# Tyrosine Phosphorylation of β-Dystroglycan at Its WW Domain Binding Motif, PPxY, Recruits SH2 Domain Containing Proteins†

Federica Sotgia,‡,§ Hyangkyu Lee,‡ Mark T. Bedford,|| Tamara Petrucci,⊥ Marius Sudol,# and Michael P. Lisanti*,‡

*Department of Molecular Pharmacology and The Albert Einstein Cancer Center, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461, Servizio Malattie Neuro-Muscolari, Università di Genova, Istituto Gaslini, Largo Gaslini 5, 16147 Genova, Italy, Department of Carcinogenesis, University of Texas M. D. Anderson Cancer Center, Smithville, Texas 78957, Laboratorio di Biologia Cellulare, Istituto Superiore di Sanita', Viale Regina Elena 299, Roma 00161, Italy, and Department of Medicine, Mount Sinai School of Medicine, One Gustave Place, New York, New York 10029*

*Received June 15, 2001; Revised Manuscript Received August 22, 2001*

ABSTRACT: β-Dystroglycan is a ubiquitously expressed integral membrane protein that undergoes tyrosine phosphorylation in an adhesion-dependent manner. However, it remains unknown whether tyrosine-phosphorylated β-dystroglycan interacts with SH2 domain containing proteins. Here, we show that the tyrosine phosphorylation of β-dystroglycan is constitutively elevated in v-Src transformed cells. We next reconstituted this phosphorylation event in vivo by transiently coexpressing wild-type c-Src with a fusion protein containing full-length β-dystroglycan. Our results demonstrate that Src-induced tyrosine phosphorylation of β-dystroglycan is strictly dependent on the presence of a PPxY motif at its extreme C-terminus. In the nonphosphorylated state, this PPxY motif is normally recognized as a ligand by the WW domain; phosphorylation at this site blocks the binding of certain WW domain containing proteins. Using a GST fusion protein carrying the cytoplasmic tail of β-dystroglycan, we identified five SH2 domain containing proteins that interact with β-dystroglycan in a phosphorylation-dependent manner, including c-Src, Fyn, Csk, NCK, and SHC. We localized this binding activity to the PPxY motif by employing a panel of β-dystroglycan-derived phosphopeptides. In addition, tyrosine phosphorylation of β-dystroglycan in vivo resulted in the coimmunoprecipitation of the same SH2 domain containing proteins, and this binding event required the β-dystroglycan C-terminal PPxY motif. We discuss the possibility that tyrosine phosphorylation of the PPxY motif within β-dystroglycan may act as a regulatory switch to inhibit the binding of certain WW domain containing proteins, while recruiting SH2 domain containing proteins.

Dystrophin is the protein product of the Duchenne muscular dystrophy (DMD) gene (*1*, *2*) and is tightly associated with the sarcolemmal membrane (*3*). Dystrophin forms a complex with a series of specific dystrophin-associated glycoproteins, termed DAGs. One major component of this complex is dystroglycan (*4*). Interestingly, the expression of dystroglycan itself is ubiquitous and is not simply restricted to muscle cells.

Dystroglycan provides a continuous link between laminin-2 in the extracellular matrix and dystrophin that is attached to the intracellular cytoskeleton (*5*). Dystroglycan begins as a precursor protein that is proteolytically cleaved into two interacting subunits, α- and β-dystroglycan (*6*). α-Dystroglycan is a heavily glycosylated extrinsic membrane protein that interacts directly with laminin-2; in contrast, β-dystroglycan is an integral membrane glycoprotein that can bind both to dystrophin and to the ubiquitous dystrophin homologue, namely, utrophin. The dystrophin-anchoring site on β-dystroglycan is localized to the extreme C-terminus of β-dystroglycan at amino acids 880−895 (*7*, *8*).

Recent studies have shown that α-dystroglycan can function as an agrin receptor, suggesting that it may play a role in neuromuscular synapse formation (*9*, *10*). In addition, several lines of evidence suggest that β-dystroglycan is part of a membrane-anchored signal transduction complex that interacts with the SH3 domain of Grb-2. Grb-2 is an adaptor protein that helps to initiate the Ras-MAP kinase signal transduction cascade and is involved in controlling cytoskeletal organization (*11*). Disruption of the dystrophin−glycoprotein complex underlies the molecular pathogenesis of a variety of forms of muscular dystrophy. This suggests that this extracellular matrix−cytoskeletal linkage is critical for maintaining the structural integrity of the sarcolemma (*12*).

Dystrophin and utrophin are known to bind β-dystroglycan through 15 residues at the C-terminus of β-dystroglycan (*7*,

† This work was supported by grants from the NIH, the Muscular Dystrophy Association (MDA), the Susan G. Komen Breast Cancer Foundation, and the American Heart Association (AHA), as well as by a Hirschl/Weil-Caulier Career Scientist Award (all to M.P.L.). F.S. was a recipient of a fellowship from Telethon-Italia. M.S. is supported by grants from the NIH and the Muscular Dystrophy Association (MDA).

* Corresponding author. Tel: (718) 430-8828. Fax: (718) 430-8830. E-mail: lisanti@aecom.yu.edu.

‡ Albert Einstein College of Medicine.

§ Università di Genova.

|| University of Texas M. D. Anderson Cancer Center.

⊥ Istituto Superiore di Sanita'.

# Mount Sinai School of Medicine.

10.1021/bi011247r CCC: $20.00 
Published on Web 11/08/2001

*8*, *13*, *14*). Interestingly, the dystrophin−β-dystroglycan and utrophin−β-dystroglycan interactions occur primarily through the WW domain of dystrophin (or utrophin) (*14*−*17*). The WW domain is a small domain of 38−40 semiconserved amino acids that is widely distributed among various structural, regulatory, and signaling proteins (*18*, *19*). The WW domain is named after two highly conserved tryptophan (W) residues spaced ∼20−22 amino acids apart. Various WW domains have been implicated in mediating protein−protein interactions by binding to peptide sequences containing Pro-rich motifs, such as PPxY (*20*, *21*).

More recent studies have shown that β-dystroglycan undergoes tyrosine phosphorylation in an adhesion-dependent manner (*13*). Mapping studies tentatively localized this phosphorylation event to the tyrosine residue within its C-terminal PPxY motif. In accordance with this idea, tyrosine phosphorylation of the PPxY motif blocks the interaction of β-dystroglycan with the WW domains of dystrophin and utrophin (*13*, *17*). Taken together, these results predict that tyrosine phosphorylation of the PPxY motif within β-dystroglycan could act as a regulatory switch to inhibit the binding of WW domain containing proteins, while promoting the recruitment of SH2 domain containing proteins. However, experimental evidence to support the latter part of this hypothesis is lacking (*22*).

Here, we show that the tyrosine phosphorylation of β-dystroglycan is constitutively elevated in v-Src transformed cells. We reconstituted this phosphorylation event in vivo by transiently coexpressing wild-type c-Src with a fusion protein containing full-length β-dystroglycan. Our results demonstrate that Src-induced tyrosine phosphorylation of β-dystroglycan is strictly dependent on the presence of a PPxY motif at its extreme C-terminus. Using a GST fusion protein carrying the cytoplasmic tail of β-dystroglycan, we identified five SH2 domain containing proteins that interact with β-dystroglycan in a phosphorylation-dependent manner, including c-Src, Fyn, Csk, NCK, and SHC. Finally, tyrosine phosphorylation of β-dystroglycan in vivo resulted in the coimmunoprecipitation of the same SH2 domain containing proteins, and this binding event required the β-dystroglycan C-terminal PPxY motif.

## MATERIALS AND METHODS

*Materials.* Antibodies and their sources were as follows: anti-caveolin-1 IgG [mouse mAb cl 2297 (*23*) was the generous gift of Dr. Roberto Campos-Gonzalez, BD Transduction Labs, Lexington, KY], anti-β-dystroglycan IgG (mouse mAb, Novocastra), anti-placental alkaline phosphatase IgG (rabbit pAb, Zymed Laboratories Inc.), anti-phosphotyrosine IgG (rabbit pAb, BD Transduction Labs), and anti-phosphotyrosine IgG (mouse mAb PY20, BD Transduction Labs). Antibodies directed against SH2 domain containing proteins were purchased from BD Transduction Labs, with the exception of antibodies to c-Abl, Grb7, and c-Met, which were purchased from Santa Cruz Biotech, Inc. A mAb directed against c-Src was purchased from Upstate Biotechnology, Inc. The cDNA encoding human c-Src WT in the pUSEamp CMV-based vector was purchased from Upstate Biotechnology, Inc. NIH 3T3 cells expressing v-Src were provided by Drs. Shalloway and Dehn (Cornell University, Ithaca, NY) (*24*). A variety of other reagents were purchased commercially: cell culture reagents were from Gibco-BRL; glutathione−Sepharose 4B was from Pharmacia Biotech. Custom synthesis of biotinylated tyrosine-phosphorylated peptides was performed by Genemed Synthesis, Inc. (Irvine, CA).

*Construction of cDNAs.* The construction of the alkaline phosphatase-tagged β-dystroglycan (AP-βDG) was as previously described (*15*). Briefly, AP-βDG is a fusion protein carrying the full-length β-dystroglycan molecule (including its transmembrane and cytoplasmic domain) fused to the ectodomain of alkaline phosphatase (AP).

*Cell Culture and Transfection.* NIH 3T3 cells were grown in DME supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% donor bovine calf serum (*25*, *26*). Cos-7 cells were grown in DME supplemented with glutamine, antibiotics (penicillin and streptomycin), and 10% fetal calf serum (*27*). Constructs were transiently transfected into Cos-7 cells alone or in combination with c-Src using the Effectene transfection reagent (Qiagen), as per the manufacturer's instructions, and analyzed 48 h post-transfection.

*Immunoblotting.* Samples were separated by SDS−PAGE under reducing conditions and transferred to nitrocellulose membranes (Schleicher and Schuell). The protein bands were visualized with Ponceau S (Sigma). Membranes were blocked with 5% low-fat dried milk in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20). Blots were then incubated at room temperature for 1 h with primary antibody and then incubated with a secondary antibody conjugated with horseradish peroxidase (BD Transduction Labs). Bound IgG's were detected using a chemiluminescent substrate (Pierce).

*Construction and Purification of GST Fusion Proteins.* The construction of the GST−β-DG fusion protein (corresponding to amino acids 787−895) was as described previously (*7*, *15*). This fusion protein was expressed into two different *Escherichia coli* strains [either BL21 (DE3) for nonphosphorylated GST−β-DG or TKB1 for tyrosine-phosphorylated GST−βDG]. The TKB1 strain is a derivative of BL21 (DE3) that harbors a plasmid-encoded IPTG-inducible tyrosine kinase gene [the Elk receptor tyrosine kinase domain; Stratagene, Inc. (*28*)]. After induction of expression through addition of 5 mM isopropyl β-D-galactoside (IPTG, Sigma), GST fusion proteins were affinity purified on glutathione−agarose beads, using the detergent sarcosyl for initial solubilization (*29*).

*Detection of SH2 Domain Proteins That Bind Tyrosine-Phosphorylated β-Dystroglycan.* Purified nonphosphorylated and tyrosine-phosphorylated GST−βDG fusion proteins were immobilized on glutathione−agarose beads and incubated with lysates from normal NIH 3T3 cells. These lysates were generated with IP buffer containing a battery of phosphatase and protease inhibitors [10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 60 mM octyl glucoside, 50 mM NaF, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, pepstatin A (1 μg/mL), and 1 tablet of complete protease inhibitor cocktail (Boehinger Mannheim)]. After rotating overnight at 4 °C, the beads were washed three times with TNET buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100), separated by SDS−PAGE, and transferred to nitrocellulose membranes. Bound proteins were visualized by immunoblotting with a panel of

Table 1: Molecules Tested for Phosphotyrosine-Specific Binding to GST−β-DG-C2

| protein | expected molecular mass (kDa) | protein | expected molecular mass (kDa) |
|---|---|---|---|
| SH2 catalytic | | SH2 adaptors | |
| c-Abl | 120 | Crk | 40 |
| BMX | 80 | Grb7 | 56 |
| Btk | 77 | NCK | 47 |
| Csk | 50 | PI3-kinase | 85 |
| FAK | 125 | PTP1C/SHP1 | 68 |
| Fyn | 59 | SHC | 66/52 |
| Jak1 | 130 | SHcC | 69/55 |
| Jak2 | 130 | tensin | 215 |
| Lck | 56 | Vav | 95 |
| Lyn | 56 | | |
| Ntk | 56 | other | |
| PLC-γ | 148 | caveolin-1 | 22−24 |
| Src | 60 | Met | 140 |
| ZAP70 kinase | 70 | | |

antibodies directed against 24 known SH2 domain containing proteins (see Table 1).

*βDG Peptide "Pull Down" Assay.* NIH 3T3 cells were lysed in 1 mL of IP buffer containing phosphatase and protease inhibitors. After preclearing, biotinylated βDG-derived phosphopeptides (either βDG-Y1/Y2, βDG-pY1/Y2, or βDG-Y1/pY2) were added prebound to streptavidin−agarose beads. After incubation for 4 h at 4 °C, the beads were washed four times with PBS, and the samples were separated by 10% SDS−PAGE and transferred to nitrocellulose membranes. Blots were probed with antibodies to c-Src, Fyn, Csk, NCK, SHC, and caveolin-1. To generate streptavidin beads containing prebound biotinylated peptides, the beads (50 μL) were incubated overnight with a 1 mL solution containing ∼50 μg/mL of peptide dissolved in TNET buffer.

*Coimmunoprecipitation Assay.* Coimmunoprecipitation studies were performed essentially as we previously described, with minor modifications (*30*). Briefly, Cos-7 cells were cotransfected with the cDNAs encoding AP-βDG and c-Src. Forty-eight hours post-transfection, the cells were lysed in IP buffer containing phosphatase and protease inhibitors (detailed above) and subjected to immunoprecipitation with protein A−Sepharose CL-4B (Amersham Pharmacia Biotech). Briefly, lysates were first precleared by addition of 50 μL of a 1:1 slurry of protein A−Sepharose in TNET buffer (defined above). After 45 min of preclearing at 4 °C, samples were centrifuged for 1 min at 10000*g*, and the supernatants were transferred to fresh tubes. Then, 50 μL of protein A−Sepharose was added together with rabbit anti-alkaline phosphatase IgG (Zymed, Inc.) After incubation for 4 h at 4 °C, the immunoprecipitates were washed three times with TNET buffer, and the samples were separated by 10% SDS−PAGE and transferred to nitrocellulose membranes. Blots were then probed with mAbs directed against phosphotyrosine (PY20), c-Src, Fyn, Csk, NCK, SHC, or caveolin-1. Similar experiments were carried out, comparing the coimmunoprecipitation activity of WT and ΔPPxY forms of AP-βDG.

## RESULTS

*Tyrosine Phosphorylation of β-Dystroglycan Is Constitutively Elevated in v-Src Transformed Cells.* Recent studies have shown that β-dystroglycan undergoes tyrosine phosphorylation in an adhesion-dependent manner (*13*). In addition, a more recent report indicated that β-dystroglycan phosphorylation is inhibited by herbimycin A, which could suggest the involvement of the Src family of tyrosine kinases (*17*). Therefore, we became interested in the phosphorylation state of β-dystroglycan in v-Src transformed cells.

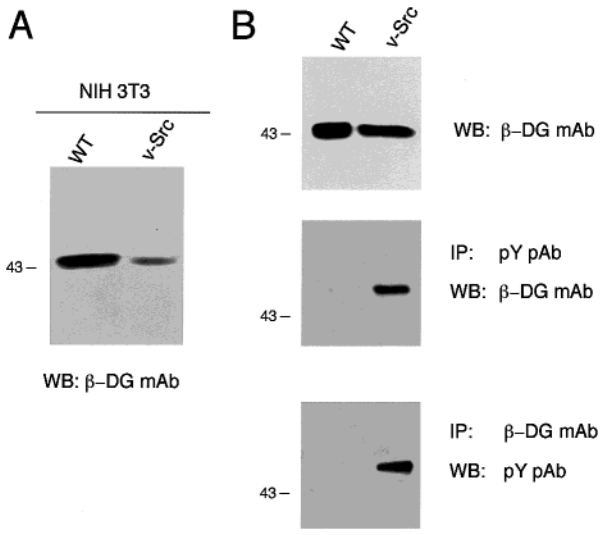


FIGURE 1: Tyrosine phosphorylation of β-dystroglycan is elevated in v-Src transformed NIH 3T3 cells. (A) Expression of endogenous β-dystroglycan in normal and v-Src transformed NIH 3T3 cells. Lysates were prepared from a given cell type and subjected to SDS−PAGE, followed by Western blotting with anti-β-dystroglycan IgG. Note that β-dystroglycan levels are decreased by ∼3−4-fold in v-Src transformed NIH 3T3 cells, as compared with normal NIH 3T3 cells. Each lane contains an equivalent amount of total protein. (B) Tyrosine phosphorylation of endogenous β-dystroglycan in normal and v-Src transformed NIH 3T3 cells. Cell lysates from wild-type and v-Src transformed NIH 3T3 cells were prepared and normalized so that they contained equivalent amounts of β-dystroglycan (upper panel). These lysates were then subjected to immunoprecipitaton with anti-β-dystroglycan IgG and blotting with anti-phosphotyrosine IgG or visa versa (middle and lower panels). Note that indeed the tyrosine phosphorylation of β-dystroglycan is dramatically elevated in v-Src transformed NIH 3T3 cells. Also, a characteristic upward mobility shift was observed for tyrosine-phosphorylated β-dystroglycan.

First, we examined the expression of endogenous β-dystroglycan in normal and v-Src transformed NIH 3T3 cells by Western blot analysis. Figure 1A shows that β-dystroglycan levels are decreased by ∼3−4-fold in v-Src transformed NIH 3T3 cells, as compared with normal NIH 3T3 cells. As tyrosine phosphorylation of a given substrate can induce its degradation, one possibility is that the tyrosine phosphorylation of β-dystroglycan is elevated in v-Src transformed cells.

To test this hypothesis directly, cell lysates from wild-type and v-Src transformed NIH 3T3 cells were prepared and normalized so that they contained equivalent amounts of β-dystroglycan (Figure 1B, upper panel). These lysates were then subjected to immunoprecipitaton with anti-β-dystroglycan IgG and blotting with anti-phosphotyrosine IgG or visa versa. Figure 1B (middle and lower panels) shows that indeed the tyrosine phosphorylation of β-dystroglycan is dramatically elevated in v-Src transformed NIH 3T3 cells. A characteristic upward mobility shift was observed for tyrosine-phosphorylated β-dystroglycan; a similar upward mobility shift has been reported previously for the tyrosine

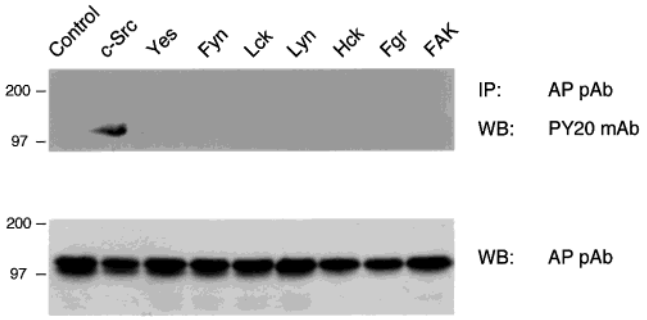


FIGURE 2: c-Src preferentially phosphorylates a fusion protein containing full-length $\beta$-dystroglycan. Cos-7 cells were cotransfected with c-Src and an alkaline phosphatase-tagged form of $\beta$-dystroglycan (AP-$\beta$DG). The tyrosine phosphorylation of AP-$\beta$DG was monitored by immunoprecipitation with anti-AP IgG and Western blotting with anti-phosphotyrosine IgG. Note that coexpression of AP-$\beta$DG with c-Src successfully reconstitutes this phosphorylation event. In contrast, when AP-$\beta$DG was coexpressed with other known tyrosine kinases (c-Yes, Fyn, Lck, Lyn, Hck, Fgr, and FAK), little or no phosphorylation was observed. The expression of AP-$\beta$DG is shown as a control for equal loading.

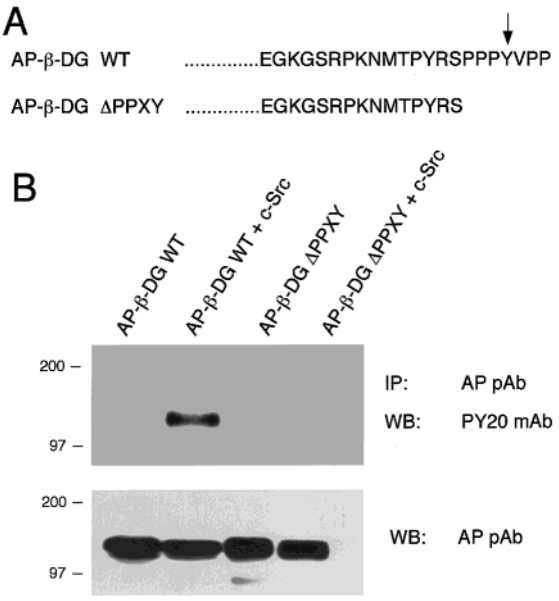


FIGURE 3: Src-induced phosphorylation of $\beta$-dystroglycan is dependent on the presence of a PPxY motif at the C-terminus of $\beta$-dystroglycan. (A) Sequence and description of the two different AP-$\beta$DG constructs that were utilized. An arrow points at the relevant tyrosine residue (Y) within the C-terminal PPxY motif. (B) Cos-7 cells were cotransfected with c-Src and an alkaline phosphatase-tagged form of $\beta$-dystroglycan (AP-$\beta$DG; WT or $\Delta$PPxY). The tyrosine phosphorylation of AP-$\beta$DG was monitored by immunoprecipition with anti-AP IgG and Western blotting with anti-phosphotyrosine IgG. Note that the Src-mediated phosphorylation of $\beta$-dystroglycan is strictly dependent on this C-terminal PPxY sequence, as AP-$\beta$DG $\Delta$PPxY fails to undergo tyrosine phosphorylation. This observation is consistent with the idea that c-Src phosphorylates $\beta$-dystroglycan at this C-terminal tyrosine residue.

phosphorylation of $\beta$-dystroglycan induced by treatment with vanadate, a tyrosine phosphatase inhibitor (*13*).

*Src-Induced Phosphorylation of $\beta$-Dystroglycan Is Dependent on the Presence of a PPxY Motif at the C-Terminus of $\beta$-Dystroglycan.* To further examine the relationship between c-Src and $\beta$-dystroglycan, we next used a heterologous expression system. Cos-7 cells were cotransfected with c-Src and an alkaline phosphatase-tagged form of full-length $\beta$-dystroglycan (AP-$\beta$DG). AP-$\beta$DG is a fusion protein carrying the full-length $\beta$-dystroglycan (including its transmembrane and cytoplasmic domains) fused to the ectodomain of alkaline phosphatase. The tyrosine phosphorylation of AP-$\beta$DG was monitored by immunoprecipition with anti-AP IgG and Western blotting with anti-phosphotyrosine IgG.

Figure 2 shows that we successfully reconstituted this phosphorylation event. Interestingly, when AP-$\beta$DG was coexpressed with other known tyrosine kinases (c-Yes, Fyn, Lck, Lyn, Hck, Fgr, and FAK), little or no phosphorylation was observed. These results indicate that this phosphorylation event is relatively specific for c-Src.

As previous studies on the adhesion-dependent phosphorylation of $\beta$-dystroglycan have tentatively localized this phosphorylation event to a tyrosine residue at the extreme C-terminus of $\beta$-dystroglycan (*13*), we next generated a mutant lacking this tyrosine residue (Figure 3A). Importantly, this tyrosine residue is located within the PPxY motif that is the WW domain ligand for dystrophin and utrophin (*13*, *14*, *31*). Tyrosine phosphorylation at this site has been shown to prevent the binding of $\beta$-dystroglycan to dystrophin and utrophin (*13*).

Figure 3B shows that the Src-dependent phosphorylation of $\beta$-dystroglycan is strictly dependent on this C-terminal PPxY sequence, as AP-$\beta$DG $\Delta$PPxY fails to undergo tyrosine phosphorylation. This observation is consistent with the idea that c-Src phosphorylates $\beta$-dystroglycan at this C-terminal tyrosine residue (*13*).

*Identification of SH2 Domain Containing Proteins That Bind to the Cytoplasmic Tail of $\beta$-Dystroglycan in Vitro.* One known function of tyrosine phosphorylation is to confer binding to SH2 domain containing proteins. To identify SH2 domain proteins that bind to tyrosine-phosphorylated $\beta$-dystroglycan, we used an established in vitro binding approach. Briefly, we prepared nonphosphorylated and tyrosine-phosphorylated $\beta$-dystroglycan from bacteria (see Materials and Methods; Figure 4A,B). These purified GST$-\beta$DG (787$-$895) fusion proteins were then incubated with lysates prepared from NIH 3T3 cells. After extensive washing, the bound material was subjected to Western blot analysis with a panel of antibodies directed against SH2 domain containing proteins (listed in Table 1).

Figure 4C shows that, of the 24 proteins surveyed, five SH2 domain containing proteins bound specifically, including c-Src, Fyn, Csk, NCK, and SHC. Importantly, all five of these SH2 domain containing proteins bound to tyrosine-phosphorylated GST$-\beta$DG but not to GST alone (not shown) or nonphosphorylated GST$-\beta$DG.

Interestingly, caveolin-1 also bound to GST$-\beta$-DG in a phosphorylation-dependent manner. As we have previously shown that a central WW domain within caveolin family members binds to the C-terminal PPxY motif within $\beta$-dystroglycan (*30*), this is the first example where tyrosine phosphorylation of the PPxY motif enhances binding. Alternatively, tyrosine-phosphorylated caveolin-1 may be indirectly bound to $\beta$-DG via another SH2 domain containing protein that is bivalent.

*A $\beta$DG-Derived Phosphopeptide Interacts with SH2 Domain Containing Proteins.* To assess whether the PPxY motif and its surrounding sequence is sufficient for binding of SH2 domain containing proteins, we next generated a panel of biotinylated $\beta$DG-derived phosphopeptides (Figure 5A). NIH 3T3 cell lysates were prepared and incubated with

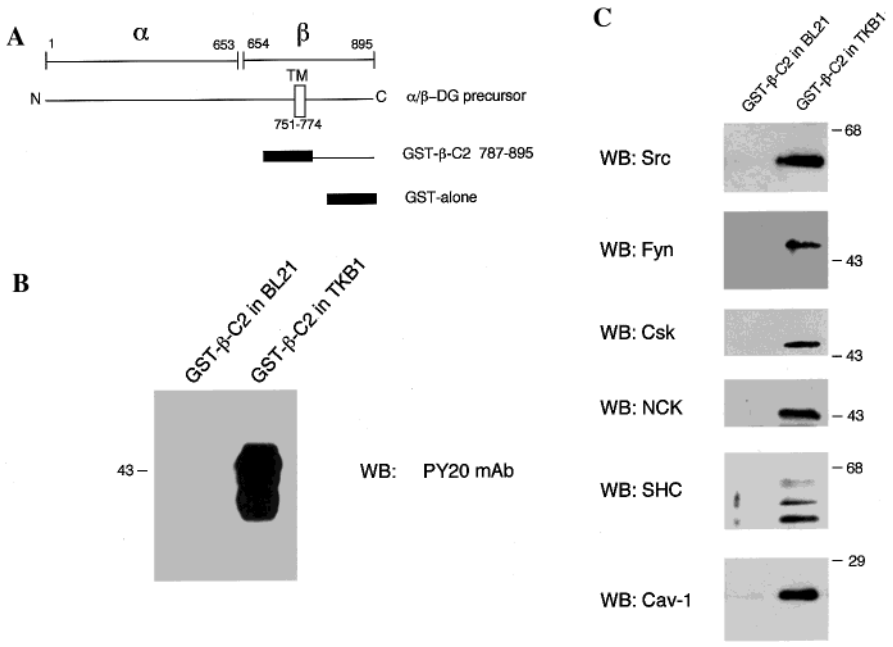


FIGURE 4: Identification of SH2 domain containing proteins that bind to the cytoplasmic tail of β-dystroglycan in a phosphorylation-dependent manner. (A) Schematic representation of precursor dystroglycan (α and β) and the GST−β-dystroglycan fusion protein utilized. Note that this region of dystroglycan (residues 787−895) contains five tyrosine residues. (B) A GST fusion protein carrying the cytoplasmic domain of β-dystroglycan was purified from normal bacteria (BL21) or from a bacterial strain harboring a tyrosine kinase (TKB1). Note that anti-phosphotyrosine IgG (mAb PY20) recognized only tyrosine-phosphorylated β-dystroglycan produced in the TKB1 strain, despite equal protein loading. (C) Nonphosphorylated and tyrosine-phosphorylated GST−βDG fusion proteins were prepared and incubated with cell lysates derived from normal NIH 3T3 cells. After binding, washing, and elution, the eluates were subjected to immunoblot analysis with antibodies directed against 24 different SH2 domain containing proteins (listed in Table 1). Note that, of the proteins surveyed, five SH2 domain containing proteins bound specifically, including c-Src, Fyn, Csk, NCK, and SHC. Importantly, all five of these SH2 domain containing proteins bound to tyrosine-phosphorylated GST−βDG but not to GST alone (not shown) or nonphosphorylated GST−βDG. Interestingly, caveolin-1 also bound to GST−βDG in a phosphorylation-dependent manner.

these biotinylated phosphopeptides prebound to streptavidin beads. After washing, these precipitates were subjected to Western blotting to detect bound SH2 domain containing proteins.

Figure 5B shows that only the βDG-derived phosphopeptide [βDG-Y1/pY2; KNMTP**Y**RSPPP(**pY**)VPP] effectively pulls down c-Src, Fyn, Csk, NCK, SHC, and caveolin-1. In contrast, two other closely related βDG-derived peptides [βDG-Y1/Y2, KNMTP**Y**RSPPP**Y**VPP, and βDG-pY1/Y2, KNMTP(**pY**)RSPPP**Y**VPP] showed no binding activity under identical conditions. These results provide additional support for the idea that tyrosine phosphorylation of the C-terminal βDG-PPxY motif is critical for its interaction with SH2 domain containing proteins and caveolin-1.

*Src-Induced Phosphorylation of β-Dystroglycan in Vivo Recruits SH2 Domain Containing Proteins: A Requirement for the C-Terminal PPxY Motif.* We next reconstituted the interaction of β-dystroglycan with SH2 domain containing proteins in vivo. Cos-7 cells were transfected with the cDNA encoding AP-βDG WT, in the presence or absence of c-Src. Lysates were then subjected to immunoprecipitation with anti-AP IgG, followed by Western blotting with antibodies directed against SH2 domain containing proteins. As a negative control for these studies, we also transfected Cos-7 cells with AP-βDG ΔPPxY, in the presence or absence of c-Src. Importantly, we have shown that AP-βDG ΔPPxY does not undergo tyrosine phosphorylation when coexpressed with c-Src (Figure 3).

Figure 6 shows that β-dystroglycan coimmunoprecipitates with c-Src, Fyn, Csk, NCK, and SHC. This binding is strictly dependent on tyrosine phosphorylation, as AP-βDG ΔPPxY fails to coimmunoprecipitate these SH2 domain containing proteins. Virtually identical results were obtained with caveolin-1, in accordance with the in vitro binding data presented in Figure 4C and Figure 5.

It should also be noted that, as a additional negative control, we examined the coimmunoprecipitation of FAK under these conditions; FAK did not bind to tyrosine-phosphorylated GST−βDG (Table 1). As predicted, FAK did not coimmunoprecipitate with βDG WT in the presence of c-Src (not shown). Thus, tyrosine phosphorylation of β-dystroglycan recruits the binding of a certain specific subset of SH2 domain containing proteins both in vitro and in vivo.

## DISCUSSION

Recent studies have shown that β-dystroglycan is tyrosine phosphorylated in an adhesion-dependent manner (*13*). The tyrosine phosphorylation of β-dystroglycan occurred in adherent cultures of HeLa cells but not in suspended cells, suggesting a possible role in cellular processes that require regulation of cell−matrix interactions and the cytoskeletal connections associated with them. Furthermore, using a phosphorylation state-specific monoclonal antibody, the site of tyrosine phosphorylation has been mapped to the tyrosine in the β-dystroglycan C-terminal WW domain binding motif PPxY. In the nonphosphorylated state, this PPxY motif is normally recognized as a WW domain ligand (*31*, *32*). The phosphorylation at this site was able to impede the interaction of β-dystroglycan with the WW domains of dystrophin and utrophin (*13*). Thus, the WW domain interaction could be regulated in a tyrosine-dependent manner similar to that of the SH3 domain interaction. However, it remains unknown

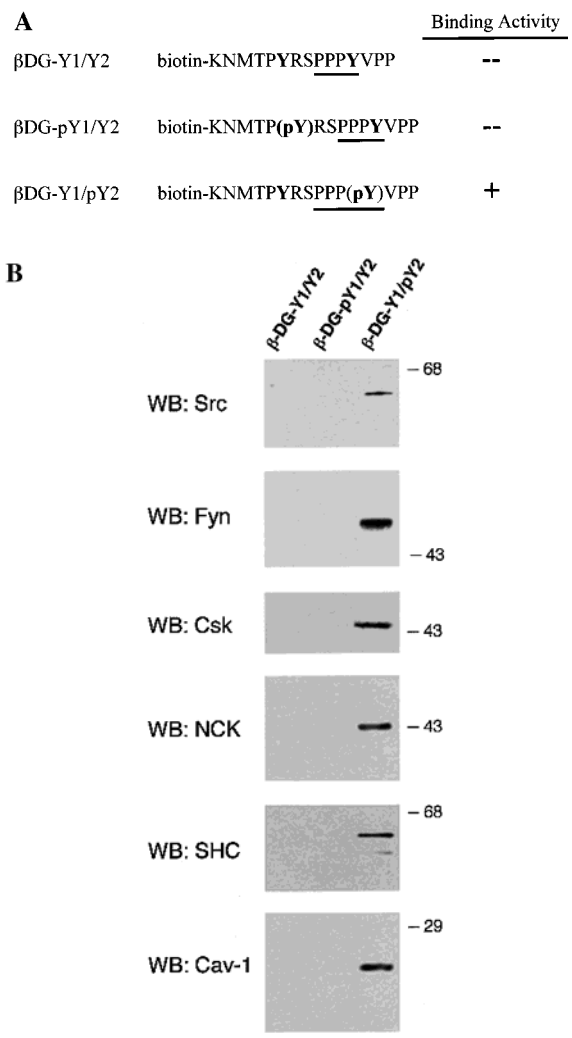


FIGURE 5: A $\beta$DG-derived phosphopeptide interacts with SH2 domain containing proteins. (A) Sequences of the biotinlyated $\beta$DG-derived phosphopeptides used in panel B. The PPxY motif is underlined, and tyrosine residues are shown in bold. (B) NIH 3T3 cell lysates were prepared and incubated with biotinylated phosphopeptides prebound to streptavidin beads. After washing, these precipitates were subjected to Western blotting with mAbs directed against c-Src, Fyn, Csk, NCK, SHC, or caveolin-1. Note that the $\beta$DG-derived phosphopeptide ($\beta$DG-Y1/pY2) effectively pulls down c-Src, Fyn, Csk, NCK, SHC, and caveolin-1. In contrast, two other closely related $\beta$DG-derived peptides ($\beta$DG-Y1/Y2 and $\beta$DG-pY1/Y2) showed no binding activity under identical conditions.

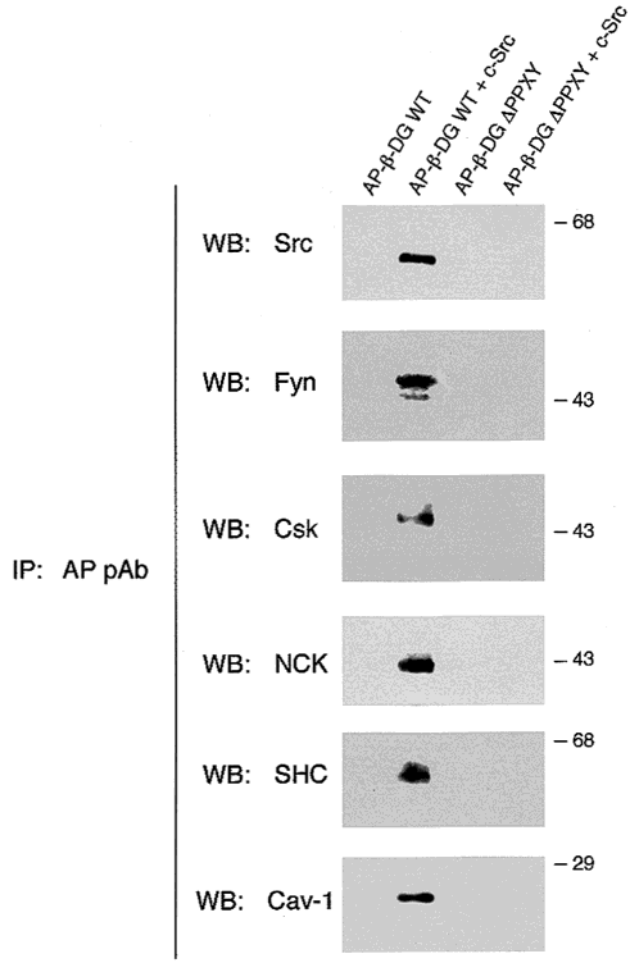


FIGURE 6: Src-induced phosphorylation of $\beta$-dystroglycan in vivo recruits SH2 domain containing proteins: a requirement for the C-terminal PPxY motif. Cos-7 cells were transfected with the cDNA encoding AP-$\beta$DG WT, in the presence or absence of c-Src. Lysates were then subjected to immunoprecipitation with anti-AP IgG, followed by Western blotting with antibodies directed against SH2 domain containing proteins. As a negative control for these studies, we also transfected Cos-7 cells with AP-$\beta$DG $\Delta$PPxY, in the presence or absence of c-Src. Note that $\beta$-dystroglycan coimmunoprecipitates with c-Src, Fyn, Csk, NCK, and SHC. This binding is strictly dependent on tyrosine phosphorylation, as AP-$\beta$DG $\Delta$PPxY fails to coimmunoprecipitate these SH2 domain containing proteins. Virtually identical results were obtained with caveolin-1. Thus, tyrosine phosphorylation of $\beta$-dystroglycan recruits the binding of SH2 domain containing proteins both in vitro and in vivo.

whether the tyrosine phosphorylation of the PPxY motif within $\beta$-dystroglycan could act as a binary regulatory switch to inhibit the binding of WW domain containing proteins and to promote interactions with SH2 domain containing proteins.

In this report, we show that the tyrosine phosphorylation of $\beta$-dystroglycan is constitutively elevated in v-Src transformed cells. We reconstituted this phosphorylation event in vivo by transiently coexpressing wild-type c-Src with a fusion protein containing full-length $\beta$-dystroglycan. Our results demonstrate that Src-induced tyrosine phosphorylation of $\beta$-dystroglycan is strictly dependent on the presence of a PPxY motif at its extreme C-terminus. Using a GST fusion protein carrying the cytoplasmic tail of $\beta$-dystroglycan, we identified five SH2 domain containing proteins that interact with $\beta$-dystroglycan in a phosphorylation-dependent manner, including c-Src, Fyn, Csk, NCK, and SHC. Finally, tyrosine phosphorylation of $\beta$-dystroglycan in vivo resulted in the endogenous coimmunoprecipitation of the same SH2 domain containing proteins, and this binding event required the $\beta$-dystroglycan C-terminal PPxY motif.

It has been recently demonstrated that caveolin-3 is able to interact with the C-terminal PPxY motif of $\beta$-dystroglycan and this interaction occurs trough a WW-like domain in the caveolin-3 protein. Thus, this interaction may be able to regulate competitively the recruitment of dystrophin to the sarcolemma. Interestingly, here we show that caveolin-1 binding to $\beta$-dystroglycan appeared to be phosphorylation dependent. These results strongly support the hypothesis that tyrosine phosphorylation of the PPxY motif within $\beta$-dystroglycan could act as a binary regulatory switch to inhibit the binding of certain WW domain containing proteins, while promoting the recruitment of other WW domain (or, more precisely, WW-like domain) containing proteins and of SH2 domain containing proteins. It would be of interest to determine the crystal structure of the WW-like domain of caveolin-3 and compare it to known structures of the SH2

domain. Perhaps new aspects of molecular convergence could be revealed by such an analysis.

In contrast to SH2 and SH3 domains, WW domains have been shown to be quite diverse in their abilities to recognize cognate ligands (*21*). Indeed, at least four groups of WW domains exist, each displaying a unique predilection for short proline-rich or proline-containing ligands. One of the subgroups of the WW domain was shown to interact with phosphorylated peptides containing phospho-Ser-Pro or phospho-Thr-Pro motifs (reviewed in ref *33*), and another group of WW domains bind PPR motifs (*34*). This proline- and arginine-rich motif can be bound and competed for by both WW domains and SH3 domains. Furthermore, many of these common protein ligands harbor proline-rich motifs which are flanked by RG repeats that represent targets for the type I protein arginine *N*-methyltransferase. The methylation of arginine residues flanking proline-rich motifs has no effect on WW domain binding, but this modification dramatically reduces the binding of certain SH3 domains (*35*). Thus post-translational modifications of WW domain ligands can alleviate competitive binding through arginine methylation (*2*) and also switch binding preferences from WW to SH2 domains through tyrosine phosphorylation (this study). It is likely that the intrinsic molecular plasticity of the three antiparallel β strands that give rise to WW domains will provide an explanation for the diverse ligand predilection of this small protein module (*36*).

It is interesting to note that phosphorylation of β-dystroglycan on tyrosine is achieved by c-Src kinase, whereas other related and muscle-expressed kinases such as c-Yes, for example, did not score positively in this assay. Another level of specificity is apparent in the SH2 domain interactions showing that only 5 out of the 24 SH2 domain containing proteins examined were able to recognize phosphorylated β-dystroglycan. In thinking about SH2 domain binding to the very C-terminus of phosphorylated β-dystroglycan, one should consider that perhaps this complex differs from other SH2 domain−ligand complexes. For most of the SH2 domains, high-affinity binding is provided by the phosphotyrosine residue itself and by residues carboxy terminal to it that follow the consensus pYxxφ, where φ is a hydrophobic amino acid (*37*, *38*). However, the φ amino acid (proline in the case of β-dystroglycan) is the carboxy-terminal end amino acid, rather than being a part of the polypeptide chain. Therefore, it is likely that the caboxy-terminally located pYxxφ sequences may interact with their cognate SH2 domains in a somewhat different manner.

We think that the importance of our study is in its contribution to the "changing image" of dystrophin/utrophin from static cytoskeletal proteins to multicomponent signaling proteins that are able to assemble functional complexes in a dynamic and regulated way. Reports on the identification of "bona fide" modular signaling domains in the C-terminal domain of dystrophin (*31*, *39*) and the interaction of the Grb2 SH3 domain with β-dystroglycan (*40*, *41*) further support this hypothesis.

## REFERENCES


1. Hoffman, E. P., Brown, R. H., and Kunkel, L. M. (1987) *Cell 51*, 919−928.
2. Koenig, M., Hoffman, E. P., Bertelson, C. J., Monaco, A. P., Feener, C., and Kunkel, L. M. (1987) *Cell 50*, 509−517.
3. Campbell, K. P., and Kahl, S. D. (1989) *Nature 338*, 259−262.
4. Ervasti, J. M., and Campbell, K. P. (1991) *Cell 66*, 1121−1131.
5. Ervasti, J. M., and Campbell, K. P. (1993) *J. Cell Biol. 122*, 809−823.
6. Ibraghimov-Beskrovnaya, O., Ervasti, J. M., Leveille, C. J., Slaughter, C. A., Sernett, S. W., and Campbell, K. P. (1992) *Nature 355*, 696−702.
7. Rosa, G., Ceccarini, M., Cavaldesi, M., Zini, M., and Petrucci, T. C. (1996) *Biochem. Biophys. Res. Commun. 233*, 272−277.
8. Jung, D., Yang, B., Meyer, J., Chamberlain, J. S., and Campbell, K. P. (1995) *J. Biol. Chem. 270*, 27305−27310.
9. Campanelli, J. T., Roberds, S. L., Campbell, K. P., and Scheller, R. H. (1994) *Cell 77*, 663−674.
10. Gee, S. H., Montanaro, F., Lindenbaum, M. H., and Carbonetto, S. (1994) *Cell 77*, 675−686.
11. Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G., and Campbell, K. P. (1995) *J. Biol. Chem. 270*, 11711−11714.
12. Campbell, K. P. (1995) *Cell 80*, 675−679.
13. James, M., Nuttall, A., Ilsley, J. L., Ottersbach, K., Tinsley, J. M., Sudol, M., and Winder, S. J. (2000) *J. Cell Sci. 113*, 1717−1726.
14. Tommasi di Vignano, A., Di Zenzo, G., Sudol, M., Cesareni, G., and Dente, L. (2000) *FEBS Lett. 471*, 229−234.
15. Rentschler, S., Linn, H., Deininger, K., Bedford, M. T., Espanel, X., and Sudol, M. (1999) *Biol. Chem. 380*, 431−442.
16. Huang, X., Poy, F., Zhang, R., Joachimiak, A., Sudol, M., and Eck, M. J. (2000) *Nat. Struct. Biol. 7*, 634−638.
17. Ilsley, J. L., Sudol, M., and Winder, S. J. (2001) *Cell. Signalling 13*, 625−632.
18. Bork, P., and Sudol, M. (1994) *Trends Biochem. Sci. 19*, 531−533.
19. Sudol, M., Chen, H. I., Bougeret, C., Einbond, A., and Bork, P. (1995) *FEBS Lett. 369*, 67−71.
20. Chen, H. I., and Sudol, M. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 7819−7823.
21. Sudol, M., and Hunter, T. (2000) *Cell 103*, 1001−1004.
22. Sudol, M. (1996) *Trends Biochem. Sci. 21*, 161−163.
23. Song, K. S., Scherer, P. E., Tang, Z.-L., Okamoto, T., Li, S., Chafel, M., Chu, C., Kohtz, D. S., and Lisanti, M. P. (1996) *J. Biol. Chem. 271*, 15160−15165.
24. Bagrodia, S., Taylor, S. J., and Shalloway, D. (1993) *Mol. Cell. Biol. 13*, 1464−1470.
25. Koleske, A. J., Baltimore, D., and Lisanti, M. P. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1381−1385.
26. Engelman, J. A., Wycoff, C. C., Yasuhara, S., Song, K. S., Okamoto, T., and Lisanti, M. P. (1997) *J. Biol. Chem. 272*, 16374−16381.
27. Scherer, P. E., Tang, Z.-L., Chun, M. C., Sargiacomo, M., Lodish, H. F., and Lisanti, M. P. (1995) *J. Biol. Chem. 270*, 16395−16401.
28. Larose, L., Gish, G., Shoelson, S., and Pawson, T. (1993) *Oncogene 8*, 2493−2499.
29. Frangioni, J. V., and Neel, B. G. (1993) *Anal. Biochem. 210*, 179−187.
30. Sotgia, F., Lee, J. K., Das, K., Bedford, M., Petrucci, T. C., Macioce, P., Sargiacomo, M., Bricarelli, F. D., Minetti, C., Sudol, M., and Lisanti, M. P. (2000) *J. Biol. Chem. 275*, 38048−38058.
31. Rentschler, S., Linn, H., Deininger, K., Bedford, M. T., Espanel, X., and Sudol, M. (1999) *Biol Chem. 380*, 431−442.
32. Chen, H. I., and Sudol, M. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 7819−7823.
33. Sudol, M., Sliwa, K., and Russo, T. (2001) *FEBS Lett. 490*, 190−195.
34. Bedford, M. T., Sarbassova, D., Xu, J., Leder, P., and Yaffe, M. B. (2000) *J. Biol. Chem. 275*, 10359−10369.

35. Bedford, M. T., Frankel, A., Yaffe, M. B., Clarke, S., Leder, P., and Richard, S. (2000) *J. Biol. Chem. 275*, 16030−16036.
36. Zarrinpar, A., and Lim, W. A. (2000) *Nat. Struct. Biol. 7*, 611−613.
37. Songyang, Z., Gish, G., Mbamalu, G., Pawson, T., and Cantley, L. C. (1995) *J. Biol. Chem. 270*, 26029−26032.
38. Sudol, M. (1998) *Oncogene 17*, 1469−1474.
39. Winder, S. J. (2001) *Trends Biochem. Sci. 26*, 118−124.
40. Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G., and Campbell, K. P. (1995) *J. Biol. Chem. 270*, 11711−11714.
41. Russo, K., Di Stasio, E., Macchia, G., Rosa, G., Brancaccio, A., and Petrucci, T. C. (2000) *Biochem. Biophys. Res. Commun. 274*, 93−98.

BI011247R