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25. McClintock [B. McClintock, *Cold Spring Harbor Symp. Quant. Biol.* **16**, 13 (1951)] has proposed that increasing dosage of *Ac* causes a delay in the timing of *Ac* action such that transposition occurs only late in the development of the tissue. Schwartz and Echt [D. Schwartz and C. Echt, *Mol. Gen. Genet.* **187**, 410 (1982)], however, have interpreted this dosage effect as being correlated with the physiological state of the cells rather than with the developmental stage of the tissue. In any case, this dosage effect is an example of element-controlled regulation.
26. Although anthocyanin pigment is localized in the vacuole of intact plant cells and should not normally diffuse from cell to cell, lightly colored cells were observed with *bz2::mu1*, *bz2::Ds2*, and *c2::Spm* at the periphery of each spot. The origin of this halo is not known; however, it did not interfere with the sector measurements because the video imaging system was calibrated (object versus background) so that only the dark purple cells were counted as part of a spot.
27. We thank A. Britt for the *c2::Spm* stock and for discussions. Supported by a Chaim Weizmann fellowship (A.A.L.), grants from the National Institute of General Medical Sciences of the NIH (GM 32422) and the USDA (89-37280-4840) to V.W., and an NIH institutional grant to Stanford University for the video imaging equipment.

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Binding of Transforming Protein, P47$^{gag\text{-}crk}$, to a Broad Range of Phosphotyrosine-Containing Proteins

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Although the oncogene product of CT10 virus, P47$^{gag\text{-}crk}$, does not itself phosphorylate proteins at tyrosine residues, it elevates phosphotyrosine in transformed cells. The P47$^{gag\text{-}crk}$ oncoprotein contains SH2 and SH3 domains, which are conserved in several proteins involved in signal transduction, including nonreceptor tyrosine kinases. P47$^{gag\text{-}crk}$ bound in vitro to phosphotyrosine-containing proteins from *crk*-transformed cells and from cells transformed by oncogenic tyrosine kinases. The association between P47$^{gag\text{-}crk}$ and p60$^{v\text{-}src}$, a phosphotyrosine-containing protein, was abolished by dephosphorylation of p60$^{v\text{-}src}$. This suggests that the SH2 and SH3 regions function to regulate protein interactions in a phosphotyrosine-dependent manner.

MANY PROTEINS INVOLVED IN SIGnal transduction contain common domains, named SH2 and SH3 (*src* homology 2 and 3), which were originally found in the regulatory regions of nonreceptor tyrosine kinases (*1–3*). Although regulatory functions for these domains were suggested from mutational analyses, their biological function is still unclear (*4*). Because P47$^{gag\text{-}crk}$ consists almost entirely of SH2 and SH3 domains (*5*), which are indispensable for transformation by this oncogene product (*6*), analysis of transformation by P47$^{gag\text{-}crk}$ should provide an understanding of the function of the SH2 and SH3 regions.

Several cellular phosphoproteins from CT10-transformed cells coimmunoprecipitate with P47$^{gag\text{-}crk}$ and antibodies to Gag (anti-Gag) or Crk (anti-Crk) (*7*). These cellular phosphoproteins are identical, as judged by V8 protease mapping, to those precipitated from lysates of the same cells, with an antibody to phosphotyrosine (anti-phosphotyr) (*7*). These results suggest that the proteins phosphorylated on tyrosine are bound to P47$^{gag\text{-}crk}$.

To examine the specificity of the interaction among these proteins, we performed immunoprecipitations in the presence of an excess of exogenously added P47$^{gag\text{-}crk}$. ^{32}P-labeled cellular proteins of 135, 120, 94, 87, and 65 to 75 kD were precipitated from lysates of *crk*-transformed 3Y1 cells (Crk-3Y1) (*8*) by anti-phosphotyr (Fig. 1, lane 4). P47$^{gag\text{-}crk}$ that was immunoprecipitated with antibody to p19^{gag} (anti-p19^{gag}) was associated with nearly all of the phosphotyrosine-containing proteins (Fig. 1, lane 3). The only protein detectable by anti-phosphotyr but not coimmunoprecipitated with anti-p19^{gag} was the 120-kD protein (Fig. 1, lane 5). The 120-kD protein was probably difficult to detect by immunoprecipitation with anti-p19^{gag} because it was weakly associated with P47$^{gag\text{-}crk}$. Therefore, we used beads coupled to recombinant P47$^{gag\text{-}crk}$ by means of anti-p19^{gag} (P47$^{gag\text{-}crk}$–anti-p19^{gag} complex) (*9*) to coimmunoprecipitate the 120-kD protein from the fraction of the lysate not bound by anti-p19^{gag} alone (Fig. 1, lane 6). If the cell lysate was first treated with anti-phosphotyr, no phosphoproteins were detected in supernatant subjected to immunoprecipitation by anti-p19^{gag} or the P47$^{gag\text{-}crk}$–anti-p19^{gag} complex (Fig. 1, lanes 7 and 8). These results suggest that P47$^{gag\text{-}crk}$ associates with phosphoproteins that contain phosphotyrosine and with few, if any, phosphoproteins that contain only phosphoserine or phosphothreonine.

To see whether P47$^{gag\text{-}crk}$ bound a broad spectrum of phosphotyrosine-containing proteins, we examined the binding of purified P47$^{gag\text{-}crk}$ in vitro to proteins phos-

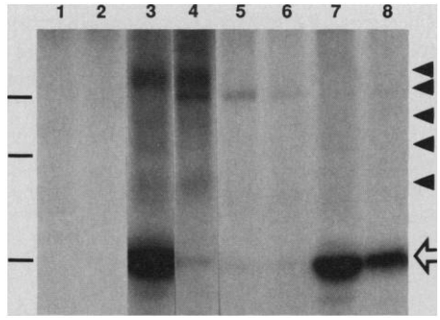

Fig. 1. Association of phosphotyrosine-containing proteins with P47$^{gag\text{-}crk}$ (*18*). [^{32}P]Orthophosphate-labeled proteins from 3Y1 (lanes 1 and 2) and *crk*-transformed 3Y1 (Crk-3Y1) cells (lanes 3 to 8) were precipitated with anti-p19^{gag} (3C2 monoclonal antibody (*19*) (lanes 1 and 3) or anti-phosphotyr (*20*) (lanes 2 and 4). Protein (50 μg) from Crk-3Y1, which contained ~0.01 μg of P47$^{gag\text{-}crk}$, was used in each lane. Supernatants from these immunoprecipitations were immunoprecipitated with a second set of antibodies: supernatant of anti-p19^{gag} immunoprecipitation (lane 3) was precipitated with anti-phosphotyr (lane 5) or with anti-p19^{gag} coupled with 0.5 μg of recombinant P47$^{gag\text{-}crk}$ produced in insect cells (P47$^{gag\text{-}crk}$–anti-p19^{gag} complex) (*9*) (lane 6); supernatant of anti-phosphotyr immunoprecipitation (lane 4) was precipitated with anti-p19^{gag} (lane 7) or the P47$^{gag\text{-}crk}$–anti-p19^{gag} complex (lane 8). Immunoprecipitates were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (10% gel). Arrowheads indicate the major phosphotyrosine-containing proteins in Crk-3Y1 cells (from top of gel): 135, 120, 94, 87, and 65 to 75 kD. P47$^{gag\text{-}crk}$ is indicated by the open arrow. Bars at the left of the figure are molecular size markers: 110, 84, and 47 kD.

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phorylated on tyrosine residues by oncogenic nonreceptor (v-*src* and v-*yes*) and receptor (v-*erb*-B) tyrosine kinases. Chicken embryo fibroblasts (CEF) were infected with transforming retroviruses carrying these oncogenes, Rous sarcoma virus (RSV) [v-*src* (*10*)], Y73 virus [v-*yes* (*11*)], and avian erythroblastosis virus (AEV) [v-*erb*-B (*12*)] as well as CT10. Lysates of transformed CEF were subjected to immunoprecipitation with anti-p19^{gag} alone or P47$^{gag\text{-}crk}$–anti-p19^{gag} complex. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and detected by immunoblotting with anti-phosphotyr (Fig. 2A). Tyrosine-phosphorylated proteins present in transformed cells expressing the tyrosine kinases were coprecipitated with the P47$^{gag\text{-}crk}$–anti-p19^{gag} complex. Except for P90$^{gag\text{-}yes}$, anti-p19^{gag} antibody alone did not coimmunoprecipitate proteins containing phosphotyrosine (Fig. 2A, Y73, lane 2). Phosphotyrosine-containing proteins in CT10-transformed cells were immunoprecipitated by anti-p19^{gag} because of their association with P47$^{gag\text{-}crk}$ expressed from CT10 virus. Association between P47$^{gag\text{-}crk}$ and phosphotyrosine-containing proteins was not blocked by phosphotyrosine, which competed for anti-phosphotyr (Fig. 2B). Therefore, P47$^{gag\text{-}crk}$ must recognize conformational determinants that are dependent on tyrosine phosphorylation rather than tyrosine phosphate itself.

To show that phosphorylation of tyrosine was essential for binding of proteins by P47$^{gag\text{-}crk}$, we studied the interaction between P47$^{gag\text{-}crk}$ and p60$^{v\text{-}src}$. The p60$^{v\text{-}src}$ protein, which is partially phosphorylated at Tyr^{416} in vivo by autophosphorylation (*13*), was immunoprecipitated with an antibody to p60$^{v\text{-}src}$ (anti-Src). This immune complex was then incubated with purified P47$^{gag\text{-}crk}$. Binding of P47$^{gag\text{-}crk}$ to p60$^{v\text{-}src}$–anti-Src complex was detected by immunoblotting with anti-Crk, and was enhanced after additional incubation with adenosine triphosphate (ATP) and Mg^{2+} in vitro (Fig. 3, lanes 2 to 4). Under this condition p60$^{v\text{-}src}$ was autophosphorylated exclusively on tyrosine (*13*). Further treatment of p60$^{v\text{-}src}$ with potato acid phosphatase greatly reduced binding to P47$^{gag\text{-}crk}$ (Fig. 3, lanes 5 and 6). These results show that P47$^{gag\text{-}crk}$ bound to p60$^{v\text{-}src}$ in a phosphotyrosine-dependent manner.

The ability of P47$^{gag\text{-}crk}$ to bind phosphotyrosine-containing proteins can be attributed to the c-*crk*–derived region of this protein. The *gag*-encoded proteins of avian retroviruses immunoprecipitated with anti-p19^{gag} did not associate with phosphotyrosine-containing proteins from transformed cells (Fig. 2A). In addition, Crk protein lacking Gag can associate with phosphotyrosine-containing proteins as well as P47$^{gag\text{-}crk}$ (*7*).

Physical interaction of molecules plays an important role in signal transduction. We propose that a function of the *crk* oncogene product, consisting almost entirely of SH2 and SH3 domains, is to modulate protein-protein interactions in response to tyrosine phosphorylation. Two proteins that contain SH2 and SH3, phosphatidylinositol-specific phospholipase C (PLC-γ) (*2*) and *ras* p21 guanosine triphosphatase (GTPase)–activating protein (GAP) (*3*) (Fig. 4), are phosphorylated by the platelet-derived growth factor (PDGF) receptor and by the epidermal growth factor (EGF) receptor after ligand stimulation, which induces autophosphorylation of these receptors on tyrosine residues (*14*). This autophosphorylation appears to be essential for the physical association of PLC-γ with the PDGF or EGF receptor (*15*). Cellular nonreceptor tyrosine kinases, which contain SH2 and SH3 regions (Fig. 4), are negatively regulated by tyrosine phosphorylation of their extreme COOH-termini (*16*), whereas NH_2-terminal regions of nonreceptor tyrosine kinases, which contain SH2 and SH3 regions, are closely apposed to their COOH-terminal catalytic domains (*17*). Mutations in the SH2 and SH3 regions and in the COOH-terminus near the site of tyrosine phosphorylation activate these tyrosine kinases (*4*, *16*). This suggests that the SH2 and SH3 regions might associate intramolecularly with a phosphotyrosine residue at the extreme COOH-terminus and thereby regulate tyrosine kinase activity.

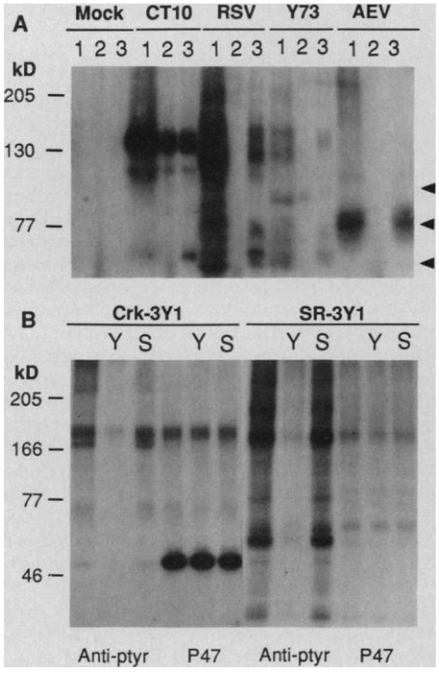

Fig. 2. (**A**) P47$^{gag\text{-}crk}$ binding of substrates for various oncogenic tyrosine kinases. CEF infected with various transforming avian retroviruses were lysed and incubated with antibodies as described in the legend to Fig. 1. Total proteins (lane 1) and those immunoprecipitated with anti-p19^{gag} (lane 2) or with the P47$^{gag\text{-}crk}$–anti-p19^{gag} complex (lane 3) were separated by SDS-PAGE (7.5% gel), transferred to Immobilon (Millipore), immunoblotted with anti-phosphotyr, and visualized by ^{125}I-labeled protein A (Amersham). Arrowheads indicate, from top to bottom, P90$^{gag\text{-}yes}$, gp68$^{erb\text{-}B}$, and p60^{src}. Bars indicate molecular size markers. (**B**) [^{32}P]Orthophosphate-labeled proteins from Crk-3Y1 and *src*-transformed 3Y1 cells (SR-3Y1) (*21*) were precipitated with either anti-ptyr or the P47$^{gag\text{-}crk}$–anti-p19^{gag} complex (P47) in the absence or presence of 10 mM phosphotyrosine (Y) or phosphoserine (S), analyzed by SDS-PAGE (7.5% gel), and visualized by autoradiography. Bars indicate molecular size markers.

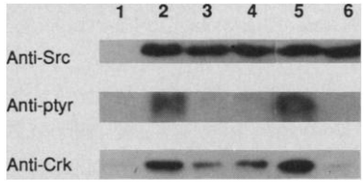

Fig. 3. Effect of tyrosine phosphorylation on the association between p60$^{v\text{-}src}$ and P47$^{gag\text{-}crk}$ (*22*). Cell lysates of SR-3Y1 were incubated without (lane 1) or with anti-Src (lanes 2 to 6). Immune complexes were incubated in kinase buffer containing ATP and Mg^{2+} (lanes 2, 5, and 6), lacking Mg^{2+} (lane 3), or lacking ATP (lane 4). Some samples were further incubated in phosphatase buffer without (lane 5) and with potato acid phosphatase (lane 6). Immune complexes were incubated with purified P47$^{gag\text{-}crk}$ (*9*). After washing, proteins associated with these immune complexes were analyzed by SDS-PAGE (10% gel) and immunoblotting with anti-Src and anti-ptyr for p60$^{v\text{-}src}$ or anti-Crk for P47$^{gag\text{-}crk}$.

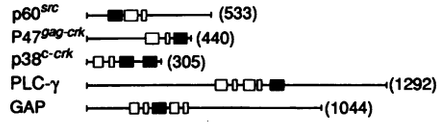

Fig. 4. Comparison of the structures of p60$^{c\text{-}src}$ (*1*, *5*), P47$^{gag\text{-}crk}$ (*5*), p38$^{c\text{-}crk}$ (*23*), PLC-γ (PLC-148) (*2*), and GAP (*3*). Black and white boxes denote the SH3 and SH2 domains, respectively. The numbers at the right indicate the total number of amino acids in each protein.

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18. Conditions for cell culture, labeling, preparation of cell lysates, and immunoprecipitation were as described (*5, 7*) with the following modifications. Cells (10^7) were labeled for 5 hours with 2.5 mCi of [^{32}P]orthophosphate (Du Pont Biotechnology Systems) in 2 ml of medium. Lysates were prepared in RIPA buffer [10 mM tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and Trasylol at 100 KIU (kallikrein inactivator units) per milliliter] containing 0.5 mM Na_3VO_4, 50 μM Na_2MoO_4, and 10 mM NaF.
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22. SR-3Y1 cells (5×10^6) were lysed in RIPA buffer and p60$^{v\text{-}src}$ was immunoprecipitated by anti-Src (monoclonal antibody 327) [L. A. Lipsich, A. J. Lewis, J. S. Brugge, *J. Virol.* **48**, 352 (1983)] and phosphorylated in kinase buffer [10 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 0.2 mM ATP] at 30°C for 20 min. Immune complexes were washed with RIPA and 50 mM Hepes (pH 7.5) and 0.1% Triton X-100 and incubated in phosphatase buffer [40 mM 1,4-piperazine diethane sulfonic acid (Pipes) (pH 6.0), leupeptin at 10 μg/ml, and Trasylol at 100 KIU/ml] with 10 μg of potato acid phosphatase (Sigma) at 30°C for 30 min. Immune complexes were washed with RIPA and incubated with 0.1 μg of purified P47$^{gag\text{-}crk}$ (9). Proteins were separated on a 7.5% SDS–polyacrylamide gel. Immunoblotting was performed as described in the legend to Fig. 2.
23. C. T. Reichman and H. Hanafusa, in preparation.
24. We thank D. Boettiger, J. S. Brugge, C. Y. Kang, and Y. Matsuura for providing cell lines and baculovirus vectors; and S. A. Kornbluth, G. A. Blobel, and D. W. Sternberg for reading of this manuscript. Supported by National Cancer Institute grant CA44356; grant 2517 from Council for Tobacco Research; and (to B.J.M.) NIH training grant AI 07233 from the National Institute of Allergy and Infectious Diseases.

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Antibodies to Clathrin Inhibit Endocytosis But Not Recycling to the Trans Golgi Network in Vitro

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Mannose 6-phosphate receptors carry newly synthesized lysosomal enzymes from the trans Golgi network (TGN) to prelysosomes and then return to the TGN to carry out another round of lysosomal enzyme delivery. Although clathrin-coated vesicles mediate the export of mannose 6-phosphate receptors from the TGN, nothing is known about the transport vesicles used to carry these receptors back to the TGN. Two different in vitro assays used in this study show that an antibody that interferes with clathrin assembly blocks receptor-mediated endocytosis of transferrin, but has no effect on the recycling of the 300-kilodalton mannose 6-phosphate receptor from prelysosomes to the TGN. These results suggest that the transport of mannose 6-phosphate receptors from prelysosomes to the TGN does not involve clathrin.

NEWLY SYNTHESIZED, SOLUBLE LYsosomal enzymes undergo a unique posttranslational modification that facilitates their delivery to lysosomes (*1*). As lysosomal enzymes traverse the secretory pathway, their N-linked oligosaccharides acquire one or two mannose 6-phosphate (man6P) residues. The presence of man6P enables lysosomal enzymes to bind to man6P receptors in the TGN; the receptor-ligand complexes are then thought to be carried in clathrin-coated vesicles to prelysosomes. The acidic interior of prelysosomes (also termed "late endosomes") triggers the release of the enzymes from man6P receptors, and lysosomal enzymes later appear in lysosomes. Meanwhile, man6P receptors are carried back to the TGN to complete the transport cycle. A small fraction of man6P receptors are also present at the cell surface, where they can bind extracellular lysosomal enzymes and deliver them to lysosomes by conventional receptor-mediated endocytosis.

We have recently reconstituted the recycling of the 300-kD man6P receptor from late endosomes to the TGN in a cell-free system (*2*). The assay takes advantage of the localization of sialyltransferase to the trans Golgi and TGN (*3*) and utilizes a mutant cell line [Chinese hamster ovary (CHO) clone 1021] in which glycoproteins are not sialylated (*4*). Man6P receptors (metabolically labeled with [^{35}S]methionine), present in late endosomes in a mutant cell extract, acquire sialic acid residues when they are transported to the TGN of wild-type Golgi complexes, which are present in reaction mixtures. The acquisition of sialic acid by man6P receptors in this system reflects a vesicular transport process, since it is dependent on time, temperature, adenosine triphosphate (ATP), and cytosol and also requires guanosine triphosphate (GTP) hydrolysis (*2*). Furthermore, man6P receptors and sialyltransferase remain in sealed membrane compartments throughout the reaction, and nonspecific membrane fusion is ruled out by several criteria (*2*).

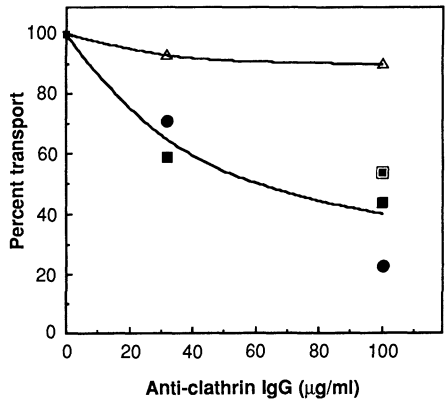

Fig. 1. Anti-clathrin IgG inhibits endocytosis but not man6P receptor recycling to the TGN in vitro. Endocytosis (closed symbols) was measured as described in Table 1; the different symbols refer to three independent endocytosis experiments and include the data presented in Table 1. Transport of 300-kD man6P receptors from endosomes to the TGN (open triangles) was assessed in three independent experiments by transport-coupled sialylation of man6P receptors (*2*) in reaction volumes of 200 μl; a typical result is shown. Endosome-TGN reactions were preincubated with antibodies for 30 min at 0°C. Samples were then warmed to 37°C for 2 hours. In control endosome-TGN reactions in the absence of antibody, 10 to 20% of total man6P receptors acquired sialic acid. Antibody preincubation had no effect on the inhibition observed for endocytosis. Data are presented as the percent transport observed relative to transport measured in the presence of a control IgG.

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