Jpn. J. Cancer Res. **90**, 1096–1103, October 1999

Epidermal Growth Factor-dependent Dissociation of CrkII Proto-oncogene Product from the Epidermal Growth Factor Receptor in Human Glioma Cells

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Human glioma cells frequently overexpress epidermal growth factor receptor (EGFR). We found that the CrkII proto-oncogene product was associated with the EGFR in human glioma cells in the absence of epidermal growth factor (EGF). EGF stimulation of glioma cells induced the phosphorylation of tyrosine 221 of the CrkII protein, which correlates with its dissociation from the EGFR. By contrast, Shc and Grb2 were inducibly associated with the EGFR in response to EGF stimulation of glioma cells. In A431 cells, epidermoid carcinoma cells which overexpress EGFR, CrkII was tyrosine-phosphorylated and associated with the EGFR in an EGF-dependent manner. Therefore, the dissociation of CrkII from the EGFR upon stimulation with EGF appears to be specific to glioma cells. The Cbl oncogene product was also tyrosine-phosphorylated in U87MG glioma cells upon EGF stimulation. However, unlike in other cell lines, CrkII was not inducibly bound to Cbl in U87MG glioma cells. Thus, EGF-dependent binding of CrkII to phosphotyrosine-containing proteins appears to be suppressed in glioma cells. To evaluate the physiological role of dissociation of CrkII from EGFR, we expressed the CrkII-23 mutant in glioma cells. CrkII-23 mutant, which was isolated as a suppressor gene of the EGF-dependent transformation of NRK cells, binds constitutively to EGFR. We found that expression of CrkII-23 inhibited the anchorage-independent growth of the glioma cells in the presence of EGF. Taken together, these data implicate EGF-dependent dissociation of CrkII from EGFR in the oncogenicity of human glioma cells.

Key words: Adaptor protein — Crk — Epidermal growth factor — Glioma — Tyrosine kinase

The rearrangement and amplification of several oncogene products and growth factor receptors including H-Ras, Src, Fos, c-Myc, platelet-derived growth factor receptor, and epidermal growth factor receptor (EGFR) have been reported in human glioma cell lines.[1, 2] EGFR abnormality is found in 40% of glioblastomas. It has been reported that the rearrangement of the EGFR correlates with poor prognosis of the patients bearing glioblastoma.[1, 2] Deletion of the extracellular domain of the EGFR stimulates the catalytic activity of EGFR tyrosine kinase and promotes tumor formation.[3] Studies with both glioma cell lines and primary tumor tissues strongly suggest that the EGFR function as an important autocrine loop supporting proliferation of human glioma, especially in high-grade glioma.[4]

v-Crk was identified originally as an oncogene product of a chicken retrovirus, CT10, and is an adaptor protein that consists mostly of Src Homology (SH) 2 and SH3 domains.[5] By alternative splicing, the human and rat *crk* genes yield two forms of mRNA that are translated to the CrkI and CrkII proteins, respectively.[6, 7] The CrkII protein is expressed ubiquitously from embryo to adult, suggesting a fundamental role for CrkII.[8] Activation of several cell surface receptors, including EGFR, induces SH2 domain-mediated binding of CrkII to phosphotyrosine-containing proteins. These include EGFR, platelet-derived growth factor receptor, TrkA, c-Cbl, Shc, PLC-γ, paxillin, and $p130^{Cas}$ [reviewed in ref. 9]. The amino-terminal SH3 domain of Crk binds to proteins of 180, 170, 160, and 135–145 kDa.[10, 11] The principal binding proteins are the 135–145 kDa C3G, a guanine nucleotide exchange protein for Rap1,[12, 13] and the 180 kDa DOCK180 protein.[14]

We previously reported the isolation of an NRK cell mutant, NRK23.[6] NRK23 can be transformed by most oncogenes except v-*erb*B, which encodes a truncated form of the EGFR, suggesting that a signaling mechanism specific to EGFR is impaired in NRK23 cells. Further study revealed that two mutations in one allele of the *crk* gene caused the transformation-defectiveness in NRK23 cells. The mutant CrkII protein, designated as CrkII-23, but not the wild-type CrkII, associates with the EGFR in the

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absence of epidermal growth factor (EGF). This result suggests that constitutive binding of CrkII to EGFR may inhibit the transformation of NRK cells.[15]

To study the involvement of CrkII in the development of human cancer, we utilized human glioma cell lines, which often exhibit rearrangement and activation of EGFR.[16] We found that CrkII constitutively binds to EGFR in human glioma cells and dissociates from EGFR upon EGF stimulation.

MATERIALS AND METHODS

Cell lines All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), unless otherwise described. U87MG (ATCC HTB-14), T98G (ATCC CRL-1690), U118MG (ATCC CRL-7712), U138MG (ATCC HTB-16), U251MG, U373MG (ATCC HTB-17), A172 (ATCC CRL-1620), and KG-1-C (JCRB 0236) are derived from human glioma cells. A431 (ATCC CRL-1555) and DLD-1 (JCRB 9094) are derived from epidermoid carcinoma and adenocarcinoma, respectively, and are known to overexpress EGFR. GOTO (JCRB 0612) and colo 201 (JCRB 0226) are derived from neuroblastoma and carcinoid, respectively, and are used as cell lines that lack the expression of EGFR.

Antibodies The anti-CrkII monoclonal antibody, 3A8, and the anti-phospho-CrkII antibody were previously described.[17,18] The anti-Crk monoclonal antibody, horseradish peroxidase-conjugated anti-phosphotyrosine antibody RC20, and anti-p130^{Cas} antibody were purchased from Transduction Laboratories (Lexington, KY). The monoclonal antibody against EGFR was from MBL (Nagoya); anti-EGFR polyclonal antibody was from Amersham (Buckinghamshire, England). The anti-c-Cbl was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

EGF stimulation and immunoblotting analysis Cells were serum-starved overnight, incubated with or without 50 ng/ml EGF (Amersham) at 37°C for 3 min, lysed with lysis buffer (10 m*M* Tris-HCl [pH 7.5], 5 m*M* EDTA, 150 m*M* NaCl, 1% Triton X-100, 0.5 m*M* Na_3VO_4, 10 m*M* NaF), and centrifuged for 20 min at 15,000*g*. Protein concentrations were determined with a BCA kit (Pierce, Rockford, IL). Equal amounts of the cleared cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 3% bovine serum albumin (BSA) and probed with primary antibodies, followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Then, bound antibodies were visualized by using the ECL chemiluminescence system (Amersham).

Immunoprecipitation experiments Equal amounts of cell lysates were incubated with a mixture of protein A-Sepharose, protein G-Sepharose (Pharmacia Biotech, Tokyo), and various antibodies at 4°C for 30 min. For the immunoprecipitation with anti-phospho-CrkII antibody, cell lysates were adjusted to 1% SDS, denatured at 95°C for 3 min, diluted ten times with the lysis buffer, and incubated with anti-phospho-CrkII antibody. The immune complexes were separated by SDS-PAGE and analyzed by immunoblotting as described above.

Expression of CrkII and CrkII-23 in glioma cells pCXN2-CrkII and pCXN2-CrkII-23 encode the wild-type rat CrkII and CrkII-23 mutants, respectively.[15] U373MG and T98G cells were transfected with pCXN2-CrkII or pCXN2-CrkII-23 by the use of Lipofectamine (Gibco BRL, Gaithersburg, MD), and selected with G418 at a concentration of 800 μg/ml. Isolated colonies were examined for the expression of CrkII or CrkII-23 by immunoblotting. Cell lines that expressed comparable levels of CrkII or CrkII-23 were used for the subsequent analysis.

Anchorage-independent growth of glioma cells Analysis of anchorage-independent growth in poly-2-hydroxyethyl-methacrylate (HEMA)-coated dish has been previously described.[19] Briefly, 10^4 cells were suspended in 100 μl of DMEM containing 1% FBS and 25 n*M* EGF or 1% FBS alone. Cells were then seeded to each well of 96-well tissue culture plates pre-coated with poly-HEMA. After various periods of incubation, 10 μl of Tetracolor One (Seikagaku Co., Tokyo) was added to each well for 1 h and $A_{450/690}$ was measured on a microplate reader.

RESULTS

Tyrosine phosphorylation of CrkII upon EGF stimulation of glioma cells All glioma cell lines that we tested expressed EGFR, but the level of expression varied among various cell lines (Fig. 1A). Some cell lines, such as U138MG, expressed as much EGFR as A431 cells, which are known to overexpress EGFR.[20] In U373MG cells, a truncated form of the EGFR was also detected. Truncation of EGFR is often accompanied with the activation of its kinase activity; however, we did not observe any remarkable increase in the level of phosphotyrosine-containing proteins in U373MG (Fig. 1B). EGF stimulation of these glioma cells induced remarkable tyrosine phosphorylation of many cellular proteins. The most prominent tyrosine-phosphorylated protein appeared to be EGFR itself (Fig. 1C).

Tyrosine phosphorylation of CrkII We next examined the expression of CrkII in human glioma cells (Fig. 2A) and noticed that the slower-migrating form of CrkII, also known as p44CrkII,[7] was increased after EGF stimulation in the glioma cells (Fig. 2A). It has been shown that the Abl tyrosine kinase phosphorylates tyrosine 221 of CrkII and reduces its mobility on SDS-polyacrylamide gels.[21]

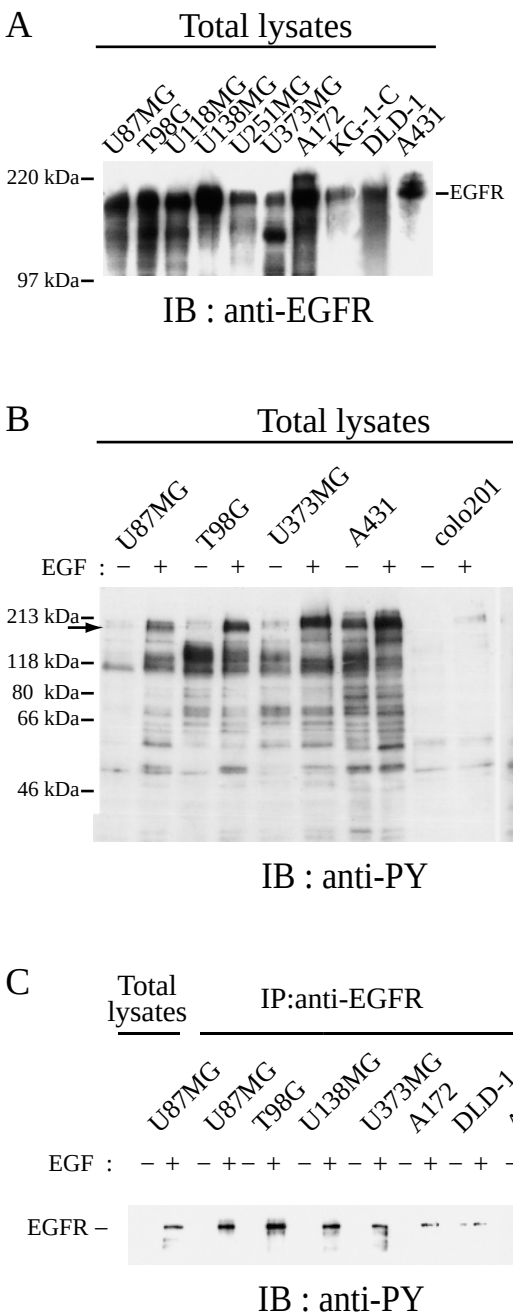

Fig. 1. Expression of EGFR in human glioma cell lines. Human glioma cells were serum-starved overnight, incubated with or without 50 ng/ml EGF at 37°C for 3 min and lysed in lysis buffer. Equal amounts of the cleared cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting with (A) the anti-EGFR antibody or (B) the anti-phosphotyrosine (PY) antibody. C, equal amounts of lysates were immunoprecipitated with the anti-EGFR antibody and then separated by SDS-PAGE and analyzed by immunoblotting with the anti-PY antibody. The arrow in B denotes EGFR.

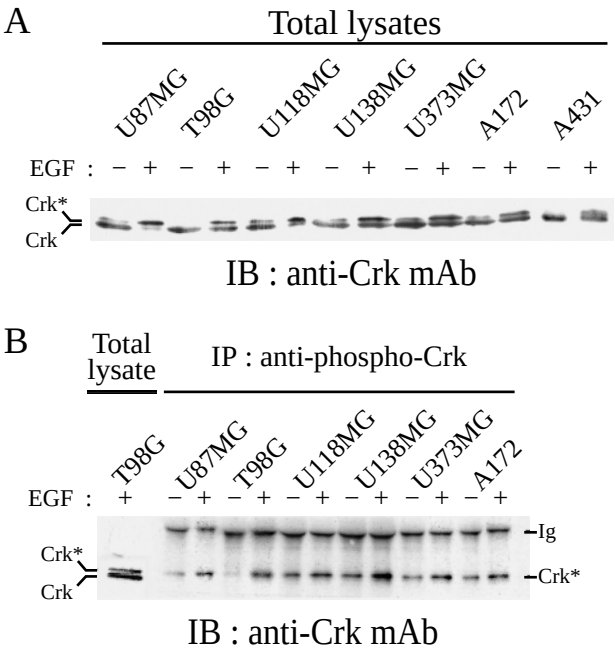

Fig. 2. Tyrosine phosphorylation of CrkII. Human glioma cells were serum-starved overnight, incubated with or without 50 ng/ml EGF at 37°C for 3 min and lysed in lysis buffer. A, equal amounts of the cleared cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with the anti-CrkII monoclonal antibody (Crk mAb). B, cells were heat-denatured and immunoprecipitated with the anti-phospho-CrkII antibody, followed by SDS-PAGE and immunoblotting with the anti-CrkII mAb. Crk* denotes the slower-migrating form of CrkII.

We examined whether EGF stimulation also induced phosphorylation of tyrosine 221 by the use of an antibody specific to tyrosine 221-phosphorylated CrkII.[18] The amount of tyrosine 221-phosphorylated CrkII increased after EGF stimulation in all glioma cell lines (Fig. 2B). The degree of the increase varied among the cell lines tested.

Dissociation of CrkII from the EGFR We next examined the association of CrkII with EGFR. In many of the glioma cell lines, U87MG, T98G, U373MG, and A172, CrkII coimmunoprecipitates with the EGFR before, but not after, EGF stimulation (Fig. 3A). This finding was confirmed by probing anti-CrkII immunoprecipitates with an anti-EGFR antibody (Fig. 3B). Although the quantity of EGFR-associated CrkII varied between cells, none of them showed EGF-dependent binding of CrkII to the EGFR. In contrast, in A431 epidermoid carcinoma cells, CrkII binds to EGFR in an EGF-dependent manner.

Association of Shc and Grb2 with EGFR We next examined whether Shc and Grb2 associate with the EGFR upon EGF stimulation of human glioma cell lines. Shc consists of three isoforms of 46, 52, and 66 kDa. We

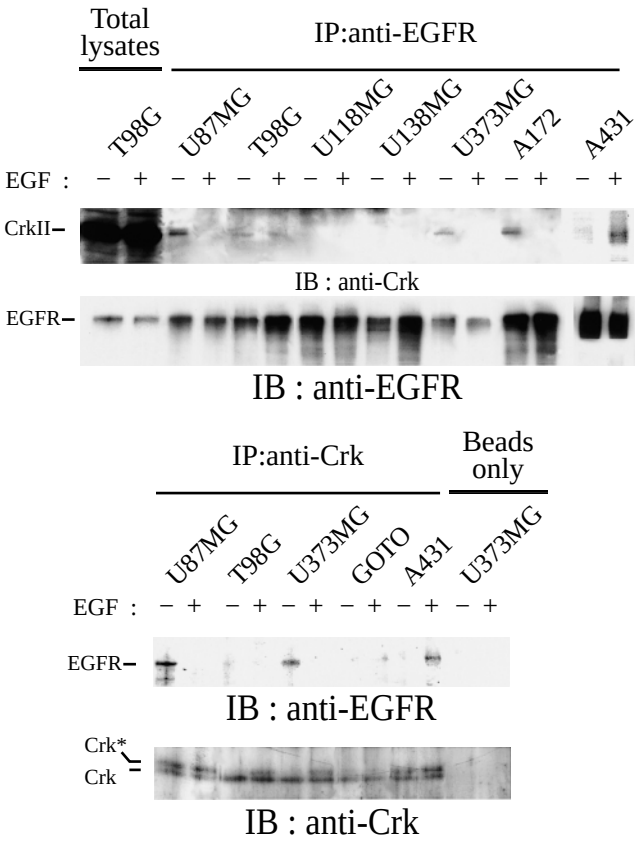

Fig. 3. Binding of CrkII to EGFR. Human glioma cells were serum-starved overnight, incubated with or without 50 ng/ml EGF at 37°C for 3 min and lysed in lysis buffer. A, total cell lysates and immunoprecipitates with the anti-EGFR antibody were separated by SDS-PAGE and analyzed by immunoblotting with either the anti-CrkII mAb or the anti-EGFR antibody. B, equal amounts of the cleared cell lysates were immunoprecipitated with the anti-Crk monoclonal antibody (3A8), followed by SDS-PAGE and immunoblotting with either the anti-EGFR antibody or the anti-CrkII monoclonal antibody. Crk* denotes the slower-migrating form of CrkII.

found that the 66 kDa form was expressed most abundantly in glioma cells and A431 cells, while the 52 kDa isoform was dominant in colo201 and DLD-1 cell lines, which are derived from colon cancer (Fig. 4A). EGF stimulation induced tyrosine phosphorylation and binding of Shc to the EGFR in the glioma cell lines (Fig. 4, B and C). Binding of 66 kDa Shc protein to EGFR could not be detected because it co-migrated with the immunoglobulin heavy chain used for the precipitation of EGFR. Similarly to Shc, Grb2 bound to EGFR after EGF stimulation in both glioma cells and A431 cells (Fig. 4, D and E). These results indicate that only CrkII among adaptor proteins examined dissociates from EGFR upon EGF stimulation.

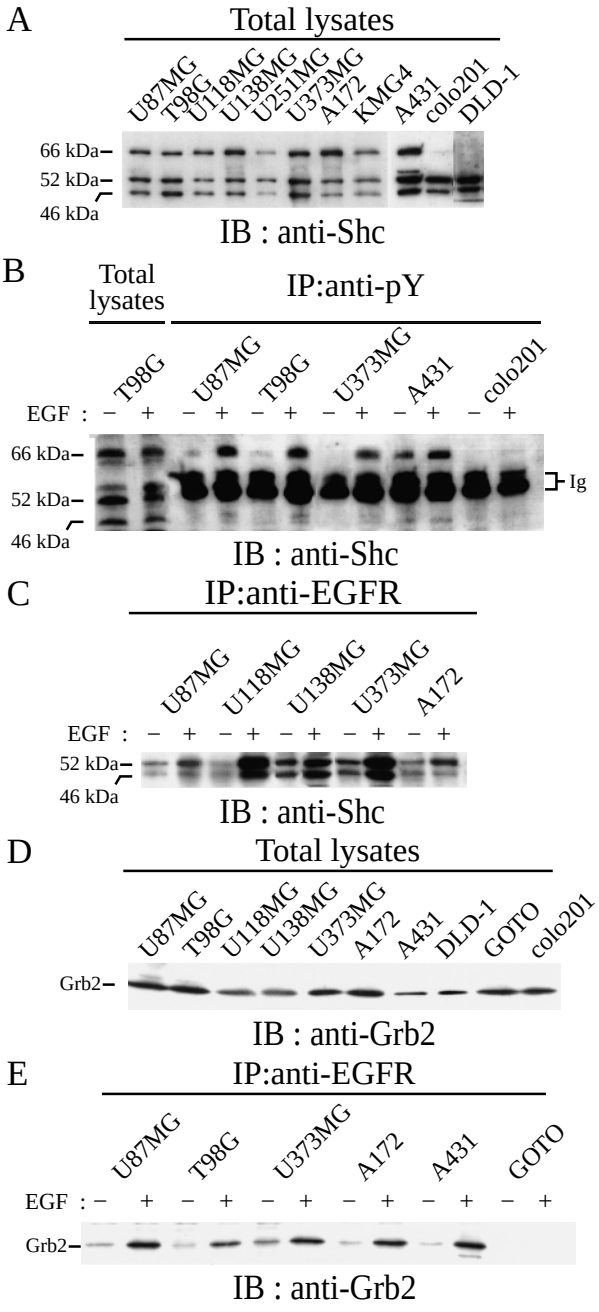

Fig. 4. Binding of Shc and Grb2 to EGFR in human glioma cell lines. Human glioma cells were serum-starved overnight, incubated with or without 50 ng/ml EGF at 37°C for 3 min and lysed in lysis buffer. A, equal amounts of the cleared cell lysates without EGF stimulation were analyzed by SDS-PAGE and immunoblotting with the anti-Shc antibody. The three isoforms of Shc are indicated on the left. Equal amounts of the cleared cell lysates were immunoprecipitated with either the anti-PY antibody (B) or the anti-EGFR antibody (C) and probed with the anti-Shc antibody. Similarly, cleared cell lysates (D) and immunoprecipitates with the anti-EGFR antibody (E) were separated by SDS-PAGE and analyzed by immunoblotting with the anti-Grb2 antibody.

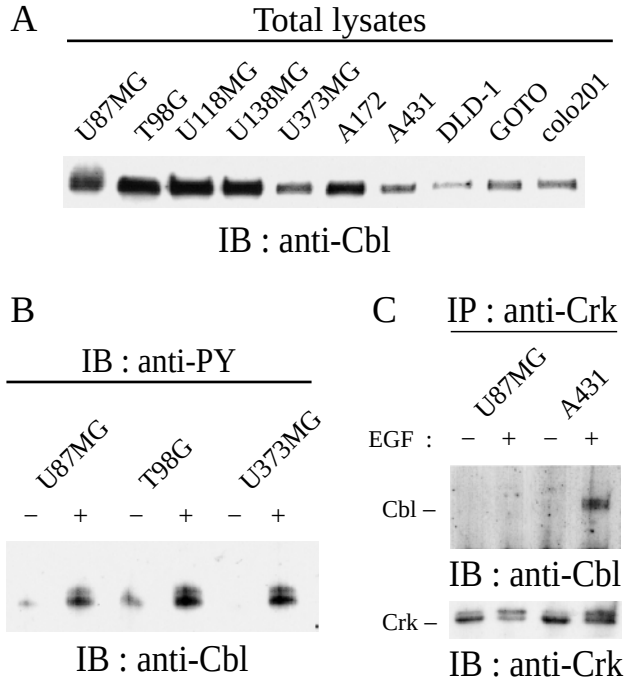

Fig. 5. Binding of CrkII to Cbl in human glioma cell lines. A, equal amounts of the cleared cell lysates of human glioma cells were separated by SDS-PAGE and analyzed by immunoblotting with the anti-Cbl antibody. B, glioma cells were serum-starved overnight, incubated with or without 50 ng/ml EGF at 37°C for 3 min and lysed in lysis buffer. Equal amounts of the cleared cell lysates were immunoprecipitated with the anti-PY antibody, separated by SDS-PAGE, and analyzed by immunoblotting with the anti-Cbl antibody. C, similarly, U87MG glioma cells and A431 epidermoid carcinoma cells were serum-starved overnight, incubated with or without 50 ng/ml EGF at 37°C for 3 min and lysed in lysis buffer. Equal amounts of the cleared cell lysates were immunoprecipitated with the anti-Crk mAb (3A8), separated by SDS-PAGE and analyzed by immunoblotting with either the anti-Cbl antibody or the anti-Crk monoclonal antibody.

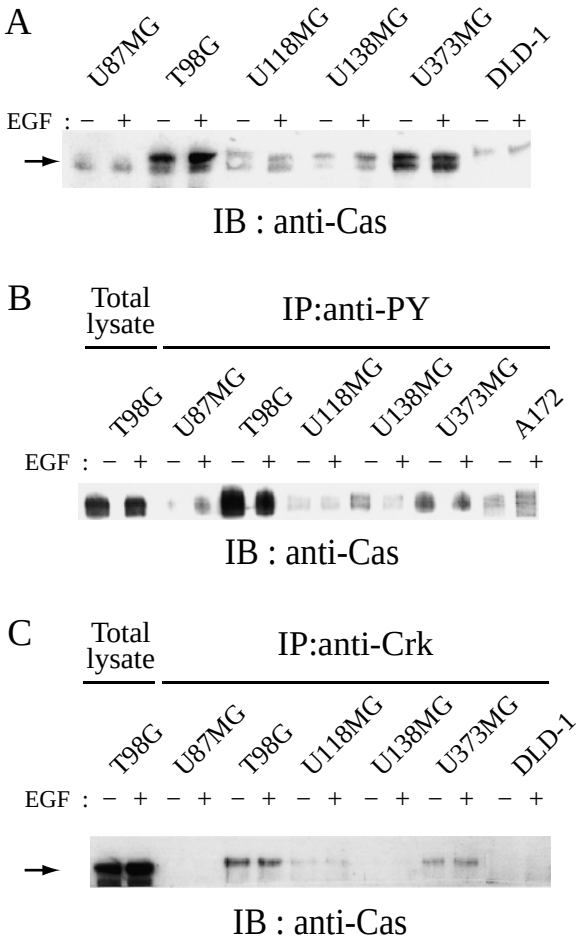

Fig. 6. Binding of CrkII to p130^{Cas}. Human glioma cells were serum-starved overnight, incubated with or without 50 ng/ml EGF at 37°C for 3 min and lysed in lysis buffer. A, equal amounts of the cleared cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-p130^{Cas}. Equal amounts of the cleared cell lysates were immunoprecipitated with either the anti-PY antibody (B) or the anti-Crk monoclonal antibody 3A8 (C), separated by SDS-PAGE, and analyzed by immunoblotting with the anti-Cas antibody.

Binding of CrkII to Cbl CrkII binds to the Cbl protein upon EGF stimulation of NIH 3T3 cells.[22, 23] Thus, we examined the binding of CrkII to Cbl in glioma cells. Cbl was expressed in all cell lines we tested (Fig. 5A). Three representative cell lines were further examined, and in these cells we found EGF-dependent tyrosine phosphorylation of Cbl (Fig. 5B). We could not detect EGF-dependent binding of Cbl to CrkII in U87MG glioma cells, whereas in A431 cells, EGF clearly induced binding of Cbl to CrkII (Fig. 5C). This result may suggest that EGF-dependent binding of CrkII to phosphotyrosine-containing proteins is impaired in human glioma cells.

Binding of CrkII to p130^{Cas} Two proteins associated with focal adhesions, p130^{Cas} and Paxillin, are known to bind to the SH2 domain of CrkII. We examined the binding of CrkII to these proteins in glioma cells in the absence or presence of EGF. The level of expression and tyrosine phosphorylation of p130^{Cas} varied among the cell lines tested (Fig. 6, A and B) and the p130^{Cas} expression level correlated with the quantity of the CrkII binding (Fig. 6C). Similar data was obtained with Paxillin (unpublished data). Therefore, the lack of CrkII binding to EGFR and Cbl is not due to the impaired function of the SH2 domain of CrkII in human glioma cells.

Growth retardation of the glioma cells expressing the CrkII-23 mutant Finally, the role of dissociation of CrkII from the EGFR was assessed by the use of CrkII-23

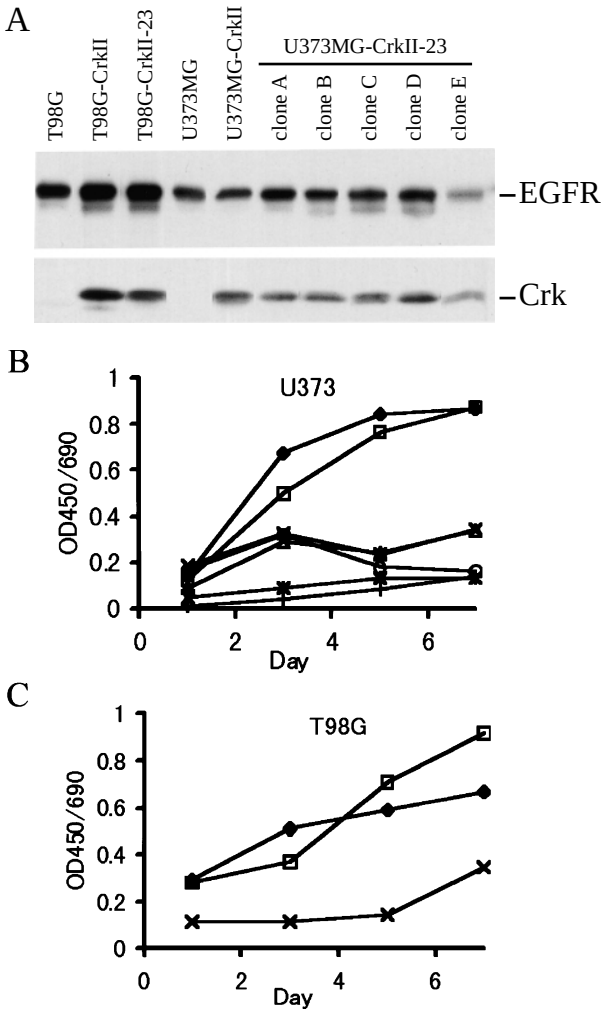

Fig. 7. Anchorage-independent growth of glioma cells expressing the CrkII-23 mutant. T98G and U373 human glioma cells were transfected with pCXN2-myc-CrkII or pCXN2-myc-CrkII-23 and selected with G418. After the cloning of several isolated colonies, cell lines expressing similar levels of CrkII or CrkII-23 were further analyzed. A, cleared lysates of the cell lines denoted on the top were separated by SDS-PAGE and analyzed by immunoblotting with the anti-EGFR antibody or the anti-Crk mAb. B and C, U373- or T98G-derived cells were grown in DMEM containing 1% FBS and 25 n*M* EGF on the 96-well tissue culture plates pre-coated with poly-HEMA. Growth of the cells was quantitated by the use of Tetracolor One as described in the text. Symbols used in the figure are ◆ mock, □ CrkII, △ CrkII-23A, × CrkII-23B, ∗ CrkII-23C, ○ CrkII-23D, + CrkII-23E.

mutant, which binds to the EGFR in the absence of EGF.[6] We expressed wild-type (w.t.) CrkII and CrkII-23 mutant in U373 and T98G glioma cells (Fig. 7A). Cell lines expressing similar levels of w.t. CrkII or CrkII-23 were further analyzed for cell growth. The growth rate of CrkII-23-expressing U373 cells was slightly slower than that of the parental cells or cells expressing the w.t. CrkII in culture dishes (unpublished data). However, the difference between these cell lines was more obvious when anchorage-independent growth in poly-HEMA-coated dish was examined in the presence of EGF (Fig. 7B). We did not see any remarkable difference in the anchorage-independent growth between the parent cells and the w.t. CrkII-expressing cells. In contrast, all five CrkII-23-expressing U373MG cells grew slower than the parent cells in poly-HEMA-coated dishes. We obtained similar results for T98G-derived clones (Fig. 7C). Thus, CrkII-23 inhibits EGF-dependent anchorage-independent growth of human glioma cells, suggesting that EGF-dependent dissociation of CrkII is required for anchorage-independent growth of glioma cells.

DISCUSSION

Among several cell lineages expressing EGFR that we tested, only in glioma cells was CrkII associated with EGFR in the absence of EGF. Because the basal level of tyrosine phosphorylation of EGFR was low in glioma cell lines (Fig. 1B), it is unlikely that CrkII binds to EGFR via an SH2-phosphotyrosine interaction in unstimulated glioma cells. The CrkII-23 mutant, which is constitutively bound to EGFR in unstimulated cells, contains two amino-acid substitutions in the second SH3 domain of CrkII, SH3(C).[6] Mutation of neither SH2 nor SH3(N) of CrkII-23 abolished the constitutive binding of CrkII-23 to EGFR.[15] Thus, a protein that interacts with the CrkII SH3(C) might contribute to the EGF-independent binding of CrkII to the EGFR. Currently no proteins have been shown to bind to the SH3(C) of CrkII. However, proteins that are expressed specifically in glial cells have not been tested for such interaction. Thus, it is possible that a glial cell-specific CrkSH3(C)-interacting protein mediates EGF-independent binding of CrkII to EGFR.

We have shown that EGF stimulation induces transient binding of CrkII to EGFR in NIH 3T3 cells.[18] Firstly, CrkII binds to the activated and tyrosine-phosphorylated EGFR. Subsequently, tyrosine-phosphorylated CrkII dissociates from EGFR. It has been shown that the phosphorylation of Tyr221 of CrkII induces dissociation of CrkII from c-Abl.[21] In this study, we have confirmed that Tyr221 was phosphorylated upon EGF stimulation in human glioma cells. Thus, dissociation of CrkII from the EGFR in glioma cell lines may occur through the same mechanism.

EGF-dependent dissociation from EGFR has already been reported for the zinc finger protein, ZPR1.[24] ZPR1 is bound to the EGFR in unstimulated cells through its zinc finger domain and dissociates from the EGFR upon EGF stimulation. Tyrosine phosphorylation of the EGFR has

been shown to be responsible for the release of ZPR1. Because CrkII does not have a zinc finger domain, and because the dissociation of CrkII correlates with the phosphorylation of CrkII, the mechanism of EGF-dependent dissociation of CrkII from the EGFR may be remarkably different from that of ZPR1.

A major protein bound to the amino-terminal SH3 domain of CrkII, SH3(N), is C3G, which is a guanine nucleotide exchange protein for Rap1.[12, 13] Rap1 negatively regulates the Ras-MAP kinase cascade.[25] Recently, it has been demonstrated that insulin-dependent recruitment of CrkII-C3G complex increased the GTP-bound active Rap1, implicating CrkII-C3G complexes in the negative regulation of Ras-MAP kinase cascade.[26] We have found that C3G is expressed and associated with CrkII in human glioma cell lines (unpublished data); therefore, the binding of CrkII to EGFR may recruit C3G to the plasma membrane, which, in turn, would suppress the Ras-MAP kinase cascade. It has been reported that expression of Rap1 is significantly increased in human glioma cells.[27] Therefore, suppression of Rap1 signaling may be an essential step in glioma development.

Taking into account these observations, we propose the following model. CrkII binds to EGFR probably through SH3(C) in glial cells, suppressing cell growth by means of the C3G-Rap1 cascade. Autocrine or paracrine activation of the EGFR in glioma induces phosphorylation of Tyr221 of CrkII, which triggers the dissociation of CrkII-C3G complex from EGFR, shutting off the C3G-Rap1 pathway. Expression of CrkII-23 mutant in glioma cells significantly inhibited the anchorage-independent growth of these cells. Because CrkII-23 has been shown to bind to EGFR in the absence of EGF, this observation strongly supports our proposal that EGF-induced dissociation of CrkII from EGFR is an important step for the EGF-dependent malignant transformation. However, our model does not detract from the significance of the previous reports that the Grb2-Sos-Ras-MAP kinase cascade is activated by EGF in glioma cell lines[4]; rather, it is likely that activation of the Ras pathway and inactivation of the Rap1 pathway cooperatively accelerate cell growth of glioma cells.

In conclusion, CrkII oncogene product is associated with EGFR in human glioma cell lines. Upon stimulation by EGF, CrkII is phosphorylated on tyrosine and dissociates from EGFR. Because CrkII is bound to C3G, an activator of Rap1, the EGF stimulation may play a role in the suppression of Rap1 activity in glioma cells.

(Received June 2, 1999/Revised July 26, 1999/Accepted July 27, 1999)

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