimulated cells, v-Crk bound p $^{145/\text{MET}}$ much more strongly than either c-Crk or SH2-Crk. GST-v-Crk is reported to bind phosphotyrosine motifs with higher affinity than does GST-c-Crk [8,15].

It was next addressed whether Grb2 could bind Crk species *in vitro*, because the Grb2-Shc complex is known to interact with the beta-subunit of MET and to recruit other effectors via its SH3 flanking domains. In addition, Crk proteins in PC12 cells have been found to associate with Shc [9]. To test this, binding reactions were carried out using GST-Crk proteins reacted with HGF/SF-treated or -untreated lysates and immunoblotted with anti-Grb2. We were able to detect a clear association of SH3-Crk with Grb2 (Figure 2B). However, GST-v-Crk and GST-c-Crk as well as SH2-Crk were found not to bind Grb2 in multiple experiments using GST-v-Crk and GST-c-Crk originating from two different laboratories. This discrepancy in Grb2 binding by the full-length GST-Crk is currently under further investigation.

Figure 3 Effects of cytochalasin D and wortmannin on HGF/SF-induced Crk phosphorylation

A431 cells were serum-starved and pretreated with either cytochalasin D (1–5 μ M) or wortmannin (5 nM and 50 nM) as described in the Materials and methods section. HGF/SF (50 ng/ml) was then added for 15 min and lysates were prepared and immunoprecipitated (IP) with anti-Crk antibody. Immunoblotted species were then probed with PY99 (**A**) and, after stripping, with anti-Crk (**B**). Lane 2 represents phosphorylated Crk species from A431 cells treated with 50 ng/ml HGF/SF, whereas in lane 5 HGF/SF was added at 100 ng/ml. The autoradiogram shown in (**A**) was quantified by densitometric scanning of the bands recognized by PY99. Values shown represent the means $\pm$ S.E.M. of three independent experiments and are expressed as fold increases in tyrosine phosphorylation above control (lane 1) (**C**). Cells were pretreated either with cytochalasin D (Cyto D; 1–5 μ M) or with LY294002 (2–25 μ M) and lysates were immunoprecipitated with anti-Crk and probed with PY99 (**D** and **E**). Phosphorylated bands were quantified and are expressed as fold increases in tyrosine phosphorylation above control (**A**, lane 1). Positive and negative controls for (**E**) are shown in (**A**, lanes 1 and 2).

We then stripped and re-probed the blot shown in Figure 1(A), with anti-Grb2 antibody (Figure 2C). Grb2 was detected in Crk immunoprecipitates and association of Grb2 with Crk was dependent on HGF/SF treatment. We suggest that Grb2 and Crk associate *in vivo* and that their association reaches maximum levels 30–60 min post-stimulation with HGF/SF (Figure 2C).

Cytochalasin D and wortmannin do not affect HGF/SF-induced phosphorylation of Crk

PI-3K is activated upon HGF/SF stimulation and integrates the proliferating and scattering signal of HGF/SF. A431 cells were treated with wortmannin, a fungal metabolite, which at nanomolar concentrations inhibits PI-3K catalytic activity [21]. The cells were preincubated for 2 h with wortmannin (5 or 50 nM). HGF/SF was then added for 15 min (Figure 3A).

As revealed by densitometric scanning (Figure 3C), 5 nM wortmannin did not affect CrkII phosphorylation, whereas

Figure 4 Crk species are associated with the detergent-insoluble cell fraction

Detergent-soluble and insoluble fractions of A431 cells, stimulated or not with HGF/SF (50 ng/ml) for the indicated times, were prepared as described in the Materials and methods section. All the detergent-soluble and insoluble fractions were resolved by SDS/PAGE and were immunoblotted with the anti-Crk antibody. Arrows indicate Crk species.

50 nM wortmannin increased CrkII phosphorylation. CrkII in several cell species appears as a 40/42 doublet. In our model CrkII also appeared as a doublet (Figure 1B). The gel-shift observed in Figure 3(A) (lanes 4 and 6) may be assigned to deficient phosphorylation of the 42 kDa subtype. Re-probing of the Figure 3(A) blot with anti-Crk antibody confirmed that the immunoprecipitates contained similar amounts of CrkII (Figure 3B).

To explore the potential involvement of PI-3K further, another set of A431 cultures was treated with LY294002, a specific inhibitor of PI-3K, at 2–25 μ M in the presence of HGF/SF. Crk immunoprecipitates were immunoblotted and probed with the anti-phosphotyrosine antibody. Densitometric scanning of the phosphorylated Crk species showed that LY294002 did not affect HGF/SF-induced phosphorylation of Crk (Figure 3E), suggesting that PI-3K does not lie upstream in the pathway linking MET with CrkII.

Cytochalasin D, which inhibits actin polymerization, was used to investigate whether disruption of the cytoskeleton could affect tyrosine phosphorylation of CrkII in response to HGF/SF (Figure 3D). Cytochalasin D, at 1–2 μ M concentration, causes disruption of the F-actin cytoskeleton in A431 cells. At 1 μ M, cytochalasin D did not seem to affect tyrosine phosphorylation of Crk (Figure 3A, lane 3). However, cytochalasin D at concentrations $> 2 \mu$ M caused a significant decrease in tyrosine phosphorylation of CrkII (Figures 3A, 3C and 3D). This effect is illustrated in Figure 3(D), in which A431 lysates from cells treated with HGF/SF and cytochalasin D at 1–5 μ M were immunoprecipitated with anti-Crk and immunoblotted with the anti-phosphotyrosine antibody. The revealed Crk phosphorylated species underwent densitometric scanning and values were expressed as fold increases in tyrosine phosphorylation compared with unstimulated immunoprecipitates (Figure 3D).

We then determined the distribution of Crk species in the detergent-soluble and detergent-insoluble cellular fractions (Figure 4). Most of the Crk was found to associate with the detergent-soluble fraction. Association of CrkII with both fractions was constitutive and no substantial translocation from one compartment to the other was observed following stimulation with HGF/SF. Densitometric scanning indicated that approximately 10% of CrkII was associated with the detergent-insoluble fraction prior to HGF/SF stimulation. Following HGF/SF stimulation, this proportion averaged 18%.

Figure 5 Effect of wild-type and dominant-negative Grb2 expression on CrkII phosphorylation in response to HGF/SF

(A) A431 cells were transiently transfected with Grb2 constructs (see the Materials and methods section) and after starvation were stimulated with HGF/SF (50 ng/ml) for 15 min. Grb2 constructs included wild-type Grb2 (wt), Grb2 lacking the SH2 domain (R36K), Grb2 lacking both SH3 domains (W36193K), Grb2 lacking N-SH3 (W36K) and Grb2 lacking C-SH3 (W193K). Cell lysates were immunoprecipitated (IP) with anti-Crk and immunoblotted with PY99. Under Grb2 IP, percentage phosphorylation of Crk is shown in lysates from Grb2-transfected (wt or R36K) A431 cells immunoprecipitated with anti-Crk and immunoblotted with PY99. Bars represent means $\pm$ S.E.M. of Crk2 phosphorylation of at least two identical, independent, experiments. Quantification of tyrosine phosphorylation was carried out by scanning densitometry. Values are expressed as percentages of the maximum response (untransfected A431 cells treated for 15 min with HGF/SF). Panel (B) shows a representative autoradiograph illustrating the results analysed in (A) under Crk IP; 5 and 15 represent the number of min in the presence of HGF/SF (control untransfected A431 cells). (C) A representative blot of Grb2 immunoprecipitates probed with PY99, from cells treated as in panel (A). Ptyr, phosphotyrosine.

Expression of dominant-negative Grb2 does not alter Crk tyrosine phosphorylation in response to HGF/SF

The finding showing co-immunoprecipitation of Crk and Grb2, as well as the *in vitro* association of N-SH3-Crk with Grb2, prompted us to investigate the potential of Grb2 to mediate Crk recruitment to p^{145/MET}. For this purpose, dominant-negative Grb2 expression mutants were used, lacking either the SH2 or SH3 domains (Figure 5). A431 cells were transiently transfected with the wild-type or dominant-negative Grb2 constructs, serum-starved and stimulated with HGF/SF (50 ng/ml). Cell lysates were immunoprecipitated with anti-Crk antibody, separated by SDS/PAGE, and immunoblotted with the anti-phosphotyrosine antibody (Figures 5A and 5B). The autoradiographs were scanned and Crk phosphorylation was expressed as the per-

Figure 6 [³H]Thymidine incorporation into DNA of A431 cells transfected with CrkII constructs

A431 cells were stably transfected with wild-type or dominant-negative CrkII constructs and stimulated with HGF/SF. Incorporation of [³H]thymidine was measured as described in the Materials and methods section. DNA synthesis is expressed as c.p.m./well and bars represent means $\pm$ S.E.M. from at least two independent experiments. (A) Bar 1, parental A431 cells treated with 50 ng/ml HGF/SF; bar 2, parental A431 cells treated with 10% (v/v) FCS; bar 3, A431 cells treated as in bar 1 in the presence of anti-HGF monoclonal antibody (1 mg/ml). Bars 4–7 represent values of DNA synthesis in A431 cells treated with 50 ng/ml of HGF/SF, transfected with wt CrkII (bar 4), with CrkII lacking the SH2 domain (2R38K) (bar 5), with CrkII lacking the N-SH3 domain (2W170K) (bar 6) and with CrkII lacking the C-SH3 domain (2W276K) (bar 7). Bars 8–11 represent DNA synthesis in A431 cells transfected with Crk constructs (see bars 4–7) and stimulated with 50 ng/ml HGF/SF in the presence of anti-HGF antibody (1 mg/ml). (B) Bar 12, parental A431 cells treated with 100 ng/ml HGF/SF. Bars 13–16 represent values of DNA synthesis in A431 cells transfected with CrkII wt and dominant-negative constructs and treated with 100 ng/ml of HGF/SF. Bar 13, A431 expressing wt CrkII; bar 14, A431 expressing 2R38K; bar 15, A431 expressing 2W170K; bar 16, A431 expressing 2W276K. Bar 17 represents DNA synthesis in parental A431 cells treated as in bar 12 in the presence of anti-HGF antibody (as in Figure 3A).

centage of that observed with HGF/SF-stimulated untransfected A431 cells. As shown in Figure 5(A), Crk phosphorylation in Grb2 transient-transfectants was not significantly altered compared with untransfected and HGF/SF-stimulated cells. This suggested that Grb2 binding to the beta-subunit of MET via its SH2 domain was not a prerequisite for CrkII phosphorylation. Re-probing with anti-Crk confirmed similar amounts of Crk in the immunoprecipitates (results not shown).

Figure 5(C) shows Grb2 immunoprecipitates from cells transfected with R36KGrb2 (mutant lacking the SH2 domain), stimulated with HGF/SF and immunoblotted with anti-MET antibody. These immunoprecipitates failed to show association of Grb2 with MET species (results not shown). This result confirmed that the SH2-Grb2 domain is required for association

Figure 7 Dominant-negative SoS counteracts the effect of CrkII in HGF/SF-induced proliferation

An A431/CrkII-expressing cell line was transfected with wild-type SoS or dominant-negative ΔSoS and stimulated with 10–100 ng/ml of HGF/SF. Lysates were immunoprecipitated (IP) with anti-SoS and were also immunoblotted with anti-SoS to confirm expression of the constructs (A). The blot shown in (A) was stripped and re-probed with anti-Grb2 (B). Lysates from A431/CrkII or A431/SoS and A431/ΔSoS cell lines were immunoprecipitated with anti-MET and probed with PY99 (C). A431 parental or cells expressing CrkII were transfected with either SoS or ΔSoS. The cell lines were stimulated with HGF/SF and pulse-labelled with [³H]thymidine, as described in the legend to Figure 6. DNA synthesis is expressed as c.p.m./well and bars represent means ± S.E.M. from at least three independent experiments (D). Bar 1, A431 parental cells stimulated with 50 ng/ml of HGF/SF; bar 2, A431/CrkII treated with 50 ng/ml of HGF/SF; bars 3 and 4, A431 parental and A431/CrkII expressing cells treated with 100 ng/ml of HGF/SF; bars 5 and 6, A431/SoS and A431/CrkII/SoS cells treated with 100 ng/ml of HGF/SF; bar 7, A431/CrkII/ΔSoS cells treated with 100 ng/ml HGF/SF; bar 8, A431/CrkII/ΔSoS cells treated with 50 ng/ml HGF/SF; bar 9, A431/ΔSoS cells treated with 100 ng/ml HGF/SF. amSoS, anti-mSoS immunoprecipitates.

with MET. On the other hand, in Grb2 immunoprecipitates from A431 cells transfected with Grb2 constructs lacking either N- or C-SH3-Grb2, Grb2 was found to associate with MET (results not shown). The R36K mutant also inhibited phosphorylation of endogenous Grb2 in response to HGF/SF, further confirming that the SH2 domain was necessary for interaction with p¹⁴⁵^{MET} (Figure 5C).

Overexpression of CrkII enhances HGF/SF-induced mitogenesis

The effect of exogenous CrkII expression on A431 proliferation in response to HGF/SF was then explored (Figure 6). A431 cells were transfected with either wild-type or dominant-negative CrkII mutants and after selection stable cell lines were generated. Cells were serum-starved for 48 h, treated with various amounts of HGF/SF (10–100 ng/ml) and then pulse-labelled with [³H]thymidine (Figures 6A and 6B). Stable CrkII transfectants were shown to exhibit increased proliferation compared with their untransfected counterparts, whereas 2R38K/A431 transfectants (Crk mutant lacking the SH2 domain) exhibited lower rates of proliferation in response to HGF/SF (Figure 6A). The Crk effect was dependent on HGF/SF, as shown by the presence of anti-HGF antibody in the medium (Figure 6A, bars 8–11).

This effect was further shown when HGF/SF was added at 100 ng/ml (Figure 6B). At this concentration, HGF/SF was able to induce proliferation of A431 CrkII-transfectants at approximately 1.6-fold compared with untransfected cells (Figure 6A, bars 8 and 9). These data suggested that overexpression of CrkII positively affected the HGF/SF-induced mitogenesis and established CrkII as a mediator of the HGF/SF proliferating signal. Similarly, the Crk effect was also dependent on HGF/SF.

On the other hand, A431 cells overexpressing CrkII lacking either N-SH3 or C-SH3 showed levels of [³H]thymidine incorporation lower than those observed in untransfected and HGF/SF-stimulated counterparts.

Dominant-negative SoS inhibits CrkII-induced proliferation in response to HGF/SF

In this experimental line, the involvement of Grb2–SoS in CrkII-dependent proliferation of A431 cells was investigated. For this purpose, a full length mSoS expression construct and a mutant lacking the guanine nucleotide exchange domain of mSoS were imported into CrkII-expressing A431 cells. The ΔSoS mutant appeared to act in a dominant-negative manner and to decrease guanine nucleotide exchange activity towards Ras in Chinese Hamster Ovary cells expressing the insulin receptor [19]. Stable cell lines were generated and expression of exogenous SoS was verified by immunoblotting anti-SoS immunoprecipitates with anti-SoS (Figure 7A). Both full-length mSoS and ΔSoS were able to associate with Grb2, suggesting that deletion of the guanine nucleotide exchange domain did not abolish binding of the Grb2 SH3 domains with the proline-rich C-terminal region of SoS (Figure 7B) [36,37]. Lysates from CrkII/SoS or CrkII/ΔSoS cells stimulated with HGF/SF were then immunoprecipitated with anti-MET and immunoblotted with anti-phosphotyrosine antibodies (Figure 7C). Expression of either full-length SoS or ΔSoS did not modify tyrosine phosphorylation of the MET receptor.

The proliferating capacity of the CrkII/SoS and CrkII/ΔSoS cells in response to HGF/SF was then examined. Cell proliferation in HGF/SF-stimulated CrkII/SoS cells was not significantly induced compared with their CrkII-expressing counterparts (Figure 7D, bars 4 and 6). However, expression of SoS in parental A431 cells increased the proliferating response to HGF/SF (Figure 7D, bar 5).

On the other hand, expression of ΔSoS inhibited HGF/SF-induced proliferation in both CrkII-overexpressing cells and their normal counterparts (Figure 7D, bars 7–9).

DISCUSSION

The C-terminus of the beta-subunit of MET contains a multifunctional docking site extended within the Y¹³⁴⁹VHVNATY¹³⁵⁶ VNV motif [22], also conserved in the MET-related receptors, Sea and Ron [23]. We have identified CrkII as an adaptor phosphorylated on tyrosine after HGF/SF stimulation and further explored whether phosphotyrosine residues within the beta-subunit of the MET receptor could mediate interactions with CrkII, via its SH2 domain. CrkII was found to co-immunoprecipitate with p¹⁴⁵^{MET}, and in binding assays Crk-SH2 was found to associate with the beta-subunit. The preferred phosphotyrosine recognition motif of the Crk-SH2 domain is YXXP [24] and MET contains at least two such motifs (Y¹¹QLP⁴⁴ and Y⁶⁵⁴VDP⁶⁵⁷) for potential interaction.

Grb2 binds preferentially to the Y¹³⁵⁶VNV motif of the MET docking site and mediates activation of MAPK via SoS and Ras [25–27]. Grb2 co-precipitated with CrkII in a phosphorylation-dependent manner and the GST–Crk–N-SH3 fusion protein was

found to bind Grb2 species in a phosphorylation-independent manner. We have been unable to determine whether Crk-N-SH3 formed a complex by binding Grb2 individually or through the proline-rich sequences within the Grb2-SoS complex. Similar interactions between Crk-SH3 and a conserved proline-rich motif were reported in EGF receptor substrate 15 ('Eps15') [28]. In both Crk immunoprecipitates and in binding assays using GST-Crk fusion proteins, SoS species were found to associate with Crk and N-SH3-Crk in a manner identical to that observed for Grb2 (results not shown).

Phosphorylation of Crk by $p^{145/\text{MET}}$ could be justified via interaction of N-SH3-Crk with Grb2, known to be recruited to the MET docking site via its SH2 domain. We explored this possibility by transiently transfecting A431 cells with wild-type and dominant-negative Grb2 expression constructs. Overexpression of either wild-type Grb2 or Grb2 lacking SH2 or SH3 domains in A431 cells did not alter CrkII phosphorylation in response to HGF/SF. These data rule out the possibility that Grb2 mediates Crk recruitment to the MET beta-subunit. We speculate that SH3-Crk binds to the Grb2-SoS complex and contributes to the activation of the Ras/MAPK pathway in a similar way to that for Grb2. Binding of Grb2-SH2 to phosphotyrosine motifs is reported not to modify the binding affinity of the Grb2-SH3 domains to SoS (non-allosteric coupling) [29,30]. However, it has been suggested that the SH2 and the SH3 domains of Crk may operate interdependently, thus facilitating new interactions in which the adjacent domain may participate [28]. As a result of such regulation, it is possible that binding of Crk-SH3 to Grb2 may facilitate a subsequent interaction between Crk-SH2 and phosphotyrosine motifs within the MET domain.

Stimulation of A431 cells expressing exogenous wild-type CrkII or dominant-negative CrkII mutants with HGF/SF has revealed that CrkII enhances the proliferating response to HGF/SF. Other reports have shown that Crk is a positive effector of IGF-I-induced mitogenesis via interaction with insulin receptor substrate-1 and 4PS [8]. v-Crk was also implicated in prolonged Ras/MAPK activation due to nerve growth factor (NGF) stimulation, promoting an increased rate of NGF receptor internalization, with preferential association of v-Crk with cytoskeletal components [31]. A proportion of CrkII was found to localize in the detergent-insoluble fraction in our studies, implying that CrkII may integrate MET signals to downstream effectors located in the cytoskeleton. Others have suggested that the significant amount of v-Crk present in the cytoskeletal fraction is correlated with translocation of p130 Crk-associated substrate to this compartment and is dependent upon the integrity of Crk-SH2 [32]. The importance of the Crk-SH2 domain was further substantiated in studies using a dominant-negative v-crk SH2 mutant which prevented interaction of v-Crk with the F-actin and microtubule network, resulting in inhibition of neurite elongation [31].

We suggest that CrkII is a mediator of the HGF/SF proliferating signal and that its association with Grb2 may constitute a step that amplifies activation of the Ras/MAPK pathway, given our finding that dominant-negative SoS was able to revert CrkII-induced DNA synthesis in response to HGF/SF.

Another major pathway shown to be essential for trans-formation by the MET oncogene is operated via activation of JNK in a Grb2-SH3-dependent manner [33]. Crk seems to play an important role in the activation of JNK, as Crk-SH2 or Crk-SH3 dominant-negative mutants are able to block growth factor-induced activation of JNK [34]. Crk participates in the activation of JNK via a mechanism that involves binding to DOCK180, and leads to increased GTP-bound Rac1 [35]. Therefore it seems that Crk is an important effector involved in the amplification of

initiatory growth factor signals. Potential sites of interaction between SH2-Crk and $p^{145/\text{MET}}$ are currently under investigation in our laboratory.

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