Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 12177–12182, October 1996
Biochemistry

CrkII signals from epidermal growth factor receptor to Ras

(adapter/Grb2/signal transduction/NRK cells/oncogenic transformation)

SHINAE KIZAKA-KONDOH*, MICHIYUKI MATSUDA†, AND HIROTO OKAYAMA*‡

*The Okayama Cell Switching Project, Exploratory Research for Advanced Technology, Research Development Corporation of Japan, Tanaka Monzen-cho, Sakyo-ku, Kyoto 606, Japan; †Department of Pathology, National Institute of Health, Toyama, Shinjuku-ku, Tokyo 162, Japan; and ‡Department of Biochemistry, Faculty of Medicine, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

Communicated by Paul Berg, Stanford University Medical Center, Stanford, CA, August 8, 1996 (received for review May 13, 1996)

ABSTRACT **A rat fibroblast mutant defective in oncogenic transformation and signaling from epidermal growth factor receptor to Ras has been isolated. The mutant contains dominant negative-type point mutations in the C-terminal SH3 domain of one *crk*II gene. Among the adapters tested, the mutant is complemented only by *crk*II cDNA. Expression of the mutated *crk*II in parent cells generates the phenotype indistinguishable from the mutant cell. Yet overexpression or reduced expression of Grb2 in the mutant before and after complementation with *crk*II have little effect on its phenotype. We conclude that adapter molecules are highly specific and that the oncogenic growth signal from epidermal growth factor receptor to Ras is predominantly mediated by CrkII in rat fibroblast.**

In *Drosophila melanogaster* and *Caenorhabditis elegans* signals from the Sevenless and Let-23 receptors are transferred to the Ras protein, respectively, via the Drk and Sem-5 adapters which contain the SH2 and SH3 Src homology domains (1–3). Grb2 has been identified as a Drk/Sem-5 homologue in mammals (4, 5). It is thought to act as the main adapter for signaling from the epidermal growth factor receptor (EGFR) to Ras because Grb2 interacts physically with EGFR and Sos, a Ras guanine nucleotide-releasing protein (GNRP) and overexpressed Grb2 can activate Ras in a EGF-dependent manner in NIH 3T3 cells overexpressing EGFR (6–9). In addition, Grb2 interacts with many receptor-type and nonreceptor-type tyrosine kinases and GNRPs, suggesting that Grb2 is involved in signal transduction from a variety of receptors and other signaling molecules (10).

Crk is a member of an adapter family possessing typical conserved SH2 and SH3 domains (11), having been identified initially as a product of the oncogene carried by the avian retroviruses CT10 and avian sarcoma virus 1 (ASV-1) (12, 13). Two cellular homologues, CrkI and CrkII, are produced from the same gene by alternative splicing and expressed in human and mouse cells (14, 15). CrkII is a 40–42 kDa protein containing one SH2 and two SH3 domains and is expressed in virtually all tissues and cells whereas CrkI is a 28-kDa protein lacking one SH3 domain and is expressed at a lower level and in limited cell-types (14). Both CrkI and CrkII bind to many of tyrosine-phosphorylated proteins that bind to Grb2—e.g., EGFR, Abl, and Sos (16–19). Nck, another putative adapter, also binds to many of the tyrosine-phosphorylated proteins to which Grb2 and Crk also bind (20).

NRK-49F (NRK), a cell line derived from normal rat kidney fibroblasts, is reversibly transformed by EGF or platelet-derived growth factor (PDGF) in the presence of transforming growth factor β (TGF-β). We have studied intracellular oncogenic signal cascades by using a genetic analysis of NRK and its mutants. Our recent experiments indicate that NRK cells use a common cascade to mediate transforming signals from the following oncogenes: v-*erb*B, activated c-*erb*B-2, v-*fms*, v-*ras*, v-*fes*, v-*fos*, v-*mos*, v-*src*, polyoma middle T antigen, simian virus 40 large T antigen, and human papillomavirus E6E7, as well as from EGF and PDGF. This cascade branches off to anchorage-dependent and anchorage-independent growth signals, the latter of which is essential for oncogenic transformation (21–23).

Recently we isolated a NRK cell mutant that is defective in signaling from EGFR to Ras. We identified the gene defective in this mutant as the *crk*II gene. In this paper, we show that in this cell the oncogenic signal from EGFR to Ras is mediated predominantly by CrkII.

MATERIALS AND METHODS

Cells, Mutant Isolation and Assays for Growth in Soft Agar, Proliferation, and Focus Formation. The NRK-49F cell line (obtained from the American Type Culture Collection) was maintained in DMEM supplemented with 5% fetal calf serum. When required, growth factors were added at final concentrations of 5% fetal calf serum, 5 ng/ml for EGF, 5 ng/ml for PDGF, and/or 0.5 ng/ml for TGF-β to DMEM. Mutants were isolated following mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) as described (21). Assays for growth in soft agar, and proliferation and focus formation were done as described (23).

cDNA Cloning. Wild-type rat *crk*I and *crk*II cDNAs were cloned with a human *crk*II cDNA as a probe (14). The mutant *crk*II cDNA was synthesized and amplified by reverse transcription of mutant cell (NRK-23) mRNA followed by PCR with three sets of *crk*II specific primer (primer 1, nucleotides −25 to −8; primer 2, 473-492; primer 3, 249-265; primer 4, 760-783; primer 5, 661-681; primer 6, 1018-1037). PCR products were then cloned.

Genomic DNA Analysis. Genomic DNA was isolated from NRK-23 as described (24), digested with the *Eco*RI and *Xba*I restriction enzymes, and resolved by agarose gel electrophoresis. DNA containing the 4-kb *Eco*RI–*Xba*I fragment of c-*crk*, which corresponds to the nucleotides 305–777 of *crk*II cDNA and part of an intron, was eluted from the gel and subcloned. The clones containing the *Eco*RI–*Xba*I c-*crk* genomic fragment were identified by colony hybridization and sequenced.

DNA Transfection and Colony Assay. Rat *crk*I, *crk*II, *grb2* (5), and *nck* cDNAs (25) were subcloned into the pMEXneo expression vector. The mutant type *crk*II cDNA (crkII-23) was inserted into pEF/hygI, a derivative of pEF-BOS (26). DNA transfection was done as described (27).

Immunoprecipitation and Western Blot Analysis. Western blot analysis was done as described (23). Crk and Grb2 proteins

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; hEGF, human EGF; TGF-β, transforming growth factor-β; ET, EGF plus TGF-β; GNRP, guanine nucleotide-releasing protein; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; NRK, normal rat kidney; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. of rat *c-crk*, D44481).

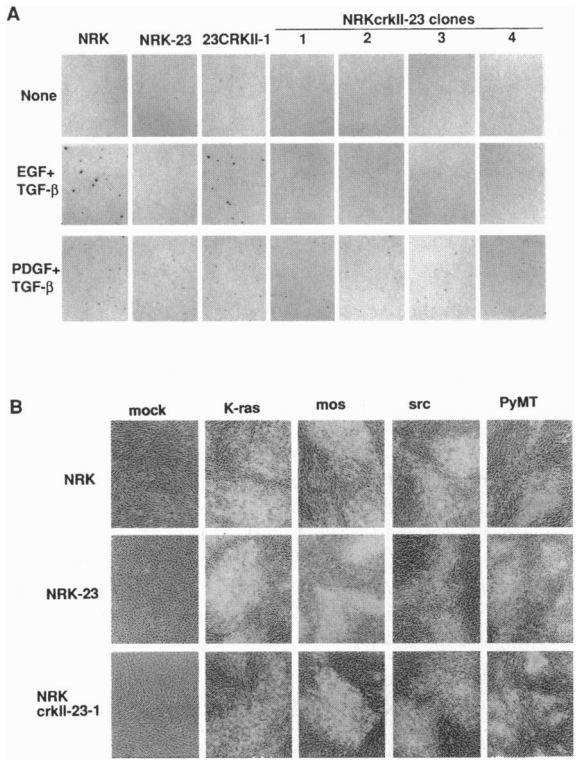

FIG. 1. Phenotypes of mutant 23, NRK and their transfected clones. 23CRKII-1, a clone of NRK-23 transfected with rat *crk*II cDNA; NRKcrkII-23-1 to -4, clones of NRK transfected with the mutant type *crkII* (*crk*II-23) cDNA. (*A*) Growth factor-stimulated colony formation in soft agar. Cells (1×10^4) were inoculated into soft agar containing the growth factors indicated and incubated for 2 weeks. (*B*) Focus formation by the oncogene-carrying retroviruses. NRK, NRK-23, and NRKcrkII-23-1 cells were infected with the oncogene-carrying retroviruses and incubated for 10 days.

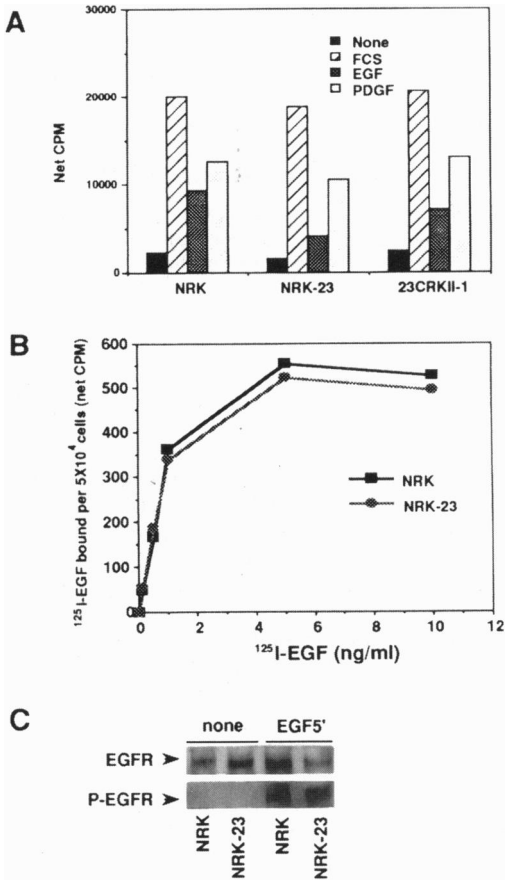

FIG. 2. Properties of NRK-23 and its complemented clones. (*A*) Proliferative responses to growth factors. For 23CRKII-1, see the legend to Fig. 1. The proliferative responses are shown by net cpm of [^{3}H]thymidine incorporated into DNA. Each experiment was done in triplicate and the average numbers of net cpm were shown in the figure. (*B*) ^{125}I-labeled EGF binding assay. The specific binding of ^{125}I-labeled hEGF to 5×10^4 cells of NRK or NRK-23 was shown as net cpm. Each experiment was done in duplicate and the average numbers of net cpm were shown in the figure. (*C*) EGF-dependent EGFR phosphorylation. Cells were starved for serum for 24 hr, stimulated for 5 min with 10 ng/ml EGF, lysed, and immunoprecipitated with an anti-hEGFR antibody. EGFR and tyrosine-phosphorylated EGFR (P-EGFR) were detected by Western blotting with anti-hEGFR and antiphosphotyrosine antibodies, respectively.

were detected with anti-Crk and anti-Grb2 antibodies (Transduction Laboratories, Lexington, KY), respectively. The anti-Crk antibody recognizes both CrkI (28 kDa) and CrkII (40 and 42 kDa). Phosphorylated and unphosphorylated Erks were detected by the Phototape Western Detection System using tyrosine-phosphorylated Erk-specific and anti-Erk antibodies (Biolabs, Bererly, MA). For EGFR phosphorylation analysis, EGFR was immunoprecipitated from cell lysate (1 mg protein) with an anti-human EGFR (anti-hEGFR) antibody (Upstate Biotechnology, Lake Placid, NY) and detected with anti-hEGFR or anti-phosphotyrosine antibody (Transduction Laboratories).

Analysis of Ras-Bound GDP/GTP. NRK, NRK-23, and NRK-23CRKII-1 cells grown in 60-mm dishes were starved for 24 hr in serum-free DMEM. Cells were washed with phosphate-free MEM (GIBCO) and labeled with 100 μCi/ml of [^{32}P]orthophosphate for 3 hr (1 Ci = 37 GBq). After stimulation with EGF, guanine nucleotides bound to Ras were analyzed (28).

^{125}I-Labeled hEGF Binding Assay. Cells (2×10^5) were seeded in 24-well plates. After 2 days incubation, cells were washed twice with binding buffer (Hanks' balanced salt solution containing 0.1% bovine serum albumin). Cells were then incubated with radioiodinated EGF (Amersham) at 23°C for 60 min, washed with binding buffer, lysed, and counted (29). All experiments were performed in duplicate.

RESULTS

Characterization of Mutant NRK-23. To identify intracellular oncogenic signal cascades, we used MNNG treatment to generate NRK mutants that are unable to be transformed by EGF plus TGF-β (ET) (21, 22). Among those, one designated NRK-23 had a reduced sensitivity to ET, v-*erb*B, and activated c-*erb*B-2, but retained a full sensitivity to v-K-*ras* and all other oncogenes including v-*fms*, v-*fos*, v-*fes*, v-*mos*, v-*src*, simian virus 40 large T antigen, polyoma middle T antigen, v-*raf*, and adenovirus E1A (Fig. 1 and Table 1). In a proliferation assay, NRK-23 showed a 2-fold reduced response to EGF, but a full response to PDGF and serum (Figs. 1 and 6). The defect in NRK-23 was unlikely to be in EGFR because the mutant had the same level of EGF binding capacity and EGF-induced EGFR phosphorylation as NRK (Fig. 2 *B* and *C*), was unable to be transformed by v-*erbB* (Table 1) or rescued by expression of a EGFR cDNA (data not shown). These data suggest that NRK-23 had a defect in a pathway from EGFR to Ras since NRK-23 was fully sensitive to *ras* oncogene.

EGF-Induced Ras Activation Is Defective in NRK-23. We examined EGF-induced Ras and Erk activation biochemically. The GTP and GDP forms of p21^{ras} were immunoprecipitated after stimulation with EGF and quantified. In NRK, upon stimulation, the proportion of Ras-bound GTP rapidly increased to 21% at 5 min and returned to a basal level within

Table 1. Sensitivity of mutant NRK-23 and its clones to transformation by growth factors and oncogenes

				NRKcrkII-23			
	NRK	NRK-23	23CRKII-1	1	2	3	4
	No. of colonies/10^4 viable inoculates in soft agar*						
None	0	0	0	0	0	0	0
EGF + TGF-β	4950	117	3495	71	112	127	22
PDGF + TGF-β	3351	1450	2636	3371	2626	1431	2430
	No. of foci/dish†						
Mock	0	0	0	0	0	0	0
K-*ras*	1920	1840	1905	1895	1800	1685	1615
mos	1490	1320	1385	1445	1635	1370	1490
src	855	875	840	920	965	815	825
PyMT	1295	1020	1020	1240	1120	1020	1190
	No. of transformed colony/no. of total colony(%)‡						
Neo/hyg	0/676	0/583	0/115	0/470	0/569	0/620	0/594
	(<0.15)	(<0.17)	(<0.9)	(<0.2)	(<0.2)	(<0.2)	(<0.2)
v-*erb*B	12/218	0/221	21/226	0/218	0/321	0/180	0/209
	(5.5)	(<0.5)	(9.3)	(<0.5)	(<0.3)	(<0.6)	(<0.5)
c-*erb*B-2	46/358	11/271	110/781	9/273	15/312	6/198	5/244
	(12.8)	(4.1)	(14.1)	(3.3)	(4.8)	(3.0)	(2.0)
fms	52/415	26/261					
	(12.5)	(10.0)					
fes	13/103	34/160					
	(12.6)	(21.3)					
fos	468/1404	420/1320					
	(33.3)	(31.8)					
SV40-T	154/491	129/386					
	(31.4)	(33.4)					
raf	74/271	17/154					
	(27.3)	(11.0)					
E1A	17/63	37/110					
	(27.0)	(33.6)					

PyMT, polyoma middle T antigen; SV40-T, simian virus 40 large T antigen.

*Soft agar colony formation was assayed in duplicate. The number of colonies (>0.1 mm in diameter) was counted 2–3 weeks after soft agar inoculation.

†Focus assay with oncogene-carrying retroviruses were done in duplicate. Ten days to 2 weeks after infection, transformed foci were counted and the average numbers of foci are shown.

‡Cells transfected by plasmid DNAs were cultured with the growth medium containing G418 (400 μg/ml) or hygromycin B (hyg) (100 μg/ml) for 2–3 weeks and then their morphology were examined.

30 min (Fig. 3*A*; see Fig. 5*C*). NRK-23 slightly responded to EGF with a similar time course, but the increase was less than a third of NRK cells (Fig. 3*A*). EGF-induced activation of Erks in NRK-23 was also strikingly reduced (Fig. 3*B*). In both assays, the response of NRK and NRK-23 to EGF was essentially same with or without the addition of TGF-β (data not shown). We conclude that NRK-23 has a defect in signaling from EGFR to Ras.

CrkII Rescues Mutant NRK-23. An adapter seemed to be a good candidate for the factor defective in NRK-23. We therefore tested several putative adapter genes for their ability to phenotypically complement NRK-23. The *crk*I, *crk*II, *grb2*, and *nck* cDNAs were inserted into an expression vector and transfected into NRK-23. *crk*II restored oncogenic response in 15% of the G418-resistant colonies, whereas *crk*I had only a slight activity, while *grb2* and *nck* virtually none (Table 2). The putative adapter genes themselves had no detectable oncogenic activity to NRK or NRK-23 (Table 2). Of eight independent stable transfectants that were examined, there was good correlation between the restoration of the transformability by ET and the elevated level of CrkII expression (Fig. 3*C*). The complemented cells were also fully capable of oncogenic responses to v-*erb*B and activated c-*erb*B-2 and of a proliferative response to EGF. Data on one representative complemented clone (23CRKII-1) are shown in Fig. 1*A*, Fig. 2*A*, Fig. 3 *A* and *B*, and Table 1. If CrkII is indeed the factor defective in NRK-23, activation of Ras and Erks should also be restored in these cells. Fig. 3 *A* and *B* shows that Ras and Erks were activated to the same extent in the parent NRK and 23CRKII cells. These results strongly suggest that CrkII is defective in NRK-23.

One Crk Allele from NRK-23 Has Point Mutations. To identify possible mutations in the *c-crk* gene in NRK-23, *crk*II cDNAs were cloned from both NRK-23 and NRK. Sequence analysis revealed that NRK-23 expressed two different *crk*II mRNAs: one identical to the *crk*II mRNA in parent NRK and the other with point mutations at nucleotides 731 and 757 (Fig. 4*A*). The presence of these two mutations was fully confirmed by direct sequencing of reverse-transcribed preamplified *crk*II mRNA (Fig. 4*B*) and by sequencing a cloned genomic c-*crk* gene fragment corresponding to that region (Fig. 4*C*). These point mutations resulted in two amino acid changes from glutamine to arginine and lysine to glutamic acid in the C-terminal SH3 domain (Fig. 4*D*) leading to inactivation of CrkII function (Table 3). The presence of double mutations in one gene, such as this, is not unusual since MNNG is a direct alkylating agent known to act predominantly at the replicating points, frequently generating closely spaced multiple mutations (30).

Mutations Are Dominant Negative. Despite the presence of the wild-type *crk* gene, NRK-23 was defective in signaling from EGFR to Ras. These data indicated that the mutation in *crk*II of NRK-23 was likely to be a dominant-negative type. This proved to be the case because transfection of NRK cells with mutant *crk*II cDNA (*crk*II-23) yielded cells that were unable to be transformed by ET. Four nonresponding colonies (NRKcrkII-23-1 to -4) were isolated and examined for CrkII expression and NRK-23-related phenotypes. They expressed 3- to 5-fold higher levels of CrkII (mutant plus wild-type CrkII)

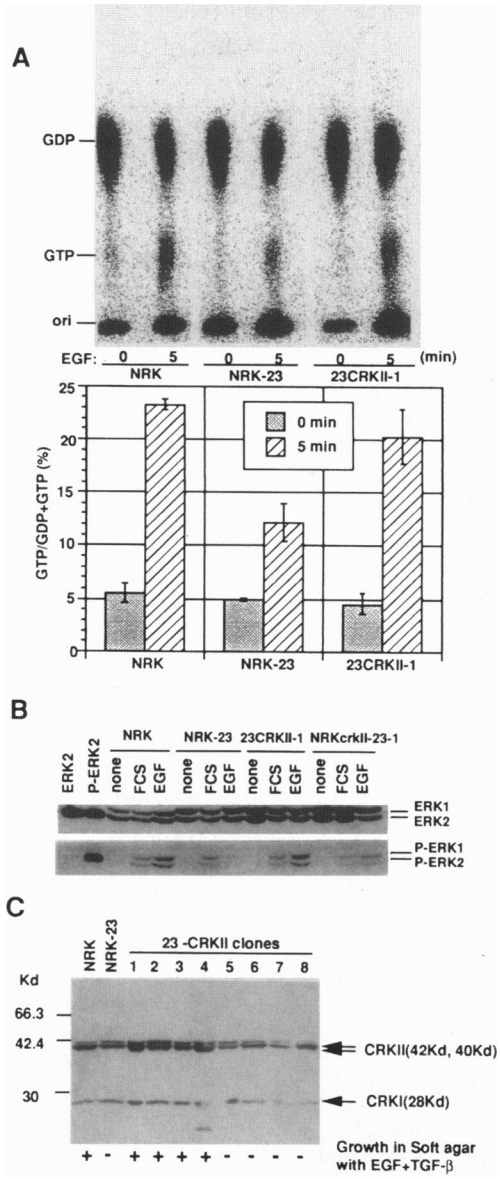

FIG. 3. Ras and Erk activation in NRK and NRK-23. (*A*) Ras activation. NRK, NRK-23, and 23CRKII-1 cells were starved for serum and labeled with [^{32}P]orthophosphate. After stimulation with EGF for 5 min, guanine nucleotides bound to Ras were analyzed. (*Lower*) Graph shows mean values with error bars obtained from three independent experiments. (*Upper*) One representative experiment is shown. Ori, GDP, and GTP are the origin of the chromatography and the spots of GDP and GTP, respectively. (*B*) Activation of Erks. For NRKcrkII-23-1, see the legent to Fig. 5. NRK, NRK-23, 23CRKII-1, and NRKcrkII-23-1 cells were serum starved for 48 hr and stimulated with 5% fetal calf serum or 10 ng/ml EGF for 10 min. Total cell lysates (15 μg) were analyzed by Western blotting with anti-Erk antibody (*Upper*) or antibody specific to tyrosine-phosphorylated Erk (*Lower*). ERK2 and P-ERK2 were control proteins for nonphosphorylated and phosphorylated ERK2, respectively. (*C*) Crk expression in NRK-23 clones transfected with rat *crkII* cDNA. 23CRKII-1 to -8, clones of NRK-23 transfected with rat *crk*II cDNA.

than the cells transfected with the empty vector (Fig. 5*A*). The phenotype of the *crk*II-23-expressing NRK cells was similar to NRK-23, in both oncogenic (Fig. 1 and Table 1) and proliferative responses (Fig. 5*B*). Furthermore, just like in NRK-23, activation of both Ras and Erks was markedly reduced in the *crkII*-23-expressing NRK cells (Figs. 3*B* and 5*C*). We conclude that the inability of NRK-23 to signal from the EGFR is a consequence of a dominant-negative type mutation in one c-*crk* allele.

Table 2. Phenotypic complementation of mutant NRK-23 by CrkII

	No. of transformed colony/no. of G418-resistant colony (%)	
	NRK (−ET)*	NRK-23 (+ET)†
Neo	0/490 (<0.2)	2/1120 (0.18)
Nck	0/480 (<0.2)	0/496 (<0.2)
Grb2	0/160 (<0.6)	0/618 (<0.2)
CrkI	0/267 (<0.4)	4/950 (0.42)
CrkII	0/386 (<0.3)	89/534 (16.7)

NRK and NRK-23 cells were transfected with an expression vector containing an adapter cDNA and cultured in G418-containing growth medium for 2 weeks.

*G418-resistant colonies were examined for their morphology in the absence of ET.

†G418-resistant colonies were cultured in ET-containing medium for 5 to 7 days, and transformed and flat colonies were counted. All colonies of NRK cells were transformed in ET-containing growth medium.

Overexpression and Reduced Expression of Grb2 Have Little Effect on 23's Phenotype. Since Grb2 has been thought to play a major role in mediating signals from EGFR to Ras, it is important to know what role Grb2 may play in the CrkII-mediated signaling in NRK cells. To address this point, we examined the effect of reduced expression or overexpression of *grb*2 on the NRK-23 phenotypes. Two independent colonies (23A$^-$18 and 23A$^-$40) having 4- to 6-fold lower expression of Grb2 were established by expression of antisense *grb*2 RNA (Fig. 6). The proliferative response to EGF of these clones was slightly reduced form that of NRK-23. Furthermore, 2- to 3-fold overproduction of Grb2 was unable to rescue the NRK-23's ability to respond to ET (23A$^+$5 and 23A$^+$7). By contrast, expression of *crk*II in 23A$^-$18 and 23A$^-$40 effectively restored their ability to be transformed by ET (12–14% of stably transfected colonies regained response); their proliferative response to EGF was also restored to that of NRK, despite the fact that the level of Grb2 was reduced (Fig. 6). The lack of significant phenotypic changes by reduction or overproduction of Grb2 is not due to some possible mutations in the EGF receptor because the same amount of Grb2 and Shc was coimmunoprecipitated with EGFR in both NRK and NRK-23 cells (data not shown). These results indicate that Grb2 has no significant role in oncogenic signaling from EGFR although it may play some role in proliferative signaling from EGFR.

DISCUSSION

One emerging feature of signal transduction from various receptor tyrosine kinases to small G proteins, particularly to Ras, is that it might be mediated by broad-specificity adapter molecules (10, 31). Such a claim seems to be exemplified by biochemical data demonstrating that the putative adapter molecules Grb2, Nck, and Crk bind to a variety of receptor

Table 3. Dominant negative effect of mutant type *crk*II

	No. of transformed colony/no. of hyg-resistant colony (%), NRK-23 (+ ET)	No. of flat colony/no. of hyg-resistant colony (%), NRK (+ ET)
Hyg	2/650 (0.31)	0/136 (<0.73)
*crk*II-23	4/1181 (0.34)	65/619 (10.5)

NRK cells were transfected with *crk*II-23 or an empty vector, and cultured in hygromycin B (hyg)-containing medium for 2 weeks. Hyg-resistant colonies were then cultured in ET-containing medium for 5–7 days, and transformed and flat colonies were counted.

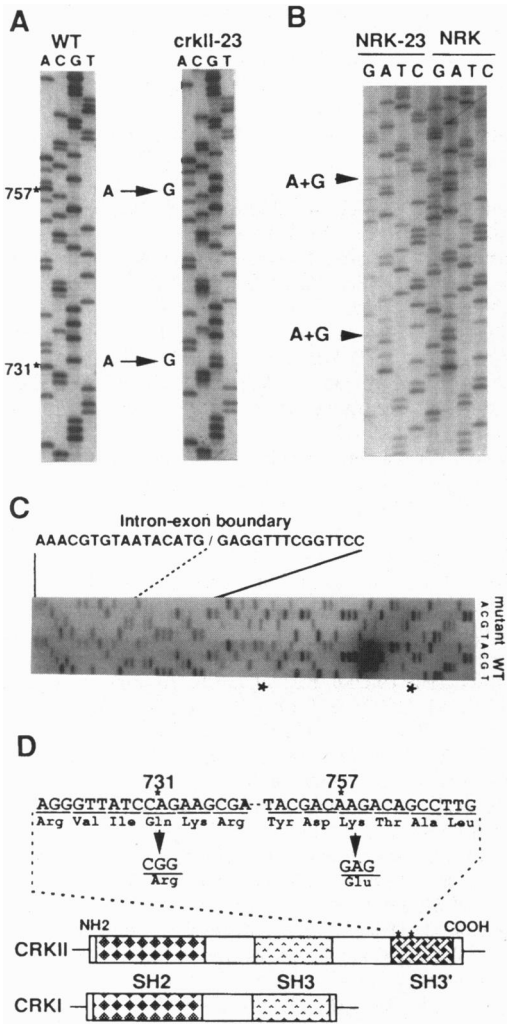

FIG. 4. Sequence analysis of *crk*II cloned from NRK-23. (*A*) Sequences of the mutated region of the wild-type and mutant-type *crk*II cDNAs. The mutated bases are indicated by nucleotide numbers from the initiation codon for *crk*II (GenBank accession no. D44481). (*B*) Direct sequencing of the C-terminal SH3 region of reverse transcripts of *crk*II mRNA. (*C*) *crk* genomic DNA sequencing. The mutated bases are indicated by asterisks. (*D*) Schematic representation of the rat *c-crk* cDNA structure and the point mutations.

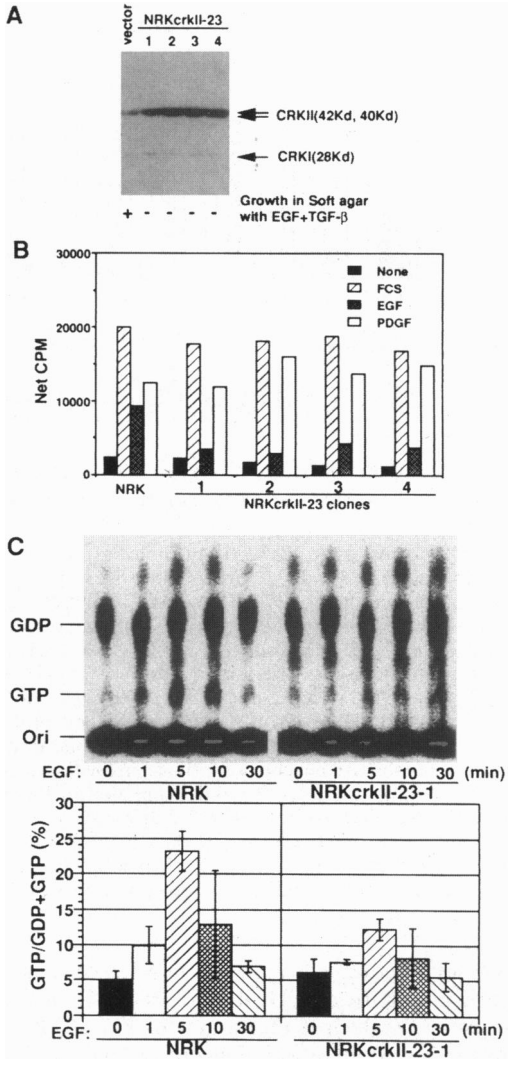

FIG. 5. Analysis of NRKcrkII-23 clones. (*A*) Western blot analysis. NRKcrkII-23-1 to -4, clones of NRK transfected with *crk*II-23 cDNA. Vector, NRK transfected with empty vector. (*B*) Proliferative responses to growth factors. Proliferative responses are shown by net cpm of [^{3}H]thymidine incorporated into DNA. Each experiment was done in triplicate, and the average numbers of net cpm were shown in the figure. (*C*) Ras activation. NRK and NRKcrkII-23-1 cells were serum-starved, stimulated with EGF for the indicated time, and analyzed for the guanine nucleotides bound to Ras as described. (*Lower*) Graph shows mean values with error bars obtained from three independent experiments.

tyrosine kinases and GNRP *in vitro* and/or *in vivo*, each displaying a considerably overlapping binding spectrum (10, 19). The molecular genetical data we present here suggest that adapter molecules are more specific, each likely mediating signals from a limited number of discrete receptor tyrosine kinases or tyrosine-phosphorylated proteins. In fact, our data show that the oncogenic signal from EGFR to Ras is predominantly mediated by CrkII in the NRK fibroblast cell line despite the presence of Grb2 (Fig. 3). More strikingly, CrkII is only partially involved in signaling from the c-*erb*B-2 receptor tyrosine kinase in spite of its close similarity to EGFR, and at most insignificantly involved in signaling from the PDGF receptor to Ras (Table 1).

Just like for signaling from Drk/Sem-5 to Ras in *D. melanogaster* and *C. elegans* (1–3), GNRP must be required for the CrkII to Ras signaling. Candidates for this are still Sos and C3G as previously reported (32). The Crk and v-Crk proteins associate with Sos and C3G as Grb2 does (18, 33), and expression of C3G induces Ras activation in COS cells although the same molecule promotes the guanine nucleotide exchange of antagonistic Rap1/Krev1/Smg25 *in vitro* (34).

CrkI physically interacts with endogenous and exogenous oncogenic Abl more effectively than CrkII (19, 35). Therefore, CrkI, and less effectively CrkII, are thought to mediate signal from Abl. Some amino acid substitutions in the SH2 or SH3 domains of CrkI effectively block Abl-induced Erk activation, whereas the same substitution mutants of CrkII show little effect on Abl-induced Erk activation but enhance EGF-induced Erk's activation (36). This suggests that the mode of Crk's involvement in Abl-induced Ras activation may be significantly different from that in EGF-induced Ras activation. The closely spaced two point mutations in the C-terminal SH3 domain effectively convert CrkII to an inactive, dominant-negative CrkII molecule, which efficiently blocks the action of normal CrkII. This illuminates the importance of this domain in signal transduction although it previously drew little attention and has been thought to play only some regulatory role (15).

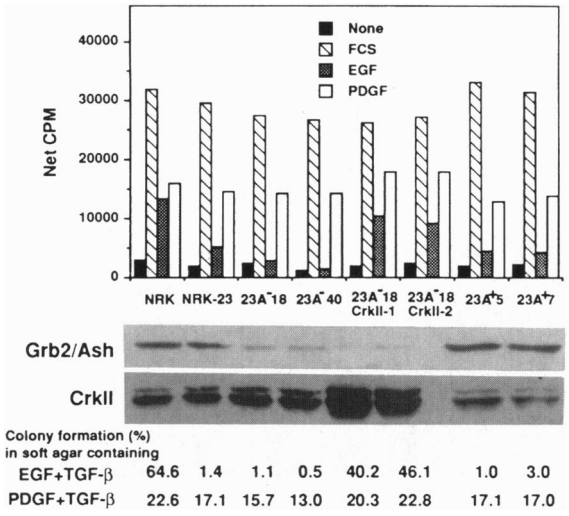

FIG. 6. Phenotypes of the NRK-23 cell clones expressing low and high levels of Grb2. 23A^{+}5 and 23A^{+}7 were those overexpressing Grb2 obtained by transfection with *grb2* cDNA. 23A^{-}18 and 23A^{-}40 were those expressing a reduced amount of Grb2 obtained by transfection with the pEF/hygI vector containing the 390-bp *Xba*I–*Sca*I fragment of *grb2* cDNA in the antisense orientation followed by G418 selection as described (23). 23A^{-}18CrkII-1 and -2 are clones of NRK-23A^{-}18 transfected with *crk*II cDNA. (*Top*) Graph shows their proliferative responses to growth factors. Each experiment was done in triplicate and the average numbers of net cpm were shown in the figure. (*Middle*) Shown are the levels of Grb2 and CrkII expression in these clones analyzed by Western blot with anti-Grb2 and anti-Crk monoclonal antibodies. (*Bottom*) Percentage of their colony formation in soft agar containing the indicated growth factors. Each experiment was done in duplicate and the average numbers of colonies were shown in the figure.

Our previous studies indicated that in NRK cells part of the signals originating from EGFR and PDGFR merge to a common pathway which is the target for the action of most oncogenes and is essential for oncogenic growth, and that the rest of the signals induce general anchorage-dependent growth (21–23). The presence of two distinct proliferative signals is particularly evident for signaling from PDGFR as indicated by two mutants (21). The results presented here further confirm the presence of two distinct proliferative signals and suggest that the signal for oncogenic growth from EGFR is predominantly mediated by CrkII.

Grb2 seems to play some role in a growth signaling pathway as reduced Grb2 expression in NRK-23 resulted in a reduction of the proliferative response to EGF. The nature of the growth signaling pathway in which Grb2 may be involved is unclear at present, but there are two possibilities. One is that a proliferative signal might branch off from the EGF receptor and be transduced via a Ras-independent pathway, just like one from PDGFR (21). The second is that cells might contain two parallel systems for signaling form EGFR to Ras: one for transient and the other for prolonged signaling, and that Grb2 might be involved in transient signaling whereas CrkII in prolonged signaling, the latter of which is essential for oncogenic signal transduction. Since a slight activation of Ras still occurs in NRK-23 and NRKcrkII-23 cells in response to EGF, the latter possibility seems to be more likely.

We thank T. Takenawa and K. Matuoka for *ash* cDNA, M. Nakafuku for Y13-259 antibody, and T. Tanaka and H. Hirai for unpublished data about the genomic structure of the mouse c-*crk*. This work was supported in part by research grants to H.O. from the Ministry of Education, Science and Culture of Japan, Human Frontier Science Program, and Asahi Glass Foundation.

1. Clark, S. G., Stern, M. J. & Horvitz, H. R. (1992) *Nature (London)* **356,** 340–344.
2. Olivier, J. P., Raabe, T., Henkenmeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E. & Pawson, T. (1993) *Cell* **73,** 179–191.
3. Simon, M. A., Dodson, G. S. & Rubin, G. M. (1993) *Cell* **73,** 169–177.
4. Lowenstein, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E. Y., Bar-Sagi, D. & Schlessinger, J. (1992) *Cell* **70,** 431–442.
5. Matuoka, K., Shibata, M., Yamakawa, A. & Takenawa, T. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 9015–9019.
6. Buday, L. & Downward, J. (1993) *Cell* **73,** 611–620.
7. Gale, N. W., Kaplan, S., Lowenstein, E. J., Schlessinger, J. & Bar-Sagi, D. (1993) *Nature (London)* **363,** 88–92.
8. Li, N., Batzer, A., Daly, R., Yajnik, V., Skolnik, E., Chardin, P., Bar-Sagi, D., Margolis, B. & Schlessinger, J. (1993) *Nature (London)* **363,** 85–88.
9. Rozakis-Adcock, M., Fernely, R., Wade, J., Pawson, T. & Bowtell, D. (1993) *Nature (London)* **363,** 83–85.
10. Pawson, T. (1995) *Nature (London)* **373,** 573–580.
11. Reichman, C. T., Mayer, B. J., Keshav, S. & Hanafusa, H. (1992) *Cell Growth Differ.* **3,** 451–460.
12. Mayer, B. J., Hamaguchi, M. & Hanafusa, H. (1988) *Nature (London)* **332,** 272–275.
13. Tsuchie, H., Chang, C. H. W., Yoshida, M. & Vogt, P. K. (1989) *Oncogene* **4,** 1281–1284.
14. Matsuda, M., Tanaka, S., Nagata, S., Kojima, A., Kurata, T. & Shibuya, M. (1992) *Mol. Cell. Biol.* **12,** 3482–3489.
15. Ogawa, S., Toyoshima, H., Kozutsumi, H., Hagiwara, K., Sakai, R., Tanaka, T., Hirano, N., Mano, H., Yazaki, Y. & Hirai, H. (1994) *Oncogene* **9,** 1669–1678.
16. Birge, R. B., Fajardo, J. E., Mayer, B. J. & Hanafusa, H. (1992) *J. Biol. Chem.* **267,** 10588–10595.
17. Margolis, B., Silvennoinen, O., Comoglio, F., Roonprapunt, C., Skolnik, E., Ullrich, A. & Schlessinger, J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 8894–8898.
18. Matsuda, M., Hashimoto, Y., Muroya, K., Hasegawa, H., Kurata, T., Tanaka, S., Nakamura, S. & Hattori, S. (1994) *Mol. Cell. Biol.* **14,** 5495–5500.
19. Feller, S. M., Ren, R., Hanafusa, H. & Baltimore, D. (1994) *Trends Biochem. Sci.* **19,** 453–458.
20. Meisenhelder, T. & Hunter, T. (1992) *Mol. Cell. Biol.* **12,** 5843–5856.
21. Masuda, A., Kizaka-Kondoh, S., Miwatani, H., Terada, Y., Nojima, H. & Okayama, H. (1992) *New Biol.* **4,** 489–503.
22. Kume, K., Jinno, S., Miwatani, H., Kizaka-Kondoh, S., Terada, Y., Nojima, H. & Okayama, H. (1992) *New Biol.* **4,** 504–511.
23. Kizaka-Kondoh, S., Sato, K., Tamura, K., Nojima, H. & Okayama, H. (1992) *Mol. Cell. Biol.* **12,** 5078–5086.
24. Okayama, H. (1987) *Methods Enzymol.* **151,** 434–444.
25. Lehmann, J. M., Riethmuller, G. & Johnson, J. P. (1990) *Nucleic Acids Res.* **18,** 1048.
26. Mizushima, S. & Nagata, S. (1990) *Nucleic Acids Res.* **18,** 5322.
27. Chen, C. & Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752.
28. Muroya, K., Hattori, S. & Nakamura, S. (1992) *Oncogene* **7,** 277–281.
29. Carpenter, G. & Cohen, S. (1976) *J. Cell Biol.* **71,** 159–171.
30. Cerda-Olmedo, E., Hanawalt, P. C. & Guerola, N. (1968) *J. Mol. Biol.* **33,** 705–711.
31. Schlessinger, J. & Bar-sagi, D. (1994) *Cold Spring Harbor Symp. Quant. Biol.* **59,** 173–179.
32. Tanaka, S., Morishita, T., Hashimoto, Y., Hattori, S., Nakamura, S., Shibuya, M., Matuoka, K., Takenawa, T., Kurata, T., Nagashima, K. & Matsuda, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 3443–3447.
33. Teng, K. K., Lander, H., Fajardo, J. E., Hanafusa, H., Hempstead, B. L. & Birge, R. B. (1995) *J. Biol. Chem.* **270,** 20677–20685.
34. Gotoh, T., Hattori, S., Nakamura, S., Kitayama, H., Noda, M., Takai, Y., Kaibuchi, K., Matsui, H., Hatase, O., Takahashi, H., Kurata, T. & Matsuda, M. (1995) *Mol. Cell. Biol.* **15,** 6746–6753.
35. Ren, R., Ye, Z.-S. & Baltimore, D. (1994) *Genes Dev.* **8,** 783–795.
36. Tanaka, M., Gupta, R. & Mayer, B. J. (1995) *Mol. Cell. Biol.* **15,** 6829–6837.