JOURNAL OF VIROLOGY, Aug. 1990, p. 3581–3589
0022-538X/90/083581-09$02.00/0

Vol. 64, No. 8

Mutagenic Analysis of the v-*crk* Oncogene: Requirement for SH2 and SH3 Domains and Correlation between Increased Cellular Phosphotyrosine and Transformation

BRUCE J. MAYER† AND HIDESABURO HANAFUSA*

Laboratory of Molecular Oncology, The Rockefeller University, 1230 York Avenue, New York, New York 10021

Received 8 February 1990/Accepted 25 April 1990

We have constructed a series of mutants with deletion, linker insertion, and point mutations in the v-*crk* oncogene of avian sarcoma virus CT10. The v-*crk* gene contains no apparent catalytic domain, but does contain two blocks of homology to putative regulatory domains, termed SH2 and SH3, found in a variety of proteins implicated in signal transduction. Infection with CT10 causes a dramatic increase in the level of tyrosine phosphorylation of several cellular proteins. We found that mutation of either the SH2 or SH3 domain of v-*crk* reduced or eliminated transforming activity, whereas mutation of regions outside the conserved domains had no effect. Deletion of amino-terminal *gag* sequences caused a partial loss of transforming activity and a change in subcellular distribution of the *crk* protein. In all cases, there was an absolute correlation between increased cellular phosphotyrosine and transformation.

The *crk* oncogene of avian sarcoma virus CT10 encodes a 47-kilodalton (kDa) *gag* fusion product, termed P47$^{gag\text{-}crk}$ (20). A second *crk*-encoding avian sarcoma virus was recently described that has a genetic structure very similar to CT10 (33). Although the v-*crk* gene has no sequence similarity to any known catalytic domain, it contains two blocks of sequence, the SH2 and SH3 domains, which are found in a wide variety of proteins. These domains are thought to regulate the activity of proteins in which they are found, perhaps by interacting with catalytic domains or with other cellular proteins (21a, 24).

The SH2 domain (the B and C boxes of Stahl et al. [29]) is composed of one region of approximately 50 amino acids (B box) and a second region of 10 amino acids (SH2′ or C box) separated by a variable region. It was originally identified as a common N-terminal domain in all nonreceptor protein-tyrosine kinases (28) and was subsequently found in two isoforms of phosphatidylinositol-specific phospholipase C (PLD-γ and PLC-IV) (10, 29, 30) and the *ras* GTPase activator protein (32, 34), as well as v-*crk*. The SH3 domain (A box), consisting of approximately 50 amino acids, is found in a wider variety of proteins, including most nonreceptor tyrosine kinases; PLC-γ and PLC-IV; the GTPase activator protein; the cytoskeletal matrix proteins fodrin, myosin-I, and actin-binding protein ABP1b; the *Saccharomyces cerevisiae* proteins Cdc25 and Fus1, and neutrophil cytosolic oxidase factor p47 (9, 18, 24, 27). Whenever enzymatic activity has been examined (in the case of the tyrosine kinases, PLCs, and the GTPase activator protein), the SH2 and SH3 domains have been shown to be unnecessary (2, 3, 10, 17, 19). Since the SH2 and SH3 domains are found independently in at least one protein and are present in various combinations in others, we presume that they have modular and independent functions.

CT10-transformed cells have greatly elevated phosphotyrosine levels on at least three cellular proteins compared with normal control cells (20). We have shown that P47$^{gag\text{-}crk}$ can tightly associate with these phosphotyrosine-containing proteins (21, 21a) and that tyrosine kinase and serine-threonine kinase activity also coimmunoprecipitate with P47$^{gag\text{-}crk}$ (20, 21, 21a). These data led us to propose a model in which the v-*crk* product increases tyrosine phosphorylation of cellular substrate proteins by mediating the formation of ternary complexes consisting of P47$^{gag\text{-}crk}$, substrate proteins, and endogenous protein kinases (21a).

We have constructed a series of mutant v-*crk* genes to determine which domains of the protein are required for transforming activity and to correlate transforming activity with biochemical parameters in mutant-infected cells. We present evidence that the SH2, SH2′, and SH3 domains are all required for full transforming activity and that N-terminal *gag* sequences also contribute to transforming activity, presumably by affecting subcellular localization. In all cases, increased cellular phosphotyrosine correlated with transforming activity, suggesting that this increase is central to the mechanism of transformation by v-*crk*.

MATERIALS AND METHODS

Cells and virus. Chicken embryo fibroblasts (CEF) were prepared and maintained essentially as described previously (14). Virus stocks were prepared by cutting pCT10 or its derivatives with *Sca*I, mixing with *Sst*I-cleaved pUR2AV helper virus DNA (22), ligating briefly, and transfecting 1 μg of each plasmid per 6-cm dish of CEF by calcium phosphate coprecipitation (6, 36). Stocks of Rous sarcoma virus (RSV)-derived mutant viruses were prepared as described previously (6, 16). Transfected cells were routinely maintained in medium containing 0.375% agar at 40°C. CEF transfected with wild-type (wt) pCT10 were generally fully transformed by the third passage (12 to 14 days posttransfection).

To assay anchorage independence, CEF were trypsinized 2 days posttransfection and 10^6 cells were plated per 10-cm bacterial plastic dish in 10 ml of medium containing 11% calf serum, 1% chicken serum, and 0.4% agar on a layer of 15 ml of the same medium containing 0.7% agar. The plates were incubated at 37°C.

* Corresponding author.
† Present address: The Whitehead Institute for Biomedical Research, Cambridge, MA 02142.

Tumorigenicity was assayed by injection of 0.1 ml of virus in each wing web of newborn (2 to 5 days posthatching) white leghorn chickens (SPAFAS, Inc.). To coculture tumor cells, tumor tissue was minced and then incubated with 0.25% trypsin in buffered isotonic saline at 37°C with gentle shaking for 20 min. Dissociated cells and small clumps were poured off, pelleted, and added to a feeder layer of freshly plated CEF. Cultures were transferred once to allow virus spread.

Construction of pCT10. A nonpermuted CT10 viral genome was constructed from the original permuted molecular clone, p10-282 (20), as outlined in Fig. 1. First, the 0.3-kilobase (kb) *Acc*I fragment of p10-282 was deleted by restriction digestion, end filling, and religation to generate p10-82Δ. The 3.2-kb *Sca*I-*Eco*RI fragment of p10-82Δ was combined in a three-part ligation with the 1.3-kb *Eco*RI-*Pst*I and the 1.8-kb *Pst*I-*Sca*I fragments of p10-282 to generate pCT10. This construct contains the entire nonpermuted CT10 genome with two long terminal repeats in a pBluescript SK(−) vector (Stratagene Inc.). It retains the full biological activity of the original molecular clones.

Construction of v-*crk* mutants. p10-ΔSH2 was constructed by ligating an *Sph*I 8-mer linker to the end-filled 5.9-kb *Eco*RI-*Sty*I fragment of pCT10. p10-ΔSH3 was constructed by blunt-end self ligation of the end-filled 6.0-kb *Acc*I-*Eco*RI fragment of pCT10. p10-BSP was made by ligation of an *Sph*I 8-mer linker to *Bgl*II-cut, end-filled pCT10 DNA. Similarly, p10-ESP was made by insertion of an *Sph*I 8-mer linker into the end-filled *Eco*RI site of pCT10, p10-MH was made by insertion of a *Hin*dIII 12-mer linker into the end-filled *Mst*II site of pCT10, and p10-SMH was made by insertion of a *Hin*dIII 12-mer linker into one of the two *Sma*I sites of pCT10.

Point mutants were generated by using the bacterial strain and protocols provided with the Mutagene kit (Bio-Rad Laboratories). The 0.23-kb *Pst*I-*Eco*RI fragment of pCT10 was cloned into M13mp18 and M13mp19, and recombinant, uracil-substituted bacteriophages were prepared in *Escherichia coli* CJ236. This DNA was used as template for oligonucleotide-directed mutagenesis. The mutagenic oligonucleotides were 5′-CTTCTTGTT*TAA*CGACTCGGG-3′, which creates a novel *Hpa*I site and changes Arg-273 of v-*crk* to Asn, and 5′-GACGATGTA*TC*GCGAGACGC-3′, which creates a novel *Nru* site and changes His-294 to Arg. The nucleotides in italics are different from the wild-type CT10 sequence. Progeny clones were grown up, sequenced to confirm mutations, and reinserted into pCT10 by ligation of the *Pst*I-*Eco*RI fragment to the 6.1-kb *Eco*RI-partial *Pst*I fragment of pCT10.

gag-deleted mutants were constructed by using a replication-competent RSV vector system (6). p10-SHB was constructed by ligating the 0.7-kb *Sfi*I-*Hae*II fragment of pCT10 to a *Bam*HI adaptor (5′-CGGATCCGGCGC-3′ plus 5′-CG GATCCGTCA-3′), digesting with *Bam*HI, and ligating to *Bgl*II-cleaved pNR200 (16). p10-NSC was made in two steps. First, hybrid vector pNR111, consisting of the 5.8-kb *Bgl*II-*Cla*I fragment of pNR200 ligated to the 1.8-kb *Cla*I-*Bgl*II fragment of pXD11-1 (6), was constructed. Then the *Bam*HI-linkered 0.8-kb *Sty*I-*Hae*II fragment of pCT10 was inserted into the *Bgl*II site of pNR111 to generate p10-NSC.

Protein analysis. Isotopic labeling, cell lysis, immunoprecipitation, and immunoblotting have been described previously (12, 20, 21a). Polyclonal rabbit anti-phosphotyrosine serum (anti-ptyr) (12, 35) and anti-*crk* serum (21a) have been previously described.

Crude membrane (P100) and cytosolic (S100) fractions

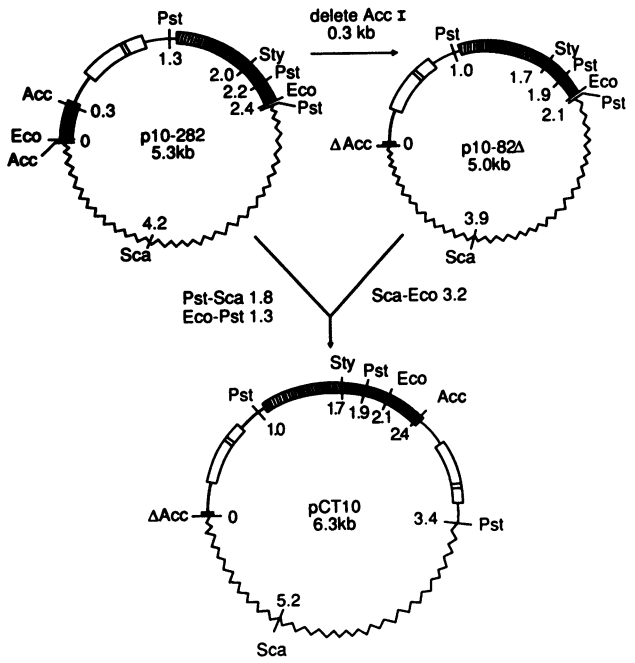

FIG. 1. Construction of pCT10. Symbols: ∿∿∿, pBluescript SK (−) vector sequences; ■, cellular proto-oncogene-derived sequences; ▨, *gag* sequences; □, viral long terminal repeat. Numbers denote kilobase pairs from the original *Acc*I site in the vector. Abbreviations for restriction enzymes: Acc, *Acc*I; ΔAcc, destroyed *Acc*I site; Pst, *Pst*I; Eco, *Eco*RI; Sty, *Sty*I; Sca, *Sca*I.

were prepared as follows. A 10-cm dish of infected cells was scraped in hypotonic buffer (10 mM NaCl, 20 mM Tris [pH 7.4], 1 mM disodium EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, 0.1 mM Na_2MoO_4, 1% trasylol), gently pelleted, resuspended in 0.5 ml of fresh hypotonic buffer, and incubated on ice for 10 min. Cells were broken in a Dounce homogenizer and briefly spun at low speed to pellet nuclei and unbroken cells. Supernatant was spun at 100,000 × *g* for 30 min at 4°C. The pellet (P100) was dissolved in 0.5 ml of hypotonic buffer containing 1% sodium dodecyl sulfate; sodium dodecyl sulfate was added to the supernatant (S100) to 1%. Fractions were boiled for 5 min and spun in a microcentrifuge at room temperature for 10 min, and 20 μl of each fraction supernatant was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting.

RESULTS

Construction of v-*crk* mutants. We have used molecularly cloned CT10 DNA to construct mutants with deletion, linker insertion, and oligonucleotide-directed point mutations in the v-*crk* gene. Since the original molecular clones were circularly permuted at the *Eco*RI site within the SH2′ domain of v-*crk*, to facilitate the generation of mutants we constructed a plasmid containing the nonpermuted CT10 genome with two long terminal repeats (Fig. 1). This plasmid, pCT10, is the parental plasmid of all the mutants described here.

The mutants that were constructed are diagrammed in Fig. 2. The entire N-terminal half of the c-*crk*-derived sequences, which encodes the SH2 and SH2′ domains (ΔSH2), or the entire C-terminal portion encoding the SH3 domain and the

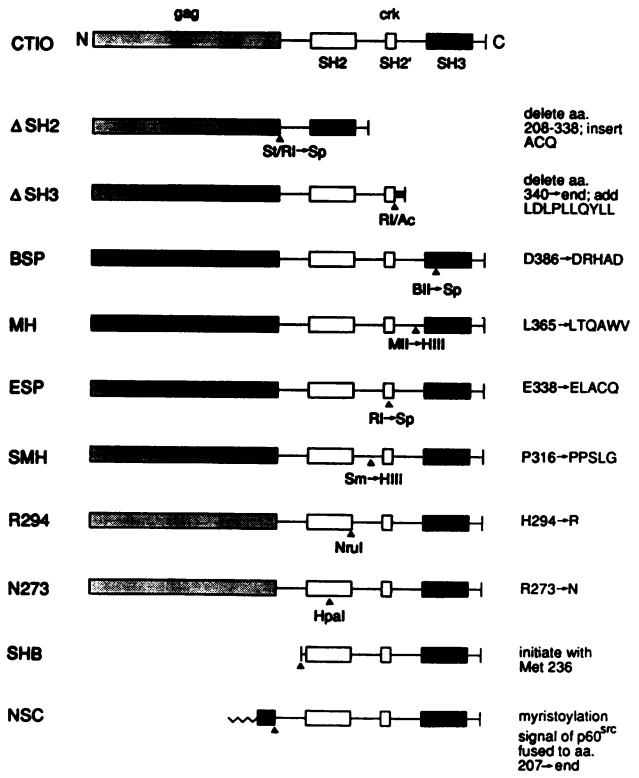

FIG. 2. In vitro-constructed mutants of the v-*crk* gene. Viral *gag* sequences ( ), SH2 and SH2′ domains ( ), and the SH3 domain ( ) are indicated. Restriction sites used for or created as a result of mutagenesis are identified (see Materials and Methods). Abbreviations: St, *Sty*I; RI, *Eco*RI; Sp, *Sph*I; Ac, *Acc*I; BII, *Bgl*II; MII, *Mst*II; HIII, *Hin*dIII; Sm, *Sma*I. Amino acid (aa) changes in the various mutants are indicated on the right in the single-letter amino acid code. Amino acid numbers are as in reference 20; wt v-*crk* encodes 440 amino acids.

last few amino acids of SH2′ (ΔSH3) was deleted. When convenient restriction sites were available, linker insertion mutants resulting in the in-frame insertion of four or five amino acids were generated. These insertions were in the amino-terminal part of SH3 (BSP), in the region between SH2′ and SH3 (MH), within the SH2′ block (ESP), and in the region between SH2 and SH2′ (SMH). Since there are no convenient restriction sites in SH2, two mutants with point mutations at well-conserved residues within this domain were made by oligonucleotide-directed mutagenesis. The histidine at position 294 was changed to arginine (R294), and the arginine at position 273 was changed to asparagine (N273). The arginine at position 273 is absolutely conserved in all known SH2 domains and is within the very highly conserved FLV*R*XS hexapeptide. The histidine at position 294 is conserved in all but one SH2 domain and is at the C-terminal boundary of the SH2 homology.

Biological activity of v-*crk* mutants. The biological activity of the mutants was assayed by transfection of pCT10-derived DNA, along with UR2AV helper virus DNA, into CEF. In this system, expression of the mutant *crk* gene product is dependent on replication of the mutant CT10 virus. UR2AV DNA is cotransfected to provide essential replicative gene products in *trans* to the replication-defective CT10-derived virus. Since all mutants contain the same long terminal repeat and *cis*-acting replicative sequences (except for two mutants that are expressed in an RSV-derived vector [see below]), the efficiency of replication of the mutants should be similar to that of wt CT10.

TABLE 1. Biological activity of v-*crk* mutants

Virus	Protein expression[a]	Morphological alteration[b]	Anchorage independence[c]	Tumorigenicity[d]
CT10 + UR2AV	+++	++	++	++ (4/4 by 13 dpi)
ΔSH2 + UR2AV	+	−	−	− (0/4 by 34 dpi)
ΔSH3 + UR2AV	−	−	−	− (0/4 by 34 dpi)
BSP + UR2AV	++	−	+	± (1/4 by 34 dpi)[e]
MH + UR2AV	+++	++	++	++ (4/4 by 16 dpi)
ESP + UR2AV	++	−	−	? (2/4 by 34 dpi)[f]
SMH + UR2AV	+++	++	++	++ (4/4 by 13 dpi)
R294 + UR2AV	++	−	−	− (0/4 by 23 dpi)
N273 + UR2AV	++	−	−	− (0/4 by 23 dpi)
UR2AV	−	−	−	− (0/4 by 34 dpi)

[a] Protein levels assayed by immunoblot with anti-*crk* serum (Fig. 4).
[b] Assayed in agar-overlaid monlayer culture after the third passage.
[c] Assayed in agar suspension (Fig. 3).
[d] Assayed by injection of each wing web of newborn chickens (number of sites with palpable tumors/total number of sites injected; dpi, days postinjection).
[e] Only tumor induced was small and slow-growing; cocultured tumor cells were not morphologically altered in monolayer culture.
[f] Tumor cocultures were morphologically altered in monolayer culture, unlike the parental 10-ESP mutant.

Three criteria for the transforming activity of the mutant *crk* viruses were assayed: morphological alteration and anchorage-independent growth of infected CEF and tumorigenicity in newborn chickens. Table 1 summarizes these results. The only mutants that retained full transforming activity were SMH and MH, which had linkers inserted into the nonconserved regions between the SH2 and SH2′ domains or between the SH2′ and SH3 domains. These viruses were indistinguishable from parental CT10 by all criteria examined. The BSP mutant, containing a linker in the SH3 domain, had a very weak transforming phenotype. Infected cells were indistinguishable from helper-infected controls in monolayer culture, but were able to form small colonies in soft agar (Fig. 3). In addition, one of two chickens (one of four sites) injected with virus developed a small, slow-growing tumor. Virus recovered from this tumor had the properties of the parental BSP mutant, suggesting that the tumor was induced by the authentic mutant. The remaining mutants had no detectable transforming activity in monolayer culture or agar suspension. The tumorigenic activity of the ESP virus was shown to be due to generation of fully transforming variants, since virus recovered from tumors induced morphological alteration and anchorage-independent growth, unlike the parental mutant.

Since retroviruses generate frequent mutants through replication errors, one often observes either the loss of sequences that are not selected for (such as nontransforming oncogene sequences) or accumulation of mutants that are selected for (changes that activate the transforming potential of nontransforming genes) (5, 15; B.J.M. and H.H., unpublished observations). This may be especially true with replication-defective viruses such as CT10, since the defective sarcoma virus is at a selective disadvantage compared with replication-competent helper virus until the culture becomes fully infected. To minimize the effect of viral mutation and selection during culture, it is therefore useful to have a transformation assay that does not depend on viral spread and full infection. For this reason we assayed the ability of mutant-transfected CEF to form colonies when plated in

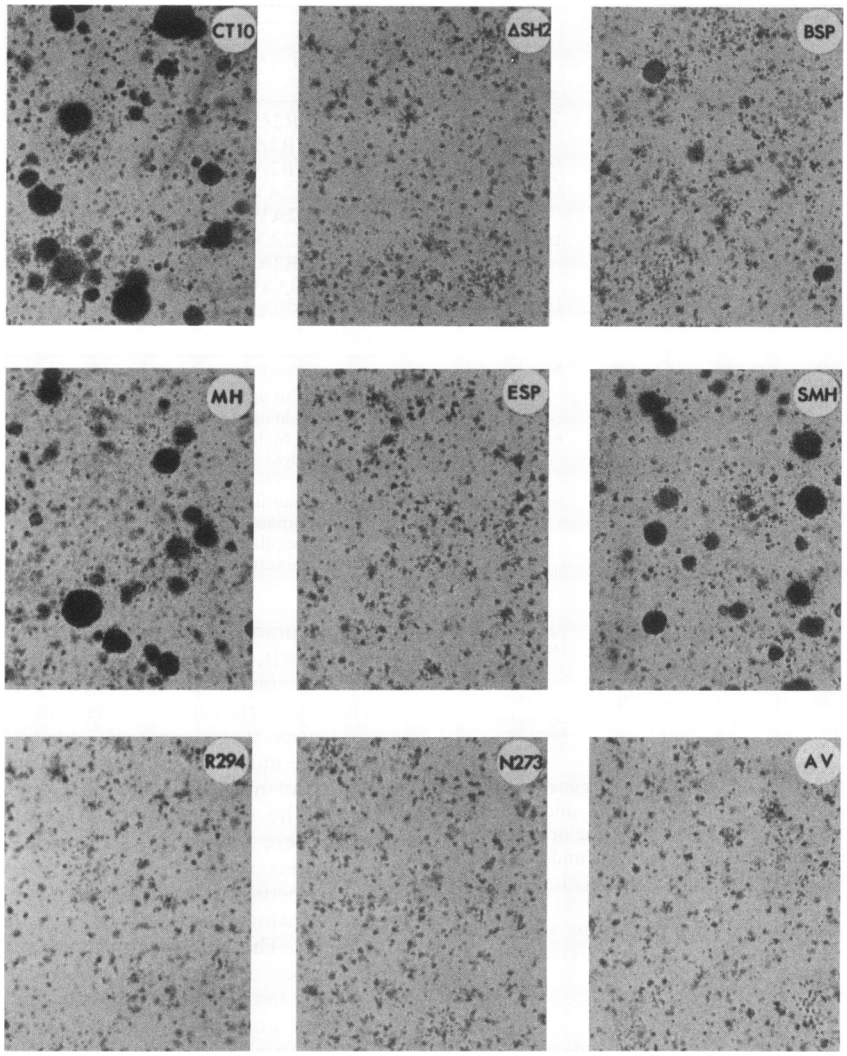

FIG. 3. Induction of anchorage-independent growth by v-*crk* mutants. CEF were transfected with pCT10-derived DNA plus UR2AV helper virus DNA as marked, plated in soft-agar suspension 2 days posttransfection, and incubated at 37°C. Photomicrographs were taken 28 days after plating. Magnification, ×25.

agar suspension 2 days posttransfection (Fig. 3). At most, one round of viral replication would have occurred by this time, so the effect of mutation is expected to be negligible. The results of this assay were qualitatively similar to those in which later-passage, fully infected cells were used (data not shown). This colony formation assay is also designed to detect anchorage-independent growth of a small number of transformed cells in a large population of normal cells.

Mutant v-*crk* proteins. We used immunoblotting to examine the level of mutant v-*crk* proteins expressed in transfected cultures (Fig. 4A). In all cases except ΔSH3, mutant protein of the expected molecular weight was observed. The level of expression of nontransforming mutants was lower than that of wt or fully transforming mutants, presumably owing to selection against replication of the nontransforming mutant viruses. We conclude that the lower levels of protein reflect wt levels of expression in a subpopulation of cells, and not lower, perhaps "subthreshold" levels of expression in all cells, for the following reasons. First, indirect immunofluorescence with an anti-*crk* antiserum demonstrated that a lower percentage of cells expressed the nontransforming

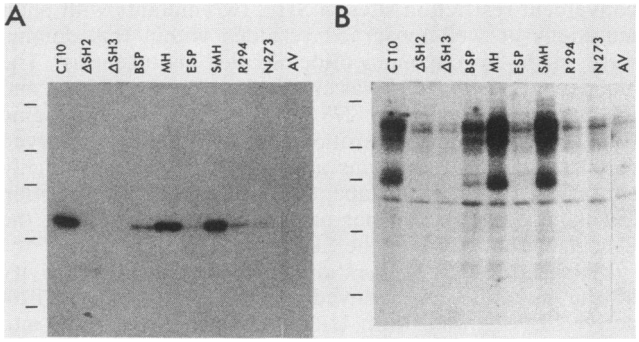

FIG. 4. *crk* proteins and phosphotyrosine-containing proteins of CEF transfected with v-*crk* mutants. (A) Anti-*crk* immunoblot; (B) anti-ptyr immunoblot. Approximately 30 μg of protein from CEF transfected with mutants as indicated was separated on 8.5% gels and immunoblotted. Markers (lines on the left of the gels): 220, 100, 68, 43, and 27 kDa.

mutant proteins, but that the level of expression in these cells was comparable to wt levels (data not shown). Second, we examined the steady-state levels of v-*crk* mRNA in mutant-infected cultures by Northern (RNA) blotting, and in all cases the level of mRNA was proportional to the levels of mutant protein observed by immunoblotting (data not shown). Since the mutations that we introduced were in most cases minor, we do not expect large differences in viral transcription rate or stability; we therefore assume that the reduced mRNA levels seen in cultures transfected with the nontransforming mutants indicate that a small number of cells were infected with the mutants. Since mRNA levels were proportional to protein levels, we also assume that there were no major differences in protein half-life, although we have not rigorously excluded this possibility by pulse-chase experiments. When mutant protein was detected, we conclude that there was no gross defect in viral replication relative to wt CT10 and that the lack of biological activity observed in the colony formation assay (Fig. 3) was due to lesions in the v-*crk* proteins of the mutant viruses. Since we saw no protein or RNA expression of the ΔSH3 mutant, no conclusion could be drawn about the biological properties of this mutant.

Phosphotyrosine levels in mutant-transfected CEF were assayed by immunoblotting with anti-ptyr (12, 35). Phosphotyrosine levels correlated absolutely with transforming activity (Fig. 4B). The fully transforming mutants had phosphotyrosine patterns similar to wt CT10, the partially transforming mutant BSP had phosphotyrosine levels only slightly above control levels, and the nontransforming mutants had no detectable elevation in phosphotyrosine levels. These results are consistent with a central role for elevated phosphotyrosine levels in transformation by v-*crk*. We also examined the association of mutant *crk* proteins with cellular phosphoproteins. Wt P47$^{gag\text{-}crk}$ binds to the major phosphotyrosine-containing proteins of infected cells, of apparent molecular masses 135 to 155, 120, and 70 kDa (21a). The fully transforming mutants, SMH and MH, bound to these proteins as well as wt P47$^{gag\text{-}crk}$, whereas the partially transforming mutant, BSP, bound much lower levels and the nontransforming mutants bound no detectable phosphoproteins (Fig. 5). This experiment also demonstrates that the mutant *crk* proteins, like wt P47$^{gag\text{-}crk}$, were highly phosphorylated.

Construction of *gag*(−) *crk* mutants. To assess the contribution of viral *gag* sequences to the biological activity of P47$^{gag\text{-}crk}$, we also constructed two *gag*-deleted [*gag*(−)] mutants. For these mutants we used a replication-competent RSV-derived vector (6, 16) in which the truncated *crk* gene is inserted at the position occupied by the *src* gene in wild-type RSV. These constructs do not require cotransfection with helper virus to replicate, and should therefore express the mutant *crk* proteins more efficiently than in the case of the replication-defective, CT10-derived mutants described above.

The two *gag*(−) constructs have different translation start sites (Fig. 2). A c-*crk* cDNA has been cloned, and the sequence revealed that the cellular product initiates at the methionine homologous to methionine 236 of v-*crk*. The region of v-*crk* between the *gag-crk* junction and methionine 236 is derived from intron and 5′ untranslated sequences and is not found in the c-*crk* product (C. T. Reichman, B. J. Mayer, S. Keshav, and H. Hanafusa, submitted for publication). The mutant SHB was constructed to use the normal c-*crk* initiator methionine (amino acid 236 of v-*crk*) and encode a 205-amino-acid product. The second mutant, NSC,

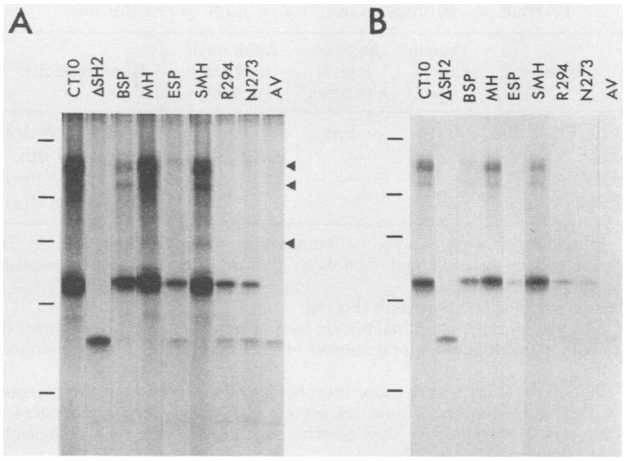

FIG. 5. Phosphotyrosine-containing proteins associated with mutant v-*crk* proteins. (A) CEF infected with mutant virus were labeled in vivo with $^{32}P_i$, lysed, and immunoprecipitated with anti-*crk* serum. Mutants are as marked; lane AV contains UR2AV helper virus alone. The gel was 8.5% polyacrylamide. Positions of molecular mass markers are indicated on the left: 220, 100, 68, 43, and 27 kDa. Bands of 130, 120, and 70 kDa proteins are indicated by arrowheads on the right. (B) Same gel as in panel A, washed in 1 M KOH at 55°C for 2 h to enhance detection of phosphotyrosine-containing proteins (4).

was constructed to initiate translation with the amino-terminal 14 amino acids of p60^{src}, which are fused to the C-terminal 234 amino acids of v-*crk*. These 14 *src*-derived amino acids are sufficient to direct myristoylation of heterologous proteins (25), so this construct is expected to direct the synthesis of a myristoylated, 252-amino-acid product. Myristoylation is often sufficient to direct tight association of proteins with the plasma membrane (25, 31); we hoped that by using the *src* amino terminus we could target this *gag*(−) *crk* mutant to membranes, as well as take advantage of a translational start site that is known to work efficiently in a retroviral context.

Biological and biochemical properties of *gag*(−) mutants. The biological properties of these two mutants are summarized in Table 2. Both mutants had partially transforming phenotypes. The myristoylated NSC mutant induced subtle morphological alteration in monolayer culture and very small colonies in suspension (Fig. 6), although in both cases it was much less potent than wt CT10 virus. When injected into chickens it induced tumors with high frequency, but when virus was isolated from tumors it was found to be as transforming as wt CT10; furthermore, the tumor-derived *crk* protein could be shown by immunoblotting and immunoprecipitation to have regained viral *gag* sequences (data not shown). This was presumably due to deletion of the sequences between *gag* and *crk*, in some cases mediated by eight nucleotides of viral *gag* sequence present in the NSC *crk* gene. Owing to this recombination, we cannot assess the tumorigenic potential of the authentic NSC mutant, although it must necessarily be less than that of the *gag*-containing recombinants. The SHB mutant was somewhat more transforming than NSC in both monolayer culture and suspension (Fig. 6), although still less so than the wt strain. It induced tumors in chickens, but the latency period was longer and the tumors were slower growing than in the case of wt CT10. No recombination with *gag* was observed with this mutant, so we assume the tumors were induced by the authentic *gag*(−) mutant.

TABLE 2. Biological activity of *gag*(−) *crk* mutants

Virus	Protein expression[a]	Morphological alteration[b]	Anchorage independence[c]	Tumorigenicity[d]
CT10 + NR200	+++	++	++	++ (4/4 by 8 dpi)
NSC	++	+	+	? (6/6 by 23 dpi)[e]
SHB	++	+	+	+ (8/8 by 23 dpi)[f]
NR200	−	−	−	− (0/6 by 28 dpi)

[a] Protein levels were assayed by immunoblot with anti-*crk* serum (Fig. 7).
[b] Assayed in agar-overlaid monolayer culture after the second passage (Fig. 6).
[c] Assayed in agar suspension (Fig. 6).
[d] Assayed by injection of each wing web of newborn chickens (number of sites with palpable tumors/total number of sites injected; dpi, days postinjection).
[e] Tumor cocultures were more morphologically altered than the parental NSC cells in monolayer culture; *crk* protein had recovered *gag* sequences.
[f] Tumors were relatively slow-growing; cocultures were morphologically similar to parental SHB cells.

The *gag*(−) mutant proteins were expressed at relatively high levels, comparable to wt P47$^{gag\text{-}crk}$ (Fig. 7A). The levels of phosphotyrosine in cells infected with these mutants, however, were reduced compared with those in wt-infected cells (Fig. 7B). Although in some experiments the tyrosine phosphorylation of the 135- to 155- and the 120-kDa proteins of NSC-infected cells was comparable to that in the wt strain, the 70-kDa protein phosphotyrosine level was always lower (Fig. 7B; data not shown). It was generally difficult to see any elevation of 120- and 135- to 155-kDa protein

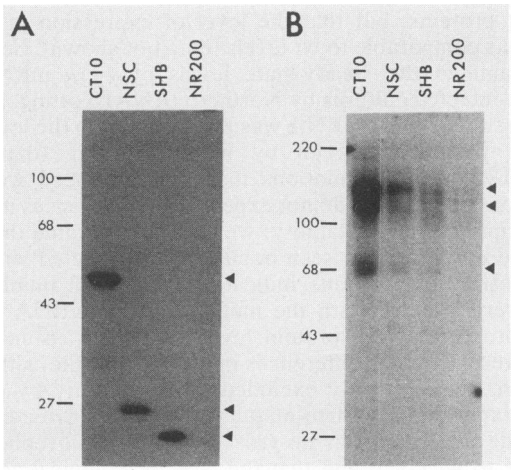

FIG. 7. *crk* proteins and phosphotyrosine-containing proteins in CEF transfected with *gag*-deleted *crk* mutants. CEF were transfected with viral DNA as marked, lysates were prepared, and proteins were separated on 10% (A) or 7.5% (B) polyacrylamide gels. Proteins were transferred to nitrocellulose, and filters were probed with anti-*crk* serum (A) or anti-ptyr (B). Apparent molecular masses of protein standards (in kilodaltons) are marked on the left. Positions of CT10, NSC, and SHB proteins (A) and 130-, 120-, and 70-kDa proteins (B) are indicated by arrowheads on the right.

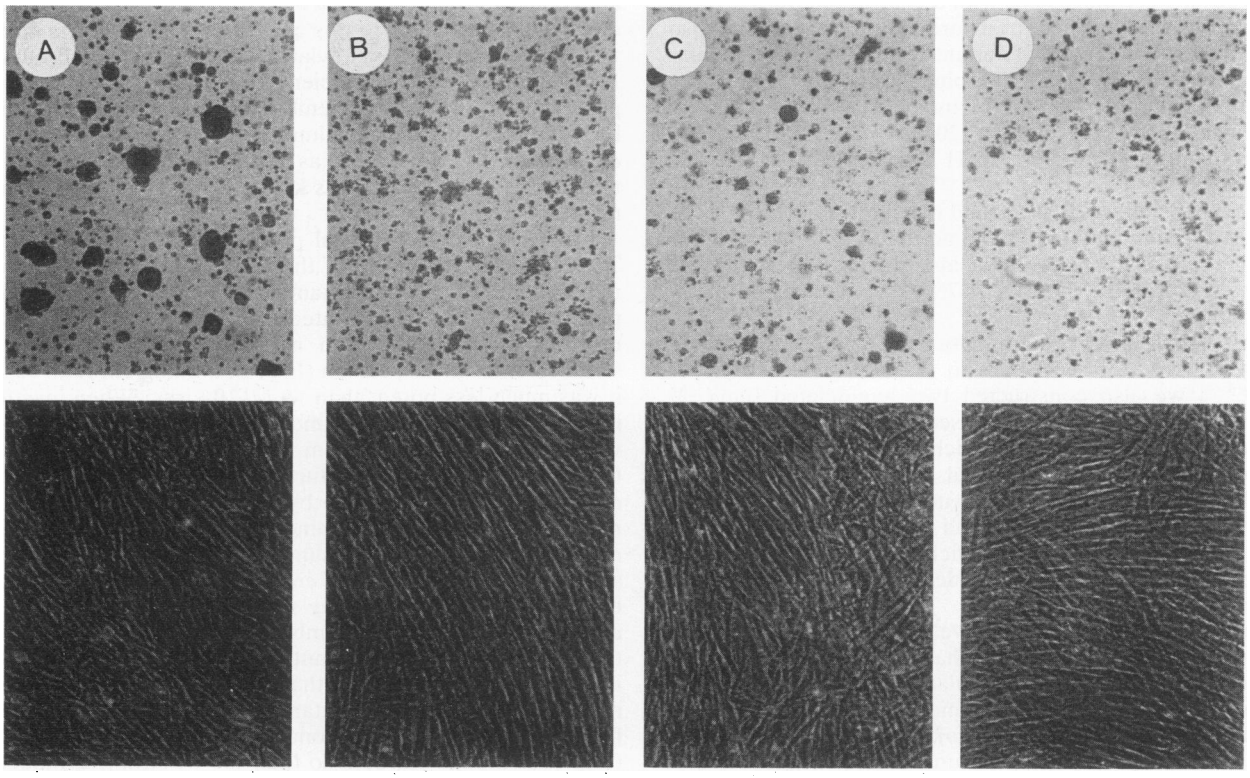

FIG. 6. Induction of anchorage-independent growth and morphological alteration in CEF infected with *gag*-deleted v-*crk* mutants. CEF were transfected with CT10 plus NR200 (CT10) (A), NSC (B), SHB (C), or NR200 helper virus (D). In the top row, transfected cells were plated in suspension 2 days posttransfection as for Fig. 3, and photomicrographs were taken 18 days after plating. Magnification, ×25. In the bottom row, transfected CEF were maintained in monolayer culture under soft agar at 40°C, and phase-contrast photomicrographs were taken after the second passage. Magnification, ×200.

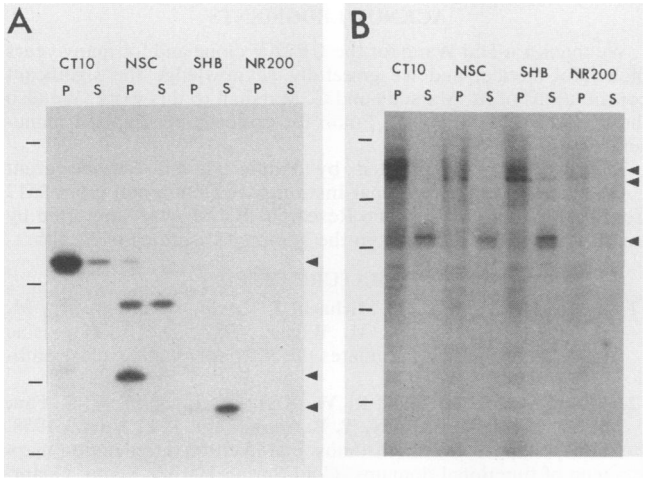

FIG. 8. Subcellular fractionation of CEF transfected with *gag*-deleted *crk* mutants. CEF transfected with virus as marked were fractionated into P100 crude membrane fraction (P) and S100 cytosolic fraction (S), as described in Materials and Methods, and separated on 10% (A) or 7.5% (B) polyacrylamide gels. Proteins were transferred to nitrocellulose filters and probed with anti-*crk* serum (A) or anti-ptyr (B). Positions of molecular mass markers are indicated on the left: 220, 100, 68, 43, 27, and 18 kDa. Positions of CT10, NSC, and SHB proteins (A) and 130-, 120-, and 70-kDa proteins (B) are indicated by arrowheads on the right.

phosphotyrosine content in SHB-infected cells, although the 70-kDa protein clearly had elevated phosphotyrosine levels relative to controls.

Subcellular distribution of *gag*(−) mutant proteins. Since mutants NSC and SHB were identical to wt v-*crk* in the c-*crk*-derived portion of the gene and since immunoblotting demonstrated that the truncated proteins were expressed at relatively high levels, it was surprising that these mutants were not fully transforming. A likely function for viral *gag* sequences is to direct the subcellular localization of proteins to which it is fused. We therefore examined the subcellular distribution of wt P47$^{gag\text{-}crk}$ and the two *gag*(−) mutants. The majority of wt P47$^{gag\text{-}crk}$ partitions in the crude membrane (P100) fraction, although a significant proportion is found in the cytosolic (S100) fraction (Fig. 8A). The NSC protein is found almost exclusively in the P100 fraction, similar to many other myristoylated proteins (23, 31). The upper bands in the NSC lanes are *crk* proteins from variant viruses that had recombined with helper and recovered N-terminal *gag* sequences. As expected, labeling of infected cells with [^{3}H]myristic acid demonstrated that the authentic NSC *crk* protein was myristoylated (data not shown). The SHB protein, in contrast, is almost exclusively cytosolic in distribution (Fig. 8A). Unlike wt P47$^{gag\text{-}crk}$, therefore, neither of the two *gag*(−) mutants provides significant amounts of *crk* protein in boyh the soluble and the membrane-associated compartments.

When the P100 and S100 fractions of CT10-infected cells were analyzed by anti-ptyr immunoblotting, most of the phosphotyrosine-containing proteins were found in the membrane fraction (Fig. 8B), consistent with previous results with tyrosine kinase oncogenes (13). The majority of the 70-kDa phosphotyrosine-containing protein, however, was found in the cytosolic fraction. If, as we suspect, binding to *crk* protein is required for tyrosine phosphorylation of these proteins, these data suggest a role for *crk* protein in both the membrane and cytosolic compartments. The results with the two *gag*(−) mutants were ambiguous. The sum of cytosolic plus membrane phosphoproteins did not give the same pattern as when mutant-infected cells were lysed directly by boiling in sodium dodecyl sulfate (Fig. 7B); these results might be due to the lengthy manipulations required for fractionation, since similar results have been observed in several experiments. However, there are some indications (for example, the higher 70-kDa protein phosphotyrosine in the cytosol of SHB-infected cells compared with NSC-infected cells) that phosphotyrosine levels are more elevated in the cellular compartment in which the *crk* protein is found. When the data of Fig. 7 and 8 are taken together, our results demonstrate that *crk* mutants lacking *gag* have a different subcellular localization than the wt protein and that these mutants do not induce the full extent of tyrosine phosphorylation seen with the wt strain; the presence of *crk* protein in the cytosol or membrane fraction correlates with increased phosphotyrosine in that compartment.

DISCUSSION

In the work described in this paper, we analyzed a series of in vitro-constructed v-*crk* mutants to determine what sequences are required for transformation by this novel oncogene. The v-*crk* gene has sequence homology to two domains, termed SH2 and SH3, found in a variety of proteins implicated in signal transduction (24). We found that both domains are required for full transformation by v-*crk*; in contrast, mutation of the spacer regions between these domains had no effect on transforming activity. This result highlights the importance of SH2 and SH3 domains, which may play a central role in mediating the assembly of multiprotein complexes during normal signal transduction (21a, 24; M. Matsuda and H. Hanafusa, submitted for publication).

We have proposed that v-*crk* might elevate the intracellular phosphotyrosine content of critical cellular proteins by binding in a ternary complex with substrate proteins and endogenous protein-tyrosine kinases (21a). In this model, it is reasonable to assume that the SH2 and SH3 domains independently bind to either kinases or their substrates, but that both domains are required for assembly of ternary complexes. This is consistent with the mutagenic data presented here and with the sequence data for a number of other proteins, which implies that the SH2 plus SH2′ domains and the SH3 domain must have modular and independent functions (24). Our results are incompatible with a model in which either SH2 or SH3 is solely responsible for the biological activity of v-*crk*.

The fact that the SH3 linker insertion mutant, BSP, retains some transforming activity could imply that this domain is less critical than SH2 plus SH2′ for transformation. However, the linker in this mutant is inserted into a region of the SH3 domain that is extremely variable when other sequences are compared; in fact, the *S. cerevisiae cdc25* gene product has a five-amino-acid insertion precisely at the homologous spot in its SH3 domain (1, 27). This mutation may therefore alter the conformation insufficiently to completley abolish SH3 function. Further mutation of highly conserved SH3 residues is required to resolve this question. In contrast, the total lack of biological activity of the two SH2 point mutants (N273 and R294) inplies a critical function for the mutated residues or an extreme sensitivity to conformational change.

We have found that in all cases the degree of increased cellular phosphotyrosine levels in mutant-infected cells cor-

related with the degree of transformation. This is consistent with involvement of increased tyrosine phosphorylation in a mechanism whereby v-*crk* can alter cellular growth control. The most potentially useful result is that partially transforming mutants increase phosphotyrosine in a partial manner and never give the full extent of tyrosine phosphorylation seen in wt CT10-transformed cells. Since only three major phosphotyrosine-containing proteins are observed, even with wt v-*crk*, it may be possible to correlate the degree of phosphorylation of individual proteins with various transformation parameters.

The requirement for retroviral *gag* sequences for full transforming activity is surprising, since the *gag*-deleted mutant viruses express high levels of the c-*crk*-derived portion of v-*crk*. Retroviral *gag* has previously been shown to be important for the transforming activity of other oncogenes, including *abl* and *fps*, which encode tyrosine kinases (7, 11, 26); deregulation of enzymatic activity, protein stabilization, and altered subcellular localization have been proposed to explain this requirement. We have found that avian *gag* (which, unlike mammalian *gag*, is not myristoylated [8]) results in partitioning of $P47^{gag\text{-}crk}$ into both the membrane and cytosolic fractions, although the majority of the protein is found on the membrane. Our experiments show that v-*crk* protein that is localized almost exclusively to either the membrane (NSC) or cytosol (SHB) cannot fully transform cells. Phosphotyrosine-containing *crk*-binding proteins are found in both compartments (21a; see above). We assume that increased phosphotyrosine levels on these proteins are due to interaction with the v-*crk* protein and that the *gag*-deleted mutants cannot interact productively with all of the cellular substrate proteins owing to their subcellular localization.

A problem with the replicating retroviral expression system used in these studies is the high viral mutation rate and apparently strong selection for transforming viruses. This leads to a lower percentage of cells expressing nontransforming mutants than the wt and to frequent generation of fully transforming revertants or pseudorevertants from less-transforming parental mutants. The small percentage of cells expressing nontransforming or weakly transforming mutant proteins made it impossible to assay these mutants for *crk*-associated tyrosine and serine-threonine kinase activities as previously observed with the wt strain (20, 21, 21a), since even with highly expressed wt v-*crk* these activities are relatively low and difficult to detect.

These problems with the retroviral system can be circumvented in the future by expression of mutant genes in mammalian cell lines and by in vitro reconstitution experiments with mutant *crk* proteins. wt v-*crk* can transform murine 3T3 cells and rat 3Y1 cells when highly expressed via an integrated selectable vector, and these *crk*-expressing cell lines have similar biochemical properties to CT10-infected CEF (C. Marshall and H. Hanafusa, unpublished data). Mutant *crk* genes could be expressed at levels comparable to wt v-*crk* in mammalian cell lines, allowing a direct comparison. More promising is the finding that v-*crk* protein expressed in bacteria, or in insect cells via a baculovirus vector, can bind to the phosphotyrosine-containing proteins and to the tyrosine kinase activity of v-*crk*-transformed cells (21a; Matsuda and Hanafusa, submitted). The nontransforming mutants described here should prove extremely useful in such reconstitution experiments to elucidate the functions of the SH2 and SH3 domains and to identify the proteins whose binding to *crk* is required for transforming activity.

ACKNOWLEDGMENTS

We thank Lu-Hai Wang for the UR2AV clone and for many years of helpful advice, and we gratefully acknowledge the significant contributions of M. Matsuda and C. Marshall to this work. We also thank S. Kornbluth and Y. Fukui for critically reading the manuscript.

This work was supported by Public Health Service grant CA44356-03 from the National Institutes of Health and grant 2517 from the Council for Tobacco Research. B.J.M. was supported by training grant AI07233-14 from the National Institutes of Health.

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