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Two Species of Human *CRK* cDNA Encode Proteins with Distinct Biological Activities

MICHIYUKI MATSUDA,[1,2]* SHINYA TANAKA,[1] SATOSHI NAGATA,[1] ASATO KOJIMA,[1] TAKESHI KURATA,[1] AND MASABUMI SHIBUYA[3]

Department of Pathology, National Institute of Health, 2-10-35, Kamiosaki, Shinagawa-ku, Tokyo 141[1]; Cell and Information Group, PRESTO, Research Development Corporation of Japan, Gojo-Karasuma, Shimokyo-ku, Kyoto 600[2]; and Department of Genetics, The Institute of Medical Science, The University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108,[3] Japan

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Two distinct human *CRK* cDNAs, designated *CRK*-I and *CRK*-II, were isolated from human embryonic lung cells by polymerase chain reaction and by screening of a human placenta cDNA library, respectively. *CRK*-I differed from *CRK*-II in that it lacked a 170-nucleotide sequence, suggesting that *CRK*-I and *CRK*-II were the products of alternative splicing. The amino acid sequences deduced from these two cDNAs differed in the carboxyl termini and contained one SH2 and either one or two SH3 domains. RNase protection analysis demonstrated both *CRK*-I and *CRK*-II mRNAs in various human cells. Three *CRK* proteins, of 42, 40, and 28 kDa, were identified in human embryonic lung cells by means of antibodies against the SH2 region and the SH3 region of the bacterially expressed *CRK*-I protein. Transient expression of *CRK*-I and *CRK*-II cDNAs in COS7 cells showed that the former encoded the 28-kDa protein and the latter encoded the 40- and 42-kDa proteins. All human cell lines so far examined expressed the 40-kDa protein; however, expression of the 28- and the 42-kDa proteins was variable. In a comparison of the biological activity of the two human CRK proteins, both proteins were stably expressed in rat 3Y1 cells. All cell lines expressing *CRK*-I protein showed altered morphology, proliferated in soft agar, and grew as massive tumors in nude mice. Although *CRK*-II-expressing cells showed a slight morphologic change, they did not make colonies in soft agar or grow in nude mice. These results demonstrate that the two species of human *CRK* cDNA encode proteins which differ in their biological activities.

The v-*crk* oncogene has been isolated from chicken retroviruses CT10 and ASV-1 (22, 43). The v-*crk* oncogene product shares homologous amino acid sequences, designated the SH2 and SH3 domains (14, 28), with many molecules involved in signal transduction, such as nonreceptor-type tyrosine kinases (22, 33), *ras* GTPase-activating protein (42, 44), phospholipase C-γ (4, 38, 40), and an 85-kDa subunit of phosphatidylinositol kinase type I (5, 27, 36). The c-*crk*-derived region of v-*crk* consists mainly of the SH2 and SH3 regulatory domains and does not have any known catalytic domains (32). In addition to its transforming activity, v-*crk* has two peculiar features. First, cells transformed by v-*crk* show an elevated level of phosphotyrosine, despite a lack of tyrosine kinase activity in v-Crk protein (22, 23). Second, v-Crk associates with a broad range of phosphotyrosine-containing proteins (19, 24). Both of these properties have been attributed to the SH2 domain of v-Crk, although some enhancing effect of SH3 has been suggested (20, 21, 25).

Analysis of the c-*crk* gene product is essential for understanding the mechanism of transformation by v-*crk*. Reichman and Hanafusa isolated a chicken c-*crk* cDNA and showed that its product had an additional SH3 domain (a total of two) and still lacked any known catalytic domains (32). Kriz and Knopf isolated a human homolog of chicken c-*crk* (*CRK*) and demonstrated that its product resembled chicken v-*crk* more than c-*crk* in terms of its carboxyl end and, as a consequence, in terms of the number of SH3 domains (13). We have isolated two distinct human *CRK* cDNAs that correspond to chicken v-*crk* and c-*crk*, and we have found that they encode proteins with differing transforming activities.

MATERIALS AND METHODS

Cloning of human *CRK* cDNAs. Human *CRK* cDNA was first isolated from human embryonic lung cells by the polymerase chain reaction (PCR) (34). A partial nucleotide sequence of a human *CRK* cDNA covering the putative coding region was provided by J. Knopf. A set of two primers was designed to include a *Bam*HI restriction site and either the amino- or the carboxyl-terminal region of the CRK protein as follows: 5′-CGGGATCCATGGCGGGCAACT TCGACTCGG-3′ for the 5′ end (primer 1) and 5′-CCG GATCCAGCTCACCGACCTCCAATCAGA-3′ for the 3′ end (primer 2). Total RNA was prepared from human embryonic lung cells by the cesium trifluoroacetate cushion procedure according to the protocol of the manufacturer (Pharmacia Biotechnology). cDNAs were transcribed from 1 μg of total RNA with Moloney murine leukemia virus reverse transcriptase at 42°C for 30 min, followed by PCR. The temperatures and cycle times used were 95°C (60 s), 60°C (60 s), and 72°C (60 s) for 35 cycles each. A 630-bp product of PCR was gel purified, cleaved by *Bam*HI, and ligated into pUC118 (Toyobo Inc.). The recombinants were designated p*CRK*-I. The nucleotide sequences of four independent clones were determined by the dideoxy-chain termination method and compared with each other. A nucleotide identical in more than three clones was characterized as a "correct" nucleotide. By this definition, one clone had

* Corresponding author.

correct nucleotides throughout the sequence, and it was used further as a *CRK*-I cDNA.

We used the *CRK*-I cDNA as a probe to screen a λgt11 human placenta cDNA library. Two independent clones covering the coding region were obtained. The cDNA fragments were subcloned into pUC118 and sequenced by the dideoxy-chain termination method. The nucleotide sequences of these two clones were identical to each other but differed from that of *CRK*-I cDNA. Therefore, we designated the cDNAs obtained from the library *CRK*-II.

Analysis of RNA. Total RNA was prepared as described above, and poly(A)$^+$ RNA was subsequently isolated by oligo(dT)-cellulose chromatography (Pharmacia Biotechnology). *CRK*-I cDNA was labeled with [α-^{32}P]dCTP using random primers and Klenow enzyme (Boehringer Mannheim Biochemicals) and was used for Northern (RNA) analysis.

We performed an RNase protection assay using an RNase protection kit (Ambion, Inc.) according to the manufacturer's protocol. A 607-bp *Eco*RI-*Pvu*II fragment of p*CRK*-II was ligated with an *Eco*RI and *Sma*I fragment of pGEM-2 (Promega Corp.) to generate pGEM2hC16 (see Fig. 2). Radiolabeled antisense RNA was transcribed from *Eco*RI-linearized pGEM2hC16 with [α-^{32}P]UTP and T7 RNA polymerase. The labeled antisense RNA was gel purified and hybridized with 1 μg of mRNA, 10 μg of total RNA, or 10 μg of yeast RNA at 42°C for 16 h. After digestion with RNase A and RNase T_1, protected fragments were analyzed on a 5% denaturing polyacrylamide gel.

Construction of expression plasmids. Bacterial expression vectors were constructed as follows. A 260-bp fragment containing the SH3 region of *CRK*-I was amplified by PCR with primer 3 (5′-CCGGATCCAGGCAGGGTAGT-3′), which was designed to have a *Bam*HI restriction site and corresponded to positions 464 to 480, and with primer 2 (see Fig. 1). The product was cleaved by *Bam*HI and subcloned into the same site of the bacterial expression vectors, pET3a (39) and pGEX2T (37), to generate pET*CRK*(SH3) and pG*CRK*(SH3), respectively. A 370-bp fragment containing the SH2 region of *CRK* was similarly amplified by PCR with primer 1 and primer 4 (5′-CGAGATCTGGAAACTGGTTC TATCA-3′), which was designed to have a *Bgl*II restriction site and corresponded to positions 467 to 449. The products cleaved by *Bam*HI and *Bgl*II were subcloned into the *Bam*HI site of pGEX2T to generate pG*CRK*(SH2).

Vectors for transient expression of *CRK* cDNAs were constructed as follows. A 620-bp *Bam*HI fragment containing whole *CRK*-I cDNA was subcloned into a *Bgl*II site of pcDL-SRα296(*Bgl*II), which was derived from pcDL-SRα296 and contained the *Bgl*II site instead of the original *Pst*I cloning site (41). The resulting plasmid was designated pV*CRK*-I. Because the nucleotide sequence of *CRK*-II was identical to that of *CRK*-I except at its 3′ end, a 1,140-bp *Eco*RI fragment of *CRK*-II covering the 3′ end was exchanged with the corresponding *Eco*RI fragment of pV*CRK*-I to generate pV*CRK*-II.

Vectors for stable expression were constructed in a manner similar to that of the construction of the transient expression vectors. The 620-bp *Bam*HI fragment of *CRK*-I was subcloned into the same site of the expression vector pMEXneo and designated pM*CRK*-I. The 1,140-bp *Eco*RI fragment of *CRK*-II was exchanged with the corresponding *Eco*RI fragment of pM*CRK*-I to generate pM*CRK*-II.

Another set of stable-expression vectors carrying the hygromycin resistance gene (*HmB*^{r}) was constructed. *Sal*I fragments of pV*CRK*-I and pV*CRK*-II covering the regions necessary for expression were subcloned into the same site of pChmB-pL, which is a derivative of pCHD2L and contains an *HmB*^{r} gene (9). The resulting vectors were designated pH*CRK*-I and pH*CRK*-II.

Preparation of anti-CRK protein antibodies. A polyclonal antibody against the SH3 region of CRK-I protein was obtained by immunization of rabbits with bacterial fusion proteins. Recombinant proteins were produced from pET-*CRK*(SH3) and pG*CRK*(SH3) as described elsewhere (37, 39). A fusion protein containing glutathione S transferase and the *CRK* SH3 region was produced from pG*CRK*(SH3), purified by glutathione-Sepharose beads as described elsewhere (37), and inoculated into rabbits subcutaneously. An 8-kDa *CRK*(SH3) protein generated from pET*CRK*(SH3) was gel purified and inoculated subcutaneously as the first booster injection. The 8-kDa recombinant protein was also purified by sequential chromatography of DEAE-Sepharose and C2 reverse-phase chromatography and inoculated intravenously as the second and third booster injections.

Monoclonal antibodies against the *CRK* SH2 region were produced essentially as described elsewhere (8). A fusion protein containing *CRK* SH2 was produced from pG*CRK*(SH2), purified on a glutathione-Sepharose column, and used as immunogen. Details of the methods used and the characterization of these monoclonal antibodies will be reported elsewhere.

Cells and transfection. Cells were cultured in Dulbecco's modified Eagle medium or in RPMI 1640 supplemented with 10% fetal calf serum. The human cell lines used in the experiments were as follows: osteosarcoma cell line 143B (American Tissue Culture Collection [ATCC] CRL 8803); neuroblastoma cell line GOTO (35); primary human embryonic lung cells; cervical cancer cell line HeLa (ATCC CRL 2); epidermal cancer cell line A431 (ATCC CRL 1555); glioblastoma cell line T98G (ATCC CRL 1690); T-cell cell line H9 (29); and COLO 201 (ATCC CCL 224), COLO 320DM (ATCC CCL 222), and DLD-1 (ATCC CCL 221), which are colon cancer cell lines. Cells used for transfection were rat 3Y1 fibroblasts (12) and COS7 monkey kidney cells (ATCC CRL 1651). Crk-3Y1 is a 3Y1 cell line in which chicken v-*crk* is constitutively expressed from a pMEXneo-derived vector (17). Transfection of expression vectors was performed by the calcium phosphate precipitation method. Rat 3Y1 cells transfected with pMEXneo-derived vectors were selected by 0.5 mg of Geneticin (GIBCO Laboratories) per ml. Cells transfected with pChmB-pL-derived vectors were selected by 0.05 mg of hygromycin B (Boehringer Mannheim) per ml.

Anchorage-independent growth and tumorigenicity in nude mice. To assay anchorage-independent growth, 10^4 cells per 6-cm dish were plated in 3 ml of medium containing 10% fetal bovine serum and 0.4% agar over another layer of 3 ml of the same medium containing 0.55% agar. Tumorigenicity was assayed by subcutaneous inoculation of nude mice with 5×10^6 cells of Geneticin-selected cell lines.

Immunoblotting. Cell cultures were washed with Dulbecco's phosphate-buffered saline and lysed immediately with lysis buffer (50 mM Tris hydrochloride [pH 7.5], 5 mM EDTA, 2% sodium dodecyl sulfate [SDS]). Protein concentration was assayed by BCA kit (Pierce Chemical Co.) and adjusted to 2 mg/ml by the addition of lysis buffer. The cell lysates were combined with an equal volume of 2× Laemmli's sample buffer. A total of 10 μg of proteins was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membranes (Millipore Corp.). The membranes were preincubated with PBS containing 0.05% Tween 20 and 2% ovalbumin for 1 h and

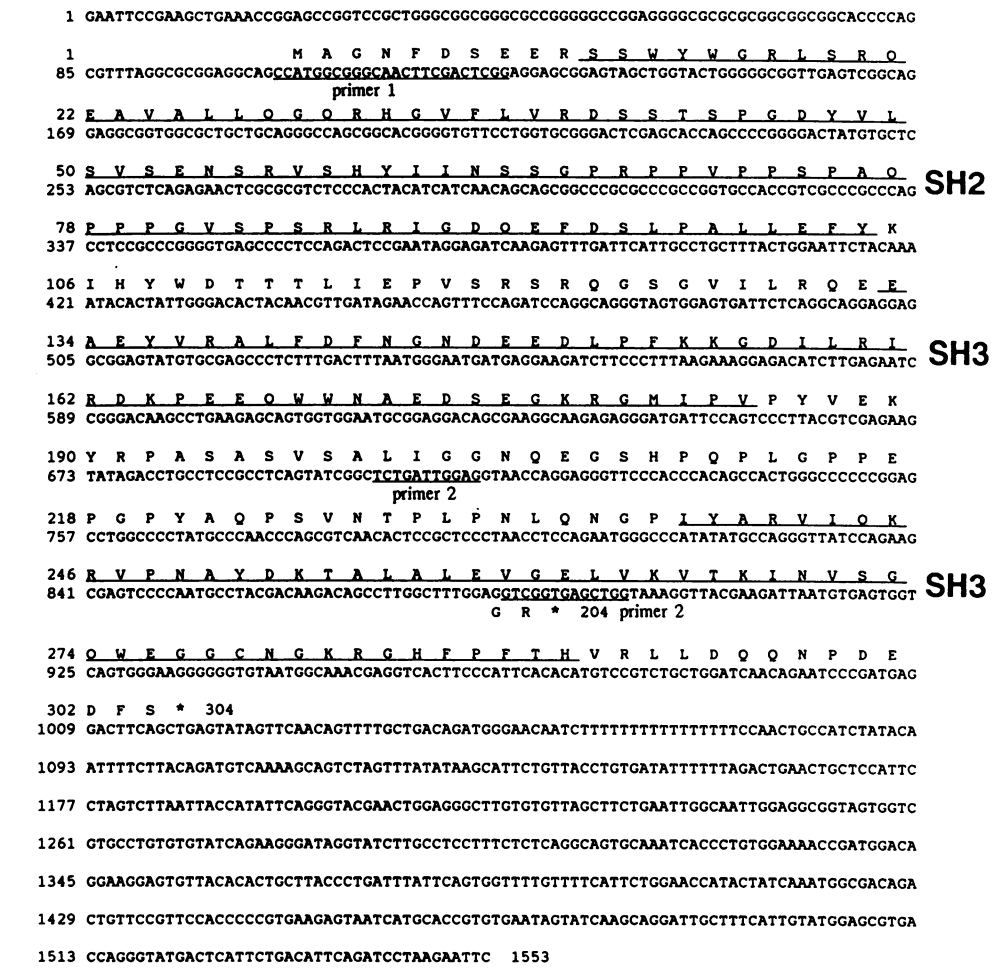

FIG. 1. Human *CRK*-II cDNA and protein sequences. Lines under the nucleotide sequences indicate the binding sites of the primers used for amplification of *CRK*-I cDNA. Note that the primer 2 binding site is split by the putative intron sequence (nt 713 to 882) that appears in the *CRK*-II cDNA. The other sequence of *CRK*-I was identical to that of *CRK*-II cDNA. The deduced amino acid sequence for the *CRK*-I amino terminus is shown below the primer 2 binding site. Lines under the amino acid sequence denote the SH2 and SH3 regions.

then with antiphosphotyrosine monoclonal antibody (PY20 [ICN Pharmaceuticals Inc.]) or anti-*CRK*(SH3) antibody for 2 h. The membranes were subsequently rinsed in PBS containing 0.05% Tween 20 and incubated with anti-mouse or anti-rabbit antibody conjugated with alkaline phosphatase (TAGO Inc.) for 1 h, followed by washing in PBS. The antibodies bound to the membrane were visualized with the chromogenic substrate bromochloroindolyl phosphate-nitroblue tetrazolium (8). In some experiments, peroxidase-conjugated anti-mouse antibody (TAGO Inc.) and the ECL chemiluminescence system (Amersham Corp.) were used for detection of mouse antibodies.

Immunoprecipitation. One of the anti-*CRK* SH2 monoclonal antibodies, 3A8, was covalently bound to protein A-Sepharose beads at 1 μg/μl as described elsewhere (8). Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris hydrochloride [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10% glycerol, 50 μM Na_3VO_4, 5 μM Na_2MoO_4, 100 mM NaF), and the protein concentration was determined as described above. Cell lysates containing 200 μg of protein were incubated with 3A8-protein A beads for 2 h at 4°C. Proteins associated with the beads were separated by SDS-PAGE (14% polyacrylamide), transferred to polyvinylidene difluoride membrane, and probed with the anti-*CRK*(SH3) rabbit antibody. Antibodies bound to the membrane were visualized as described above.

Nucleotide sequence accession number. The sequence data in this report have been submitted to the DDBJ/EMBL/GenBank libraries under the accession number D10656.

RESULTS

DNA sequence analysis of human *CRK* cDNA. A 620-bp fragment of human *CRK* cDNA was obtained by PCR and designated *CRK*-I. Using this fragment as a probe, we isolated distinct cDNA fragments from a human placenta cDNA library and designated them *CRK*-II. The nucleotide (nt) sequences and deduced amino acid sequences of *CRK*-I and *CRK*-II are shown in Fig. 1. The nucleotide sequence of *CRK*-I was identical to that of *CRK*-II, except for the *CRK*-I 3′ end. This 25-base sequence of the 3′ end of *CRK*-I (nt 707 to the 3′ end) appears downstream in *CRK*-II from nt 877 to 901 (Fig. 1), suggesting that the *CRK*-I and *CRK*-II mRNAs were generated by alternative splicing. The nucleotide sequence surrounding these putative splicing sites was arbi-

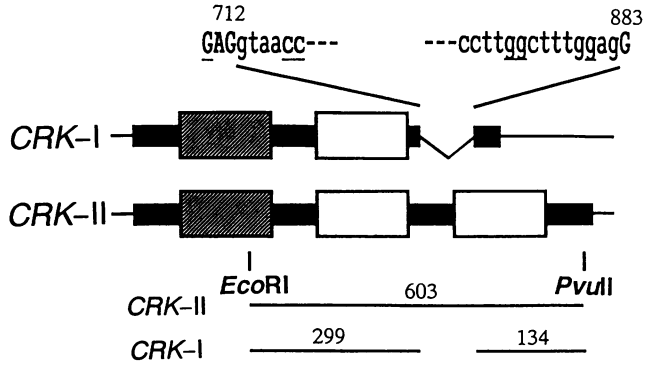

FIG. 2. Schematic representation of the *CRK* cDNA domain structure. Black bars, coding regions; hatched boxes, the SH2 domains; open boxes, the SH3 domains. The nucleotide sequences encompassing the putative splicing sites are shown at the top. Uppercase letters indicate nucleotides in the exon; lowercase letters indicate those in the intron. Underlining denotes the nucleotides which are not consistent with the consensus sequences of the splicing junction. The *Eco*RI-*Pvu*II fragment indicated at bottom is used as an antisense RNA probe. Horizontal lines are fragments expected to appear in RNase protection assay; their sizes are indicated (Fig. 4).

trarily aligned to provide the best homology with splice junction consensus sequences (26) (Fig. 2). The GT-AG rule is obeyed in the putative intron sequence. The 5′ junction matches the consensus sequence in six of nine positions. The 3′ junction has a pyrimidine-rich region (8 of 10 positions) followed by a short consensus sequence. Because genomic DNA of human *CRK* has not yet been obtained, we could not conclude that these two *CRK* cDNAs were transcribed from a single gene.

The *CRK*-I and *CRK*-II cDNAs encode proteins of 204 and 304 amino acids, respectively, which have one SH2 and either one or two SH3 regions. In both proteins, the SH2 and SH3 regions occupy nearly 60% of the total amino acid sequences. The nucleotide sequence of *CRK*-I was identical to that of the human *CRK* cDNA isolated by Kriz and Knopf except for 4 nucleotides, suggesting that these two are encoded by the same gene (13). The amino acid sequence encoded by *CRK*-II cDNA has 95% homology with that of the chicken c-*crk*. A total of 8 mismatches of 20 were in subdomain iii of SH2 (14) (between SH2 and SH2′ of Mayer et al. [22]), supporting the view that this region functions as a spacer region (20, 25).

Analysis of *CRK* mRNA. Northern blotting demonstrated a single band of 4.2 kb in the poly(A)-selected RNA pool (Fig. 3). To verify the presence of the two species of *CRK* mRNA, we performed an RNase protection assay. As shown in Fig. 2, *CRK*-II mRNA should generate a 603-base fragment and *CRK*-I should yield 299- and 134-base fragments after RNase treatment. Each of the three expected fragments was detected in mRNA from the human osteosarcoma cell line 143B (Fig. 4, lane 1) and in the total RNA fraction from the human neuroblastoma cell line GOTO (lane 2) and from human embryonic lung cells (lane 3). However, the 603-base fragment was only faintly visible in the lane of human embryonic lung cells, and the background was higher when the total RNA fraction was used. None of these fragments was detected in yeast RNA. Because the efficiency of hybridization varies according to the size of the fragments, we could not assess the relative abundance of *CRK*-I and *CRK*-II mRNAs.

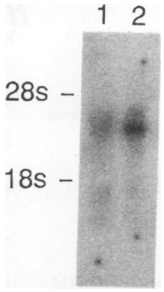

FIG. 3. Northern blot analysis of human *CRK* mRNA. Total RNA isolated from human osteosarcoma cell line 143B was fractionated into poly(A)$^-$ (lane 1) and poly(A)$^+$ (lane 2). The blot was hybridized with a radiolabeled *CRK*-I cDNA probe.

Analysis of *CRK* proteins. We used expression of the cDNAs of *CRK*-I and *CRK*-II in COS7 cells to correlate their products with endogenous CRK proteins. COS7 cells transfected with expression vectors carrying *CRK*-I and *CRK*-II cDNAs (pV*CRK*-I and pV*CRK*-II, respectively) were lysed and analyzed by immunoblotting with anti-*CRK*(SH3) antibody (Fig. 5A). Two species of protein, of 40 and 42 kDa, were detected in nontransfected monkey cells (anti-hCRK; Fig. 5A, lane 1). An additional 28-kDa protein was detected only in cells transfected with pV*CRK*-I (anti-hCRK; Fig. 5A, lane 2). The quantities of both 40- and 42-kDa proteins were increased in the cells transfected with pV*CRK*-II, indicating that *CRK*-II cDNA encoded the two proteins (anti-hCRK; Fig. 5A, lane 3).

To increase the sensitivity of detection, we first immunoprecipitated CRK proteins with an anti-*CRK*(SH2) monoclo-

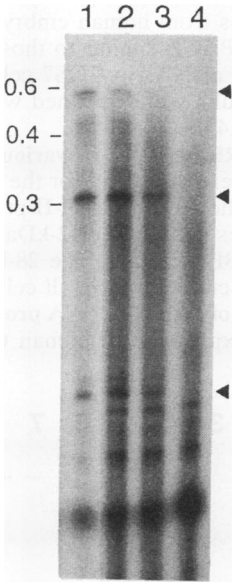

FIG. 4. RNase protection assay of human *CRK* mRNA. The [α-^{32}P]UTP-labeled antisense RNA probe was hybridized with 1 μg of poly(A)$^+$ RNA from 143B cells (lane 1), 10 μg of total RNA from GOTO (lane 2) and human embryonic lung cells (lane 3), and 10 μg of yeast RNA (lane 4). The fragments protected from RNase digestion were analyzed on a 5% denaturing polyacrylamide gel. Arrowheads indicate three fragments expected from the cDNA sequences as shown in Fig. 2. Horizontal lines represent RNA size markers.

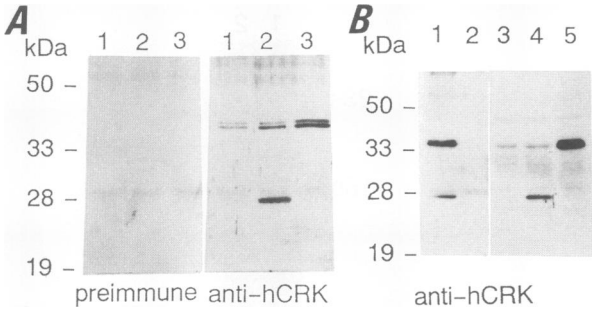

FIG. 5. Expression of *CRK* cDNAs in COS7 cells. (A) Total lysates of COS cells transfected with salmon sperm DNA (lane 1), pV*CRK*-I (lane 2), and pV*CRK*-II (lanes 3) were analyzed by immunoblotting with preimmune serum and an anti-*CRK*(SH3) antibody (anti-hCRK). (B) An anti-*CRK*(SH2) monoclonal antibody (3A8) covalently bound to protein A-Sepharose was incubated with a 2-mg RIPA lysate of human embryonic lung cells (lane 1) or RIPA only (lane 2). Proteins bound to the antibody, together with total-cell lysates of COS7 transfected with salmon sperm DNA (lane 3), pV*CRK*-I (lane 4), and pV*CRK*-II (lane 5), were further analyzed by immunoblotting with the anti-*CRK*(SH3) antibody.

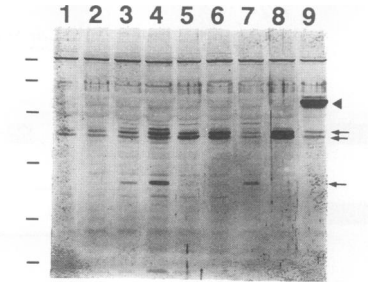

FIG. 7. Stable expression of CRK proteins in rat 3Y1 fibroblasts. Rat 3Y1 fibroblasts were transfected with expression vectors and selected with either Geneticin or hygromycin B. Cells were lysed in SDS sample buffer, and 10 μg of proteins was separated on SDS-PAGE (12% polyacrylamide) and analyzed by immunoblotting with the anti-*CRK*(SH3) antibody. Lanes: 1, 3Y1; 2, YM1; 3, YMC-I 28; 4, YMC-I 34; 5, YMC-II 1; 6, YMC-II 3; 7, YHC-I 1; 8, YHC-II 2; 9, Crk-3Y1. The arrowhead indicates chicken v-Crk. Arrows show 42- and 40-kDa products of *CRK*-II and the 28-kDa product of *CRK*-I. Horizontal lines are molecular size markers of 97, 66, 45, 31, 21, and 14 kDa.

nal antibody, and then we analyzed bound proteins by immunoblotting with the anti-*CRK*(SH3) antibody. By this method, we detected the proteins that are reactive with both antibodies against the SH2 and the SH3 regions of the CRK-I protein, eliminating the possibility of detection of cross-reactive antigens. Figure 5B, lane 1, shows the three products of the *CRK* gene, the 42-, 40-, and 28-kDa proteins, in human embryonic lung cells, from which we isolated the cDNA of *CRK*-I. The 40-kDa protein was more abundant than the other 42- and 28-kDa proteins. These proteins were not the cross-reactive products which might be present in the antibody-protein A complex (Fig. 5B, lane 2). The 42-, 40-, and 28-kDa proteins from human embryonic lung cells had mobilities on SDS-PAGE similar to those of the product of *CRK*-I and *CRK*-II cDNAs in COS7 cells (Fig. 5B, lanes 4 and 5). Similar results were obtained with a human osteosarcoma cell line, 143B.

Expression of CRK proteins in various human cell lines. Human cell lines were examined for the expression of CRK proteins (Fig. 6). The 40-kDa CRK-II protein was observed in all of the cell lines tested. The 42-kDa form was visible in cell lines A431, 143B, and H9. The 28-kDa CRK-I protein was below the detectable level in all cell lines.

Stable expression of the *CRK* cDNA products. We observed the effect of overexpression of human *CRK* cDNAs in rat 3Y1 cells. Stable cell lines overexpressing CRK proteins were isolated by selection with either Geneticin or hygromycin B. We established five *CRK*-I-expressing cells (YMC-I cells) and four *CRK*-II-expressing cells (YMC-II) by selection with Geneticin. Two cell lines each expressing *CRK*-I (YHC-I) and *CRK*-II (YHC-II) were isolated by selection with hygromycin B. Representative data on the expression of CRK proteins are shown in Fig. 7. Parent 3Y1 and YM1 (3Y1 with pMEXneo only) (Fig. 7, lanes 1 and 2) contained 42- and 40-kDa endogenous rat CRK-II proteins. YMC-I 28 (Fig. 7, lane 3), YMC-I 34 (lane 4), and YHC-I 1 (lane 7) expressed the 28-kDa CRK-I protein. In one of the *CRK*-I-overexpressing cells (YMC-I 34), the increase in the 42-kDa product of *CRK*-II was also observed. YMC-II 1 (Fig. 7, lane 5), YMC-II 3 (lane 6), and YHC-II 2 (lane 8) showed overexpression of 40- and 42-kDa CRK-II proteins. Figure 7, lane 9 shows the expression level of the chicken v-*crk* protein in the cell line Crk-3Y1. The 95-kDa proteins detected by the anti-CRK(SH3) serum reflect the presence of antibodies against glutathione S transferase, which was fused to the CRK protein to make immunogens (data not shown).

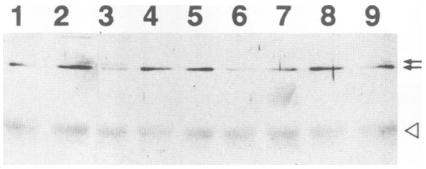

FIG. 6. Expression of CRK proteins in various human cell lines. A total of 0.2 mg of RIPA lysates was incubated with the anti-*CRK*(SH2) monoclonal antibody 3A8 covalently bound to protein A-Sepharose. Proteins bound to the antibody were analyzed by immunoblotting with the anti-*CRK*(SH3) antibody. Lanes: 1, HeLa; 2, A431; 3, 143B; 4, T98G; 5, GOTO; 6, COLO 201; 7, COLO 320DM; 8, DLD-1; 9, H9. Arrows indicate 42- and 40-kDa products of *CRK*-II. The open arrowhead indicates immunoglobulin light chain.

Figure 8 shows the morphology of these *CRK*-expressing cell lines. 3Y1 and YM1 cells (3Y1 cell lines transfected with pMEXneo) did not show any morphologic change. All of the cell lines expressing *CRK*-I (YMC-I cells) showed spindle and refractile cell morphology. The degree of these morphologic changes was more prominent in cells highly expressing *CRK*-I (YMC-I 34) than in cells expressing low levels of *CRK*-I (YMC-I 28). Cell lines expressing *CRK*-II also became slightly spindled. The cell lines expressing human *CRK*-I or *CRK*-II were assayed further for colony formation in soft agar and tumorigenicity in nude mice (Table 1). All *CRK*-I-expressing cell lines proliferated in soft agar and grew into tumors on nude mice. The expression of the 28-kDa product of *CRK*-I in these tumors was confirmed by immunoblotting (data not shown). No other cell lines grew into tumors.

Because the chicken v-*crk* induces an elevated level of phosphotyrosine, we examined the phosphotyrosine content in the *CRK*-I-expressing cells (Fig. 9). An increase in tyrosine phosphorylation of a 125-kDa protein was observed in YMC-I 34, where expression of *CRK*-I was highest. The

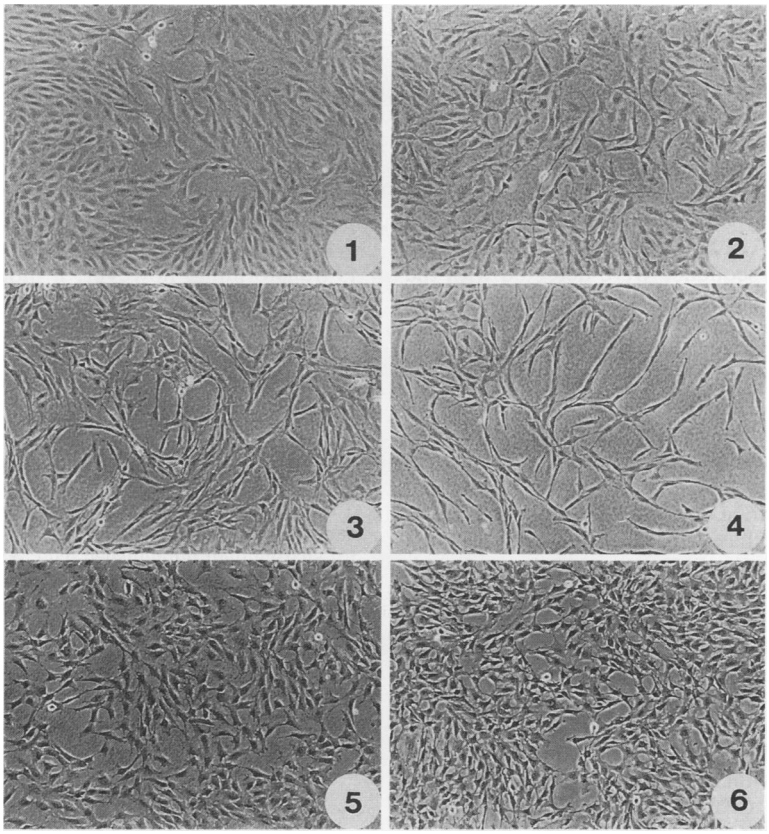

FIG. 8. Morphologic alteration of 3Y1 cells overexpressing CRK-I protein. (1) Parent 3Y1 cells; (2) A 3Y1 cell line expressing Neo^r only, YM1; (3 and 4) 3Y1 cell lines expressing CRK-I protein, YMC-I 28, and YMC-I 34; (5 and 6) 3Y1 cell lines expressing CRK-II protein, YMC-II 1, and YHC-II 2. Magnification, ×200.

apparent molecular mass was smaller than the 130- to 150-kDa protein tyrosine phosphorylated in v-Crk-transformed 3Y1 cells. The difference in the apparent mobilities may have reflected the various phosphorylation states; however, we could not examine it because of the low level of 125-kDa protein expression. In the other *CRK*-I-expressing cell lines, an increase in phosphotyrosine content was very slight. We could not detect an increase of phosphotyrosine content in *CRK*-II-expressing cells.

TABLE 1. Anchorage-independent growth and tumorigenicity in nude mice of *CRK*-expressing cells

Cell line	Vector	Colony formation[a]	Tumor formation (no. of tumors/no. of mice injected)[b]
3Y1		0	0/4
YM1	pMEXneo	0	0/10
YM2	pMEXneo	NT[c]	0/5
YMC-I 28	pM*CRK*-I	NT	3/6
YMC-I 34	pM*CRK*-I	190 ± 2	4/4
YMC-I 35	pM*CRK*-I	916 ± 68	4/4
YHC-I 1	pH*CRK*-I	22 ± 3	1/5
YMC-II 1	pM*CRK*-II	0	0/5
YMC-II 2	pM*CRK*-II	0	0/5
YMC-II 3	pM*CRK*-II	0	0/5
YHC-II 2	pH*CRK*-II	0	0/10

[a] Colonies were counted at 14 days after plating 10^4 cells per 6-cm dish. Numbers are the averages of two plates. Data are from three independent experiments.

[b] Assayed by inspection of nude mice at 20 days postinjection of 5×10^6 cells.

[c] NT, not tested.

DISCUSSION

We have cloned two species of human *CRK* cDNAs, with one SH2 and either one or two SH3 regions, and designated them *CRK*-I and *CRK*-II, respectively. Although we could not isolate *CRK*-I cDNA from a human placenta cDNA

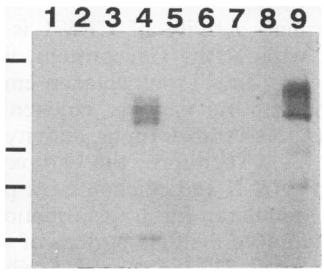

FIG. 9. Phosphotyrosine-containing proteins in *CRK*-I-expressing cells. Ten micrograms of cell lysates was separated on SDS-PAGE and immunoblotted with antiphosphotyrosine antibody. Bound antibodies were detected with an ECL chemiluminescence system. Exposure time was 5 min. Lanes: 1, 3Y1; 2, YM1; 3, YMC-I 28; 4, YMC-I 34; 5, YMC-II 1; 6, YMC-II 3; 7, YHC-I 1; 8, YHC-II 2; 9, Crk-3Y1. Horizontal lines are prestained molecular size markers of 200, 97, 69, and 46 kDa.

library, there are four findings that support the existence of the mRNA for *CRK*-I. First, the nucleotide sequence of *CRK*-I is identical, except for 4 bases, to that of the human *CRK* cDNA isolated from a colon carcinoma cell line by Kriz and Knopf (13). Second, the primer 2 used to amplify *CRK*-I cDNA crosses over the putative splicing junction of *CRK*-II. The primer contains no fewer than 13 nt before the putative splicing site (Fig. 1). This short stretch of homologous sequence is probably insufficient to amplify *CRK*-I cDNA from *CRK*-II mRNA. Third, two fragments that were predicted from the *CRK*-I sequence were detected with the RNase protection assay. Fourth, a 28-kDa protein in human embryonic lung cells, which was recognized by both anti-*CRK*(SH2) and anti-*CRK*(SH3) antibodies, had the same electrophoretic mobility as did the product of *CRK*-I cDNA.

Two proteins, of 40 and 42 kDa, were identified by the transient expression to be the products of *CRK*-II cDNA. Reichman and Hanafusa found that the more slowly migrating form of chicken c-Crk protein is a highly phosphorylated form (32). We also confirmed this by treating the CRK-II protein with alkaline phosphatase (data not shown). It must be studied further whether the SH3 region of the carboxyl-terminal side is the phosphorylation site or whether this additional SH3 regulates the phosphorylation of the other region.

The termination codon of the *CRK*-I cDNA appears at the same position as v-*crk*. Moreover, we found that the nucleotide sequence of the carboxyl-terminal region of v-*crk* also appears in the second SH3 region of chicken c-*crk*, at the corresponding position in which we found the putative splice acceptor sequence in the *CRK*-II cDNA. This nucleotide sequence of chicken c-*crk* also contains the consensus sequence of splice junctions (data not shown). These results suggest that the v-*crk* originated from another chicken c-*crk* mRNA which is related to the human *CRK*-I cDNA.

This is the first report that two molecules, one with one and the other with two SH3 domains, could be generated by alternative splicing. In chicken c-*src*, however, alternative splicing generates three species of $p60^{src}$s which differ in the amino acid sequences of SH3, resulting in the activation of their tyrosine kinase activities and transforming activities (16, 18, 31). The SH3 domain is regarded as a negative-regulatory domain in nonreceptor-type tyrosine kinases (6, 7, 10, 11, 30) and as a positive-regulatory domain in v-Crk protein (1, 25). In some molecules, SH3 is suggested to be an actin-binding site (3). Our results suggest that cells modulate the function of these SH3-containing proteins by modifying the amino acid sequence of, or even deleting, the SH3 region by alternative splicing.

The transforming activity of *CRK*-I is not surprising, because v-Crk without the Gag protein, which resembles CRK-I, still weakly transforms chicken embryo fibroblasts (25). Consistent with our findings, chicken c-Crk with two SH3 regions has less transforming activity than does that with one SH3 (32). Therefore, the carboxyl-terminal SH3 regions of both *CRK*-II and chicken c-*crk* proteins function as a negative modulator for transformation. Because the deletion or mutation of the SH3 regions of v-Crk impairs its transforming activity (21, 25), this SH3, which corresponds to the amino-terminal SH3 of human CRK proteins, appears to function as a positive regulator for transformation. Whether the number of SH3 domains is important for this ambivalent regulation or whether each SH3 has a fixed positive or negative regulatory role must be studied further.

Although an increase in the phosphotyrosine content is regarded as an essential feature of v-*crk* transformation and although all *CRK*-I-expressing cell lines showed the transforming phenotype, the *CRK*-I-expressing cell lines exhibited variable levels of increase in phosphotyrosine. The only cell line that constantly showed an increased level of phosphotyrosine was the one that had the highest *CRK*-I protein content (YMC-I 34). Therefore, the other cell lines expressing less *CRK*-I protein may not increase phosphotyrosine content sufficiently for detection by immunoblotting. It is possible that an undetectable level of increase in phosphotyrosine is sufficient for transformation. v-Crk without Gag is transforming; however, it induces much less phosphotyrosine than does wild-type v-Crk (25). In cells transformed by polyomavirus middle-T antigen, the increase in phosphotyrosine level is not detected under the usual conditions (45), even though the activation of Src family tyrosine kinases is regarded as a primary trigger of transformation (2, 15).

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