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Peptide Modulators of Src Activity in G₁ Regulate Entry into S Phase and Proliferation of NIH 3T3 cells

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Abstract

Cascades of kinases and phosphatases are regulated by selective protein-protein interactions that are essential for signal transduction. Peptide modulators of these interactions have been used to dissect the function of individual components of the signaling cascade, without relying on either the over- or underexpression of proteins. Previously, we identified RACK1 as an endogenous substrate, binding partner and inhibitor of Src tyrosine kinases. Here we utilized cell-permeable peptides that selectively disrupt or enhance the interaction of RACK1 and Src to further examine the function of RACK1. Our results provide direct physiologic evidence that RACK1 regulates growth of NIH3T3 cells by suppressing the activity of Src and other cell cycle regulators in G₁, and delaying entry into S phase. They also demonstrate the potential for using peptide modulators of Src activity as a tool for regulating cell growth, and for designing new strategies for cancer therapy that target specific protein-protein interactions.

Keywords

Src; RACK1; PKC; tyrosine kinases; G₁/S transition; cell cycle regulation

RACK1 is the founding member of a family of intracellular receptors for activated C kinase (PKC) collectively called RACKs [reviewed in ¹]. RACKs determine the specificity and function of PKC isozymes by selectively anchoring activated isozymes to specific subcellular sites, and near to specific substrates that mediate distinct cellular functions. For example, εRACK anchors activated εPKC, which protects the heart from ischemic and reperfusion damage, whereas RACK1 anchors activated βIIPKC, which mediates phenylephrine-induced cardiac hypertrophy [reviewed in ²].

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Using the yeast two-hybrid assay, we identified RACK1 as a binding partner for the Src family of tyrosine kinases [3]. Following serum or platelet-derived growth factor (PDGF) stimulation or PKC activation, we observed that RACK1 co-localizes with Src at the plasma membrane and functions as a substrate and inhibitor of Src [3–6].

Peptide modulators of selective protein-protein interactions have proven invaluable in defining the function of individual components of signaling cascades, without relying on either the over- or underexpression of proteins [reviewed in 7]. For example, peptides that selectively activate or inhibit specific PKC isozymes have been used to identify the opposing roles of δ and ϵ PKC in cardiac ischemia and reperfusion [reviewed in 2] and the δ PKC inhibitor is now used in human clinical trials, in patients with acute myocardial infarction. Moreover, a selective activator peptide of ϵ PKC confers cardioprotection in vivo, in part by reducing the incidence of lethal arrhythmias during ischemic-reperfusion injury [8]. Thus, peptides that activate ϵ PKC may be useful treatment for patients with ischemic heart disease.

Here we used peptides that selectively disrupt or enhance the interaction of RACK1 with Src, via a β IIPKC-dependent mechanism, to define the role of RACK1 in regulating growth of cells. Our results provide direct physiologic evidence that RACK1 regulates growth of NIH 3T3 cells by suppressing the activity of Src and Src-mediated cell cycle regulators in G₁ and delaying entry of cells into S phase. Our results also demonstrate the potential for using peptide modulators of Src activity as a tool for dissecting the function of Src in cells, regulating cell growth and designing new strategies for cancer therapy that target specific protein-protein interactions.

Materials and methods

Cell Culture and Cell Proliferation Assays

NIH3T3 cells were maintained in Dulbecco's modified Eagle medium (Mediatech, Herndon, VA). Cells were supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), unless otherwise stated. Methods for peptide incubations were previously described [9]. For cell proliferation assays, cells were seeded in 96-well plates (5×10^3 cells/well). Peptides and 10% Alamar Blue were added and absorbance was measured hourly for 8 h at a wavelength of 562 and 595 nm using a Vmax kinetic microplate reader (Molecular Devices, Palo Alto, CA). Fresh peptide was added every 2 h. Reduction of alamarBlue was calculated from absorbance, according to the manufacturer's protocol (Biosource, Camarillo, CA).

Peptides

Peptides β C2-4 (SLNPEWNET, corresponding to amino acids 218–226 in β PKC; 10), β IIV5-3 (QEVRN, corresponding to amino acids 645–650 in β IIPKC; 11) and β agonist (pseudo- β RACK1 or ψ β RACK1; SVEIWD, derived from the C2 regions of β PKC; 12) were conjugated to carrier peptide derived from the TAT protein (TAT_{47–57}: YGRKKRRQRRR) for delivery into cells [13].

Immunoblot Analysis, Antibodies and In Vitro Protein-Kinase Assays

Immunoprecipitates or lysates of total cellular proteins were analyzed by protein immunoblotting (after SDS-PAGE) with the specified mouse monoclonal or rabbit polyclonal antibody, as described [3–6]. Primary antibodies included mouse monoclonal antibodies to Src (MAb 327; 14), RACK1, Stat3, phosphotyrosine (Py20), Kip1/p27 (Transduction Laboratories, Lexington, KY), c-Myc (9E10), and CDK2 (D-12), (Santa Cruz Biotechnology, Santa Cruz, CA). Other primary antibodies included rabbit polyclonal antisera to CDK4 (C-22), cyclin D1 (M-20), cyclin E (M-20), cyclin A (C-19), p16 (M-156), E2F1 (KH95) (Santa Cruz Biotechnology), phospho-Rb (Ser795 or Ser807/811) and p15 INK4B (Cell signaling,

Beverly, MA). Methods to assess the phosphorylating activity of Src, CDK4 and CDK2 by kinase assays in vitro have been described [3–6].

Flow Cytometric Analysis for DNA Content and Ki-67 Expression

For analysis of G₁/S progression, cells were synchronized in G₀, released into G₁ and analyzed for cellular DNA content as described [6]. For analysis of Ki-67 expression [15], 10⁶ asynchronous cells were fixed in 70% ethanol, collected by low speed centrifugation, resuspended in PBS containing fluorescein isothiocyanate (FITC)-conjugated anti-Ki67 or mouse IgG1, and incubated for 30 min at room temperature in the dark according to the manufacturer's protocol (BD Pharmingen) before staining with propidium iodide (PI; Sigma). Cells (10⁴) were analyzed by FACScan cell sorting (Becton Dickinson, San Jose, CA). Dot plots were prepared using FlowJo software (Tree Star, San Carlos, CA).

Results

RACK1 regulates Src-mediated signaling pathways that culminate in induction of Myc

Previously, we suggested that RACK1 regulates NIH 3T3 cell growth by modulating Src activity at the G₁ checkpoint [6]. This conclusion was based largely on indirect nonphysiologic evidence, namely, by enforced overexpression or depletion of RACK1 in cells. With the development of cell-permeable peptides that selectively disrupt or enhance the interaction of Src and RACK1 in a β IIPKC-dependent manner [9], we had the opportunity to test this hypothesis directly by using an approach that does not rely on either over- or underexpression of proteins. We first confirmed our previous finding that incubation of NIH 3T3 cells with either of two peptides that disrupt the RACK1- β IIPKC complex [antagonist β C2-4, which inhibits all the classical PKC isozymes (10) or β IIV5-3, which selectively inhibits β IIPKC and not any other member of the PKC family (11)] results in disruption of the Src-RACK1 complex and thus increases Src in vitro kinase activity (9, and Fig. 1, top two panels, compare lanes 2 and 3 with 1). In contrast, incubation of cells with a peptide that increases RACK1- β IIPKC binding (agonist β Ag; 12) increases RACK1-Src binding and decreases Src in vitro kinase activity (compare lane 4 with 1).

Myc and cyclin D1 are downstream effectors of Src and positive regulators of G₁/S progression. Stat3 is a Src substrate and a cytoplasmic transcription factor that is required for Rac1 induction of Myc [6, 16]. Following activation by Src phosphorylation, Stat3 dimerizes, translocates to the nucleus and binds promoter elements of responsive genes involved in cell proliferation including *myc* and cyclin D1 [17, 18]. Thus, we next examined the influence of the peptides on Src-mediated signaling that culminates in induction of Myc and cyclin D1 (Fig. 1, lower panels). We found that incubation of cells with either antagonist peptide resulted in increased tyrosine phosphorylation of Stat3 and induction of Myc and cyclin D1 (compare lanes 2 and 3 with 1). The agonist peptide had the opposite effect (compare lane 4 with 1). These results provide direct evidence that RACK1 regulates Src-mediated signaling pathways that culminate in induction of Myc.

RACK1 Regulates Proliferation of NIH 3T3 Cells by Suppressing Src Activity

To assess the influence of the peptides on proliferation of NIH 3T3 cells, we first measured the ability of metabolically-active cells to reduce alamarblue (Fig. 2A). We observed that cells incubated with either antagonist peptide, which activate Src by disrupting its interaction with RACK1 (hence Src-activating peptides), were more metabolically active than cells incubated with carrier peptide or no peptide. In contrast, cells incubated with agonist peptide, which inhibits Src by enhancing its interaction with RACK1 (hence Src-inhibiting peptide), were less metabolically-active than control cells.

In a complementary proliferation assay, we used bivariate-flow-cytometric analysis to assess cells for expression of the nuclear proliferation antigen Ki-67 (which is expressed during G₁, S, G₂ and M, but not G₀), and for phase of the cell cycle (Fig. 2B). Ki-67 expression was nearly twice as high in cells incubated with either of the Src-activating peptides (lower panels) than in cells incubated with carrier peptide (upper left panel). In contrast, Ki-67 expression was considerably lower in cells incubated with the Src-inhibiting peptide (upper right panel) than in control cells. Moreover, most of the Src-inhibited cells were in the G₀/G₁ phase of the cycle, whereas many more of the Src-activated cells were in S and G₂/M. These results provide direct evidence that RACK1, in part via its interaction with Src, regulates growth of NIH 3T3 cells.

RACK1 Works Through Src to Regulate G₁/S Progression

Results from the flow cytometry analyses indicated that enhanced binding of RACK1 to Src prolongs the G₀/G₁ phase of the cell cycle (Fig. 2B). This suggested that RACK1, via its interaction with Src, might regulate G₁/S progression. To test this directly, we synchronized NIH 3T3 cells in G₀ with serum withdrawal for 48 h, released them into G₁ with the addition of serum, added peptide and analyzed cellular DNA content by flow cytometry (Fig. 3). After treatment of serum-starved cells for 6 h with 10% serum, approximately three quarter of cells incubated with carrier peptide or no peptide were in G₁ (77 and 75%, respectively) and few had entered S phase (13 or 10%, respectively). In contrast, only half of the cells incubated with either of the Src-activating peptides, remained in G₁ and a third had moved into S phase. Cells incubated with the Src-inhibiting peptide were arrested in G₁ (90%). These results provide direct evidence that RACK1 works through Src to regulate G₁/S progression.

By Suppressing Src Activity in G₁, RACK1 Regulates Key Cell Cycle Proteins that Control G₁/S Transition

To determine the mechanism whereby RACK1 regulates G₁/S transition, we examined the influence of the peptides on the activities of Src and Src-mediated regulators of cell cycle progression at the G₁/S boundary. G₁/S transition is controlled by two key families of proteins: cyclin-dependent kinases (CDKs) and cyclins. Cyclins bind to, and activate CDKs [19, 20]. Another critical event in cell cycle progression at G₁/S is hyperphosphorylation and inactivation of the retinoblastoma protein (pRb), which releases regulatory proteins such as E2F that stimulate transcription of target genes that are required for S phase entry and DNA replication. v-Src, the transforming homolog of c-Src, directly influences cell [21, 22, reviewed in 23]. For example, in cycle proteins that regulate G₁/S transition fibroblasts, v-Src induces rapid transit through the G₁ checkpoint and entry into S phase by simultaneously suppressing the CDK inhibitor p27 and inducing cyclins D1, E and A.

Thus, we examined the influence of the peptides on these Src-mediated regulators of G₁/S progression (Fig. 4). Cells were synchronized in G₀ by serum withdrawal for 48 h and released into G₁ by the addition of serum for 6 h. Incubation of G₁ cells with peptides that disrupt the Src-RACK1 complex resulted in increased *in vitro* kinase activities of Src, CDK4 and CDK2, induction of cyclins D1, E and A, and enhanced binding of CDKs to their activating cyclin partners (CDK4 to cyclin D1, and CDK2 to cyclin E and A) (Fig. 4A and 4B, compare lanes 2 and 3 with 1). Incubation of G₁ cells with the peptide that enhances binding of RACK1 to Src had the opposite effect: inhibiting the kinase activities of Src, CDK4 and CDK2, the expression of cyclins and the binding of CDKs to their cyclin partners (compare lane 4 with 1).

We next assessed the influence of the peptides on CDK inhibitors (CKIs), which play a key role in the function of the G₁/S checkpoint by inhibiting G₁-phase cyclin-CDK complexes [reviewed in 24]. There are two families of CKIs, the INK4 inhibitors (p16, p15, p18 and p19) and the Cip/Kip inhibitors (p27, p21 and p57). The INK4 family inhibits CDK4 and 6

activity during G₁ phase specifically, whereas the Cip/Kip family can inhibit CDK activity during all phases of the cell cycle. Both families of CDKIs have important roles in the G₁/S checkpoint. For example, p16 mediates the p53-independent G₁ arrest in response to DNA damage through abrogation of cyclin-D-CDK4 and CDK6-mediated phosphorylation of pRb [25]. We found that the Src-activating peptides inhibited expression of CDKIs p16, p15 and p27 in G₁ (Fig. 4C, compare lanes 2 and 3 with 1). In contrast, the Src-inhibiting peptide induced expression of the CDKIs in G₁ (compare lane 4 and 1).

Finally, we examined the influence of the peptides on phosphorylation of pRb and release of E2F1 (Fig. 4D). We observed that Src-activating peptides induced the hyperphosphorylation of pRb at multiple serine residues (795, 807 and 811), and induced the release of E2F1 (lanes 2 and 3). In contrast, the Src-inhibiting peptide induced the hypophosphorylation of pRb and the sequestration of E2F1 (lane 4). Together, these results provide direct evidence that RACK1, by suppressing Src activity in G₁, regulates key cell cycle proteins that control G₁/S transition.

Discussion

As part of our ongoing investigation of the function of RACK1 in cells, we sought a means of modulating RACK1's influence on Src that did not rely on the non-physiologic approach of either over- or underexpressing proteins. Cell-permeable peptides that modulate specific protein-protein interactions in cells provide such a means. Previously, we used peptide modulators of RACK1's interaction with β IIPKC and thus Src to define RACK1's influence on Src-mediated Sam68 and p190RhoGAP signaling, Rho activity and actin cytoskeleton rearrangement [9]. Here, we used the peptides to define RACK1's influence on cell growth. Our results provide direct evidence that RACK1 regulates growth of NIH 3T3 cells (Fig 2), at least in part, by suppressing the activity of Src and Src-mediated cell cycle regulators in G₁, (Figs 1 and 4) and delaying entry of cells into S phase (Fig 3). These results confirm our previous indirect evidence obtained by over- or underexpressing RACK1 [6].

Peptide modulators of protein-protein interaction show great promise in treating patients with ischemic heart disease [8, 26, reviewed in 27]. We and others showed that the specific activity of Src is strikingly elevated in some human cancers [reviewed in 28]. Thus, with a long-term goal of exploring potential new strategies for treating cancer, we started by assessing the influence of the peptide modulators of Src activity on the growth of normal cells. We found that a peptide that enhances β IIPKC's and thereby Src's interaction with RACK1 [9], suppresses Src activity at the G₁ checkpoint (Fig 4A) and slows the growth of NIH 3T3 cells (Figs 2 and 3). Peptides that disrupt β IIPKC-RACK1-Src complex [9] have the opposite effect. These results reveal a novel mechanism of cell cycle control in G₁ of cells that works via an endogenous inhibitor of Src kinases. They also demonstrate the potential for using peptide modulators of β IIPKC and thereby Src activity for designing new strategies of cancer therapy that target specific protein-protein interactions. Although a peptide with intracellular activity has not yet been approved for use in clinical medicine, if its effect is validated in animal studies, it could justify the investment in developing a drug that affects the same protein-protein interaction. Moreover, if the current clinical trials with another PKC peptide regulator are positive, stabilizing the peptides and devising means for sustained delivery may remove at least some of the barriers to the use of the peptides themselves as a drug, because they appear to be very safe and selective even when delivered chronically at high dose via an Alzet pump [29].

Previously, we demonstrated that RACK1, β IIPKC and Src function in a trimolecular complex where β IIPKC's association with RACK1 is required for Src phosphorylation of, and binding to RACK1, and for RACK1 inhibition of Src [9]. Thus, specificity of peptide effect on biological activities is determined by the selectivity of the peptides for the β IIPKC-RACK1 interaction. We have carried out numerous studies (in cell-based assays and *in vivo*) that support

the selectivity of the peptides for the isozyme they were designed for: activation of only the right isozyme (e.g. β IIPKC) is affected; other isozymes present in the cells and affected by the same stimulus (e.g. β IPKC), are unaffected by the peptide modulators [reviewed in ¹]. Furthermore, the peptide regulators for one isozyme have biological activities that are different and sometimes even opposing to peptide regulators of another isozyme [reviewed in ²]. Together, these results provide strong evidence that the peptides selectively and specifically affect biological activities involving the β IIPKC-RACK1 interaction.

Because the peptides modulate RACK1's interaction with β IIPKC, and only indirectly the interaction of RACK1 with Src, their striking effects on G₁/S progression (Fig. 3) and cell growth (Fig 2) could be due to their effect on the RACK1- β IIPKC interaction. However, our results clearly show that the peptides modulate biological activities that are known to be Src-mediated. For example, the inhibitory effect of the peptide that enhances the β IIPKC-RACK1-Src interaction on: a) tyrosine phosphorylation of the Src-specific substrates Sam68 and Stat3 (Fig 1), b) induction of the Src effectors Myc and cyclin D1 (Fig 1), and c) on Src-mediated p190RhoGAP activity and actin-cytoskeleton rearrangement [⁹], is strikingly similar to the effect observed with: a) the Src-specific tyrosine kinase inhibitors SU6656, PP2 or PP1 [reviewed in ^{23, 30}], b) dominant-negative mutants of Src [^{23, 30}], or c) overexpression of the endogenous Src inhibitor RACK1 [⁶]. Conversely, the activating effects of the peptides that disrupt the β IIPKC-RACK1-Src interaction are similar to those observed with ν -Src [^{23, 30, 31}], constitutively-active Y527F c-Src [^{23, 30}] or with depletion of RACK1 [⁶]. Thus, perturbing RACK1's interaction with Src is a major mechanism by which the peptides function to regulate cell growth.

Recently, caveolae were shown to be important for Src function downstream of the PDGF receptor [³²]. Moreover, Src activity appears to be regulated in caveolae [³³], perhaps by RACK1 [³⁴]. Thus, caveolae may serve an important function by recruiting signaling kinases and their regulators and substrates to growth factor receptor complexes.

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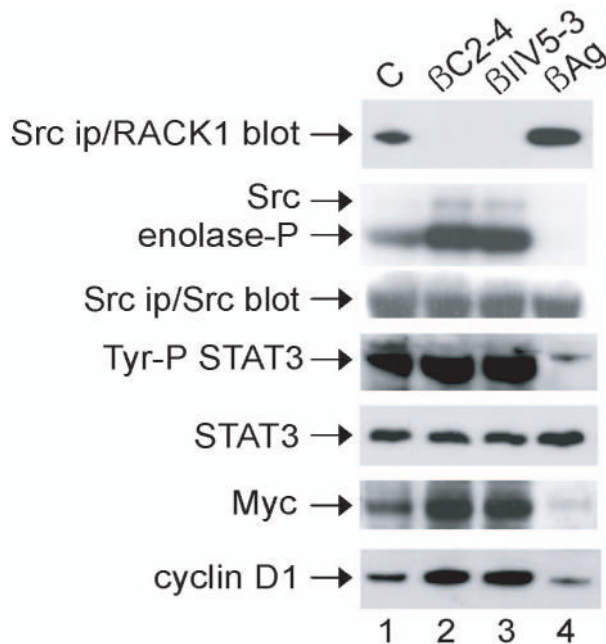


Fig. 1.

Effect of peptides on Src kinase activity and Src-mediated signaling in NIH 3T3 cells. Cells were incubated with peptide (1 μ M) as described in the Methods. Proteins were immunoprecipitated with anti-Src (MAb 327) (panels 1–3) or phosphotyrosine (Py20) (panel 4) from lysate containing 500 μ g of cellular protein and subjected to immunoblot analysis with anti-RACK1 (panel 1), Src (panel 3) or Stat3 (panel 4), or were assayed for in vitro protein-kinase activity by incubating with [γ - 32 P]ATP together with $MnCl_2$ and enolase for 10 min at 30°C (panel 2). Otherwise, lysates containing 20 μ g of total cellular protein were subjected to immunoblot analysis with anti-Stat3, Myc, or cyclin D1, as indicated (lower panels). C, Tat carrier peptide (control); β C2-4, β PKC antagonist peptide conjugated to Tat (an inhibitor of all the classical PKC isozymes); β IIV5-3, β IIPKC antagonist-selective peptide conjugated to Tat; β Ag, pseudo-RACK1 agonist peptide conjugated to Tat ($\psi\beta$ RACK1). Data are representative of 2–3 independent experiments.

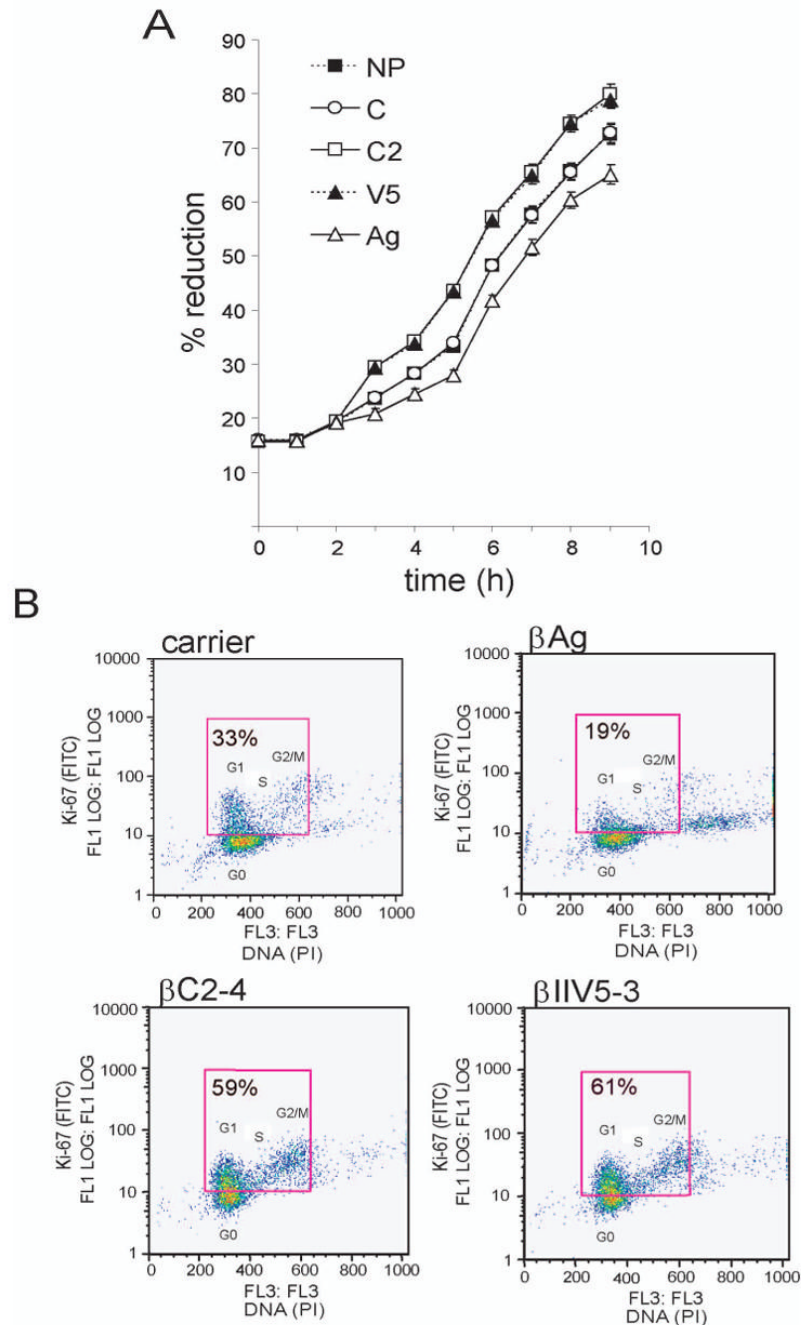


Fig. 2. Effect of peptides on proliferation of NIH 3T3 cells. (A) Effect of peptides on the metabolic activity of cells. Cells (5×10^3) were incubated with peptide ($1 \mu\text{M}$) and alamarBlue (10%), and absorbance was measured hourly at a wavelength of 562 and 595 nm. Fresh peptide was added every 2h for 8 h. Reduction of alamarBlue was calculated from absorbance, according to the manufacturer's protocol. x-axis, hours incubated; y-axis, relative reduction of alamarBlue. --■--, no peptide (NP); -○-, carrier peptide (C); -□-, β C2-4 peptide (C2); --▲--, β IIV5-3 peptide (V5); Δ , β agonist peptide (Ag). Data represent mean values \pm standard errors from 5 wells and are representative of 2 independent experiments. (B) Effect of peptides on Ki-67 expression and cell cycle distribution. Cells (2×10^6) were seeded onto 10-cm plates.

24 h later, cells were incubated with peptide for 30 min prior to fixation, stained with FITC-conjugated Ki-67 mAb and propidium iodide (PI), and analyzed by bivariate flow cytometry. x-axis, DNA content; y-axis, Ki-67-positive cell number. Ki-67-positive cells (in the G₁, S or G₂/M phase of the cycle) are located within the box. Ki-67-negative cells (in G₀) are located below the box. %, percentage of cells that are Ki-67-positive. Data are representative of 2 independent experiments.

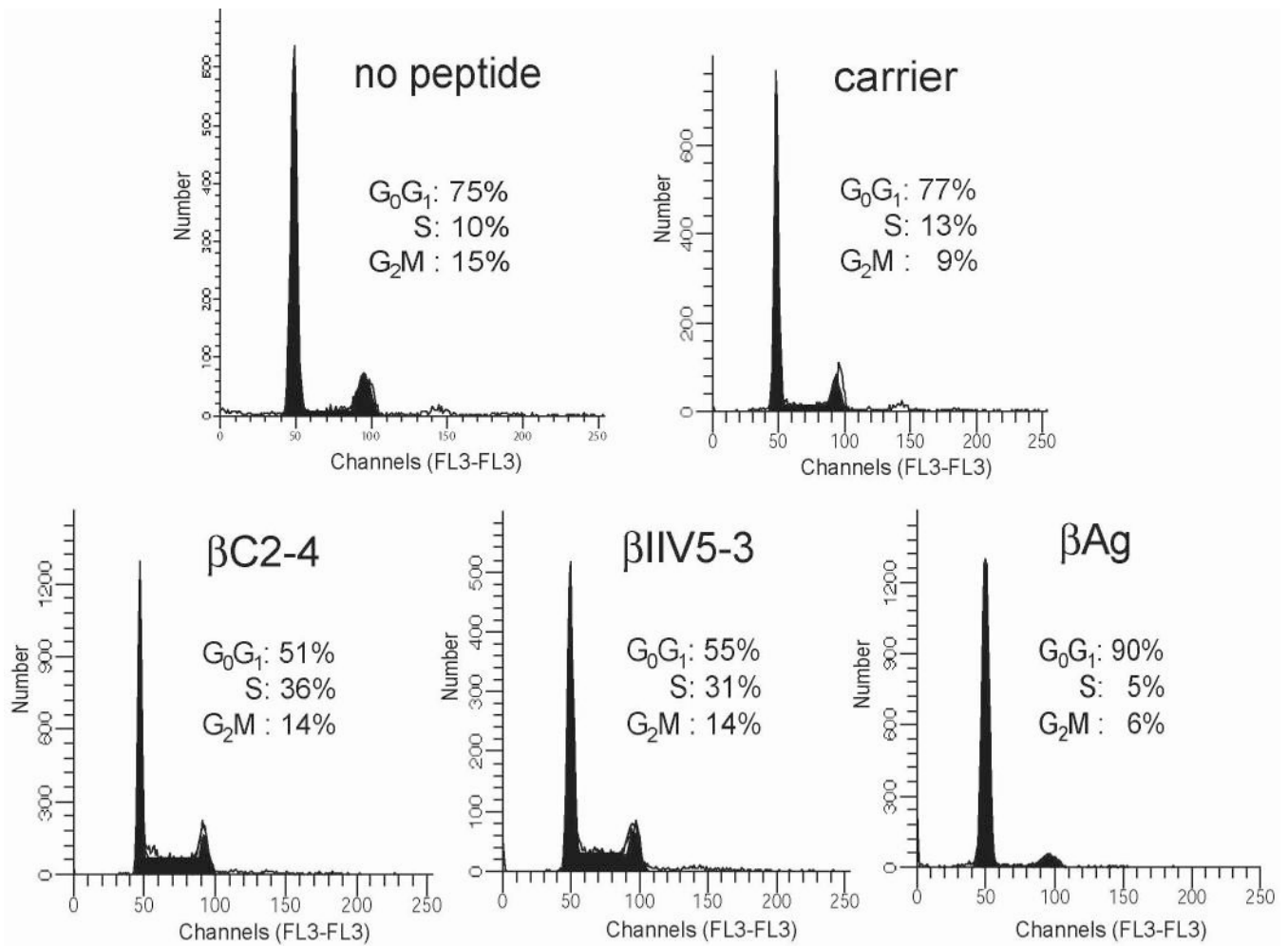
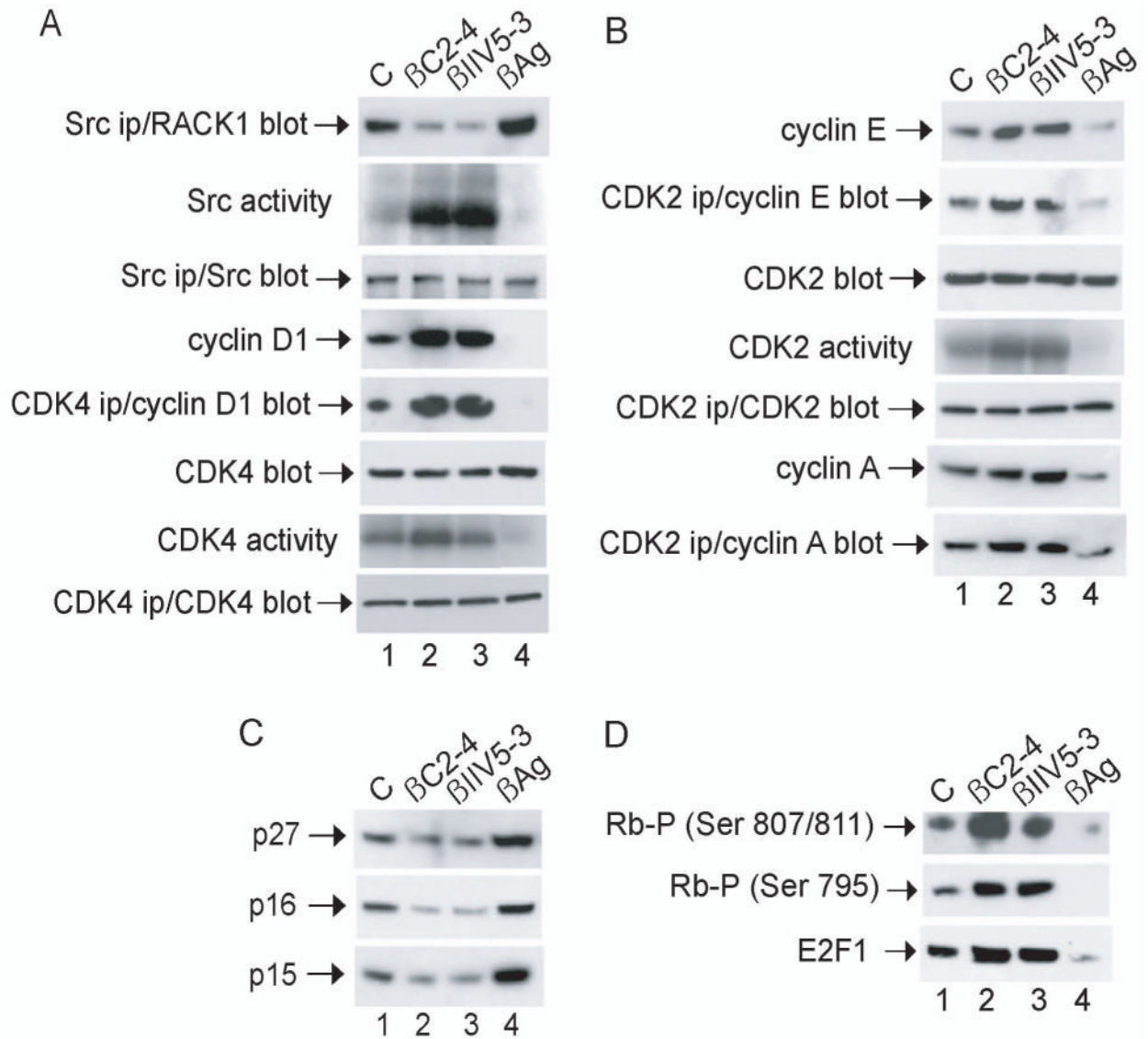


Fig. 3.

Effect of peptides on G₁/S progression in NIH 3T3 cells. Cells were synchronized in G₀ by serum starvation for 48 h and released into G₁ with addition of 10% FBS for 6 h. Cells were incubated with peptide and analyzed for cellular DNA content by flow cytometry. Data are representative of 3 independent experiments.

**Fig. 4.**

Effect of peptides on key regulators of G₁/S transition in NIH 3T3 cells. Cells were synchronized in G₀, released into G₁ and incubated with peptide as described in the legend to Fig 3. (A and B) Proteins were precipitated with anti-Src, CDK4 or CDK2 from lysates containing 500 μ g of cellular protein and analyzed for in vitro protein-kinase activity by incubating with [γ -³²P]ATP together with MnCl₂ and enolase (Src ips), MgCl₂ and Rb peptide (CDK4 ips) or MgCl₂ and histone H1 (CDK2 ips) for 10 min at 30°C, or subjected to immunoblot analysis with anti-Src, CDK4 or CDK2, respectively. Otherwise, proteins from lysates containing 20 μ g of cellular protein were subjected to immunoblot analysis with anti-CDK4, CDK2 or cyclin D1, E or A, as indicated. (C and D) Proteins from lysates containing 20 μ g of cellular protein were subjected to immunoblot analysis with anti-p16, p15, p27, phospho-Rb (Ser 795 or Ser 807/811) or E2F1, as indicated. Data are representative of 2–3 independent experiments.