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A novel signaling molecule, p130, forms stable complexes *in vivo* with v-Crk and v-Src in a tyrosine phosphorylation-dependent manner

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$p47^{v\text{-}crk}$ (v-Crk), a transforming gene product containing Src homology (SH)-2 and -3 domains, induces an elevated level of tyrosine phosphorylation of several cellular proteins. Among these proteins, a 125–135 kDa protein (p130) shows marked phosphorylation at tyrosines and tight association with v-Crk, suggesting a direct signal mediator of v-Crk. Here we report the molecular cloning of rat p130 by immunoaffinity purification. The p130 is a novel SH3-containing signaling molecule with a cluster of multiple putative SH2-binding motifs of v-Crk. Immunochemical analyses revealed that p130 is highly phosphorylated at tyrosines during transformation by $p60^{v\text{-}src}$ (v-Src), as well as by v-Crk, forming stable complexes with these oncoproteins. The p130 behaves as an extremely potent substrate of kinase activity included in the complexes and it is a major v-Src-associated substrate of the Src kinase by partial peptidase mapping. Subcellular fractionation demonstrated that the cytoplasmic p130 could move to the membrane upon tyrosine phosphorylation. The p130 (designated Cas for Crk-associated substrate) is a common cellular target of phosphorylation signal via v-Crk and v-Src oncoproteins, and its unique structure indicates the possible role of $p130^{Cas}$ in assembling signals from multiple SH2-containing molecules.

Key words: Crk/Src/Src homology-2/Src homology-3/tyrosine phosphorylation

Introduction

A group of cytoplasmic tyrosine kinases contain a conserved and non-catalytic stretch of ~100 amino acids called the Src homology (SH)-2 domain and a region of ~50 amino acids called the SH3 domain (Sadowski *et al.*, 1986; Mayer *et al.*, 1988; Koch *et al.*, 1991). SH2 and SH3 domains are also found in other cellular signaling enzymes and cytoskeleton-associated proteins. Besides many SH2/SH3-containing molecules with known catalytic or functional domains, there are several signaling molecules called 'adapter proteins' which are so small that no conserved domains seem to exist except SH2 and SH3 regions, such as Nck (Lehmann *et al.*, 1990), Grb2/Ash/Sem-5 (Clark *et al.*, 1992; Lowenstein *et al.*, 1992; Matuoka *et al.*, 1992) and Crk. Recent studies indicate that these regions regulate signals by binding their specific target proteins (Moran *et al.*, 1990; Pawson and Gish, 1992; Birge and Hanafusa, 1993). Each SH2 region binds its specific phosphotyrosine-containing proteins by recognizing a phosphotyrosine in the context of several adjacent amino acids and transduces specific signals in a phosphorylation-dependent manner (Muller *et al.*, 1992; Songyang *et al.*, 1993). The specific binding of these regions promotes the formation of signaling complexes which typically contain the autophosphorylated growth factor receptors. For example, platelet-derived growth factor (PDGF) receptor forms an *in vivo* complex with phospholipase C (PLC)-γ and GTPase activator protein (GAP) (Kaplan *et al.*, 1990; Kazlauskas *et al.*, 1990; Morrison *et al.*, 1990), and epidermal growth factor (EGF) receptor with Grb2 and Sos proteins (Egan *et al.*, 1993).

$p47^{v\text{-}crk}$ (v-Crk) is a gene product of a transforming gene v-*crk*, originally isolated from avian sarcoma viruses, CT10 (Mayer *et al.*, 1988) and ASV-1 (Tsuchie *et al.*, 1989). The structure of v-Crk shows that it encodes a fusion gene product of viral *gag* protein and a part of cellular Crk (Matsuda *et al.*, 1992; Reichman *et al.*, 1992) including one set of an SH2 and an SH3. v-Crk is therefore thought to be a mediator of cell transformation signal through protein–protein interactions, although the precise mechanism of transformation remains unclear (Mayer and Hanafusa, 1990a). v-Crk induces an elevated level of tyrosine phosphorylation of several proteins in v-Crk-transformed cells while it lacks a kinase domain, and association of v-Crk with these phosphoproteins is also observed (Matsuda *et al.*, 1990; Mayer and Hanafusa, 1990b). These phosphoproteins include phosphorylated paxillin, as identified recently (Birge *et al.*, 1993), although the most predominantly tyrosine-phosphorylated protein in v-Crk-expressing cells is an unidentified 130 kDa protein, p130 (Matsuda *et al.*, 1990; Birge *et al.*, 1992). The p130 is tightly associated with v-Crk, and the SH2 region of v-Crk is required for the association and transformation (Matsuda *et al.*, 1991, 1993). These facts suggest that p130 plays an important role in SH2-mediated signal transduction of v-Crk during cell transformation. It now appears that the identification of the role of p130, the main v-Crk-associated phosphoprotein, is critical to elucidate the mechanism of transformation by v-Crk.

Therefore we performed the isolation of rat p130 by means of immunoaffinity purification which enabled the partial determination of the amino acid sequences and the molecular cloning of rat p130. We report here the structural

and biological characteristics of p130, along with immunological and biochemical analyses of p130 in the relationship with cellular transformation.

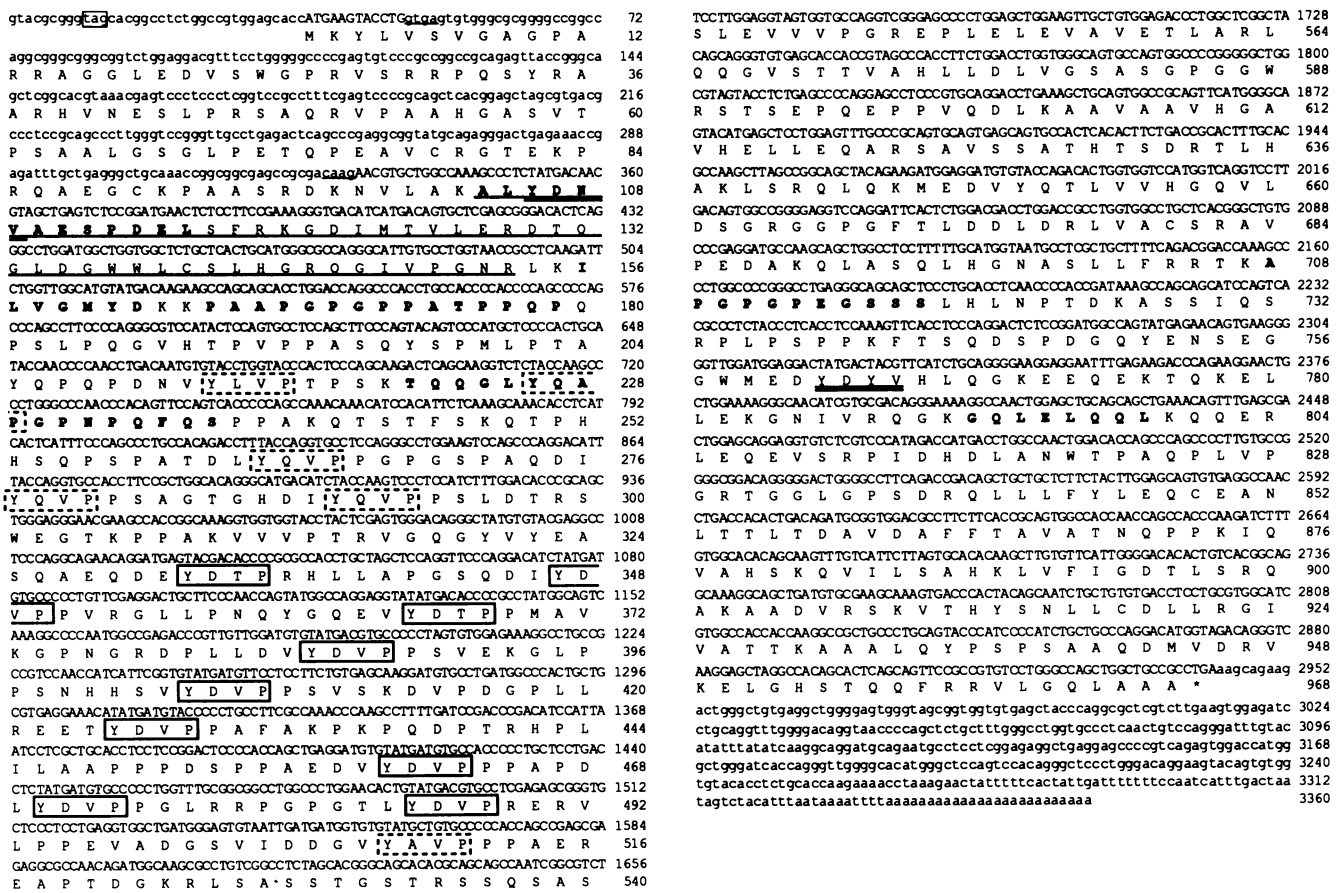
Results

cDNA cloning of rat p130

We have purified p130 from rat 3Y1 cells transfected with a v-Crk-expressing plasmid (3Y1-Crk) using two sequential procedures of immunoaffinity purification (R.Sakai, A.Iwamatsu, N.Hirano, S.Ogawa, T.Tanaka, J.Nishida, Y.Yazaki and H.Hirai, submitted; see Materials and methods). Six sets of partial peptide sequences were obtained by the amino acid sequence analysis of proteolytic fragments of the purified p130. A 384 bp fragment that encodes four out of six peptide sequences was amplified using the PCR method by degenerate primers and was used as a probe for screening a rat cDNA library.

As a result, most of cDNA clones obtained were ~3.1–3.4 kb in size and contained a full coding region with a putative initiating ATG (Kozak, 1981) and an upstream in-frame stop codon. An in-frame deletion at the N-terminal region found in eight independent clones results in an open reading frame (ORF) of 874 amino acids (short form), while full-length cDNAs found in three independent clones code 968 amino acids (long form) (Figure 1A). The predicted molecular weights of each form are 94.3 and 104.1 kDa, respectively. The deletion is probably caused by an alternative splicing because the 282 bp region lost in the short form cDNA lies between sequences which are variants of 5′ donor and 3′ acceptor splice consensus (Mount, 1982) (Figure 1A). All six amino acid

A



B

rat	p130	ALYDN VAESPD E - LS FRK G DIMTVLERDTQGLDG WW LCSLHG---- GIVP GN RLKIL
chicken	c-*src*	ALYDY ESRTET D - LS FKK G ERLQIV---NNTEGD WW LAHSLTTGQT GYIP SN YVAPS
mouse	c-*abl*	ALYDF VASGDN T - LS ITK G EKLRVLG--YNHNGE WD EAQTK-NGQ- GWVP SN YITPV
chicken	v-*crk*	ALFDF KGNDDE D - LP FKK G DILKIR---DKPEEQ WW NAEDMD-GKR GMIP VP YVEKC
human	GRB2(N)	AKYDF KATADD E - LS FKR G DILKVL--NEECDQN WY KAELN--GKD GFIP KN YIEMK
human	GRB2(C)	ALFDF DPQEDG E - LG FRR G DFIHVM---DNSDPN WW KGACH----- GQTG MF PRNYV
human	PLC-γ	ALFDY KAQRED E - LT FIK S AIIQNV---EKQDGG WW RGDYGGKKQ- LWFP SN YVEEM
bovine	GAP	AILPY TKVPDT D E IS FLK G DMFIVH---NELEDG WM WVTNLRTDEQ GLIV ED LVEEV

Fig. 1. Nucleotide and predicted amino acid sequences of rat p130 cDNA. (**A**) Nucleotide sequences and predicted amino acid sequences (single-letter code) of rat p130 cDNA are presented. Shown in small letters are non-coding regions and an alternatively deleted region with possible 5′ donor and 3′ acceptor splice sites is underlined. An in-frame stop codon preceding the initiating ATG is shown in a box. Shown in bold type are the amino acid sequences obtained from the peptide sequence analysis of purified p130. A conserved SH3 region is underlined. Characteristic clusters of four amino acids denoted as YDXP (which is a putative binding motif of the v-Crk SH2 region) are highlighted with boxes. Shown in dashed line boxes are variants of YDXP which are also clustered in the same region. Two candidates for binding motifs of the v-Src SH2 region are indicated by double underlines. (**B**) Alignment of SH3 regions (Mayer *et al.*, 1988; Lowenstein *et al.*, 1992; Booker *et al.*, 1993). The alignment of amino acid sequences of the SH3 region of p130 with those of various signal transducing molecules. Residues that are conserved in the SH3 regions are shown in bold type.

sequences determined by the peptide sequence analysis were found in the coding region (Figure 1A).

p130 is an SH3-containing signaling molecule with a cluster of SH2-binding motifs

Homology search against the Genbank and PIR databases revealed that the cDNA of p130 encodes a novel protein except that there exists a region (nucleotides 1990–2393) highly homologous to EST06380 (81% homology), a 410 bp nucleotide fragment randomly cloned from a human infant brain cDNA library (Adams *et al.*, 1993). It does not belong to any protein family with a known catalytic activity. The p130 possesses a unique SH3 region in the N-terminal domain (Figure 1A and B) and a characteristic proline-rich domain with a cluster of tyrosines having similar adjacent sequences at variable intervals. Seven YDVPs and two YDTPs surrounded by three YQVPs, one YLVP, one YQAP and one YAVP, are scattered every 15–30 amino acids in this region (Figure 1A). Various SH2 regions bind their specific phosphotyrosine-containing proteins by recognizing phosphotyrosines in the context of several (probably three) amino acids following a tyrosine. Combinations found in this region may be suitable as binding targets of a series of SH2-containing molecules including v-Crk, for the nine YDXP patterns in the center of this region are close to the binding motif of the SH2 region of v-Crk (Songyang *et al.*, 1993). They are also suitable as substrates of tyrosine kinases because most of them are preceded by one or two negatively charged amino acids (glutamic acids or aspartic acids, Figure 1A). This region is therefore called the 'substrate region'. No significant structural homology with known proteins was found in this region or in the C-terminal region which is rich in leucines and alanines.

By Northern blot analysis, mRNA of p130 was detected as an apparently single band with the size of ~3.2 kb, indicating that one of the two cDNA species is predominantly expressed (Figure 2). The amount of p130 mRNA in 3Y1-Crk was almost the same as in the original 3Y1 cells suggesting that no transcriptional activation of p130 is involved in the transformation by v-Crk. The p130 mRNA was widely expressed in all rat tissues at a high concentration, although relatively high expression was found in testis, intestine and lung (Figure 2).

Detection of the phosphorylated and unphosphorylated p130 by polyclonal antibodies

The structural characteristics and hydrophilic profile of p130 are illustrated in Figure 3. Three kinds of polyclonal antibody (αCas1, 2 and 3) were raised against three different portions of p130 (Figure 3; see Materials and methods). Each of them recognized semi-purified p130 at an expected molecular size of 125–135 kDa (Figure 4, lanes 6–8), which was also detected by 4G10, a monoclonal antibody against phosphotyrosines (Figure 4, lane 1). These antibodies, except αCas1, precipitated tyrosine-phosphorylated p130 with the similar molecular weight from lysate of 3Y1-Crk cells (Figure 4, lanes 2–4).

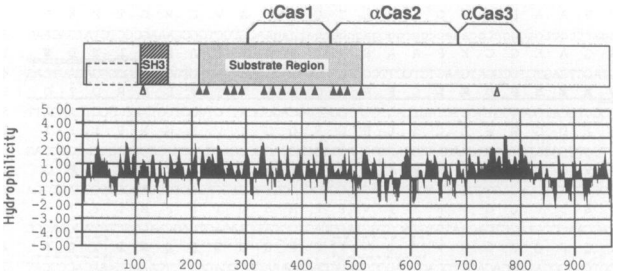


Fig. 3. The primary domain structure of p130 is shown along with three regions which are bacterially expressed as GST fusion proteins for polyclonal antibodies. The possible SH2-binding sites of the similar motifs are shown by closed triangles and two putative Src-binding site are marked by open triangles. Shown below is a hydropathy profile of p130 calculated using the MacVector program (IBI, New Haven, CT) by the method of Kyte and Doolittle (1982) with a window size of 7.

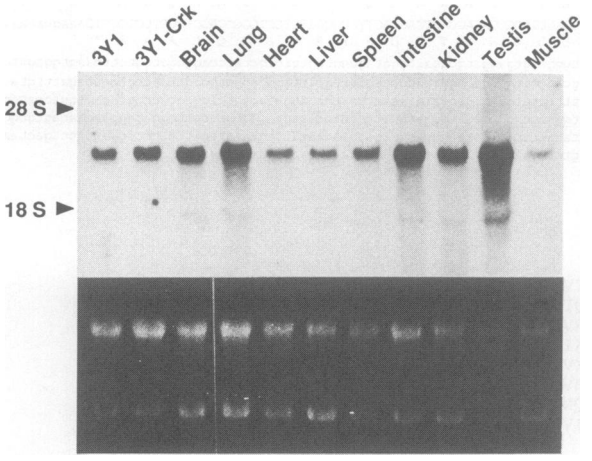


Fig. 2. The expression of p130 mRNA in rat 3Y1 cells and various rat tissues. An RNA blot containing total RNA from the indicated rat tissues and from 3Y1 cells (20 μg/lane) was hybridized with a labeled p130 total cDNA probe, washed and exposed to an X-ray film for 14 h at −70° with an intensifying screen. The positions of 28S and 18S ribosomal RNAs are shown on the left. As controls, the ribosomal RNAs of corresponding lanes stained with ethidium bromide are shown below.

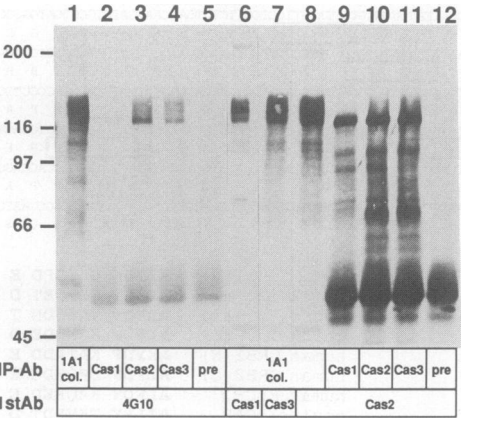


Fig. 4. Detection and immunoprecipitation of p130 in 3Y1-Crk cells by polyclonal antibodies. The p130 semi-purified from 3Y1-Crk cells by a 1A1-CNBr Sepharose 4B column was detected by Western blotting using three kinds of polyclonal antibody (αCas1, 2 and 3) raised against three parts of bacterially expressed p130 cDNA (lanes 6–8) as well as 4G10 (lane 1). On the other hand, lysates of 3Y1-Crk cells were immunoprecipitated by these antibodies or by preimmune rabbit serum (pre) and detected by 4G10 (lanes 2–5) or αCas2 (lanes 9–12). Molecular weights are given in kDa.

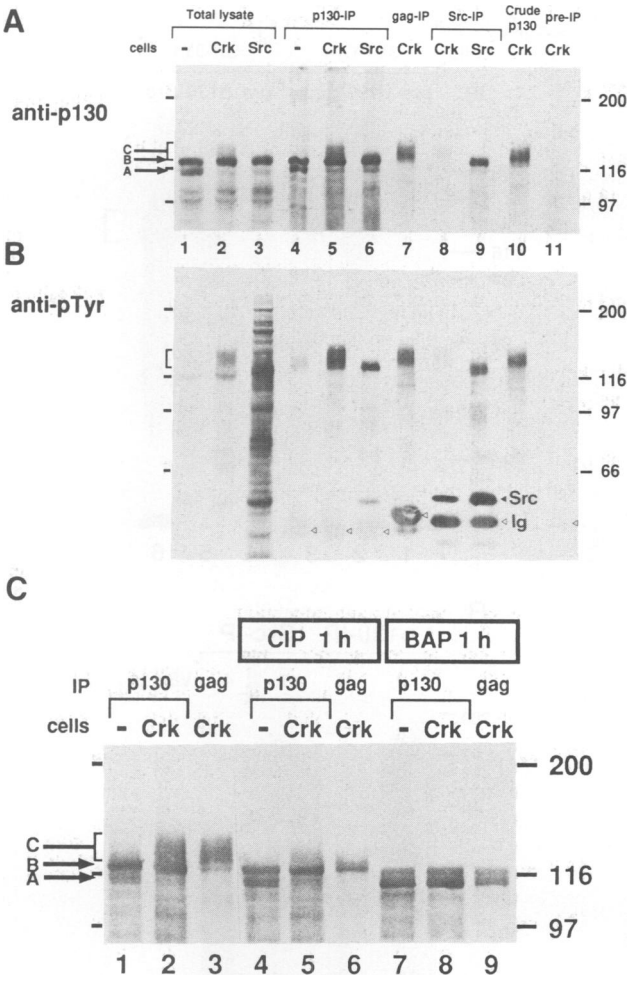


Fig. 5. Expression and tyrosine phosphorylation of p130 in rat 3Y1 transformants. Duplicated filters were analyzed by immunoblotting of p130 using αCas2 (**A**) and phosphotyrosines using 4G10 (**B**). Total cell lysates of 3Y1 (–), 3Y1-Crk (Crk) and SR-3Y1 (Src) cells (lanes 1–3) and lysates directly immunoprecipitated by αCas2 (lanes 4–6), 1A1(anti-*gag*, lane 7), an anti-Src antibody (lanes 8 and 9) and by preimmune rabbit serum for αCas2 (lane 11) were analyzed. Lane 10 shows semi-purified p130 according to the first step of immunoaffinity purification using CNBr Sepharose 4B (Pharmacia) coupled with anti-*gag* antibody, 1A1. The position of tyrosine-phosphorylated p130 (p130-C) is shown by a square bracket on the left. The positions of Src proteins (Src) and Ig heavy chains (Ig) are also indicated. (**C**) Immunoprecipitated samples were treated with protein phosphatases. Lysates of 3Y1 (−) and 3Y1-Crk (Crk) cells immunoprecipitated by αCas2 (lanes 1 and 2) and 3Y1-Crk cells by 1A1 (lane 3) were treated for 1 h at 37°C with 2 U of calf intestine alkaline phosphatase (CIP, lanes 4–6) or 4 U of bacterial alkaline phosphatase (BAP, lanes 7–9). The positions of three forms of p130 (p130-A, -B and -C) detected by αCas2 in 3Y1 cells and 3Y1-Crk cells are shown left as A, B and C, respectively. Molecular weights are given in kDa.

However, each of these three antibodies precipitated the majority of p130 at 125 kDa (Figure 4, lanes 9–11) which fails to be detected by tyrosine phosphorylation or affinity purification with v-Crk (Figure 4, lanes 2–4 and 6–8). The fact that only αCas1, which recognizes a central polypeptide of the substrate region, does not have the ability to precipitate the tyrosine-phosphorylated p130 from 3Y1-Crk lysates suggests that considerable alterations of dominant epitopes by tyrosine phosphorylation should occur in this region because the antibodies are made from non-phosphorylated bacterially expressed p130. In normal 3Y1 cells, p130 was detected as multiple bands at ~115 and 125 kDa (p130-A and p130-B, respectively) by these antibodies (Figure 5A, lanes 1 and 4). In 3Y1-Crk cells, a decreased amount of p130-A and, as described in Figure 4, an appearance of a broad band spreading at 125–135 kDa (p130-C) were observed, whereas the amount of p130-B appears to be constant (Figure 5A, lanes 2 and 5). In these three types of p130, tyrosine phosphorylation is found mostly in p130-C (Figure 5B, lanes 2 and 5), and a trace amount of phosphorylated p130 is also detected in normal 3Y1 cells by immunoprecipitation with anti-p130 antibody (Figure 5B, lane 4). By immunoprecipitating v-Crk from 3Y1-Crk lysate with anti-*gag* antibody, p130-C (the tyrosine-phosphorylated form of p130) was mainly co-precipitated, indicating that the tight association of v-Crk and p130 requires tyrosine phosphorylation of p130 (Figure 5A and B, lanes 7). By treatment with phosphatases, p130-C is converted to p130-A or p130-B (Figure 5C), suggesting that the divergent gel mobility found in p130-C is probably due to multiple phosphorylation states, while divergence in p130-A and -B may be due to other modifications including the short and long form of coding sequences found in the cDNA clones.

Marked tyrosine phosphorylation of p130 was also found in SR-3Y1, a 3Y1 cell line transformed by v-Src of Rous sarcoma virus, although the position of the phosphorylated band is lower than that of p130-C (Figure 5A and B, lanes 6). The condensed and lower band of phosphorylated p130 detected in v-Src transformant cells indicates that numbers and/or positions of phosphorylation sites are different in these cells from those in 3Y1-Crk cells. The stable association of v-Src with the tyrosine-phosphorylated form of p130 in SR-3Y1 cells was also detected (Figure 5A, lane 9). No elevation in the level of phosphorylation of p130 was found in transformants by other activated oncogenes including v-*fes*, v-*fms*, v-*fos*, v-*raf*, v-K-*ras*, v-*erb* B and SV40 (data not shown), suggesting that the mechanisms of cell transformation by these oncogenes should be at least partly different from those by v-*crk* or v-*src*.

p130 is a major substrate component of both v-Crk and v-Src

To ensure the association, v-Crk and v-Src were revealed to be included in the complex immunoprecipitated with p130 (Figure 6A, lane 5; Figure 6B, lane 3). In 3Y1-Crk cells, the binding between $p60^{c\text{-}src}$ (c-Src) and phosphorylated $p130^{Cas}$ was also detected (Figure 5A and B, lanes 8; Figure 6B, lane 2). There are two candidate motifs in p130 which can bind the SH2 region of v-Src (Songyang *et al.*, 1993). One is YDNV found in SH3 and another is YDYV found in the C-terminal region (Figure 1A). The latter motif is more probable because it is preceded by two negatively charged amino acids. Similar motifs are also found in recently cloned p110, another substrate of v-Src kinase in chicken embryo cells (Flynn *et al.*, 1993).

Several immunological and *in vitro* observations show that a 130 kDa major substrate of v-Src is phosphorylated in v-Crk-transformed cells or possesses binding activity with Crk (Kanner *et al.*, 1990, 1991; Matsuda *et al.*, 1991; Koch *et al.*, 1992). To demonstrate that the cloned p130 ($p130^{Cas}$ for Crk-associated substrate) is the main substrate

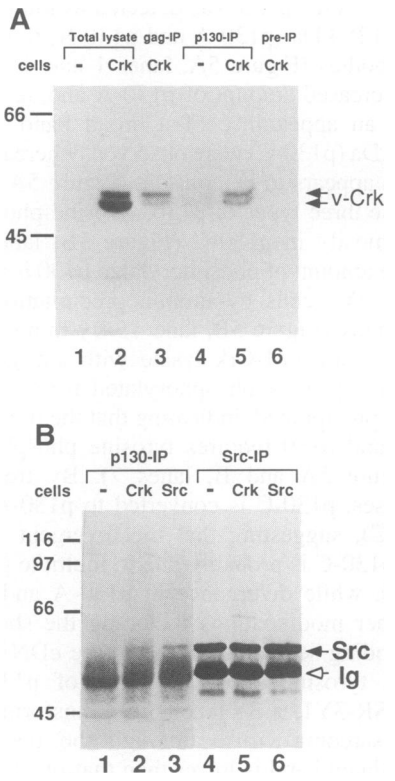


Fig. 6. Binding of p130 to v-Crk and v-Src. (**A**) Binding of p130 to v-Crk. v-Crk proteins associated with p130 were detected. Cell lysates of control 3Y1 cells (−) and 3Y1-Crk (Crk) (lanes 1 and 2) were immunoprecipitated by anti-p130 antibody (αCas2). Co-precipitated proteins were separated by 10% SDS−PAGE and analyzed by immunoblotting using anti-v-Crk antibody (αHcrk, lanes 4 and 5). As controls, v-Crk protein was directly precipitated by anti-*gag* antibody (lane 3) or was precipitated by preimmune rabbit serum (lane 6). The position of v-Crk is indicated. Molecular weights are given in kDa. (**B**) Binding of p130 to Src. Src proteins (c-Src and v-Src) associated with p130 were detected. Lysates of control 3Y1 (−), 3Y1-Crk (Crk) and SR-3Y1 cells (Src) were immunoprecipitated by anti-p130 antibody (αCas2, lanes 1–3). Co-precipitated proteins were separated by 9% SDS−PAGE and Src proteins were detected by immunoblotting using MAb 327. As controls, Src proteins in these cells were precipitated by anti-Src antibody (lanes 4–6). The positions of Src proteins (Src) and Ig heavy chains (Ig) are indicated. Molecular weights are given in kDa.

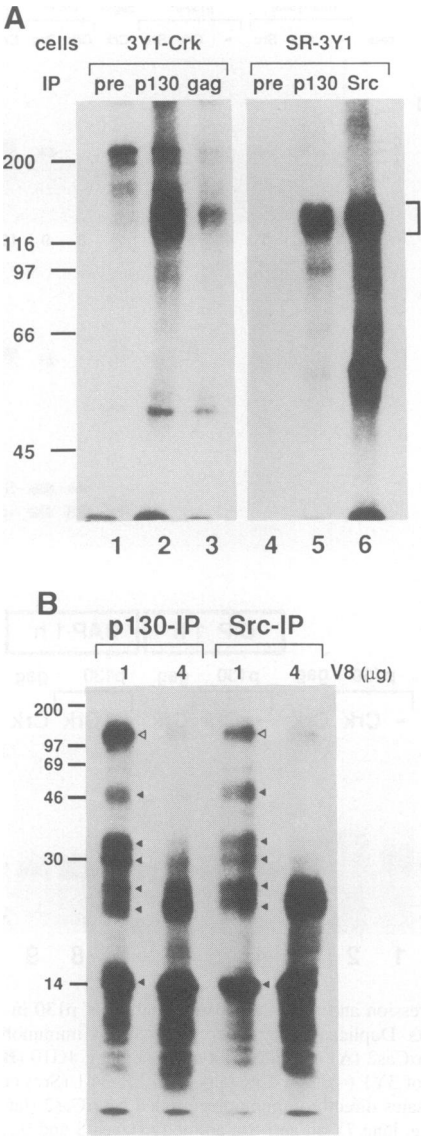


Fig. 7. *In vitro* kinase assay of p130 complexes. (**A**) p130 is a potent kinase substrate of p130 protein complexes. An immune complex kinase assay was performed on 3Y1-Crk (lanes 1–3) or SR-3Y1 cells (lanes 4–6). Proteins immunoprecipitated using anti-p130 (αCas2, lanes 2 and 5), anti-*gag* (1A1, lane 3), anti-Src (lane 6) or control preimmune serum (lanes 1 and 4) were subjected to the autophosphorylation assay. Samples were analyzed by 7.5% SDS−PAGE and the dried gel was exposed to an X-ray film for 5 h (lanes 1–3) and 30 min (lanes 4–6) at −70° with an intensifying screen. Molecular weights are given in kDa. (**B**) p130 is identical to the main substrate component of v-Src. The main phosphorylated bands at 130 kDa from lanes 5 and 6 in (A) were cut out and partially digested by V8 protease. Shown in the figure are p130 (p130-IP) and the 130 kDa main substrate of v-Src (Src-IP) subjected to partial digestion with 1 or 4 μg of *S.aureus* V8 protease. The positions of intact 130 kDa proteins are shown by open triangles and major fragments produced by V8 digestion (1 μg) are shown by closed triangles. Molecular weights are given in kDa.

of v-Src, the immune complex kinase assay was performed. Using v-Crk and v-Src transformants, p130 was proved to be an efficient substrate of kinase(s) involved in both v-Crk and v-Src complexes (Figure 7A, lanes 2 and 5). The size and broad appearance of the phosphorylated p130 is similar to the main substrate involved in the v-Src complex (Figure 7A, lanes 5 and 6). Furthermore, these molecules show identical fragments by partial protease digestion using V8 protease (Figure 7B). We finally concluded from these results that $p130^{Cas}$ is identical to the main v-Src-associated substrate. The 130 kDa band of main v-Src-associated phosphotyrosine-containing proteins in SR-3Y1 cells (Figure 5B, lane 9) shows a slight extension to the lower molecular weight when compared with that of phosphorylated $p130^{Cas}$ (Figure 5B, lane 6). According to the study using chicken cells (Cobb *et al.*, 1994), these phosphoproteins could contain a minor population of $p125^{Fak}$ (focal adhesion kinase) in addition to $p130^{Cas}$.

Transformation-dependent phosphorylation of p130 in NIH3T3 cells

To examine the relationship between the phosphorylation of $p130^{Cas}$ and cellular transformation, the tyrosine phosphorylation of $p130^{Cas}$ in mouse NIH3T3 cells transfected with c-Src, activated Src (Y527F c-Src) and v-Src was examined (Hirai and Varmus, 1990). A cotransfectant

of c-Src and v-Crk was also examined because co-expression of c-Src and v-Crk transformed these cells as reported (Sabe *et al.*, 1992), although the expression of c-Src or v-Crk alone was insufficient for the transformation of NIH3T3 cells. As shown in Figure 8, tyrosine phosphorylation of $p130^{Cas}$ was observed only in cells with transformed phenotypes, and the highest phosphorylation is found in v-Src transformants, suggesting that the transformation is closely related to the level of phosphorylation of $p130^{Cas}$ at tyrosines.

p130 moves from cytoplasm to particulate fractions by tyrosine phosphorylation

Subcellular localization of $p130^{Cas}$ was analyzed by fractionation of hypotonically lysed 3Y1 cells and 3Y1-Crk cells followed by immunoblotting of p130 and phosphotyrosines. The unphosphorylated forms of $p130^{Cas}$ (p130-A and p130-B) in 3Y1 cells and 3Y1-Crk cells were mostly detected in a cytosolic fraction using αCas2 antibody (Figure 9A). On the other hand, tyrosine-phosphorylated $p130^{Cas}$ (p130-C) found in 3Y1-Crk is detected specifically in membranous and nuclear fractions by immunoblotting of both $p130^{Cas}$ and phosphotyrosines (Figure 9A and B, lanes 2 and 4) and is not detectable in the cytosolic fraction. Due to the limitation of accuracy of subcellular fractionation, a substantial part of the membranous proteins is often detected in a nuclear fraction as well as in a membrane fraction. Since there is no structural evidence such as a nuclear localizing signal which indicates that $p130^{Cas}$ also exists in the nucleus, it is still possible that the $p130^{Cas}$ found in the nuclear fraction is leakage from the membrane fraction. However, these results indicate that tyrosine phosphorylation of $p130^{Cas}$ induces drastic changes in the cellular localization of $p130^{Cas}$ from cytoplasm to cellular membrane and possibly to nucleus, suggesting the important role of $p130^{Cas}$ as a cytoplasmic signal-transducing molecule.

Discussion

Many kinds of oncoprotein have been identified in recent years to have the ability to endow normal cells with malignant phenotypes, as for cell growth and differentiation. Most of these molecules, including various tyrosine kinases and serine threonine kinases, are shown to affect the phosphorylation of cellular proteins either directly or indirectly. Thus, specific protein phosphorylation induced by each of them is believed to be a primary event which results in cellular transformation. A substantial volume of information is available about the main target substrates of phosphorylation attacked directly by oncoproteins. For example, the phosphorylation of PLC-γ (Wahl *et al.*, 1988), GAP (Ellis *et al.*, 1990), Nck (Li *et al.*, 1992; Meisenhelder and Hunter, 1992; Park and Rhee, 1992), Vav (Bustelo *et al.*, 1992; Margolis *et al.*, 1992), Shc (Pelicci *et al.*, 1992), Syp/SH-PTP2 (Feng *et al.*, 1993) and cortactin (p80/p85; Wu and Parsons, 1993) is observed during cellular transformation or by various growth factor stimuli. Each of these SH2/SH3-containing molecules may receive several kinds of upstream signal and transduce a common set of signals which regulate cellular growth and differentiation in a phosphorylation-dependent manner.

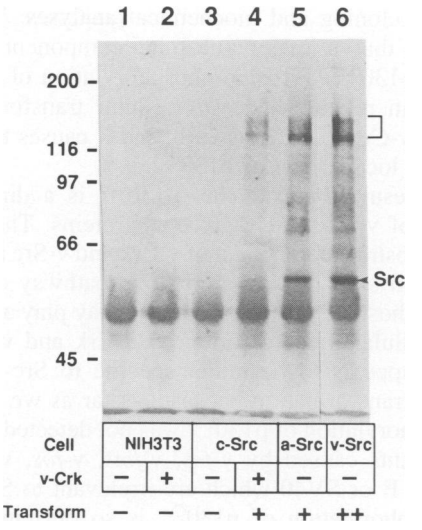


Fig. 8. Detection of phosphorylated p130 in various Src transformants. Tyrosine phosphorylation of p130 was analyzed in mouse stable transfectants of c-Src (lane 3), activated Src (Y527F c-*src*) (lane 5), v-Src (lane 6) and control NIH3T3 cells (lane 1). As the expression of v-Crk (lane 2) or c-Src alone was inadequate for the transformation of NIH3T3 cells, a cotransfectant of c-Src and v-Crk which showed transformed phenotype as expected from a previous report (Sabe *et al.*, 1992) was established and examined (lane 4). Transforming activity, as judged from morphology and tumorigenecity in nude mice, is displayed below. After immunoprecipitation by anti-p130 antibody, p130 phosphorylated at tyrosines was detected by Western blotting using 4G10. The position of tyrosine-phosphorylated p130 is indicated by a square bracket on the right. The position of Src protein is also indicated. Molecular weights are given in kDa.

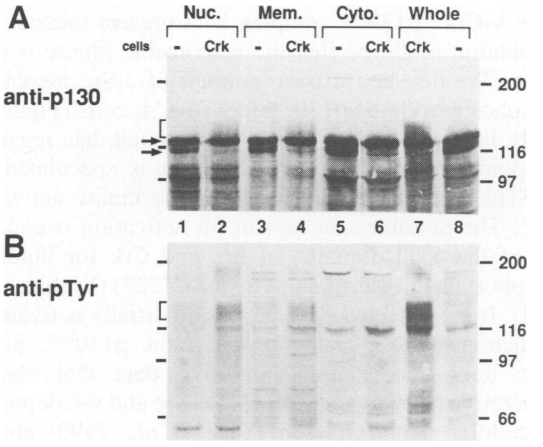


Fig. 9. Subcellular fractionation of hypotonically lysed 3Y1 (−) and 3Y1-Crk cells (Crk) was performed and the localization of p130 was analyzed. Whole cell lysate (Whole, lanes 7 and 8), nuclear P1 fractions (Nuc., lanes 1 and 2), membranous P100 fractions (Mem., lanes 3 and 4) and cytosolic S100 fractions (Cyto., lanes 5 and 6) were analyzed by Western blotting using αCas2 (**A**) or 4G10 (**B**) as first antibodies. The positions of three types of p130 (p130-A, -B and -C) are shown on the left of the upper figure. The position of tyrosine-phosphorylated p130 (p130-C) is also shown by a square bracket on the left of the lower figure. Molecular weights are given in kDa.

$p130^{Cas}$ is a common signal mediator of both v-Crk and v-Src

In this report we characterized $p130^{Cas}$, the main v-Crk-induced and v-Crk-associated phosphoprotein, by

molecular cloning and biochemical analyses. We finally confirmed that a major substrate component of v-Src kinase is p130^{Cas}. Tyrosine phosphorylation of p130^{Cas} is observed in relationship with cellular transformation in v-Src- or v-Crk-expressing cells and it causes the change in cellular localization of p130^{Cas}.

These results indicate that p130^{Cas} is a direct signal mediator of v-Crk and v-Src oncoproteins. The fact that a major substrate component of v-Crk and v-Src is identical suggests, in other words, a common pathway originating from the phosphorylation of p130^{Cas} may play a important role in cellular transformation by v-Crk and v-Src. This pathway appears to be rather specific to Src- and Crk-mediated transformation because, as far as we examined, the phosphorylation of p130^{Cas} was not detected in various transformants caused by v-*fes*, v-*fms*, v-*fos*, v-*raf*, v-K-*ras*, v-*erb* B or SV40 which are irrelevant to Src or Crk. The phosphorylation of p130^{Cas} is so outstanding once the pathway is activated that it might be a good marker for monitoring whether a cell growth signal by cellular transformation or by growth factors is utilizing the pathway.

Mechanisms of phosphorylation of p130^{Cas}

It is an interesting and important issue how p130^{Cas} shows such remarkable tyrosine phosphorylation during transformation. In the case of the v-Src−p130^{Cas} complex, it is reasonable to suppose that the kinase responsible for p130^{Cas} phosphorylation is v-Src kinase itself because it is an extremely potent kinase. The main kinase involved in the v-Crk−p130^{Cas} complex is at present unclear. One explanation might be that the responsible kinase is c-Src kinase. We detected a trace amount of c-Src associating with phosphorylated p130^{Cas} in 3Y1-Crk cells (Figure 5A and B, lanes 8). Although no experimental data regarding this point are available at present, it is speculated that the SH2 region of v-Crk activates the kinase activity of c-Src. The possible mechanism for activation is competition of the SH2 domains of Src and Crk for binding a phosphorylated C-terminal tyrosine (Y527) (Bibbins *et al.*, 1993). It is suspected that the c-Src partially activated by interaction with v-Crk phosphorylates p130^{Cas}, just as v-Src does. There are supportive data that show a cooperative role of overexpressed c-Src and v-Crk proteins for cellular transformation (Sabe *et al.*, 1992) and the transformation is suppressed by Csk, a kinase for Src C-terminal negative regulatory tyrosine.

Another possibility is that the associated v-Crk prevents phosphorylated p130^{Cas} from dephosphorylation by phosphotyrosine phosphatases. It has been demonstrated recently that the addition of bacterially expressed v-Crk protein to cell lysate containing phosphorylated p130^{Cas} protects p130^{Cas} from dephosphorylation (Birge *et al.*, 1992). It is speculated that p130^{Cas} is in equilibrium with dephosphorylation by some cellular phosphotyrosine phosphatases and phosphorylation with some kinases. By the expression of v-Crk, it may protect the phosphorylated SH2-binding sites from phosphotyrosine phosphatase activity and may cause the shift of the equilibrium to a highly phosphorylated state.

p130^{Cas} is a possible signal assembler of SH2-containing molecules

We observed that tyrosine-phosphorylated p130^{Cas} localizes in membrane and nuclear fractions by subcellular fractionation, whereas the majority of unphosphorylated p130^{Cas} localizes in cytoplasm. Considering that it is one of the most efficient SH2-binding molecules, there is no doubt that the phosphotyrosine-containing p130^{Cas} exists in association with membranous or membrane-associated SH2-containing molecules, such as Src family kinases, leaving the remainder of the p130^{Cas} in the cytoplasm as an unphosphorylated stock.

It has been mentioned that similar proteins are tyrosine phosphorylated in transformants of Src family oncogenes, including v-*src*, v-*yes* and v-*fps* (Kamps and Sefton, 1988). There is also a report to show that a 130 kDa protein, which must be identical to p130^{Cas}, associates with v-*src*, v-*fyn* and v-*fgr* oncogene products (Sartor *et al.*, 1991), indicating the possibility that several Src family kinases commonly utilize a pathway through p130^{Cas}. Since the SH2 regions of adapter molecules Nck and Crk are predicted to recognize similar amino acid stretches following a tyrosine (Songyang *et al.*, 1993), Nck is a candidate for one of the binding proteins to the substrate domain of p130^{Cas}. The substrate domain of p130^{Cas} contains a total of 15 repeats of six variants of putative SH2-binding motifs in an ~250 amino acid stretch. This region may thus recognize a set of SH2-containing molecules and bind some of them simultaneously or competitively. As no signaling molecules are known so far which contain such a structure, p130^{Cas} may play a special role in signal transduction. Judging from its structure, p130^{Cas} could (i) work as a 'signal assembler' of Src family kinases and several cellular SH2-containing proteins binding to its substrate domain and (ii) switch on and off the downstream signal through its SH3 domain depending on the proteins attached to p130^{Cas}. The structure and function of the signal-transducing molecule, p130^{Cas}, are quite unique, suggesting a novel mechanism of cellular signal transduction.

Materials and methods

Cells and antibodies

3Y1-Crk is an isolated clone of rat 3Y1 cells (Kimura *et al.*, 1975) transfected with v-*crk* cDNA (Mayer *et al.*, 1988) of an avian sarcoma virus, CT10, inserted in expression vector pMV-7 (Kirschmeier *et al.*, 1988). SR-3Y1 (Zaitsu *et al.*, 1988) is a 3Y1 cell line transformed by v-Src of Rous sarcoma virus. Mouse monoclonal antibodies against viral *gag* protein (1A1) (Potts and Vogt, 1987) and against phosphotyrosines (4G10) (Morrison *et al.*, 1990) were collected from culture supernatant of hybridoma cells. MAb 327, a monoclonal antibody against Src, was provided by J.S.Brugge (Lipsich *et al.*, 1983). A rabbit polyclonal antibody (αHcrk) against v-Crk protein expressed by the baculovirus vector system and affinity purified using anti-*gag* antibody (1A1) was produced. Stable transformants of NIH3T3 cells transfected with c-*src*, activated *src* (Y527F c-*src*) and v-*src* were established as described (Hirai and Varmus, 1990)

Purification and cDNA cloning of p130

p130 was purified from 3Y1-Crk cells by immunoaffinity columns (R.Sakai *et al.*, manuscript in preparation). Briefly, ~3×10^{10} 3Y1-Crk cells semi-confluent in a total number of 3000 culture dishes (175 cm^2) were lysed in 1% Triton buffer (10 mM Tris−HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 μg/ml

aprotinin, 1 mM Na_3VO_4 and 100 μg/ml leupeptin). Rat p130 was affinity purified from the lysate initially by a cyanogen bromide (CNBr) Sepharose 4B (Pharmacia) coupled with an anti-*gag* monoclonal antibody (1A1), and secondly by that coupled with an anti-phosphotyrosine monoclonal antibody (4G10). Purified p130 was concentrated, electrophoresed and blotted onto a ProBlott membrane (Applied Biosystems). After being visualized by ponceau-S, an isolated 130 kDa band (~4 μg) was cut out and *in situ*-digested with *Achromobactor* protease I (lysylendopeptidase; WAKO). Six sequences, consisting of eight to 12 amino acids, were determined by amino acid sequence analysis of the fragments and were used to design degenerate primers for PCR. DNA fragments were amplified from 3Y1 cells by the reverse transcriptase (RT)-PCR method using all possible combinations of degenerate primers and were analyzed as described previously (Honda *et al.*, 1993). Finally, a 384 bp fragment (nucleotides 352–735 in cDNA) was selected as a screening probe because it contained four amino acid sequences determined by sequence analysis in the same coding frame. An unamplified λ-ZAP II (Stratagene) cDNA library constructed from mRNA of rat 3Y1 cells was screened and 20 positive clones out of 5×10^5 independent clones were obtained. The longest clone was analyzed by nucleotide sequencing on both strands by nested deletions using a 373A DNA sequencer (Applied Biosystems) and Sequenase (USB). Since alternative forms at the upstream region were noticed by restriction enzyme mapping, nucleotide sequences of 5′-terminal regions of each clone were also determined.

Northern blot analysis

A 6 week-old male rat was sacrificed for tissues. The extraction of total RNA was performed as described (Chomczynsky and Sacchi, 1987). Total RNAs were separated on a 1% agarose gel by electrophoresis and transferred to a Hybond N nylon filter (Amersham). Total p130 cDNA was labeled by a Multiprime DNA Labeling System (Amersham) and used as a probe. Hybridization was carried out at 42°C in a solution containing 50% formamide, 5× SSC, 5× Denhardt's solution, 0.5% SDS, denatured salmon sperm DNA (20 μg/ml) and a ^{32}P-labeled probe. Filter was washed twice in 2× SSC/0.1% SDS, once in 0.5× SSC/0.1% SDS and once in 0.1× SSC/0.1% SDS for 20 min at 50°C preceding exposure to an X-ray film at −80°C.

Production of antibody against bacterially expressed p130

As shown in Figure 3, three *Pvu*II-digested fragments of p130 cDNA (nucleotides 936–1402, 1403–2106 and 2107–2431) were cloned in-frame in the *Sma*I site of pGEX-2T, pGEX-1 and pGEX-3X vectors (Pharmacia), respectively, and expressed in *Escherichia coli* as fusion proteins with glutathione S-transferase (GST). After purification with a glutathione−Sepharose 4B column (Pharmacia), GST fusion proteins were used for the immunization of rabbits. Antisera raised against GST fusion proteins were called αCas1, 2 and 3, corresponding to the positions of the cDNA.

Immunoprecipitation and Western blotting

For protein analysis, cells were lysed in 1% Triton buffer [10 mM Tris−HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM Na_3VO_4]. SDS−PAGE was performed as described by Laemmli (1970) using 7.5% polyacrylamide gel, unless indicated. For Western blotting of total cell lysates, samples containing 100 μg protein per lane were applied to gels. For immunoprecipitations, 250 μl of cell lysates (4 μg protein/μl) per lane were mixed with 2.5 μl of αCas2, 5 μl of 1A1, a monoclonal antibody against viral *gag* (1 μg IgG/ml) or 1μl of 2-17 (a monoclonal antibody against Src; Microbiological Associates), and incubated for 1 h on ice. As the 1A1 belongs to the mouse IgG1 group, 1 μl of a rabbit polyclonal antibody against mouse IgG was also added. Samples were rotated with protein-A−Sepharose (Sigma) for 1 h at 4°C, then beads were washed five times with 50-fold 1% Triton buffer and boiled in Sample buffer (2% SDS, 0.1 M Tris−HCl, pH 6.8, 10% glycerol, 0.01% bromophenol blue and 0.1 M dithiothreitol). Western blotting was performed as described (Towbin *et al.*, 1979) using αCas2 (1:2500), 4G10 (5 μg IgG/ml), αHcrk antibody (1:2500) or MAb 327 (1:1000) as first antibodies and a ProtoBlot Western AP System (Promega) for second antibodies and staining. For the detection of v-Crk, [^{125}I]protein A (Amersham) was used at a concentration of 0.5 μCi/ml in place of second antibodies because the molecular weight of v-Crk is close to those of Ig heavy chains. For phosphatase treatment of proteins, immunoprecipitated proteins on Sepharose gel were washed twice with 1% Triton buffer and twice with 1× AP buffer (1 mM $ZnCl_2$, 1 mM $MgCl_2$ and 10 mM Tris−HCl, pH 8.3). Proteins were then treated in 70 μl of 1× AP buffer with 2 U calf intestine alkaline phosphatase (Boehringer Mannheim) or 4 U bacterial alkaline phosphatase (Takara) for 1 h at 37°C. After the reaction, 20 μl 10% SDS was added to each gel and samples were boiled and analyzed by Western blotting.

Immune complex kinase assay and partial protease digestion

For the immune complex kinase assay, cell lysates containing 500 μg of proteins were immunoprecipitated, washed three times using 1% Triton buffer and three times using Kinase buffer (50 mM Tris−HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$ and 10 mM $MnCl_2$), and the kinase reaction was performed in 30 μl of Kinase buffer with 5 μCi of [γ-^{32}P]ATP (Amersham) at room temperature for 15 min. V8 partial digestion was performed basically as described (Cleveland *et al.*, 1977). ^{32}P-labeled proteins were cut out from the dried gel, swollen in 0.1 M Tris−HCl (pH 6.8) and applied to 15% SDS−PAGE. Sample buffer, including 1 or 4 μg of *Staphylococcus aureus* V8 protease (WAKO), was overlaid and protein digestion was performed in the stacking gel for 30 min at room temperature.

Subcellular fractionation

About 1×10^7 cells were homogenized in 0.5 ml of hypotonic suspension buffer (10 mM sodium phosphate buffer, pH 7.0, 5 mM EDTA, 1 mM DTT, 1 mM PMSF and 1 mM Na_3VO_4) using Dounce homogenizer and separated by differential centrifugation. The nuclear fraction (P1) was first separated by the centrifugation at 1000 *g* and the supernatant was further separated into membrane (P100) and cytosolic (S100) fractions by ultracentrifugation at 100 000 *g*. One tenth (1×10^6 cells) of each fraction was applied to SDS−PAGE and analyzed.

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