**ABSTRACT**

The human *crk* gene is translated into *crkI* and *crkII* by alternative splicing. *crkII* mRNA was detected both in normal brain and glioblastoma tissues, whereas *crkI* mRNA levels were quite low in normal brain and up-regulated in glioblastoma tissues. Expression of *CrkI* but not *CrkII* in glioblastoma U87MG cells induced transformation that stimulated cell migration and invasion concomitant with tyrosine phosphorylation of p130 Crk-associated substrate. N-cadherin-mediated signal transduction, which was essential for invasion by U87MG cells, was no longer required for *CrkI*-transformed cells. These results suggest that *CrkI* contributes to malignancy of glioblastoma by inducing phosphorylation of p130 Crk-associated substrate.

**INTRODUCTION**

*Crk* was originally isolated as an oncogene product of the CT10 chicken retrovirus, and it belongs to a group of adapter proteins that are comprised of SH2 and SH3 domains, which interact with phosphotyrosine and proline-rich regions, respectively (1, 2). The SH2 domain of *Crk* can bind to p130 and paxillin, and growth factor receptors. The N-terminal SH3 domain of *CrkI* can bind to C3G (guanine nucleotide exchange factor for Rap1), Sox (guanine nucleotide exchange factor for Ras), DOCK180 (which activates Rac1 after binding to the CrkII/p130 complex), c-Abl, and PI 3-K regulatory subunit (2). The human *crk* gene is translated into two products, *CrkI* (M, 28,000) and *CrkII* (M, 42,000), by alternative splicing (3). *CrkII*, which is predominantly expressed in many cell lines, consists of one SH2 and two SH3 domains with a spacer region between the SH3 domains that includes a tyrosine phosphorylation site, Tyr221 (2). On tyrosine phosphorylation, *CrkII* undergoes intramolecular binding, which results not only in blockage of CrkII SH2-mediated binding to phosphotyrosine residues in other molecules but also in reduced affinity of the *CrkII* central SH3 domain (2, 4–6). In contrast, *CrkI* consists of one SH2 and only one SH3 domain, and it lacks this tyrosine phosphorylation site. Because *CrkI* but not *CrkII* expression induces transformation in rat 3Y1 fibroblasts, *CrkI* appears to resemble the v-*crk* oncogene product not only in its structure but also in its function (2, 3). *CrkI* has been implicated in FAK-induced cell migration by coupling with p130 (6, 7); however, the expression and function of *CrkI* in tumor cells are poorly understood. In the present study, we demonstrated specific expression of *crkI* in glioblastoma tissues and analyzed the role of *CrkI* in malignancy of glioblastoma.

**RESULTS AND DISCUSSION**

**crkI and crkII mRNA Expression in Glioblastoma Tissues.** To investigate the expression of *crkI* and *crkII* mRNA in normal brain and glioblastoma tissues, we designed PCR primers to amplify the alternatively spliced regions (nucleotides 713–882 in *crkII* mRNA) that distinguish *crkII* from *crkI*. *crkII* mRNA was detected both in normal brain and glioblastoma tissues at comparable levels (Fig. 1B). In contrast, *crkI* mRNA was quite low in normal brain but up-regulated to the level of *crkII* mRNA in glioblastoma tissues (*P < 0.05*). This is, to the best of our knowledge, the first demonstration that *crkI* but not *crkII* mRNA expression is specifically up-regulated in glioblastoma tissues.

**Crk Promotes Cell Migration.** To compare the function of *CrkI* and *CrkII*, U87MG cells were cotransfected with pHA262pur puroycin-resistance plasmids (Corning Costar Corp.) as described (3). The top surfaces of the Transwell membranes were coated with 1 mg/ml Matrigel basement membrane matrix (Becton Dickinson Labware) overnight at 4°C. Serum-starved cells (2 × 10⁵) suspended in 100 μl of DMEM containing 1 mg/ml BSA and 0.5% serum were seeded into chambers with or without LY294002 (10 μM) or GC-4 (dilution 1:50) for 16 h, and then the number of cells on the bottom surface was counted.

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3 The abbreviations used are: SH, Src homology; FAK, focal adhesion kinase; PI 3-K, phosphatidylinositol 3-kinase; p130, p130 Crk-associated substrate; GAPDH, glyceroldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR.

**MATERIALS AND METHODS**

**RNA Extraction and RT-PCR Analysis.** Fresh human glioblastoma tissues were obtained from five patients, and normal brain tissues were taken from the margin of surgical specimens. The classification of human brain tumors used in this study is based on the revised WHO criteria for tumors of the central nervous system (8). All of the tumor tissues were obtained at primary resection, and none of the patients had been subjected to chemotheraphy or radiation therapy. RT-PCR was carried out using the following PCR primers based on human *crkI* and *crkII* cDNA (GenBank accession nos. NM_005206 and NM_016823, respectively): (a) *crk* 5′-primer starting at nucleotide 336 of *CrkII*, 5′-GCCCTGGCCGGGTGCCACCTTCCT-3′, and (b) *crk* 3′-primer starting at nucleotide 953 of *CrkII*, 5′-CCGTGGCATTACCCCTACTCC-3′. The PCR products amplified with these primers were 618 bp for *CrkI* and 448 bp for *CrkII*, respectively.

**Antibodies and Reagents.** Anti-phosphotyrosine (PY20), anti-p130, anti-Crk, anti-FAK, anti-paxillin, anti-N-cadherin, and anti-β-catenin antibodies were purchased from Transduction Laboratories. Anti-C3G antibody was from Santa Cruz Biotechnology. N-cadherin blocking mouse monoclonal antibody (GC-4), PI 3-K inhibitor LY294002, and DMEM were from Sigma-Aldrich. Anti-phospho-Akt (Ser473) and anti-Akt antibodies were from New England Biolabs.

**Cell Motility Assay.** U87MG (1.5 × 10⁶) cells were electroporated with 10 μg of pcDNA-CrkI or pcDNA-CrkII together with 5 μg of pRK-green fluorescent protein and 5 μg of pH262pur puromycin-resistance plasmids. The cells were subcultured at a 1:3 dilution 12 h after transfection and maintained a further 48 h in 1.5 μg/ml puromycin-containing medium. After puromycin selection, cells were replated onto 35-mm glass bottom dishes coated with 10 μg/ml fibronectin and cultured in DMEM containing 2 mg/ml BSA. Cell movements were measured using time-lapse video microscopy.

**Cell Invasion Assay.** Cell invasion was assayed using modified Boyden chambers consisting of Transwell membrane filters (Corning Costar Corp.) as described (9). The top surfaces of the Transwell membranes were coated with 1 mg/ml Matrigel basement membrane matrix (Becton Dickinson Labware) overnight at 4°C. Serum-starved cells (2 × 10⁶) suspended in 100 μl of DMEM containing 1 mg/ml BSA and 0.5% serum were cultured in chambers with or without LY294002 (10 μM) or GC-4 (dilution 1:50) for 16 h, and then the number of cells on the bottom surface was counted.
p130<sup>as</sup> was more effectively tyrosine phosphorylated in CrkI-transfected cells compared with control or CrkII-transfected cells. Increased p130<sup>as</sup> tyrosine phosphorylation is known to activate Rac1 by forming a complex with Crk and DOCK180 (7, 10, 11). These results suggest that CrkI expression promotes cell migration by activating the FAK/p130<sup>as</sup>/CrkI/DOCK180 pathway.

**Transformation of U87MG Cells by CrkI.** Fig. 3A shows the morphology of the cells stably transfected with CrkI at 12 or 72 h after incubation in DMEM with or without serum (labeled 10% fetal bovine serum and SFM, respectively). U87MG cells and mock transfectants formed branching colonies with multilayering, especially in serum-free medium, whereas stable CrkI transfectants grew dispersed. Dissociation of branching colonies was also observed in cells transiently transfected with CrkI but not CrkII (data not shown). Although CrkI-expressing cells did not form branching colonies, the expression of N-cadherin and β-catenin was not significantly altered in these cells (Fig. 3B). On the other hand, tyrosine phosphorylation of p130<sup>as</sup> and C3G was substantially augmented in CrkI-expressing cells, whereas that of FAK and paxillin was not altered (Fig. 3C). Increased tyrosine phosphorylation of p130<sup>as</sup> and C3G is known to activate Rac1 and Rap1, respectively (10–12). Rac1 is involved in both the assembly and disassembly of adherens junctions (13). C3G-dependent Rap1 activation promotes cell adhesion and spreading (14). Overexpression of CrkII promotes breakdown of adherens junctions and spreading of breast cancer epithelial T47D cells (15). Therefore, CrkI reconstitution in U87MG cells may induce activation of Rac1 and Rap1 by enhancing tyrosine phosphorylation of p130<sup>as</sup> and C3G, resulting in transformation stimulating cell migration and dissociating branching colonies.
U87MG cells were serum starved overnight, trypsinized, suspended at 4 x 10^5/ml, and replated onto Matrigel-coated dishes in the presence or absence of GC-4 (dilution 1:50) for 16 h and then homogenized and immunoblotted with anti-phospho-Akt (Blot: pAkt), anti-Akt (Blot: Akt) antibodies. In B, cell invasion assays were carried out as described in "Materials and Methods." Bars, SE from three independent experiments. *P < 0.001; **P < 0.005 versus control.

CrkI Promotes Cell Invasion. Invasive cells must coordinately regulate cell migration and survival. In fact, p130Cas/Crk coupling facilitates both cell invasion and survival in a Rac-dependent manner (16). Because N-cadherin-mediated intercellular interaction promotes cell survival by activating the PI 3-K/Akt pathway (17), treatment of glioblastoma cells with N-cadherin-blocking antibody GC-4 suppresses invasion (18). On the other hand, U87MG cells transformed by CrkI grow without cell–cell interaction as described above. Consequently, we examined the effect of N-cadherin-blocking antibody GC-4 on PI 3-K/Akt activation in CrkI-transformed cells (Fig. 4A). When mock and CrkI-transformed U87MG cells were cultured on Matrigel, Akt was phosphorylated to comparable levels. Although treatment of control U87MG cells with GC-4 antibody preferentially inhibited PI 3-K/Akt activation, CrkI-transformed cells were resistant to it. Invasion by both mock and CrkI-transformed cells was inhibited by the PI 3-K inhibitor LY294002, indicating that the PI 3-K/Akt pathway is essential for invasion. Consistent with its effects on Akt activation, GC-4 suppressed invasion of mock transfected but not CrkI-transformed cells. The PI 3-K/Akt pathway is constitutively activated in v-Crk-transformed chicken embryo fibroblasts (19). Similarly, in CrkI-transformed U87MG cells, the PI 3-K/Akt pathway is constitutively activated; moreover, N-cadherin-mediated cell–cell interaction is lost and no longer essential for invasion and survival. The expression of CrkI in U87MG cells enhanced invasiveness by 70% compared with that of control cells, which may be explained in part by enhanced migration through the activation of the FAK/p130Cas/Crk/DOCK180 pathway by CrkI as described above.

The U87MG cell line is defective in the tumor suppressor gene PTEN (phosphatase and tensin homologue deleted from chromosome 10) and expresses N-cadherin (9, 18). Inhibition of U87MG cell invasion by phosphatase and tensin homologue deleted from chromosome 10 expression or treatment with GC-4 antibody is accompanied by suppression of the PI 3-K/Akt pathway (Fig. 4A; Refs. 9 and 20). Thus, CrkI seems to be a key molecule in signaling networks that coordinateably regulate cell–cell adhesion, cell spreading, migration, and survival.

We have demonstrated here that expression of crkI but not crkII is specifically up-regulated in glioblastoma tissues, which contributes to malignancy of glioblastoma while activating p130Cas. CrkI is also involved in promoting the invasive phenotype by activating PI 3-K/Akt signaling without N-cadherin-mediated intercellular interactions. CrkI may not only be a diagnostic marker but also a molecular target for drug development against glioblastoma.

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Crkl Adapter Protein Modulates Cell Migration and Invasion in Glioblastoma

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