

# A Mass Spectrometry-based Proteomic Approach for Identification of Serine/Threonine-phosphorylated Proteins by Enrichment with Phospho-specific Antibodies

IDENTIFICATION OF A NOVEL PROTEIN, FRIGG, AS A PROTEIN KINASE A SUBSTRATE\*

Mads Grønberg‡§, Troels Zakarias Kristiansen‡, Allan Stensballe‡, Jens S. Andersen‡, Osamu Ohara¶, Matthias Mann‡||, Ole Nørregaard Jensen‡\*\*, and Akhilesh Pandey‡‡§§

Although proteins phosphorylated on tyrosine residues can be enriched by immunoprecipitation with anti-phosphotyrosine antibodies, it has been difficult to identify proteins that are phosphorylated on serine/threonine residues because of lack of immunoprecipitating antibodies. In this report, we describe several antibodies that recognize phosphoserine/phosphothreonine-containing proteins by Western blotting. Importantly, these antibodies can be used to enrich for proteins phosphorylated on serine/threonine residues by immunoprecipitation, as well. Using these antibodies, we have immunoprecipitated proteins from untreated cells or those treated with calyculin A, a serine/threonine phosphatase inhibitor. Mass spectrometry-based analysis of bands from one-dimensional gels that were specifically observed in calyculin A-treated samples resulted in identification of several known serine/threonine-phosphorylated proteins including drebrin 1,  $\alpha$ -actinin 4, and filamin-1. We also identified a protein, poly(A)-binding protein 2, which was previously not known to be phosphorylated, in addition to a novel protein without any obvious domains that we designate as Frigg. Frigg is widely expressed and was demonstrated to be a protein kinase A substrate *in vitro*. We identified several *in vivo* phosphorylation sites by tandem mass spectrometry using Frigg protein immunoprecipitated from cells. Our method should be applicable as a generic strategy for enrichment and identification of serine/threonine-phosphorylated substrates in signal transduction pathways. *Molecular & Cellular Proteomics* 1:517–527, 2002.

Signal transduction events involve transmission and amplification of signals from transmembrane receptors to the nucleus. One major conduit of information is mediated via reversible phosphorylation of proteins (1–3). In eukaryotes, phosphorylation occurs chiefly on serine, threonine, and tyrosine residues. Phosphorylation on serines and threonines is far more abundant than that on tyrosine residues. Indeed, the ratio of phosphorylation on phosphoserine:phosphothreonine:phosphotyrosine is 1800:200:1 in vertebrates (4). The overall level of phosphorylation is regulated by an interplay between the activities of protein kinases, as well as protein phosphatases within cells.

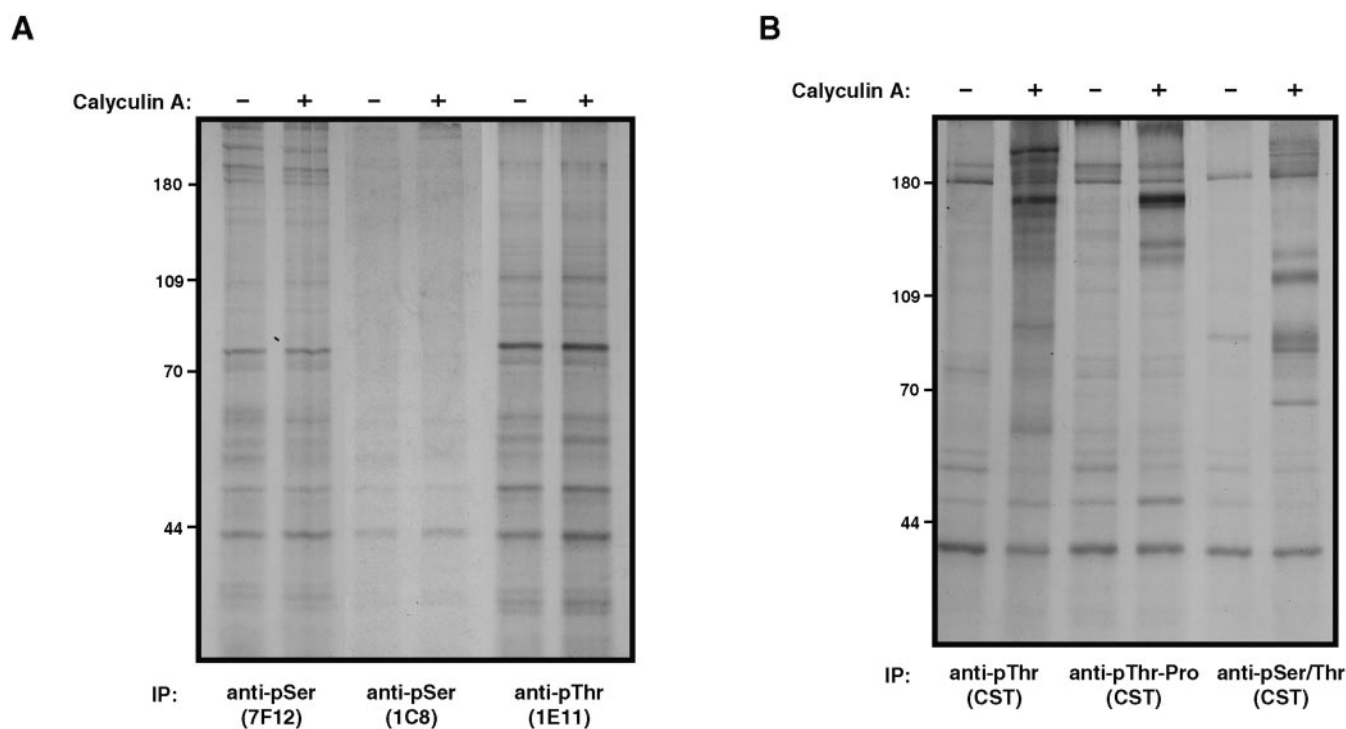
Although tyrosine phosphorylation is far less frequent than serine/threonine phosphorylation in cells, it has been studied quite intensively. One of the major reasons for this is the availability of excellent antibodies that recognize phosphorylated tyrosine residues in immunoprecipitation, as well as Western blotting experiments. In fact, detection of a protein by these antibodies generally constitutes proof that the protein is tyrosine-phosphorylated. Antibodies that specifically recognize serine/threonine-phosphorylated proteins have become available in recent years. However, a major drawback is that they cannot be used for enrichment purposes, because they do not work in immunoprecipitation experiments. Although strategies such as immobilized metal affinity chromatography have been developed for selective enrichment of phosphorylated peptides (5–8), their use to enrich for phosphorylated proteins has not yet been demonstrated. Other strategies to enrich for serine/threonine-phosphorylated proteins have been published, but they involve a series of chemical modifications that can result in loss of proteins (9–11).

In this report, we have tested several antibodies for their ability to immunoprecipitate and Western blot serine/threonine-phosphorylated proteins. We show that some of these antibodies can be used to specifically immunoprecipitate proteins phosphorylated on serine/threonine residues. We performed a large scale purification of proteins whose phosphorylation was induced by treatment of cells with calyculin A, a phosphatase inhibitor. Mass spectrometry has emerged as

From the ‡Department of Biochemistry and Molecular Biology, Center for Experimental Bioinformatics, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark, ¶Kazusa DNA Research Institute and RIKEN Research Center for Allergy and Immunology, 1532-3 Yana, Kisarazu, Chiba 292-0812, Japan, and ‡‡Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142 and Brigham and Women's Hospital, Boston, Massachusetts 02115

Received, February 18, 2002, and in revised form, July 5, 2002

Published, MCP Papers in Press, July 22, 2002, DOI 10.1074/mcp.M200010-MCP200



**FIG. 1. Testing immunoprecipitation efficiency of various anti-phosphoserine/threonine antibodies in HeLa cells.** Metabolic labeling of HeLa cells was performed as described under “Experimental Procedures.” After labeling, cells were left untreated or treated with 0.1  $\mu\text{g}/\text{ml}$  calyculin A for 30 min. **A**, the cell lysates were immunoprecipitated (IP) with monoclonal antibodies against phosphoserine (pSer, 7F12, and 1C8) or phosphothreonine (pThr, 1E11) antibodies from Alexis Biochemicals. The lack of differences between untreated and treated lanes indicates the inability of these antibodies to enrich for serine/threonine-phosphorylated proteins. **B**, the cell lysates were immunoprecipitated with antibodies against pSer/Thr or pThr or against phosphorylated threonine in the context of proline (pThr-Pro) from Cell Signaling Technology (CST). The significant differences in the patterns observed in the untreated and treated lanes by these antibodies indicate good immunoprecipitating ability.

the technique of choice for identification of proteins, as well as for analysis of phosphorylation (12, 13). Therefore, we excised bands that were only observed in calyculin A-treated cells from one-dimensional gels and used matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)<sup>1</sup> or nanoelectrospray tandem mass spectrometry for identification. Several proteins were identified from this screen including drebrin 1, poly(A)-binding protein 2, myosin heavy chain,  $\gamma$ -actin, and filamin-1. Using antibodies directed against filamin-1, we confirmed that the endogenous protein is phosphorylated on threonine residues, which is consistent with the observation that it has been shown to be a substrate of protein kinase A (PKA) based on *in vitro* studies. We also identified a novel protein as a phosphoprotein that does not contain any obvious domains from this large scale experiment. We have designated this protein as Frigg, which means “the one who loves,” after the wife of Odin, a Nordic god. Northern blot analysis showed that Frigg is widely expressed.

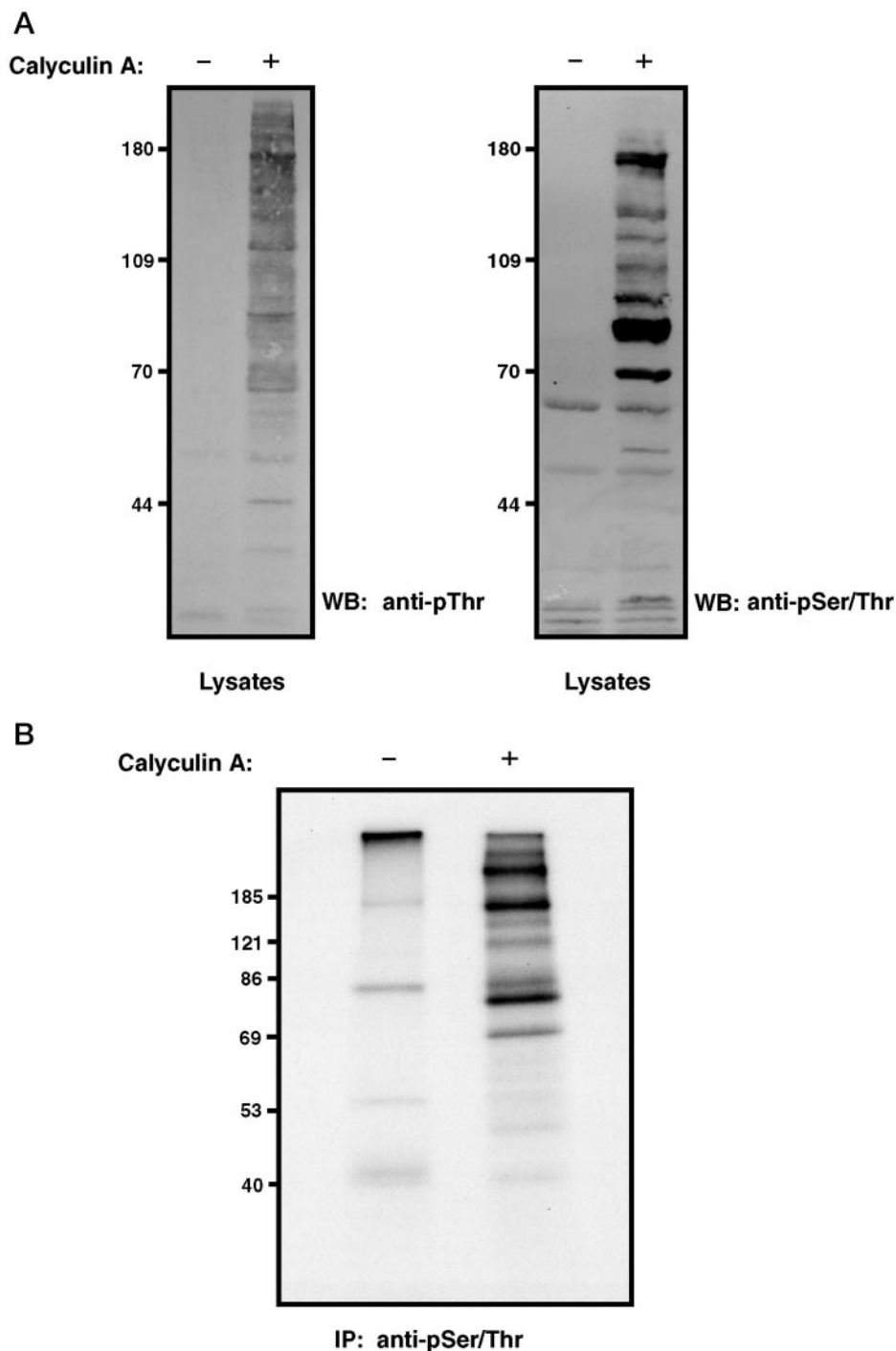
<sup>1</sup> The abbreviations used are: MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; LC-MS/MS, liquid chromatography tandem mass spectrometry; MS/MS, tandem mass spectrometry; PKA, protein kinase A; pSer, phosphoserine; pThr, phosphothreonine; PABP, poly(A)-binding protein.

Using an epitope-tagged version of Frigg, we show that it is phosphorylated upon treatment of transfected 293T cells by calyculin A and that it is a substrate of PKA by *in vitro* phosphorylation experiments. Finally, we identified several serine and threonine phosphorylation sites using immunoprecipitated Frigg protein by tandem mass spectrometry, one of which corresponds to a consensus site for PKA.

#### EXPERIMENTAL PROCEDURES

**Cell Culture and Antibodies**—HeLa and 293T cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum plus antibiotics. Anti-phosphoserine (7F12, 1C8) and anti-phosphothreonine antibodies (1E11) were from Alexis Biochemicals (San Diego, CA). Anti-phosphoserine (1C8) is a monoclonal mouse IgM antibody that recognizes a broad range of serine-phosphorylated proteins, preferring positively charged amino acids directly neighboring the phosphoserine residues. Anti-phosphoserine (7F12) and anti-phosphothreonine (1E11) are monoclonal mouse IgG antibodies. The 7F12 antibody has the same specificity as 1C8, and 1E11 recognizes a broad range of phosphothreonine-phosphorylated proteins.

Anti-pSer/Thr PKA substrate (catalog number 9621), anti-pThr (catalog number 9381), and anti-pThr-Pro (catalog number 9391) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-pSer/Thr and anti-pThr are rabbit polyclonal antibodies that were raised against a synthetic PKA substrate peptide and synthetic phosphothreonine-containing peptides, respectively. Anti-pThr-Pro is a



**FIG. 2. Serine/threonine phosphorylation of immunoprecipitated proteins.** *A*, for Western blots (WB),  $\sim 10^7$  HeLa cells were grown and serum-starved for 24 h. The cells were then treated with 0.1  $\mu\text{g/ml}$  calyculin A for 30 min to enhance phosphorylation or left untreated. Cleared cell lysates were Western blotted with anti-pSer/Thr or anti-pThr antibody as indicated. *B*, *in vivo* labeling with [ $^{32}\text{P}$ ]orthophosphate. Immunoprecipitates from labeled HeLa cells were resolved by SDS-PAGE and subjected to autoradiography as shown.

monoclonal mouse IgM antibody generated using synthetic phosphothreonine-proline peptides. Anti-FLAG antibody was from Sigma, and anti-filamin-1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

**Metabolic Labeling, Immunoprecipitation, and Western Blotting—**Metabolic labeling of HeLa and 293T cells was done with  $\sim 10^7$  cells in Dulbecco's modified Eagle's labeling medium. 0.2 mCi/ml of  $^{35}\text{S}$ -labeled cysteine and methionine (Translabel; ICN) was added and incubated for 6–12 h at 37 °C. After metabolic labeling, the cells were treated with 0.1  $\mu\text{g/ml}$  calyculin A for 30 min at 37 °C or left untreated.

The cells were lysed in lysis buffer (50 mM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 5 mM sodium pyrophosphate, and 10 mM  $\beta$ -glycerophosphate) in the presence of protease inhibitors. The lysates were centrifuged for 30 min at 4 °C at 14,000 rpm. Cleared cell lysates were incubated overnight at 4 °C with 20  $\mu\text{l}$  of polyclonal antibody (anti-pThr and anti-pSer/Thr) and 30  $\mu\text{l}$  of protein A-agarose beads or 20  $\mu\text{l}$  of monoclonal antibody (anti-pThr-Pro) and 30  $\mu\text{l}$  of protein L-agarose beads. The monoclonal antibodies from Alexis (7E12, 1C8, and 1E11) were first redissolved and subsequently incubated with cleared cell

lysates and 30  $\mu$ l of protein L agarose beads. Precipitated immune complexes were then washed three times in ice-cold lysis buffer, and the bound proteins were eluted by boiling for 6–8 min in sample buffer. The proteins were resolved by SDS-PAGE and subjected to autoradiography.

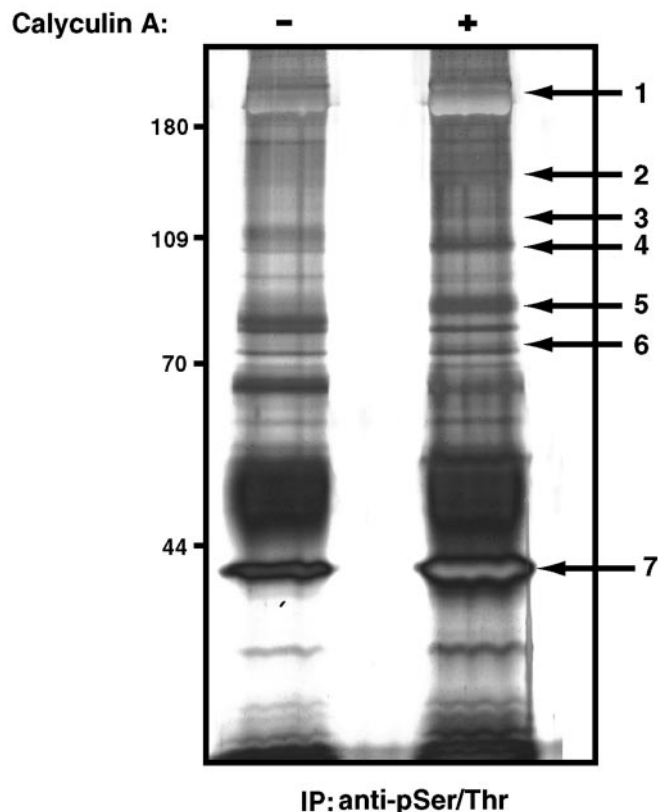
For Western blotting experiments, unlabeled cells were used but otherwise treated as described above. After SDS-PAGE, the gel was transferred onto nitrocellulose. The membranes were blocked in 5% skimmed milk powder in phosphate-buffered saline containing 0.1% Tween 20 overnight at 4 °C and incubated with the relevant antibody. The membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibody and developed using enhanced chemiluminescence detection according to the manufacturer's instructions (Amersham Biosciences).

**In Vivo Labeling with [<sup>32</sup>P]Orthophosphate**—HeLa cells grown to 90% confluence in two 10-cm dishes were starved overnight in serum-free Dulbecco's modified Eagle's medium. The medium was switched to phosphate-free medium 1 h prior to labeling. The cells were subsequently labeled for 3 h with [<sup>32</sup>P]orthophosphate (200  $\mu$ Ci/ml; Amersham Biosciences). One dish was then treated with 100 ng/ml calyculin A (Sigma) for 30 min. Cells from both untreated and treated dishes were then lysed in lysis buffer containing protease inhibitors and cleared by centrifugation at maximum speed in a microcentrifuge. Immunoprecipitation of the cleared lysate was then done with 20  $\mu$ l of anti-pSer/Thr antibody combined with 30  $\mu$ l of protein A-agarose bead solution (15- $\mu$ l bead volume) for 8 h at 4 °C. The immunoprecipitates were washed three times in lysis buffer, boiled in SDS sample buffer, and subjected to standard SDS-PAGE. The gel was then dried, and the bound material was detected by autoradiography.

**Large Scale Immunoprecipitation**—For the large scale experiment shown in Fig. 3,  $\sim 6 \times 10^8$  HeLa cells were grown and starved for 24 h. The cells were then treated with 0.1  $\mu$ g/ml calyculin A for 30 min at 37 °C or left untreated. The cells were lysed in 20 ml of lysis buffer. Cleared cell lysates were incubated with 200  $\mu$ l of anti-pSer/Thr antibody and 300  $\mu$ l of protein A-agarose beads overnight at 4 °C. Precipitated immune complexes were washed three times in lysis buffer, and bound proteins were eluted by boiling in sample buffer. The proteins were finally resolved by SDS-PAGE, and the gel was silver-stained by a method compatible with mass spectrometric analysis (14).

**Northern Blotting, cDNA Constructs, and Transfection**—Multiple tissue and cancer cell line Northern blots were purchased from CLONTECH and probed according to the manufacturer's instructions. A cDNA clone corresponding to the KIAA0668 protein was used to generate a FLAG epitope-tagged construct. The open reading frame was amplified by a PCR reaction using the primers 5'-AAAGAATTCGTGGGCGGGCTCCCATGCACTCT-3' and 5'-AAAGCGCCGCCACCATGTCCCGAAGAAGCCAGCGCCTC-3'. The PCR fragment was cut with *Not*I and *Eco*RI restriction enzymes and cloned into pCMV-Tag4a vector (Stratagene). 293T cells were transfected with 15  $\mu$ g of expression vector containing FLAG-tagged Frigg or empty vector using the calcium phosphate method. 2 h prior and 6 h after transfection the medium was changed for optimal conditions. 24 h after transfection, the cells were serum-starved for 24 h. The cells were then treated with 0.1  $\mu$ g/ml calyculin A for 30 min at 37 °C or left untreated. Cells were lysed and subjected to immunoprecipitation and Western blotting as described above.

**In Vitro Phosphorylation Assay**—Following transfection with Frigg, cells were lysed at 4 °C in lysis buffer. Cleared cell lysates were immunoprecipitated with 10  $\mu$ g of anti-FLAG antibody for 6 h at 4 °C. After incubation, the beads were washed two times in lysis buffer and two times in kinase buffer (50 mM Tris-Cl, pH 7.4, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol). Immune complexes were incubated for 30 min at 30 °C in 5  $\mu$ l of ATP mixture (300  $\mu$ M cold ATP, 45 mM MgCl<sub>2</sub>, and 20  $\mu$ Ci of

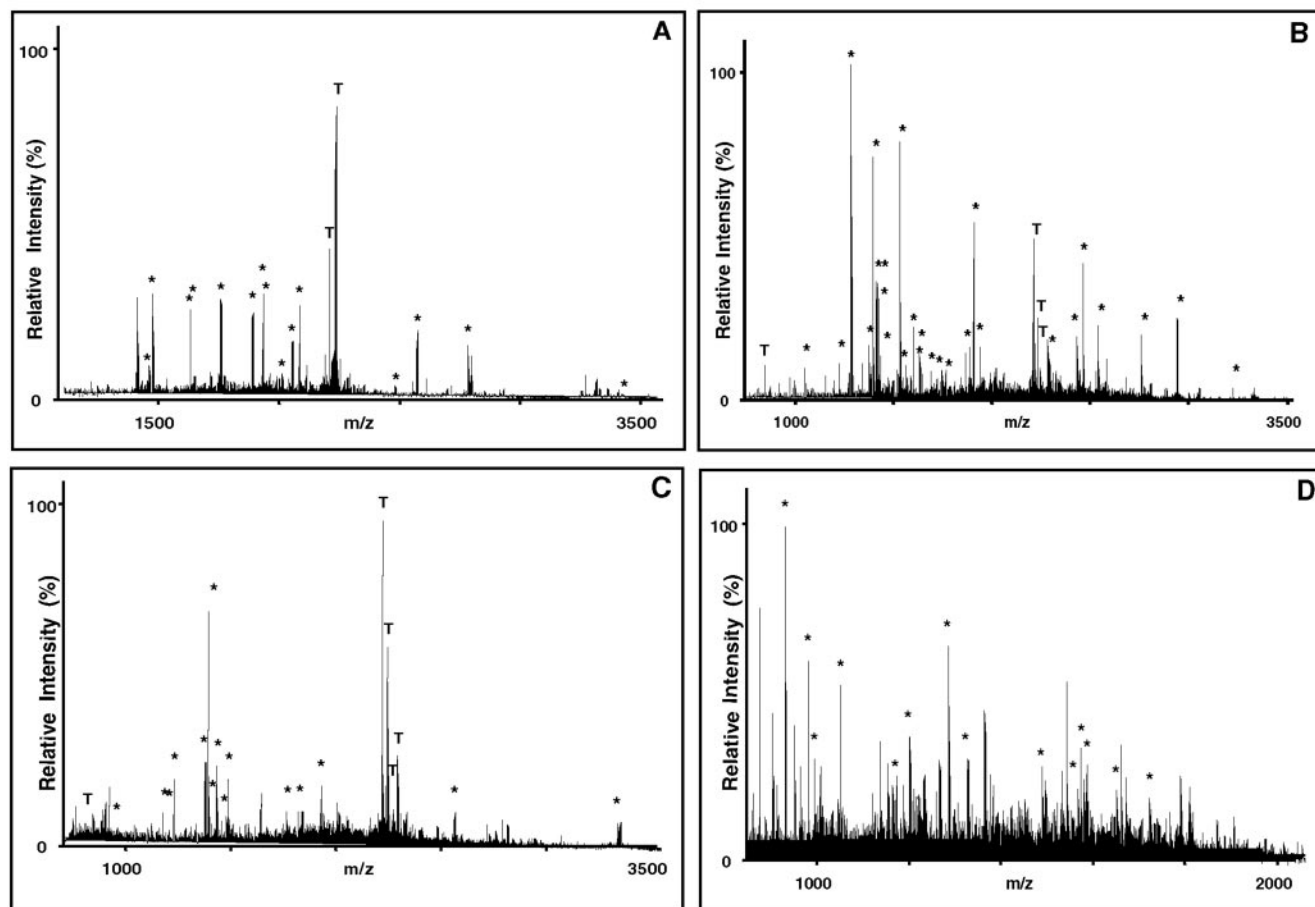


**Fig. 3. Large scale immunoprecipitation of phosphorylated proteins.** HeLa cells ( $6 \times 10^8$ ) were grown normally and then serum-deprived for  $\sim 24$  h before treating half of the cells with 0.1  $\mu$ g/ml calyculin A for 30 min. The cell lysates were immunoprecipitated (IP) with anti-pSer/Thr antibody, the immune complexes were washed, and the bound proteins were eluted by boiling and resolved by SDS-PAGE. The gel was then silver-stained and is shown in the figure. Molecular mass markers (in kDa) are indicated on the left. The arrows with numbers indicate the positions of the bands that were excised for digestion by trypsin and subsequent mass spectrometric analysis.

[ $\gamma$ -<sup>32</sup>P]ATP) and 60 units of protein kinase A (Calbiochem). Protein samples were then eluted by boiling in sample buffer and resolved by SDS-PAGE. The gel was dried and exposed on a phosphorimaging screen.

**Mass Spectrometric Analysis**—Mass spectrometric identification of proteins was done essentially according to a strategy described previously (15). The gel slices corresponding to the bands indicated in Fig. 3 were excised. After reduction and alkylation, the proteins were digested with an excess of modified sequencing grade trypsin (Promega, Madison, WI). The digestion was carried out overnight at 37 °C. After in-gel digestion, the supernatant was acidified to 5% with formic acid and loaded onto a nanoscale Poros R2 microcolumn (PerSeptive Biosystems, Framingham, MA) and desalted according to Gobom *et al.* (16).

For MALDI-TOF analysis, peptides were eluted with 1  $\mu$ l of saturated matrix solution (4-hydroxy- $\alpha$ -cyanocinnamic acid in 50% acetonitrile/2.5% formic acid). The eluate was spotted as a series of nanoliter volume droplets onto a MALDI probe. Internal mass calibration of peptide mass maps was performed using porcine tryptic autocatalytic products (*m/z* 842.51 and 2211.10). The samples for MS/MS analysis were acidified as described above. Subsequently the



**Fig. 4. Identification of serine/threonine-phosphorylated proteins by mass spectrometry.** Proteins bands indicated in Fig. 3 were subjected to in-gel digestion by trypsin and analyzed by MALDI-TOF mass spectrometry. *A*, MS spectrum of protein band 1 that was identified as filamin-1 (actin-binding protein 280). *B*, MS spectrum of protein band 2 that was identified as myosin heavy chain-A (polypeptide 9, non-muscle). *C*, MS spectrum of protein band 4 that was identified as  $\alpha$ -actinin 4. *D*, MS spectrum of protein band 5 that was identified as a hypothetical protein, KIAA0668. Peaks marked with asterisks correspond to tryptic peptides that were matched with a protein from a database search. *T* refers to trypsin autolysis products.

peptides were eluted with 40% methanol/5% formic acid directly into a nano-electrospray needle (MDS Proteomics, Odense, Denmark). Nano-electrospray tandem mass spectrometry was performed on a qTOF hybrid instrument (Micromass, Manchester, United Kingdom).

Automated nanoflow liquid chromatography/tandem mass spectrometric analysis (LC-MS/MS) was performed using a qTOF Ultima mass spectrometer (Micromass, Manchester, United Kingdom) employing data-dependent acquisition. A commercial nano-high pressure liquid chromatography system (Ultimate; LC Packings, Amsterdam, The Netherlands) was used to deliver a flow rate of 175 nL/min. Peptide samples were loaded onto a column (made using Agilent Zorbax C18 3.5  $\mu$ m; 8 cm, 75  $\mu$ m internal diameter) using a high pressure vessel. Peptide separation was accomplished using a gradient of 5–38% acetonitrile resulting in an average peak width of 15 s. Data analysis was performed using MassLynx and ProteinLynx software, and the resulting MS/MS data set was analyzed using the Mascot search engine (Matrix Science Ltd., London, United Kingdom).

#### RESULTS AND DISCUSSION

##### *Testing of Antibodies for Their Ability to Enrich Serine/Threonine-phosphorylated Proteins in Metabolically Labeled*

*HeLa Cells*—To enrich serine/threonine-phosphorylated proteins, we decided to first carry out preliminary experiments with several commercially available antibodies. HeLa cells were metabolically labeled with  $^{35}$ S and then left untreated or treated with calyculin A. Treatment with calyculin A increases the phosphorylation of several protein substrates on serine/threonine residues because of the unopposed activity of protein kinases within cells. Cell lysates were immunoprecipitated with several antibodies that were directed against phosphorylated proteins. In our experience, we find that although most of such antibodies perform well in Western blotting experiments, they are generally not useful in immunoprecipitating proteins and therefore cannot be used for enrichment purposes. As shown in Fig. 1A, three of these antibodies did not immunoprecipitate any bands that were different between untreated and calyculin A-treated cells. However, three of the antibodies showed patterns that indicated that they were specifically able to immunoprecipitate

proteins from calyculin A-treated cells (Fig. 1B). These antibodies were raised against pThr residues, pThr-Pro (which indicates that it recognizes phosphothreonine residue in the context of a following proline), or a mixture of both phosphoserine and phosphothreonine (pSer/Thr) residues, respectively, and are available commercially.

*Testing of Antibodies in Western Blotting Experiments to Detect Serine/Threonine-phosphorylated Proteins*—We decided to characterize further two of the antibodies that worked well in immunoprecipitation experiments. For this purpose, cell lysates from HeLa cells were subjected to Western blotting with anti-pThr and anti-pSer/Thr antibodies. Fig. 2A shows that these two antibodies were able to detect proteins whose phosphorylation was induced by treatment of cells with calyculin A treatment in Western blotting experiments. The profile of the bands observed was largely similar to that seen in the immunoprecipitation experiments (shown in Fig. 1B or 2B). Therefore, these two antibodies can be used for immunoprecipitation, as well as Western blotting experiments.

*Large Scale Purification of Serine/Threonine-phosphorylated Proteins and Identification by Mass Spectrometry*—A large scale experiment was next performed using  $6 \times 10^8$  HeLa cells that were serum-starved and divided into two. One half was left untreated, and the other half was treated with calyculin A for 30 min. Cell lysates were immunoprecipitated using anti-pSer/Thr antibody. The immune complexes were boiled and resolved by one-dimensional SDS-PAGE, and the gel was silver-stained to visualize proteins (Fig. 3). Seven major protein bands that were observed specifically in the calyculin A-treated cells were excised, digested by trypsin, and analyzed by MALDI-TOF mass spectrometry.

As shown in Fig. 4A, band 1 was identified as filamin-1 by MALDI-TOF mass spectrometry. It is also known as actin-binding protein 280 and is an essential component of the cytoskeleton where it promotes rearrangement of actin filaments in response to a variety of physiological and exogenous stimuli (17). In addition, filamin-1 has been shown to associate with lipid membranes, to direct intracellular trafficking, and to bind to membrane surface receptors (18–20). Filamin-1 is a known phosphoprotein with serine 2152 serving as a unique target site for PKA (17, 18, 21). We decided to test whether filamin-1 is also phosphorylated on threonine residues upon treatment of HeLa cells with calyculin A. Endogenous filamin-1 was immunoprecipitated with anti-filamin-1 antibody followed by Western blotting with anti-pThr antibody. Filamin-1 underwent efficient phosphorylation on threonine residues upon calyculin A treatment indicating that it is phosphorylated on threonine, in addition to serine, residues (Fig. 5).

*Band 2* was identified as myosin heavy chain A (non-muscle) (Fig. 4B). Myosin heavy chain-A, non-muscle, is a cellular/cytoplasmic myosin very similar in structure to the skeletal, cardiac, and smooth muscle myosins. It has been identified in a wide range of cells including yeast and mammalian cells (22). The key feature of cytoplasmic myosin is its binding to

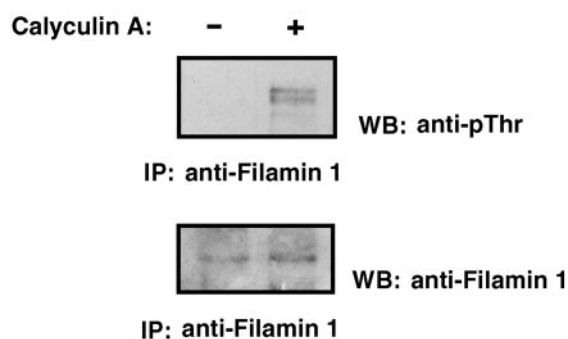


FIG. 5. **Filamin-1 is phosphorylated by calyculin A treatment.** HeLa cells were left untreated or treated with 0.1  $\mu$ g/ml calyculin A for 30 min. Cleared cell lysates were immunoprecipitated (IP) with anti-filamin-1 antibody, resolved SDS-PAGE, and Western blotted (WB) with anti-pThr antibody. The *top panel* shows a Western blot using anti-pThr antibody to detect immunoprecipitated filamin-1 from untreated or calyculin A-treated cells. The *bottom panel* is a Western blot from a parallel experiment to show equal expression of filamin-1.

actin filaments thereby generating contractile forces during cytokinesis and in maintaining cortical tension because of the hydrolysis of ATP. The function of cytoplasmic myosin is known to be regulated by its phosphorylation state (23). In *Dictyostelium*, for example, specific phosphorylation of threonine residues 1823, 1833, and 2029 is observed, which drives myosin from filamentous to a monomeric state thereby inhibiting cellular activity (24).

Tandem mass spectrometry was performed to confirm the identity of the protein migrating at 120 kDa in band 3. It was revealed to be drebrin 1 (F-actin-binding protein) (data not shown). It is an F-actin-binding protein identified originally in chicken brain as a neuron-specific developmentally regulated brain protein expressed mainly in neurons (25). Three isoforms have been identified in the chicken (26) and humans (27, 28) that are generated by alternative splicing of a single transcript (29). In addition to its ability to interact with F-actin, drebrin 1 inhibits competitively the actin binding property of tropomyosin, fascin, and  $\alpha$ -actinin (30–32). Hayashi *et al.* (30) have shown that it is highly phosphorylated *in vivo*.

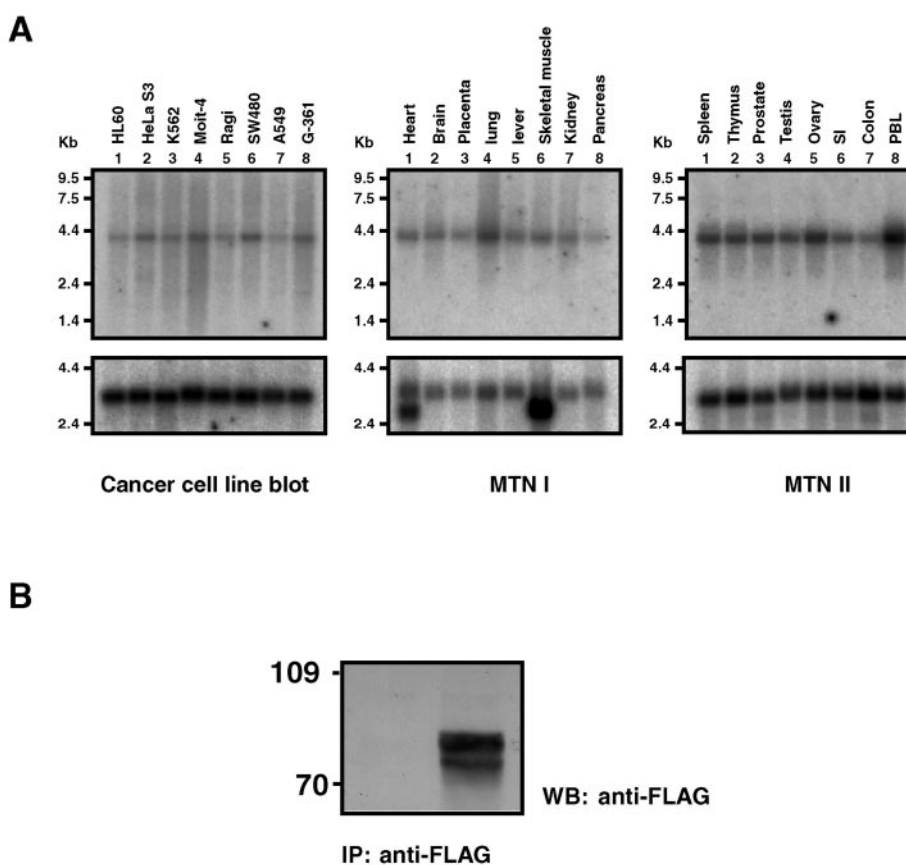
Tryptic peptides from band 4 were derived from  $\alpha$ -actinin 4 (Fig. 4C), a protein discovered originally as a component of the z-disc in skeletal muscle (22). It is composed of three domains, an N-terminal actin-binding domain, an extended rod-shaped domain, and a C-terminal region containing two EF-hands motifs (33).  $\alpha$ -Actinin is a member of actin binding superfamily of proteins, which binds to the integrin  $\beta$ 1 cytoplasmic domain and is believed to link the actin cytoskeleton to focal adhesions (34, 35). So far, four isoforms of human  $\alpha$ -actinin have been identified (designated 1 to 4), and  $\alpha$ -actinin 4 is believed to be involved in cell motility and in cancer invasion. Mukai *et al.* (33) have shown that  $\alpha$ -actinin 4 is a direct substrate for protein kinase N, which phosphorylates it in the N-terminal region. In addition, it has been shown to be

TABLE I  
A list of phosphoproteins identified by mass spectrometry

The table lists the proteins and GenBank™ accession numbers identified by mass spectrometry in the large scale experiment by enrichment with anti-pSer/Thr antibody. Data on phosphorylation and corresponding references are indicated. The band numbers correspond to the bands excised from the silver-stained gel shown in Fig. 3.

Band excised	Protein name	Phosphorylation data	Accession no.	References
Band 1	Filamin-1	Phosphorylated on serine 2152 (PKA site)	NP_001447	17, 18, 20, 21
Band 2	Myosin heavy chain A	Phosphorylated on threonine residues 1823, 1833, and 2029 in <i>Dictyostelium</i>	NP_002464	23, 24
Band 3	Drebrin 1	Phosphorylated <i>in vivo</i>	NP_004386	27, 30
Band 4	$\alpha$ -Actinin 4	Phosphorylated in N-terminal region by protein kinase N. Phosphorylated <i>in vitro</i> by FAK on residue 12	NP_004915	33, 35–37
Band 5	Frigg (KIAA0668)	Phosphorylated on serine 12, 54, and 116 (additional sites present)	BAA31643	This study
Band 6	PABP2	Phosphorylation of PABP, a protein related to PABP2, specifies the type of binding to poly(A) RNA and to other proteins	Q15097	38–41
Band 7	$\gamma$ -Actin	Phosphorylated upon stimulation of monocytes	NP_001605	47

**FIG. 6. Expression of Frigg.** A, a cancer cell line blot or multiple tissue Northern blots were probed with a <sup>32</sup>P-labeled fragment from KIAA0668 (top panels) according to the manufacturer's instructions (CLONTECH). The bottom panels show reprobing of the blots with a  $\beta$ -actin probe after stripping. Molecular weight markers in kb are shown on the left. B, transfection of 293T cells with a FLAG epitope-tagged version of Frigg. Western blot (WB) with anti-FLAG antibody revealed that the expressed protein migrates at 80 kDa, which is consistent with the size of the protein observed in the initial large scale purification experiment. IP, immunoprecipitated.

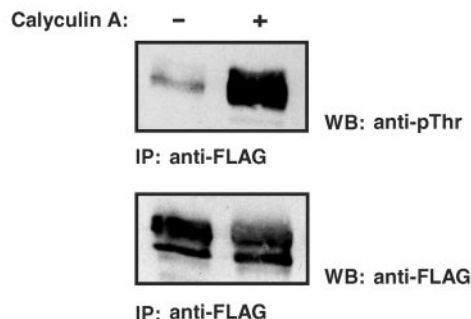


tyrosine-phosphorylated by focal adhesion kinase, *in vitro*, on residue 12 (36, 37).

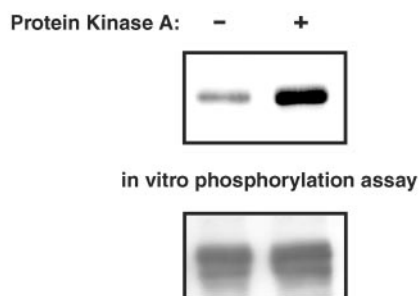
The protein in band 6 was identified by MALDI-TOF MS as poly(A)-binding protein 2 (PABP2). It is an RNA-binding protein that binds with high affinity to nascent poly(A) tails, stimulating their extension and controlling their length (38). Short expansions of the GCG trinucleotide repeat in the gene encoding PABP2 lead to oculopharyngeal muscular dystrophy

(39). PABP2 has been demonstrated to interact with SKIP and MyoD to stimulate MyoD-dependent transcription indicating a role in signal transduction pathways (40). Le *et al.* (41) have shown recently that the level of phosphorylation of PABP, a protein related to PABP2, specifies the type of binding to poly(A) RNA and to other proteins (e.g. different translation initiation factors). Because we enriched PABP2 with an anti-pSer/Thr antibody, we conclude that it is a serine-phospho-

A



B



**FIG. 7. Serine/threonine phosphorylation of Frigg by calyculin A treatment.** A, induction of KIAA0668 Ser/Thr phosphorylation by calyculin A. 293T cells were transfected with KIAA0668 plasmid using the calcium phosphate method. 24 h after transfection, cells were serum-starved for 24 h. The cells were treated with calyculin A for 30 min or left untreated. Cell lysates were immunoprecipitated (IP) with antibody against the FLAG epitope. The proteins were then resolved by SDS-PAGE and Western blotted (WB) with anti-pThr (top panel). The bottom panel shows the blot after stripping the membrane followed by reprobing with anti-FLAG to confirm equal loading. B, *in vitro* phosphorylation of Frigg by PKA. Following transfection with Frigg, cells were lysed and immunoprecipitated with anti-FLAG antibody. Precipitated immune complexes were incubated for 30 min at 30 °C in ATP mixture and PKA. Protein samples were eluted and resolved by SDS-PAGE. The dried gel was exposed on a phosphorimaging screen.

rylated protein, and its level of phosphorylation may have functional importance as is the case with PABP.

Table I shows the list of all proteins that were identified from the gel shown in Fig. 3. The tryptic peptides from the protein migrating at 80 kDa in band 5 matched a novel protein that corresponds to a cDNA fragment (KIAA0668) that was predicted to encode a truncated protein (Fig. 4D).

**Analysis and Expression of a Novel Phosphorylated Protein, Frigg**—Because the protein encoded by KIAA0668 was novel, we decided to characterize it further. Because there was no in-frame stop codon in the longest reading frame, this clone

TABLE II

A list of tryptic peptides in Frigg where exact phosphorylation sites were localized by mass spectrometry

The table lists tryptic peptides derived from Frigg with the specific phosphorylation sites identified by MS/MS marked in bold. Residue numbers correspond to the position of the indicated phosphorylated residue within Frigg sequence.

Phosphorylated amino acid	Tryptic peptide
Ser-12	LTRY <b>S</b> QGDDDGSSSSGGSSVAGSQSTL <b>F</b> K
Ser-54	RL <b>S</b> PAPQLGPSSDAHTSYSESLVHESLWFPPR
Ser-116	GTGGSESSRAS <b>L</b> GVGR

TABLE III

A list of phosphorylated tryptic peptides where phosphorylation site could not be localized by mass spectrometry

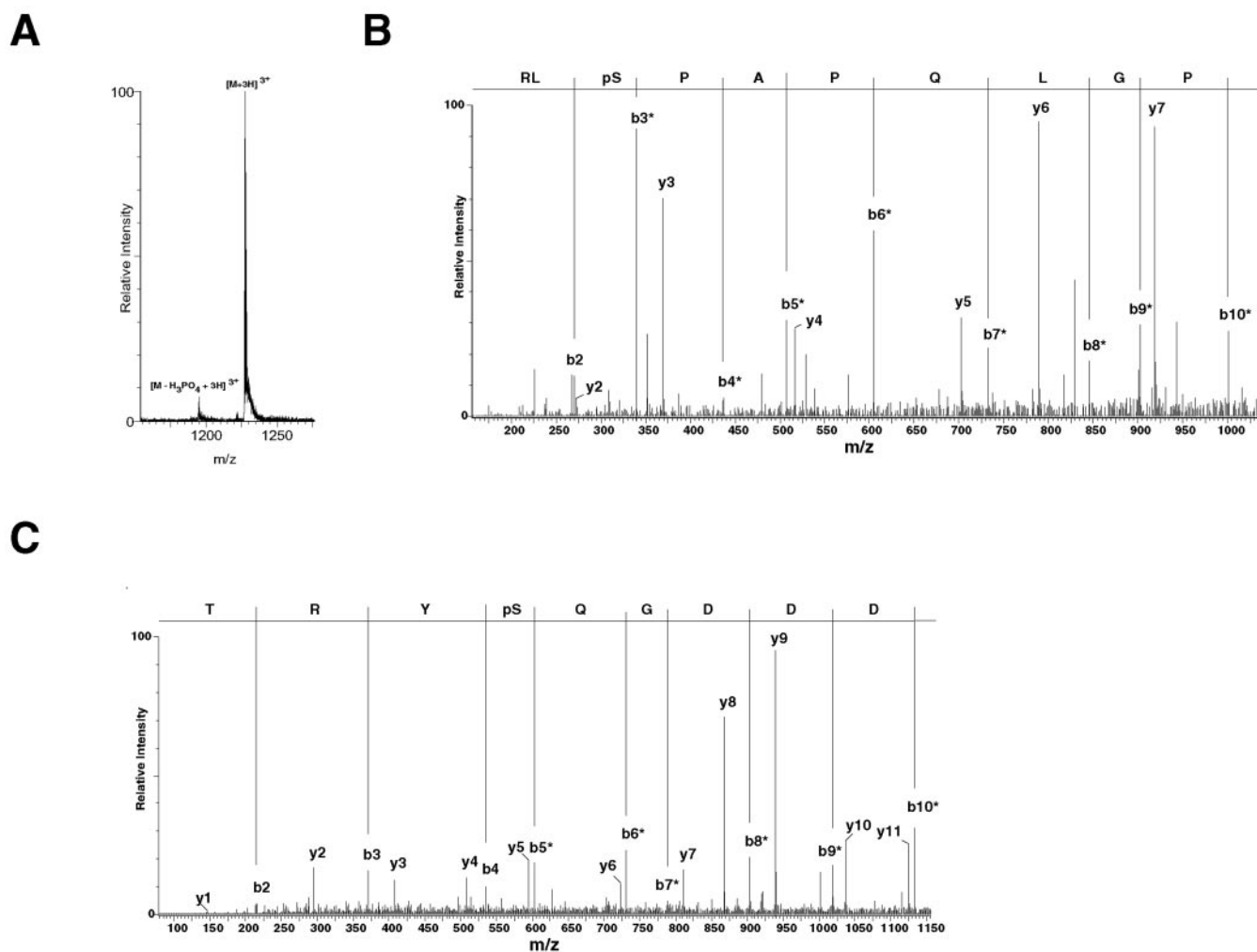
The table lists tryptic peptides derived from Frigg where specific phosphorylation sites could not be localized. The residue marked in bold in one of the tryptic peptides corresponds to one of the phosphorylation sites identified by MS/MS (see Table II).

Tryptic peptide	Number of phosphate moieties <sup>a</sup>
GTGGSESSRAS <b>L</b> GVGR	2
AGSLLWMVATSPGR	1
DSSPHFQAEQR	1
FTYDQDGEPIQTFHFQAPT <b>M</b> ATYQVV <b>E</b> LR	1

<sup>a</sup> Indicates the number of phosphate moieties on tryptic peptides identified by MS.

had been labeled with a warning of potential N-terminal truncation. Comparative genomic analysis was used for assignment of the start codon by comparing the translated sequence from this cDNA against all murine sequences (42). We found that the conservation of amino acid sequence started at the methionine residue present at position 77 indicating that this was the actual initiator codon. The open reading frame of this cDNA encoded a protein of 717 amino acids with a predicted molecular mass of 80.3 kDa. This protein was designated as Frigg after a goddess who was the wife of Odin in Nordic mythology. Examination of the protein sequence using domain prediction programs such as SMART and Pfam did not reveal the presence of any identifiable domain (43, 44). A BLAST search against the protein sequence databases showed that this protein was 37% identical to another entry, KIAA0810, which also did not possess any obvious domains. To check the expression of Frigg, a Northern blot analysis was performed. As shown in Fig. 6A, it is expressed as a single band of ~4 kb in all examined tissues and cell lines. To characterize Frigg at the level of the protein, the open reading frame was amplified by polymerase chain reaction and cloned into a C-terminal FLAG epitope-tagged vector. The expression vector was transfected into 293T cells by the calcium phosphate method, and the expressed protein was immunoprecipitated with anti-FLAG antibody. The precipitated immune complexes were then subjected to one-dimensional





**FIG. 8. Localization of phosphorylation sites on Frigg by mass spectrometry.** LC-MS/MS analysis of tryptic peptides derived from Frigg. Six phosphoserine/phosphothreonine-containing peptides were identified in the LC-MS/MS run. Specific phosphorylation sites were identified in three of the six phosphopeptides. The fragmentation pattern of the peptide is indicated (only *b* and *y* ions are indicated for simplicity). Ions labeled with an asterisk were generated from the peptide in which the phosphoserine had been converted to dehydroalanine by  $\beta$ -elimination (loss of phosphoric acid, 98 Da). A, part of the MS/MS spectrum of a triply charged peptide ion at  $m/z$  1226.93. Note the elimination of phosphoric acid ( $H_3PO_4$ ; loss of 98 Da) from the precursor ion. B, part of an MS/MS spectrum of a quadruply charged phosphoserine-containing peptide RLpSPAPQLGPSSDAHTSYSESILVHESWFFPPR ( $m/z$  920.45). The phosphorylation site was localized to the serine in position 3 in the peptide (serine 54 in Frigg). The serine positioned at 54 is part of a consensus sequence (RLS) that is phosphorylated by protein kinase A. C, part of an MS/MS spectrum of a triply charged phosphoserine-containing peptide LTRYpSQGDDGSSSSGGSSVAGSQSTLFK ( $m/z$  987.76). The phosphorylation site was localized to the serine in position 5 of the peptide (serine 12 in Frigg).

SDS-PAGE and Western blotted with anti-FLAG conjugated to horseradish peroxidase. The Western blot revealed that the expressed protein migrated at 80 kDa (Fig. 6B), which is consistent with size of the protein observed in the initial large scale purification experiment.

**Frigg Is a Novel PKA Substrate**—The phosphorylation state of epitope-tagged Frigg was next examined by treating transfected 293T cells with calyculin A. After enrichment by immunoprecipitation with anti-FLAG antibody, the precipitated proteins were separated by one-dimensional SDS-PAGE and Western blotted with anti-phosphothreonine antibody. The Western blot clearly shows that Frigg is highly phosphorylated

*in vivo* as a result of calyculin A treatment (Fig. 7A). The anti-pSer/Thr antibody used in the large scale immunoprecipitation experiment recognizes phosphoserine/threonine with an arginine in the  $-2$  position. This is the consensus sequence for protein kinase A. An *in vitro* phosphorylation assay was therefore performed to test whether Frigg was a potential substrate of PKA. Immunoprecipitated Frigg was incubated with  $\gamma$ - $^{32}P$ -labeled ATP in the absence or presence of the purified catalytic subunit of PKA for 30 min. The proteins were eluted by boiling in sample buffer and subsequently resolved by SDS-PAGE. The result of this *in vitro* phosphorylation assay was analyzed on a phosphorimaging screen. The assay

shows clearly that Frigg undergoes phosphorylation when incubated with the catalytic subunit of PKA implying that Frigg is a substrate of PKA (Fig. 7B). Because the stoichiometry of phosphorylation in such *in vitro* phosphorylation experiments can be low, we used overexpressed Frigg protein that was immunoprecipitated from calyculin A-treated cells to identify *in vivo* phosphorylation site(s). The cellular context of such an experiment is important, because *in vitro* phosphorylation experiments can sometimes give artifactual results. LC-MS/MS experiments were therefore carried out to locate specific serine/threonine phosphorylation site(s) in Frigg. Tryptic peptides were eluted in a gradient of acetonitrile ranging from 5 to 38% and analyzed by tandem mass spectrometry on a quadrupole time-of-flight mass spectrometer. Forty-nine peptides originating from Frigg were identified. Six of these were identified as phosphoserine/phosphothreonine-containing peptides (containing one or two phosphorylation sites each) by the Mascot Search program (see Table II and Table III). Based on the MS/MS data, specific residues were identified as phosphorylation sites in three of the six peptides (Table II). Phosphorylation on serine residues was confirmed by the detection of dehydroalanine in the sequence, corresponding to loss of phosphoric acid (−98 Da) by β-elimination. Phosphorylation on serine residues was found on residues 12, 54, and 116 (Fig. 8). The LC-MS/MS data indicates strongly the presence of several other phosphorylation sites in Frigg, but the lack of fragmentation of the precursor ion in some MS/MS spectra made detection of further phosphorylation site(s) impossible. Table III shows a list of such phosphopeptides that were identified as phosphorylated where the site of phosphorylation could not be localized.

#### CONCLUSIONS

Because phosphorylation is an important mechanism in a number of signal transduction events, it is important to possess the appropriate tools and techniques for a global analysis. Until now, a major limitation for studying serine/threonine-phosphorylated proteins was the lack of availability of immunoprecipitating antibodies. To circumvent this problem, a number of strategies based on chemical modification of phosphoserine and phosphothreonine residues have been developed. A drawback of these approaches is that they employ a series of chemical reactions and purification steps that may lead to loss of phosphorylated proteins. In this report, we have shown that antibodies directed against phosphoserine and phosphothreonine residues can be used for enrichment of these proteins for a global analysis as we had shown earlier for tyrosine phosphorylation signaling pathways (45). Most phosphorylation prediction programs did not predict several of the phosphorylation sites and phosphopeptides that we have identified. This occurrence of false negatives is similar to what we have reported previously in the case of tyrosine phosphorylation site substrates in the epidermal growth factor receptor signaling pathway (46).

Identification of phosphorylated proteins from such global approaches is only the first step in characterizing these signaling molecules. It is important to verify the involvement of the proteins identified from such approaches by studies *in vivo*. Such studies may be performed by using antibodies against the endogenous proteins and/or overexpression of wild-type and mutant forms of epitope-tagged proteins to examine the role of the protein in signal transduction pathways. It is hoped that these antibodies will permit a systematic analysis of all substrates involved in signaling pathways that involve serine and threonine phosphorylation events. Finally, the data on the phosphorylated residues identified from such experiments will help refine the prediction programs used for prediction of phosphorylation sites.

\* Work at the Center for Experimental Bioinformatics was supported by a generous grant from the Danish National Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by a grant from Novo Nordisk, Gentofte, Denmark.

|| To whom correspondence may be addressed. Tel.: 45-65502364; Fax: 45-65933929; E-mail: mann@bmb.sdu.dk.

\*\* To whom correspondence may be addressed. Tel.: 45-65502368; Fax: 45-65932661; E-mail: jenseno@bmb.sdu.dk.

§§ Supported by Howard Temin Award CA 75447 from the NCI, National Institutes of Health and by a travel grant from the Plasmid Foundation, Roskilde, Denmark. To whom correspondence may be addressed: Visiting Scientist, Center of Experimental Bioinformatics, University of Southern Denmark, Odense, Denmark. Tel.: 45-65502366; Fax: 45-65933018; E-mail: pandey@cebi.sdu.dk.

#### REFERENCES

1. Krebs, E. G. (1983) Historical perspectives on protein phosphorylation and a classification system for protein kinases. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **302**, 3–11
2. Pawson, T., and Scott, J. D. (1997) Signaling through scaffold, anchoring, and adaptor proteins. *Science* **278**, 2075–2080
3. Hunter, T., Alexander, C. B., and Cooper, J. A. (1985) Protein phosphorylation and growth control. *Ciba Found. Symp.* **116**, 188–204
4. Hunter, T. (1998) The Croonian Lecture 1997. The phosphorylation of proteins on tyrosine: its role in cell growth and disease. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **353**, 583–605
5. Posewitz, M. C., and Tempst, P. (1999) Immobilized gallium(III) affinity chromatography of phosphopeptides. *Anal. Chem.* **71**, 2883–2892
6. Cao, P., and Stults, J. T. (1999) Phosphopeptide analysis by on-line immobilized metal-ion affinity chromatography-capillary electrophoresis-electrospray ionization mass spectrometry. *J. Chromatogr. A* **853**, 225–235
7. Stensballe, A., Andersen, S., and Jensen, O. N. (2001) Characterization of phosphoproteins from electrophoretic gels by nanoscale Fe(III) affinity chromatography with off-line mass spectrometry analysis. *Proteomics* **1**, 207–222
8. Xhou, W., Merrick, B. A., Khaledi, M. G., and Tomer, K. B. (2000) Detection and sequencing of phosphopeptides affinity bound to immobilized metal ion beads by matrix-assisted laser desorption/ionization mass spectrometry. *J. Am. Soc. Mass Spectrom.* **11**, 273–282
9. Oda, Y., Nagasu, T., and Chait, B. T. (2001) Enrichment analysis of phosphorylated proteins as a tool for probing the phosphoproteome. *Nat. Biotechnol.* **19**, 379–382
10. Goshe, M. B., Conrads, T. P., Panisko, E. A., Angell, N. H., Veenstra, T. D., and Smith, R. D. (2001) Phosphoprotein isotope-coded affinity tag approach for isolating and quantitating phosphopeptides in proteome-wide analyses. *Anal. Chem.* **73**, 2578–2586
11. Zhou, H., Watts, J. D., and Aebersold, R. (2001) A systematic approach to

- the analysis of protein phosphorylation. *Nat. Biotechnol.* **19**, 375–378
12. Aebersold, R., and Goodlett, D. R. (2001) Mass spectrometry in proteomics. *Chem. Rev.* **101**, 269–295
  13. Resing, K. A., and Ahn, N. G. (1999) Applications of mass spectrometry to signal transduction. *Prog. Biophys. Mol. Biol.* **71**, 501–523
  14. Pandey, A., Andersen, J. S., and Mann, M. (2000) Use of mass spectrometry to study signaling pathways. *Science's STKE stke.sciencemag.org/cgi/content/full/OC\_sigtrans;2000/37/pl1*
  15. Jensen, O. N., Larsen, M. R., and Roepstorff, P. (1998) Mass spectrometric identification and microcharacterization of proteins from electrophoretic gels: strategies and applications. *Proteins* **33**, Suppl. 2, 74–89
  16. Gobom, J., Nordhoff, E., Mirgorodskaya, E., Ekman, R., and Roepstorff, P. (1999) Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* **34**, 105–116
  17. Jay, D., and Stracher, A. (1997) Expression in *Escherichia coli*, phosphorylation with cAMP-dependent protein kinase and proteolysis by calpain of a 71-kDa domain of human endothelial actin binding protein. *Biochem. Biophys. Res. Commun.* **232**, 555–558
  18. Goldmann, W. H. (2001) Phosphorylation of filamin (ABP-280) regulates the binding to the lipid membrane, integrin, and actin. *Cell Biol. Int. Rep.* **25**, 805–808
  19. Liu, G., Thomas, L., Warren, R. A., Enns, C. A., Cunningham, C. C., Hartwig, J. H., and Thomas, G. (1997) Cytoskeletal protein ABP-280 directs the intracellular trafficking of furin and modulates proprotein processing in the endocytic pathway. *J. Cell Biol.* **139**, 1719–1733
  20. Li, M., Bermak, J. C., Wang, Z. W., and Zhou, Q. Y. (2000) Modulation of dopamine D(2) receptor signaling by actin-binding protein (ABP-280). *Mol. Pharmacol.* **57**, 446–452
  21. Jay, D., Garcia, E. J., Lara, J. E., Medina, M. A., and de la Luz Ibarra, M. (2000) Determination of a cAMP-dependent protein kinase phosphorylation site in the C-terminal region of human endothelial actin-binding protein. *Arch. Biochem. Biophys.* **377**, 80–84
  22. Kreis, T., and Vale, R. (1999) *Guidebook to the Cytoskeletal and Motor Proteins* 2nd Ed., Oxford University Press, Oxford, UK
  23. Xu, X. S., Lee, E., Chen, T., Kuczmarski, E., Chisholm, R. L., and Knecht, D. A. (2001) During multicellular migration, myosin II serves a structural role independent of its motor function. *Dev. Biol.* **232**, 255–264
  24. Luo, X., Crawley, S. W., Steimle, P. A., Egelhoff, T. T., and Cote, G. P. (2001) Specific phosphorylation of threonine by the Dictyostelium myosin II heavy chain kinase family. *J. Biol. Chem.* **276**, 17836–17843
  25. Shirao, T., and Obata, K. (1985) Two acidic proteins associated with brain development in chick embryo. *J. Neurochem.* **44**, 1210–1216
  26. Shirao, T., Kojima, N., Kato, Y., and Obata, K. (1988) Molecular cloning of a cDNA for the developmentally regulated brain protein, drebrin. *Brain Res.* **464**, 71–74
  27. Shirao, T., Kojima, N., and Obata, K. (1992) Cloning of drebrin A and induction of neurite-like processes in drebrin-transfected cells. *Neuroreport* **3**, 109–112
  28. Toda, M., Shirao, T., Minoshima, S., Shimizu, N., Toya, S., and Uyemura, K. (1993) Molecular cloning of cDNA encoding human drebrin E and chromosomal mapping of its gene. *Biochem. Biophys. Res. Commun.* **196**, 468–472
  29. Shirao, T., Hayashi, K., Ishikawa, R., Isa, K., Asada, H., Ikeda, K., and Uyemura, K. (1994) Formation of thick, curving bundles of actin by drebrin A expressed in fibroblasts. *Exp. Cell Res.* **215**, 145–153
  30. Hayashi, K., Ishikawa, R., Kawai-Hirai, R., Takagi, T., Taketomi, A., and Shirao, T. (1999) Domain analysis of the actin-binding and actin-remodeling activities of drebrin. *Exp. Cell Res.* **253**, 673–680
  31. Sasaki, Y., Hayashi, K., Shirao, T., Ishikawa, R., and Kohama, K. (1996) Inhibition by drebrin of the actin-bundling activity of brain fascin, a protein localized in filopodia of growth cones. *J. Neurochem.* **66**, 980–988
  32. Ishikawa, R., Hayashi, K., Shirao, T., Xue, Y., Takagi, T., Sasaki, Y., and Kohama, K. (1994) Drebrin, a development-associated brain protein from rat embryo, causes the dissociation of tropomyosin from actin filaments. *J. Biol. Chem.* **269**, 29928–29933
  33. Mukai, H., Toshimori, M., Shibata, H., Takanaga, H., Kitagawa, M., Miyahara, M., Shimakawa, M., and Ono, Y. (1997) Interaction of PKN with alpha-actinin. *J. Biol. Chem.* **272**, 4740–4746
  34. Otey, C. A., Pavalko, F. M., and Burridge, K. (1990) An interaction between alpha-actinin and the beta 1 integrin subunit *in vitro*. *J. Cell Biol.* **111**, 721–729
  35. Otey, C. A., Vasquez, G. B., Burridge, K., and Erickson, B. W. (1993) Mapping of the alpha-actinin binding site within the beta 1 integrin cytoplasmic domain. *J. Biol. Chem.* **268**, 21193–21197
  36. Izaguirre, G., Aguirre, L., Ji, P., Aneskievich, B., and Haimovich, B. (1999) Tyrosine phosphorylation of alpha-actinin in activated platelets. *J. Biol. Chem.* **274**, 37012–37020
  37. Izaguirre, G., Aguirre, L., Hu, Y. P., Lee, H. Y., Schlaepfer, D. D., Aneskievich, B. J., and Haimovich, B. (2001) The cytoskeletal/non-muscle isoform of alpha-actinin is phosphorylated on its actin-binding domain by the focal adhesion kinase. *J. Biol. Chem.* **276**, 28676–28685
  38. Calado, A., Kutay, U., Kuhn, U., Wahle, E., and Carmo-Fonseca, M. (2000) Deciphering the cellular pathway for transport of poly(A)-binding protein II. *RNA* **6**, 245–256
  39. Brais, B., Bouchard, J. P., Xie, Y. G., Rochefort, D. L., Chretien, N., Tome, F. M., Lafreniere, R. G., Rommens, J. M., Uyama, E., Nohira, O., Blumen, S., Korczyn, A. D., Heutink, P., Mathieu, J., Duranceau, A., Codere, F., Fardeau, M., Rouleau, G. A., and Korczyn, A. D. (1998) Short GCG expansions in the PABP2 gene cause oculopharyngeal muscular dystrophy. *Nat. Genet.* **18**, 164–167
  40. Kim, Y. J., Noguchi, S., Hayashi, Y. K., Tsukahara, T., Shimizu, T., and Arahata, K. (2001) The product of an oculopharyngeal muscular dystrophy gene, poly(A)-binding protein 2, interacts with SKIP and stimulates muscle-specific gene expression. *Hum. Mol. Genet.* **10**, 1129–1139
  41. Le, H., Browning, K. S., and Gallie, D. R. (2000) The phosphorylation state of poly(A)-binding protein specifies its binding to poly(A) RNA and its interaction with eukaryotic initiation factor (eIF) 4F, eIFiso4F, and eIF4B. *J. Biol. Chem.* **275**, 17452–17462
  42. Peri, S., and Pandey, A. (2001) A reassessment of the translation initiation codon in vertebrates. *Trends Genet.* **17**, 685–687
  43. Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998) SMART, a simple modular architecture research tool: identification of signaling domains. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 5857–5864
  44. Sonnhammer, E. L., Eddy, S. R., Birney, E., Bateman, A., and Durbin, R. (1998) Pfam: multiple sequence alignments and HMM-profiles of protein domains. *Nucleic Acids Res.* **26**, 320–322
  45. Pandey, A., Podtelejnikov, A. V., Blagoev, B., Bustelo, X. R., Mann, M., and Lodish, H. F. (2000) Analysis of receptor signaling pathways by mass spectrometry: identification of Vav-2 as a substrate of the epidermal and platelet-derived growth factor receptors. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 179–184
  46. Steen, H., Kuster, B., Fernandez, M., Pandey, A., and Mann, M. (2002) Tyrosine phosphorylation mapping of the epidermal growth factor receptor signaling pathway. *J. Biol. Chem.* **277**, 1031–1039
  47. Hauschildt, S., Schwarz, C., Heine, H., Ulmer, A. J., Flad, H. D., Rietschel, E. T., Jensen, O. N., and Mann, M. (1997) Actin: a target of lipopolysaccharide-induced phosphorylation in human monocytes. *Biochem. Biophys. Res. Commun.* **241**, 670–674