Binding Specificity for RACK1 Resides in the V5 Region of βII Protein Kinase C*

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Identification of selective anchoring proteins responsible for specialized localization of specific signaling proteins has led to the identification of new inhibitors of signal transduction, inhibitors of anchoring protein-ligand interactions. RACK1, the first receptor for activated C kinase identified in our lab, is a selective anchoring protein for βII protein kinase C (βIIIPKC). We previously found that at least part of the RACK1-binding site resides in the C2 domain of βIIIPKC (Ron, D., Luo, J., and Mochly-Rosen, D. (1995) J. Biol. Chem. 270, 24180–24187). Here we show that the V5 domain also contains part of the RACK1-binding site in βIIIPKC. In neonatal rat cardiac myocytes, the βIIV5-3 peptide (amino acids 645–650 in βIIIPKC) selectively inhibited phorbol 12-myristate 13-acetate (PMA)-induced translocation of βIIIPKC and not βIIPKC. In addition, the βIIV5-3 peptide inhibited cardiac myocyte hypertrophy in PMA-treated cells. Interestingly, βIIV5-3 (646–651 in βIIIPKC), a selective translocation inhibitor of βIIPKC, also inhibited PMA-induced cardiac myocyte hypertrophy, demonstrating that both βI- and βIIIPKC are essential for this cardiac function. Therefore, the βIIV5 domain contains part of the RACK1-binding site in βIIIPKC; a peptide corresponding to this site is a selective inhibitor of βIIPKC and, hence, enables the identification of βIIPKC-selective functions.

The localization of signaling enzymes within cells is highly specific and often regulated by selective anchoring proteins (1, 2). A number of these proteins have recently been identified; some anchor and coordinate multiple enzymes in the same signaling cascade (3, 4) and can bind to their selective proteins or enzymes depending on their activation state (2). Selective localization of signaling enzymes in cells results in tethering them in the proper subcellular location for their function. Disruption of the selective protein-protein interactions between the signaling enzymes and their anchoring proteins alters the specialized localization of the signaling enzymes and thus disrupts their function (5).

We have studied the mechanism leading to selective localization of protein kinase C (PKC).1 PKC isoforms are a family of serine/threonine, phospholipid-dependent protein kinases (6) that translocate after stimulation to select subcellular sites where they bind their corresponding selective anchoring proteins, RACKs (receptor for activated C kinase) (2). RACKs bind only the active form of their respective PKCs. Our lab has identified some of the RACK-binding sites on β, ε, and δPKC and demonstrated that RACK binding is essential for both proper localization and function of these PKC isoforms (5). So far we have cloned and characterized two RACKs and demonstrated that RACK1 is selective for βIIIPKC (7, 8), whereas RACK2, also known as β’COP (a coatomer protein involved in vesicle transport) is selective for εPKC (9).

βI- and βIIIPKC, members of the classical family of PKCs, are differentially spliced products of the same gene and therefore differ only in their C-terminal variable domain, the V5 domain (10, 11). Immunofluorescence studies demonstrate that βI- and βIIIPKC are differentially localized in both their inactive and active states (12, 13) in a number of cell types. We have demonstrated that the second conserved domain in βPKC, the C2 domain, contains part of the RACK-binding site in βPKC (7, 14). The βC2 domain binds RACK1 in vitro and peptides derived from this domain inhibit this interaction (7). In addition, the C2-derived peptides block translocation of both βI- and βIIIPKC in cells (7). However, the C2 domains of βI- and βIIIPKC are identical and, therefore, cannot account for the differential localization of βI- and βIIIPKC. We hypothesize that the distinct sequences in the βIV5 and βIIV5 domains should confer the RACK-binding specificity and differential localization of these isoforms. A selective RACK for βIIIPKC has yet to be identified. We therefore used RACK1, the selective anchoring protein for βIIPKC, to test our hypothesis.

Using short peptides derived from the βIIV5 domain, we show here that unique sequences within βIIPKC contain part of the RACK1-binding site. In addition, we show that one of the V5-derived peptides functions as an isozyme-selective translocation inhibitor of βIIPKC in neonatal rat cardiac myocytes. This peptide was used to demonstrate that βIIPKC mediates phorbol 12-myristate 13-acetate (PMA)-induced cardiac myocyte hypertrophy. Of interest, a peptide-selective translocation inhibitor of βIIPKC identified in this study also inhibited PMA-induced myocyte hypertrophy, suggesting that the two isoforms are required for this function.

EXPERIMENTAL PROCEDURES

Materials—PMA was purchased from LC Laboratories. Diacylglycerol and phosphatidylserine were purchased from Avanti. Luminol, p-coumaric acid, IGEPAL detergent, saponin, and Triton X-100 were purchased from Sigma. Polyclonal anti-βIIPKC and anti-βIIIPKC antibodies were purchased from Santa Cruz Biotechnologies, and R&D Antibodies. Amylose resin was purchased from New England Biolabs. Monoclonal anti-βPKC antibodies were purchased from Seikagaku, Inc.

12-myristate 13-acetate; MBP, maltose-binding protein; GST, glutathione-S-transferase.

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1 The abbreviations used are: PKC, protein kinase C; PMA, phorbol ester; MBP, maltose-binding protein; GST, glutathione-S-transferase; COP, a coatomer protein involved in vesicle transport; IIPKC, to test our hypothesis.

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and Transduction Laboratories. Anti-RACK1 antibodies were purchased from Transduction Laboratories. The secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit and HRP-conjugated goat anti-mouse antibodies, glutathione Sepharose 4B beads, and $^3$Hphenylalanine were purchased from Amersham Pharmacia Biotech. Recombinant βIIPKC and βIIPKC were purchased from PanVera.

**Protein Expression and Purification**—Recombinant βIIPKC was purchased from PanVera, or a clone (received from Alexander Newton) was expressed in Trichoplusia ni insect cells and partially purified to homogeneity as previously described (15). Fragments of the βPKC C2 (amino acids 175–289), βV ($^3$Hphenylalanine acids 622–671), and βIV3 (amino acids 622–673) domains were expressed in Escherichia coli as fusion proteins with maltose-binding protein (MBP) using the pMAL-c2 expression vector (New England Biolabs). Bacterial pellets were resuspended in amylose column buffer (10 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol) and lysed by sonication. Fusion proteins were purified by immobilization on an amylose resin column. The column was washed with 8 column volumes of column buffer, and bound protein was eluted in 10 mM maltose in column buffer. Protein concentration was determined by Bradford assay.

**RACK1** was expressed in bacteria as a fusion protein with glutathione-S-transferase (GST) using the pGEX-4T-1 GST gene fusion expression vector (Amersham Pharmacia Biotech). Bacterial pellets were resuspended in STE buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA) and lysed by sonication. Triton X-100 was added to the lysate to a final concentration of 1%, and the mixture was incubated on ice for 30 min with occasional mixing. The lysate was then centrifuged at 12,000 × g at 15 min, and the supernatant was stored in 50% glycerol at −20 °C.

**Binding Assay**—Recombinant GST or GST-RACK1 bacterial lysate was incubated with 25 μl (~20 μl packed bead volume) of pre-equilibrated glutathione-Sepharose 4B beads, and the beads were washed with overlay wash (200 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% polyethylene glycol, 12 mM β-mercaptoethanol). For competition experiments the complex was preincubated with the PKC-derived peptides (10 μM) or protein fragments (1–2.5 μM) in overlay buffer (200 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% polyethylene glycol, 12 mM β-mercaptoethanol, 0.1% bovine serum albumin, 20 μg/ml leupeptin, 20 μg/ml soybean trypsin inhibitor, 20 μg/ml aprotinin, and 10 μg/ml phenylmethylsulfonyl fluoride) for 15 min before the addition of PKC and PKC activators. The complex was then incubated with or without PKC or PKC fragments in the presence or absence of PKC activators (2 μg/ml diacetylgluceral and 60 μg/ml phosphatidylserine and 1 mM CaCl$_2$) for 15 min at room temperature in overlay buffer. The beads were washed three times with overlay wash containing 1% IGEPAL detergent, and the third wash was used to transfer the beads to fresh Eppendorf tubes to help decrease background. Bound proteins were eluted in sample buffer, followed by SDS-polyacrylamide gel electrophoresis and Western analysis. βPKC-selective antibodies were used to detect βPKC holoenzyme and C2 and V5 fragments.

**Isolation and Permeabilization of Neonatal Rat Cardiac Myocytes**—Cardiac myocytes were isolated from 4 litter of 1-day-old Sprague-Dawley rats (each with 8–10 animals) as described previously (16). Cells were plated in 12-well plates for $^3$Hphenylalanine incorporation experiments or laminin-coated 8-well chamber slides for immunofluorescence studies. Cells were maintained in media M199 (Life Technologies, Inc.) with 10% serum after plating. For $^3$Hphenylalanine incorporation experiments, cells were transferred to serum-free media on day 3, and experiments were initiated on day 4. Immunofluorescence experiments were performed on days after a change to serum-free media on day 4. All peptides were delivered into cells via transient transfection and isolated by Western blot analysis with an antibody against the regulatory domain of PKC or PKC activators. Bound protein was eluted followed by SDS-polyacrylamide gel electrophoresis and Western blot analysis. βIIPKC was detected with a βIIPKC isozyme-selective antibody. A representative Western blot is shown. B, quantitative results from four independent experiments. C, selective binding of βIIPKC to RACK1 in vitro. Recombinant βIIPKC or βIIPKC was incubated with immobilized GST (lane 3) or GST-RACK1 (lane 4) in the presence of PKC activators. Bound protein was detected by Western analysis using an antibody against the regulatory domain of βPKC that recognizes β- and γ-PKC equally well (lanes 3 and 4). Lanes 1 and 2 contain 5 and 10 ng of β- and βIIPKC used as standards. A representative of three Western blots is shown.

**RESULTS**

**Activated βIIPKC Selectively Binds to RACK1 in Vitro**—Immunofluorescence studies demonstrated that RACK1 co-localizes with active βIIPKC in CHO cells (8) and in cardiac myocytes (7), and endogenous RACK1 and βIIPKC co-immuno-

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FIG. 1. **Dose-dependent binding of βIIPKC to RACK1 in vitro.** A, RACK1, bacterially expressed as a fusion protein with GST (GST-RACK1), was immobilized on glutathione-Sepharose 4B beads. The complex was incubated with purified βIIPKC in the presence (Active PKC) or absence (Inactive PKC) of PKC activators. Bound protein was eluted followed by SDS-polyacrylamide gel electrophoresis and Western blot analysis. βIIPKC was detected with a βIIPKC isozyme-selective antibody. A representative Western blot is shown. B, quantitative results from four independent experiments. C, selective binding of βIIPKC to RACK1 in vitro. Recombinant βIIPKC or βIIPKC was incubated with immobilized GST (lane 3) or GST-RACK1 (lane 4) in the presence of PKC activators in vitro. Bound protein was detected by Western analysis using an antibody against the regulatory domain of βPKC that recognizes β- and γ-PKC equally well (lanes 3 and 4). Lanes 1 and 2 contain 5 and 10 ng of β- and βIIPKC used as standards. A representative of three Western blots is shown.

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precipitate. Here, we determined the binding affinity of βIIPKC for RACK1 in *vitro*. RACK1, expressed as a fusion protein with GST, was immobilized on glutathione-conjugated Sepharose beads and incubated with increasing concentrations of recombinant βIIPKC in the presence of activators. Binding of activated βIIPKC to RACK1 is both dose-dependent and saturable with a half-maximal binding of 3 ± 2 nM (n = 4; Fig. 1, A and B). Furthermore, at all concentrations, binding of βIIPKC to RACK1 is at least 2-fold greater than that of βC2 (n = 3; Fig. 1C).

We previously demonstrated that the C2 domain of βPKC (βC2), identical in the two βPKC isozymes, contains part of the RACK1-binding site in βPKC (7, 14). Here, *in vitro* binding studies show that the half-maximal binding of the βC2 domain-MBP fusion protein to RACK1 is ~500 nM (Fig. 2A). Since RACK1 is selective for βIIPKC (7, 8) and its subcellular localization overlaps that of βIIPKC and not βPKC (7, 13), we reasoned that the unique sequences in the βIIV5 domain should confer specificity of βIIPKC for RACK1. This suggests that the distinct βIIV5 domain may bind RACK1 directly. We incubated the recombinant βIIV5 domain, expressed as an MBP fusion protein, with immobilized GST-RACK1 in *vitro*, as described under “Experimental Procedures” and found that βIIV5 binding to RACK1 was dose-dependent and saturable with a half-maximal binding of ~400 nM (Fig. 2B). This affinity is similar to that of the βC2 domain, having a half-maximal binding of ~500 nM (Fig. 2A). Therefore, the βIIV5 domain also contains part of the RACK1-binding site in βIIPKC.

 βC2 and βV5 Domains Compete with βIIPKC for RACK1 Binding—If part of the RACK1-binding site in βIIPKC is within the βIIV5 domain, then βIIV5 should inhibit βIIPKC binding to RACK1. Furthermore, if both C2 and V5 domains are required for βIIPKC binding to RACK1, an additive inhibitory effect may be seen when combining the βIIV5 domain along with the βC2 domain. To this end, recombinant βIIV5, βIIV5, and/or βC2 fusion proteins were preincubated with immobilized GST-RACK1, and then full-length βIIPKC was added in the presence of PKC activators. We found that the βIIV5 domain competed with βIIPKC for RACK1 binding. Furthermore, an additive effect in competition for βIIPKC binding to RACK1 was observed in the presence of both βC2 and the βIIV5 domains (Fig. 3). However, similar results were obtained when using the βIIV5 domain, both alone or in combination with the βC2 domain.

![FIG. 2. Both βC2 and βIIV5 bind to RACK1. Binding of βC2 (A) and βIIV5 (B) domains expressed and purified as fusion proteins with MBP to GST-RACK1 was determined as in Fig. 1. Bound protein was detected by Western analysis using anti-βC2 (A)- or anti-βIIV5 (B)-selective antibodies. A representative of two Western blot assays is shown.](http://www.jbc.org/)

![FIG. 3. βC2 and βV5 domains inhibit βIIPKC binding to RACK1. A, βC2 (2.5 μM), βIIV5 (1 μM), and βIIV5 (1 μM) domains were preincubated with GST-RACK1 before the addition of βIIPKC (5 nM) and PKC activators. Bound βIIPKC was detected by Western analysis using anti-βIIPKC antibodies. A representative Western blot is shown. B, quantitative results from three experiments are presented as the percent of βIIPKC bound (n = 3; *, S.E., p < 0.04; **, p < 0.001).](http://www.jbc.org/)

βIIV5 fragments (Fig. 3) was competitive with RACK1 binding. Furthermore, the C2 domain-precipitate was competitively displaced by RACK1 at a dose of 50 nM (Fig. 2A, C). Therefore, we expect that the βIIV5 domain should confer βIIPKC RACK1-binding specificity. We synthesized short peptides corresponding to the least similar sequences in the βV5 domains, since we expected that they would contain the selective RACK1-binding sequences in βIIPKC. Three peptides corresponding to unique regions were selected from each of the βI and βII V5 domains: βIIV5-1 (AGFSYNTPFEVIVN), βIIV5-2 (ARDRKRDS), βIIV5-3 (KLFIMN), and βIIV5-4 (ACGRNAE), and βIIV5-3 (QEVRN) (Fig. 4A) (note that

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2 M. M. Rodriguez and D. Mochly-Rosen, unpublished observation.
The V5-derived peptides were tested for their ability to inhibit βIIIPKC-RACK1 interactions in vitro (Fig. 4). We previously showed that disruption of intracellular PKC-RACK interactions inhibits PKC translocation and proper subcellular localization, therefore preventing PKC substrate phosphorylation and blocking downstream function (2, 5, 18).

We show here that the combination of βC2- and βIIV5-derived peptides are necessary to inhibit βIIIPKC-RACK1 interactions in vitro (Fig. 4). However, we previously demonstrated that each of the peptides derived from the RACK1-binding site in the βC2 domain (βC2-1, βC2-2, and βC2-4) is sufficient alone to inhibit translocation of both βI- and βIIIPKC in neonatal rat cardiac myocytes (7). To determine if individual peptides derived from the βIIV5 domain can also act alone as translocation inhibitors, we first determined their effects on βIIV5 fragment binding to RACK1 in vitro. We found that each of the βIIV5-1, βIIV5-2, and βIIV5-3 peptides (10 μM) alone inhibits binding of MBP-βIIIPKC fusion protein (500 nM) to RACK1 in vitro by 37, 30, and 34%, respectively (average of 3 measurements), suggesting that each peptide contains part of the RACK1-binding site in the βIIV5 domain and, therefore, may be an effective inhibitor of translocation in cells. Consequently, we set out to test the effects of the individual V5-derived peptides on βIIIPKC translocation in cells. Since none of the peptides stood out as the overall strongest inhibitor of βIIV5-RACK1 binding in vitro, we chose to start our in-cell studies with the βIIV5-3 peptide. We propose that the βIIV5-3 peptide, containing part of the RACK1-selective binding sequence in βIIIPKC, may function as isoyme-selective translocation inhibitor by binding to the isozyme-selective RACK and inhibiting translocation of βIIIPKC isozyme. The βIIV5-3 peptide was used both as a control for βIIV5-3 and a possible selective inhibitor of βIIIPKC translocation.

Neonatal rat cardiac myocytes were permeabilized in the presence or absence of 10 μM peptide and then treated with or without 10 nM PMA for 5 min (we showed previously that ~10% of the applied peptide is internalized by the cells (16)). Cells were then fixed and stained with isozyme-selective anti-βI- and anti-βIIIPKC-selective antibodies followed by a fluorescein isothiocyanate-conjugated secondary antibody, as described under “Experimental Procedures.” In norepinephrine- or PMA-treated neonatal rat cardiac myocytes, active βIIIPKC localizes in the nucleus of the cell, whereas active βIIIPKC translocates to both the perinuclear region and the cell periphery upon activation (13). In these experiments, cells were scored for the number of cells staining for active βIIIPKC or βIIIPKC at their respective sites in the cell, as previously described (7). PMA-treated cells permeabilized in the absence of peptide showed 89% of the cells staining βIIIPKC at the perinuclear region and cell periphery (89% of the cells staining βIIIPKC at their respective sites in the cell). However, when used in combination with the mixture of all three βC2-derived peptides, the three βIIV5-derived peptides together (βIIV5-1, βIIV5-2, and βIIV5-3, 10 μM of each) nearly abolished βIIIPKC binding to RACK1, whereas the combined βC2- and βIIV5-derived peptides had no effect (Fig. 4, B and C, n = 3). Therefore, the βIIV5-derived peptides provide the selectivity necessary to inhibit βIIIPKC-RACK1 binding in the presence of the βC2-derived peptides, suggesting the βIIV5 unique sequences, not present in βIIV5, correspond to the RACK1-selective binding sites within βIIIPKC.

Isoyme-selective Translocation Inhibitors of βI and βII PKC in Cardiac Myocytes—In cells it is thought that RACKs act as isoyme-selective anchoring proteins, functioning to tether specific PKC isozymes nearby their respective substrates (2, 5). We previously showed that disruption of intracellular PKC-RACK interactions inhibits PKC translocation and proper subcellular localization, therefore preventing PKC substrate phosphorylation and blocking downstream function (2, 5, 18).

δIV5-1 and δIIV5-1 comprise part of the antigenic peptides used for production of many of the commercially available anti-βI and anti-βII PKC isozyme-specific antibodies.

The V5-derived peptides were tested for their ability to inhibit βIIIPKC binding to RACK1. Additionally, three peptides derived from the C2 domain (βC2-1, βC2-2, and βC2-4), previously shown to inhibit binding of a C2 domain-containing fragment to RACK-1 (7), were also used. Similar to the mixture of all three βC2-derived peptides (βC2-1, βC2-2, and βC2-4, 10 μM each), the mixtures of the three βI- or βIIIPKC-derived peptides did not inhibit βIIIPKC holoenzyme-RACK1 interactions in vitro (Fig. 4, B and C). However, when used in combination with the mixture of all three βC2-derived peptides, the three βIIV5-derived peptides together (βIIV5-1, βIIV5-2, and βIIV5-3, 10 μM of each) nearly abolished βIIIPKC binding to RACK1, whereas the combined βC2- and βIIV5-derived peptides had no effect (Fig. 4, B and C, n = 3). Therefore, the βIIV5-derived peptides provide the selectivity necessary to inhibit βIIIPKC-RACK1 binding in the presence of the βC2-derived peptides, suggesting the βIIV5 unique sequences, not present in βIIV5, correspond to the RACK1-selective binding sites within βIIIPKC.

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playing βIPKC staining in the nucleus of the cells versus the no peptide control (89% ± 3; Fig. 5). Conversely, the βIV5-3 peptide selectively inhibited βIPKC translocation, with βIV5-3-treated cells showing 17% ± 3 of βIPKC nuclear staining (Fig. 5).

Taken together, these data demonstrate that when introduced alone into cardiac myocytes, the βIV5-3 and βIV5-5 peptides are effective isozyme-selective translocation inhibitors of βI- and βIIPKC, respectively; they prevent translocation of the corresponding isozyme with no effect on the other isozyme. Because greater than 70% inhibition of βIIPKC translocation was obtained using βIV5-3 alone, the other βIV5-derived peptides were not studied further.

βIIPKC is Essential for PMA-induced Cardiac Myocyte Hypertrophy—Previous studies demonstrate that isozyme-selective PKC translocation inhibitors can selectively inhibit isozyme function (7, 18, 19). A peptide derived from the RACK2 binding sequence in εPKC (εV1-2), which inhibits εPKC translocation, prevents phorbol ester-induced negative chronotropy in neonatal rat cardiac myocytes (19) as well as protection from ischemic insult (20). Additionally, peptides derived from the βC2 domain (βC2-1, βC2-2, and βC2-4) inhibit a βC2-mediated cellular function in Xenopus oocytes (7) and regulation of L-type calcium channels (21).

We proposed that the βIV5-derived translocation inhibitor, βIV5-3, determines βIIPKC-selective functions in primary cultures of neonatal rat cardiac myocytes. βIIPKC has recently been reported to mediate cardiac hypertrophy (22), a normal process occurring during development as well as a compensatory mechanism after an insult to the adult heart (23). We therefore set out to determine if phorbol ester-induced hypertrophy requires βIIPKC using the translocation inhibitor, βIV5-3. Cardiac hypertrophy involves an overall increase in the size of the cardiac myocyte, due primarily to increased expression of specific contractile proteins. Simpson et al. (24) demonstrate that this increased protein expression directly correlates with the size of the cell, enabling the use of total protein synthesis as a quantitative measure for increased cell size. In our experiments, hypertrophy of isolated primary neonatal rat cardiac myocytes was induced using limiting amounts of PMA (10 nM), and hypertrophy after 48 h was measured via 14C-labeled phenylalanine incorporation into protein as a measure of protein synthesis (16). Cardiac myocytes were transiently permeabilized in the presence or absence of peptide, incubated with or without 10 nM PMA in media containing 14C-labeled phenylalanine for 48 h, and total protein was harvested as described under “Experimental Procedures.” Fluorescence-activated cell sorter analysis, used to compare vehicle and PMA-treated cells by size, confirmed that 14C-labeled phenylalanine incorporation correlates with increased cardiac myocyte cell size.3 Cells treated with 10 nM PMA in the absence of peptide displayed an increase in cell size (Fig. 6B) as well as a 2-fold increase in protein synthesis (Fig. 6A). Pretreatment with the βIV5-3 or βIV5-5 translocation inhibitor peptides resulted in a 77% ± 20 and 82% ± 14 decrease in PMA-induced protein synthesis, respectively (Fig. 6A). Additionally, the βIV5-3 peptide inhibited basal hypertrophy by 26% ± 3, with a 21% ± 8 decrease observed with the βIV5-3 peptide (Fig. 6A). Fig. 6B shows phase contrast pictures of cells after pretreatment with peptides and after PMA-induced hypertrophy. Unlike the control cells, the cells treated with either the βIV5-3 peptide or the βIV5-5 peptide did not increase in size in response to PMA but, instead, were much closer in size to the non-PMA-treated control cells (Fig. 6B). Therefore, the βIV5-3 and βIV5-5 peptides inhibited the PMA-induced increase in cell size. Additionally, cells treated with a C2-derived peptide, βC2-4, previously shown to inhibit βPKC-mediated cellular functions (7, 21), did not increase in size in response to 10 nM PMA, whereas a

3 R. R. Begley and D. Mochly-Rosen, unpublished observation.
control peptide (with non-relevant sequence) had no effect on
PMA-induced cell size (data not shown). Taken together, these
data demonstrate that both βτ and βIIPKC are essential for
PMA-induced cardiac myocyte hypertrophy.

**DISCUSSION**

This study demonstrates that a domain other than the C2
domain of βIIPKC (7) is required for binding of βIIPKC to
RACK1. Using fragments and peptides derived from the V5
domain of the β and βII isoforms of PKC, we have shown that
the βIV5 domain bound RACK1 directly (Fig. 2B) and par-
tially inhibited βIIPKC binding to RACK1 (Fig. 3). Further-
more, a combination of the βC2 domain and the βIIV5 or the
βIV5 domain nearly abolished βIIPKC binding to RACK1 (Fig.
3). The RACK1 selectivity was mapped to the unique sequences
in the V5 domain. When combined with βC2-derived pepti-
des, known to contain part of the RACK1-binding site in
βIIPKC, peptides derived from the unique sequences in the
βIV5 domain selectively competed with βIIPKC binding to
RACK1 in vitro (Fig. 4). Importantly, when introduced into
cardiac myocytes, the βIV5- and βIV5-derived peptides βIV5-3
and βIV5-5, selectively inhibited translocation of their respec-
tive PKC isoforms (Fig. 5). Therefore, βIV5-3 and βIV5-5
function as isozyme-selective translocation inhibitors of βIIPKC
and of βIIPKC, respectively. We conclude that the βIIV5 do-
main contains part of the RACK1-binding site in βIIPKC. Our
data on the role of the V5 domain of the βPKCs in binding to
their respective RACKs suggest that the V5 domains of other
PKC isoforms may be important for binding to their RACKs.
Supporting this conclusion is the observation that the V5 dom-
ains of α-, β-, βII, γ, δ, and εPKC are greater than 88%
conserved between species. Therefore, a combination of the C2
and V5 domains may be useful as bait for identifying unknown
RACKs for other classical and novel PKCs.

The importance of the V5 domain of βPKC in enzyme local-
ization and function has been previously demonstrated. Over-
expression of chimeras of the regulatory and catalytic domains
of α- and βIIPKC in K562 erythroleukemia cells demonstrated
that the C-terminal 13 amino acids of βIIPKC were sufficient
to confer proper localization and function (lamin B phosphoryla-
tion) of an α/βI chimera (25, 26). Fields and co-workers (26, 27)
attributed the selectivity of this region to a direct interaction
with the nuclear membrane lipid, phosphatidylglycerol. Fur-
thermore, these authors reported that βIIPKC-phosphatidylgly-
cerol interaction was inhibited with a peptide derived from the
C-terminal 13 amino acids (26). This peptide corresponds to
our peptide labeled βIIV5-1, which we found to partially inhibit
βIV5 binding to RACK1 in vitro (see “Results”). Therefore, the
βIV5-1 sequence may be important for both βIIPKC binding to
lipids as well as for RACK1 binding. Using a number of βIIPKC
C-terminal deletion mutants lacking parts of the βIV5 do-
main, Cooper and co-workers (28) further demonstrate the
requirement of an intact βIV5 domain for proper enzyme
function. The work described here adds to the above studies
and shows that a short peptide, βIV5-3, corresponding to part of
the RACK1-binding site in the V5 domain of βIIPKC is
sufficient to inhibit βIIPKC function in cells.

Our data cannot exclude the possibility that at least part of
the effects exerted by the V5-derived peptides is due to their
interaction with the C2 domain. Newton and co-workers (29,
30) show that the βV5 region regulates calcium binding, a
function of the C2 domain (31). This suggests a direct interac-
tion between the βC2 and βV5 domains. The data presented
here demonstrating direct interactions of both the βIV5 and
βC2 fragments with RACK1 further support this hypothesis. It
is interesting to note that the half-maximal binding of the βC2
and βIV5 domains for RACK1 (400 and 500 nM, respectively,
Fig. 2, A and B) is 2 orders of magnitude higher than that of
βIIPKC holoenzyme RACK1 binding (3 nM ± 2, Fig. 1A). This
may reflect cooperativity between the βC2 and βIV5 domains
upon βIIPKC holoenzyme binding to RACK1.

Although only a combination of the βC2 and βIV5 fragments
or peptides was sufficient to completely inhibit βIIPKC binding
to PKC in vitro (Figs. 3 and 4), we found previously that each of
the βC2-derived peptides alone, βC2-1, βC2-2, and βC2-4,
inhibit translocation of both βτ- and βIIPKC in neonatal rat
cardiac myocytes (7). Furthermore, we show here that a single
peptide derived from the βV5 or βIV5 domains (βIV5-3 and
βIV5-5, respectively) is each sufficient to inhibit translocation
of its corresponding isozyme in an isozyme-selective manner
(Fig. 5). Why is a peptide from either the βC2 or the βIV5
domain sufficient to inhibit translocation of βIIPKC in cells
when in vitro interference with both βC2- and βIV5-RACK1
interactions is required to inhibit βIIPKC-RACK1 binding?
This may be due to the local relative concentrations of βIIPKC
and RACK1 in cells. Using known amounts of recombinant
proteins as standard, we estimate the intracellular concentra-
tion of βIIPKC to be ~1 nM and that of RACK1 to be ~10 nM.
Moreover, the local concentration of each may vary from one
cell compartment to the next. The intracellular concentration
of the peptide is estimated to be ~1 μM (10% of the extracellu-
lar concentration (16)), ~2 orders of magnitude above that of
RACK1. Therefore, a single peptide containing part of the
RACK1-binding site in βIIPKC may be sufficient to selectively
bind RACK1 and inhibit βIIPKC from binding in cells. Fur-
thermore, additional intracellular components not present in
an in vitro assay, such as other binding proteins and modula-
tors of βIIPKC and RACK1, may affect βIIPKC-RACK1 inter-
actions in cells. In this case, a perturbation of the βIIPK-
RACK1 interaction induced by the selective translocation
inhibitor peptide may be sufficient to prevent the translocation
of βIIPKC to its RACK in cells and, hence, prevent its cellular
function.

We also show here that complete inhibition of βIIPKC bind-
ing to PKC in vitro (Figs. 3 and 4) occurs only when the βC2
and βIV5 fragments (500 nM each) or peptides (10 μM each) are
added together; the concentration of inhibitors used in vitro
here is in great excess of that of the βIIPKC holoenzyme (5 nM).
Such a difference in relative affinities of signaling enzymes
to their anchoring proteins and a peptide inhibitor derived from
one of them for the same binding protein was previously re-
ported for several other inhibitors of protein-protein interac-
tions. For example, a concentration of 25 μM or more of a
peptide derived from the cAMP-dependent protein kinase (pro-
tein kinase A)-anchoring protein, AKAP 79, is necessary to
inhibit in vitro binding of the regulatory RII subunit of protein
kinase A to AKAP 79 (32). Yet the half-maximal binding of the
protein kinase A RII subunit to AKAP79 is obtained at a
concentration of ~1 nM (33). Therefore, to inhibit this protein-
protein interaction, a 25,000-fold excess of the inhibitory pep-
tide over the holoenzyme was required. Additionally, Iyengar
and co-workers used a 2000-fold excess of a peptide (100 μM
versus 50 nM) to inhibit G protein βγ subunit (Gβγ) binding to
adenyl cyclase in vitro (34) and ~1000-fold more peptide to
stimulate phospholipase Cβ2 to a similar extent to that seen
with full-length Gβγ (35). Therefore, when examined in in vitro
studies, it is common to see a relatively low affinity of inhibi-
tory peptides relative to the affinity of the proteins from which
they are derived.

Active βIIPKC, and not active βIIPKC, localizes to sites in
cardiac myocytes where RACK1 is located (7). These data sug-
gest that RACK1 may bind to βIIPKC, and not βIIPKC, in cells.
However, although the in vitro binding of βIIPKC to RACK1 is
greater than that of pIPKC, some binding of pIPKC to RACK1 is observed (Fig. 1C). Why does pIPKC bind to RACK1 in vitro? The lack of absolute selectivity of interaction between a kinase and its anchoring protein in vitro is a common phenomenon. For example, Baltimore and co-workers (36), investigating the differential binding specificity of the SH3 domains of Src, NCK, Grb2, and Abl to the Abl SH3 binding protein, 3BP2, found little specificity in vitro. Binding affinities were not determined in that study; however, binding of each SH3 domain to the 3BP2 SH3 binding domain appeared to be within an order of magnitude of the others when examined by an in vitro binding assay (36). Yet subsequent studies show exquisite specificity of the SH3 domains in cells. Work from the laboratories of Bar-Sagi et al. (37) demonstrates that the SH3 domains of phospholipase Cy and Grb2 determine the differential localization of the two proteins in cells (37). This specificity indicates the high selectivity of protein-protein interactions in cells in contrast to the two proteins in cells (37). This specificity indicates the high levels were elevated in hearts from a pressure overload-in

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