The regulation of the multifunctional calcium/calmodulin-dependent protein kinase II (CaMKII) by serine/threonine protein phosphatases has been extensively studied in neuronal cells; however, this regulation has not been investigated previously in fibroblasts. We cloned a cDNA from SV40-transformed human fibroblasts that shares 80% homology to a rat calcium/calmodulin-dependent protein kinase phosphatase that encodes a PPM1F protein. By using extracts from transfected cells, PPM1F, but not a mutant (R326A) in the conserved catalytic domain, was found to dephosphorylate in vitro a peptide corresponding to the autoinhibitory region of CaMKII. Further analyses demonstrated that PPM1F specifically dephosphorylates the phospho-Thr-286 in autophosphorylated CaMKII substrate. Furthermore, overexpression of PPM1F in fibroblasts caused a reduction in the CaMKII-specific phosphorylation of the known substrate vimentin (Ser-82) following calcium/mobilizing agents, CaMKII is autophosphorylated (at Thr-286 for CaMKIIα) generating a kinase with Ca2+-independent activity (autonomous activity); however, this activity rapidly declines on removal of the stimulus (2–4), suggesting that protein phosphatases may contribute to the regulation of CaM kinase. Dephosphorylation of Thr-286 by the serine/threonine protein phosphatases PP1 (5), PP2A (6), and PP2C (7) has been shown to occur in vitro, and the phosphatase(s) responsible for regulating the endogenous CaMKII activity have been identified in a variety of cell types (7–15).

Because CaMKII comprises up to 2% of the total protein in some regions of the brain, multiple investigations into the regulation of this kinase by phosphatases have been done with tissue from the central nervous system (7–12, 15). In the rat forebrain, the predominant phosphatase varies with the distinct cellular compartment; PP2A dephosphorylates soluble CaMKII, whereas PP1 acts on postsynaptic density-associated kinase (8). In the case of rat cerebellar granule cells, the predominant phosphatase activity in cytosolic extracts is PP2C (7). More limited studies have examined the regulation of CaMKII by phosphatases in non-neuronal cells, including canine vascular smooth muscle cells (16, 17), mouse pancreatic β-cells (14), and rat pancreatic acinar cells (13).

However, CaMKII regulation has not been evaluated in fibroblasts. CaMKII has been shown to be expressed in these cells based on the detection of kinase activity in the following rodent and human fibroblast cell lines: NIH 3T3 (mouse embry) (18); 3Y1 (rat embryo) (19); WI38 (human embryonic lung) (20, 21); and GM38 (human foreskin) (22). Hence, this kinase may play a critical role in the Ca2+-mediated signaling cascades that have been shown to be required for cell cycle progression in fibroblasts (23–25). In support of this possibility, treatment of proliferating NIH 3T3 cells with KN-93, a membrane-permeable synthetic inhibitor of CaMKII, induced G1 arrest (18). In addition, CaMKII contributes to the S-phase delay in the cell cycle of γ-irradiated human fibroblasts; ataxia telangiectasia fibroblasts fail to undergo this delay because of the inability of these cells to activate CaMKII following γ-irradiation (22), and normal fibroblasts treated with KN-62, a pharmacological inhibitor of CaM kinases, exhibit an “ataxia telangiectasia-like” phenotype after γ-ray treatment (26). As with it have been the focus of several studies. In response to Ca2+-mobilizing agents, CaMKII is autophosphorylated (at Thr-286 for CaMKIIα) generating a kinase with Ca2+-independent activity (autonomous activity); however, this activity rapidly declines on removal of the stimulus (2–4), suggesting that protein phosphatases may contribute to the regulation of CaM kinase. Dephosphorylation of Thr-286 by the serine/threonine protein phosphatases PP1 (5), PP2A (6), and PP2C (7) has been shown to occur in vitro, and the phosphatase(s) responsible for regulating the endogenous CaMKII activity have been identified in a variety of cell types (7–15).

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PPM1F Regulates CaMKII in Fibroblasts

has been observed with neuronal cells, the increase in autonomaous CaMKII activity was transient in the γ-irradiated normal fibroblasts, suggesting that the rapid decline in kinase activity is the result of phosphatase regulation (22). Presently, very little is known about the phosphatases that regulate CaMKII in fibroblasts. There is indirect evidence to indicate that this regulation does occur. The finding that Swiss 3T3 fibroblasts, pretreated with KN-93, were protected from apoptosis induced by nodulin-1, a cyclic pentapeptide inhibitor of PP1 and PP2A, suggests that the inhibition of these phosphatases can lead to CaM kinase deregulation and subsequent apoptosis in fibroblasts (27). We previously identified a cDNA (termed J4-3) from a subtractive hybridization screen of SV40-transformed human fibroblasts (28) that matched the 3′-untranslated region of a cDNA (KIAA0015) cloned from a human myeloid cell line (29) that contains a putative PP2C phosphatase motif. Since then, two groups of investigators have independently isolated the same cDNA from different sources and have named it hFEM-2 (30) and POPX2 (31), depending on its identified function. Both groups recognized what we had found earlier during the course of our investigation, the human sequence shares 80% identity with the rat Ca2+/calmodulin-dependent protein kinase phosphatase (rcaMKPase) (32); however, neither group fully investigated this possible function of the gene, other than to demonstrate that a partially purified hFEM-2 could dephosphorylate CaMKII in vitro (30). The rCaMKPase has characteristics similar to members of the PP2C family, including a dependence on divalent cations for activity and insensitivity to okadaic acid (OA), and has been shown to dephosphorylate as well as deactivates autophosphorylated CaMKII in vitro (33). This phosphatase substantiates the findings in previous studies (7, 8, 10, 14) that PP2C-like phosphatases play a role in regulating CaM kinase activity. To assess CaMKII regulation in fibroblasts, we determined whether J4-3, which we name PPM1F in accordance with the PP2C family designation as given by the Human Genome Nomenclature Commission, functions as CaM kinase phosphatase. Our results show that like its rat homolog PPM1F specifically dephosphorylates CaMKII in vitro. Moreover, we demonstrate by immunoprecipitation (co-IP) that the two proteins interact intracellularly and, most importantly, that PPM1F can inhibit the in vitro autophosphorylation of a CaMKII-specific substrate by the endogenous CaM kinase, indicating that PPM1F can contribute to the regulation of CaMKII in fibroblasts.

EXPERIMENTAL PROCEDURES

Cell Culture—The normal fetal human diploid bone marrow fibroblast HST4, its SV40-transformed immortal cell sublines, and HEK293T were cultured in Dulbecco’s modified Eagle’s medium/F-10 medium (Invitrogen) and 10% fetal bovine serum (Sigma) as described previously (34). Chinese hamster fibroblast CHO-K1 was cultured in Dulbecco’s modified Eagle’s medium/F-12 medium (Invitrogen) and 10% fetal bovine serum as described previously (35). Mouse NIH 3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 1 mM sodium pyruvate (Sigma) and 10% bovine calf serum (Sigma) as described previously (36). All cell lines were maintained in complete medium at 37 °C.

Cloning of PPM1F and Construction of Deletion Mutants—The PPM1F-A cDNA (full-length J4-3) was generated from an SV40-transformed nonimmortal human diploid fibroblast cell line by reverse transcription with an oligo(dT) primer by using the SuperScript™ Preamplification System (Invitrogen) followed by PCR with DNA primers flanking the largest open reading frame of the cDNA by using Taq polymerase (Roche Diagnostics). PPM1F-A was cloned into pIREs2EGFP (Clontech), and an HA epitope tag was introduced at the C-terminal end by ligating a double-stranded oligo to the BstAPI (SibEnzyme) site overhang. In a 4-amino acid truncated protein that we termed PPM1F-B. The construct carrying the HA epitope-tagged PPM1F-B (PPM1F-B-HA) under the control of a CMV promoter was named pPPM1F-B-HA/IG. The CMV-IE promoter/PPM1F-B-HA/IRE5/EGFP/SV40 poly(A) cassette was subcloned in a vector carrying a Pac gene under the control of an SV40 origin-defective early promoter resulting in the pPPM1F-B-HA/IGPac construct. The PPM1F cDNA was generated from the ovarian cancer cell line SKOV3 using the same protocol as described for PPM1F-A and was cloned into pVAx1 (Invitrogen). The nontagged version of the PPM1F Δ13–94 mutant was generated by the ligation of MscI and filled in EcoRI overhang (New England Biolabs) within the PPM1F cDNA. The PPM1F cDNA was ligated into the SalI (Invitrogen) site of pCMV-Myc (Clontech). As a consequence of generating the construct pCMV-Myc-PPM1F, an additional 33 amino acids, including the Myc epitope tag, are present at the N terminus of the Myc-PPM1F protein. All subsequent deletion mutants of PPM1F were generated by using pCMV-Myc as the vector and consequently contain the additional amino acids unless otherwise specified. The PPM1F Δ13–94 gene was subcloned into the SalI site to give the Myc-PPM1F Δ13–94 mutant. The ligation of the SrfI (Stratagene) overhang from PPM1F with that of SalI from the vector generated the Δ1–148 mutant that has only 23 of the 33 additional amino acids mentioned earlier. The large internal deletion mutant Δ148–397 was constructed by ligating the two small (86 and 33 amino acids) pieces from the Δ300–454 deletion mutant in PPM1F that were the farthest apart from each other. To generate the Δ300–454 deletion mutant, the filled in overhangs of BssHII (New England Biolabs) from PPM1F and NotI (New England Biolabs) from the vector were ligated together. As a result of this ligation, the stop codon fell within the vector sequence 7 amino acids downstream of the PPM1F sequence. The remainder of the C-terminal deletion mutants of PPM1F, which include Δ411–454, Δ417–454, Δ419–454, Δ422–454, Δ433–454, and Δ441–454, were constructed by Expand High Fidelity PCR (Roche Diagnostics) by using a common 5′ DNA primer corresponding to PPM1F sequence directly proximal to the open reading frame together with an Xhol restriction site. Specific 3′ primers encode six amino acids immediately downstream of the stop codon, a stop codon at the position of the truncation and a BglII (Roche Diagnostics) restriction site. The missense mutant R326A was generated by the same PCR method by using a 5′ primer containing a conversion of the AGA codon (976–978 bp of the PPM1F cDNA) to that of GCA as well as a HindIII (New England Biolabs) restriction site, and a 3′ primer corresponding to the distal open reading frame sequence, including the stop codon together with a BstAPI restriction site. The digested PCR product was incorporated into the PPM1F cDNA by replacing the original HindIII/BstAPI fragment. All PPM1F constructs were sequenced in their entirety to verify that no other mutations were present. DNA primers and sequencing were provided by the Molecular Resource Facility of the UMDNJ-New Jersey Medical School.

Preparation of Phosphatase Assay Substrate—AutoCaMtid3 (Invitrogen) at a concentration of 100 μM was phosphorylated in vitro with 500 ng of purified CaMKII (Upstate Biotechnology, Inc.), as described previously (37), with the addition of 1 mM CaCl2 and 20 μM/l calmodulin to the reaction mixture (175-μl ATP, 6,000 Ci/mmol, PerkinElmer Life Sciences). The reaction was diluted with 3X phosphatase reaction buffer containing 33.3 mM Tris-HCl (pH 7.5) and 0.33 mM EGTA (pH 7.5) to a final concentration of 25 μM 32P-AutoCaMtid3. Labeled peptide was separated from kinase protein by centrifugation through Microcon-10 microconcentrators (Millipore).

Phosphatase Assays—Depending on the experimental conditions, phosphatase assays were performed to evaluate the activity of exogenous PPM1F in a variety of cell types. For the time course analysis by assaying the immunoprecipitation experiments, CHO-K1 were seeded at 7.5 × 104 cells per 60-mm plate and then transiently transfected using Lipofectamine2000™ (Invitrogen) with 10 μg of pPPM1F-B-HA/IG or vector control plasmid for 5 h. These cells were then harvested 40 h later for the preparation of the phosphatase deletion mutants of PPM1F, HEK293T cells were seeded at 3.5 × 106 cells per 60-mm plate and then transiently transfected overnight using Lipofectamine2000™ with 10 μg of pCMV-Myc-PPM1F or with specific deletion mutants. These cells were then harvested 18 h post-transfection for assay. Cell extract was prepared in a manner similar to that described by Dhawan et al. (35) with exclusion of phosphatase inhibitors from the extraction buffer (referred to as modified PIPES-buffered extraction solution). For each sample, an aliquot was used for protein estimation with the Bio-Rad protein assay and for Western blot analysis with the mouse monoclonal anti-HA epitope or

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anti-Myc epitope antibodies (Santa Cruz Biotechnology, 0.2 µg/ml) and with mouse monoclonal anti-α-actinin antibody (Sigma, 1:1000) to ensure that equal amounts of protein were loaded. The blots were then stained with horseradish peroxidase-conjugated rat anti-mouse χ light chain-specific IgG (Zymed Laboratories Inc.) as the secondary antibody. Enhanced chemiluminescence was performed by using Western Light-ninging Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). To determine the activity directly in lysates, the extract supernatant was diluted 1:4 in excess extraction buffer. Phosphatase reactions were initiated with the addition of an aliquot of diluted supernatant to a 30 °C preincubated reaction mixture consisting of the following: 7.5 µM AutoCaMtk3 with 8-fold excess of CaMKII-specific inhibitors (PPM1F–H9262, PPM1F–H9251, and PPM1F–H9004) (39) and was incubated for 1 h at 37 °C. The immunoprecipitate was centrifuged and washed three times at 4 °C as follows: once with ice-cold modified RIPA buffer containing 0.1 M sodium chloride, and then twice with ice-cold modified RIPA buffer containing 0.15 M sodium chloride. The immunoprecipitate was resuspended in modified RIPA buffer and SDS loading buffer subjected to Western blot analysis with rabbit polyclonal anti-CaMKII and anti-Myc epitope-specific antibodies. For each sample, 20 µg of cell lysate was analyzed by Western blot with the same anti-CaMKII antibody.

CoMKII-specific Phosphorylation of Vimentin—For the experiments with endogenous CaMK kinase, NIH 3T3 cells were seeded at 1.5 × 10^5 cells per 100-mm plate (3 plates per plasmid) and then were transiently transfected with pCMV-CaMKII-(1-492) (gift from Dr. Thomas R. Soderling) and 10 µg of pCMV-PPM1F-HA/IGPac or vector. Eighteen hours post-transfection, cells from each 100-mm plate were transferred into a single 150-mm plate with complete medium containing 4 µg/ml puromycin (Sigma). Twelve hours prior to cell harvesting, surviving cells from all three plates of the same plasmid were consolidated into a single 60-mm plate containing selection medium. For the experiments with the endogenous CaMK kinase, the cells, after 48 h of puromycin selection, were treated with 1 µM ionomycin or MeSO (for 5 min at 37 °C). Cell extracts were prepared as described previously by Kitani et al. (42) with the additional step of passing each Nonidet P-40-insoluble pellet through a 25-gauge syringe needle (at least four times). An aliquot from each sample was removed prior to the addition of SDS loading buffer and was used for protein estimation with the DC Protein Assay kit (Bio-Rad). For the experiments with the PCV-PPM1F vector, cells were transiently transfected with 3 µg of pSV-Sv-CaMKII (1-290) (a gift from Dr. Thomas R. Soderling) and 10 µg of pCMV-PPM1F-HA/IGPac or vector. Twelve hours prior to cell harvesting, surviving cells from all three plates of the same plasmid were consolidated into a single 60-mm plate containing selection medium. For the experiments with the endogenous CaMK kinase, the cells, after 48 h of puromycin selection, were treated with 1 µM ionomycin or MeSO (for 5 min at 37 °C). Cell extracts were prepared as described previously by Kitani et al. (42) with the additional step of passing each Nonidet P-40-insoluble pellet through a 25-gauge syringe needle (at least four times). An aliquot from each sample was removed prior to the addition of SDS loading buffer and was used for protein estimation with the DC Protein Assay kit. Ten micrograms of each extract was subjected to Western blot analysis with goat anti-vimentin antiserum (Sigma, 1:1000 dilution) and then with horseradish peroxidase-conjugated rabbit anti-goat IgG (Sigma) as the secondary antibody. The amount of sample extract loaded on subsequent gels was normalized according to the uninduced vector sample. Duplicate Western blots were generated and stained with mouse monoclonal anti-phospho-vimentin (Ser-32) antibody (1:1000 dilution) (43) and mouse monoclonal anti-α-actinin antibody (1:1000 dilution) (44), gift from Dr. Masaki Inagaki (Aichi Cancer Center Research Institute, Nagoya, Aichi, Japan), or with anti-vimentin antiserum. RESULTS


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we found that the protein is being expressed in these cells (data not shown). Because of the lack of specific inhibitors and the failure of the PPM1F-454C antibody to immunoprecipitate sufficient levels of endogenous phosphatase, it was not possible to discriminate among PPM1 activities in these cells, and consequently, the endogenous level of PPM1F-A activity could not be assessed.

While these studies were ongoing, the putative PPM1F-A sequence was found to share 80% identity with rCaMKPase (32), suggesting that PPM1F could act as a CaM kinase phosphatase. The rCaMKPase has been shown to dephosphorylate specifically and to deactivate CaMKI, -II, and -IV (45). We focused our efforts on CaMKII as a potential substrate in HSF4 based on the fact that it is a multifunctional kinase and is widely expressed. The expression of CaMKII in HF lysates was assessed by an in vitro CaMKII-specific assay, and we found that the kinase is present in these cells at a level of activity (data not shown) similar to that reported for the normal lung fibroblast cell line WI38 (20).

Because both proteins are present in HF, we proceeded to determine whether CaMKII could be a substrate for PPM1F. Our first approach was to use [32P]-AutoCaMtide3 as the substrate for in vitro phosphatase assays. An analogous peptide substrate, which also contained the Thr-286 autophosphorylation site of CaMKIIα, had been identified (37) and characterized (33) rCaMKPase as a CaM kinase phosphatase in vitro. A C-terminal hemagglutinin (HA) epitope-tagged PPM1F-A was generated in the mammalian expression plasmid pRES2EGFP (see “Experimental Procedures”) and was termed PPM1F-B-HA. The resulting construct (pPPM1F-B-HA/Ig) and the parent vector were transfected separately into CHO-K1 cells, and extracts were prepared 40 h post-transfection. Because cell lysates contained endogenous CaMKII as well as several phosphatases, we included CaM-KIINtide to block CaMKII phosphorylation of AutoCaMtide3 and OA to inhibit the majority of protein serine/threonine phosphatases with the notable exception of those belonging to the PPM1 class. The substrate for the phosphatase assay was generated by the kinase reaction using commercially available CaMKII with AutoCaMtide3 in the presence of [γ-32P]ATP. The final product of the phosphatase reaction, released [32P]PO4, was determined by the difference between filter-bound substrate with total incorporation (starting control) and the residual [32P] following exposure to lysate (experimental sample). The data in Fig. 1 were calculated after normalization of the activity of each sample to its total protein concentration. An increase in total phosphatase activity was observed as a result of PPM1F-B-HA transfection. Furthermore, in the presence of 10 μM OA and 10 μM CaM-KIINtide, the lysate from cells overexpressing PPM1F-B-HA showed 45% released [32P]PO4 after 2.5 min as compared with 0% from vector-transfected cells, indicating that PPM1F is directly or indirectly responsible for the dephosphorylation of [32P]-AutoCaMtide3. This type of analysis was used to test phosphatase activity of various full-length and deletion mutant clones of PPM1F generated during the course of this investigation (data summarized in Table I), and expression of each protein was verified by Western blot analysis of cell extracts.

Other studies have identified an OA-resistant/Mg2+-dependent phosphatase in the extracts of different cell types able to dephosphorylate CaMKII (7, 8, 14). To address whether PPM1F was dephosphorylating the peptide directly or acting through another phosphatase in the lysate, PPM1F-B-HA was immunoprecipitated by using anti-HA antibody, and the immunoprecipitate was tested for phosphatase activity in vitro in the presence of 10 μM OA. The PPM1F-B-HA immunoprecipitate released 70% of the incorporated radioactivity, whereas there was no radioactivity released with the vector sample (Fig. 2), indicating that PPM1F is directly responsible for the removal of [32P]PO4 from AutoCaMtide3. These data therefore show that PPM1F can function as a phosphatase when overexpressed in mammalian cells and support the possibility that the phospho-Thr-286 of CaMKIIα could be a target for dephosphorylation by this phosphatase.

### Table I

Summary of phosphatase and CaMKII binding activities of the various PPM1F mutants

| Phosphatase reaction substrate | CaMtide | CaMKII protein | CaMKII binding |
|-------------------------------|---------|----------------|----------------|
| **Non-Myc Tag PPM1F**         |         |                |                |
| PPM1F                         | +       | ND             | ND             |
| Δ13–94                        | −       | ND             | ND             |
| PPM1F-(S2C)/(S124N)           | +       | ND             | ND             |
| PPM1F-B-HA                    | +       | ND             | NL             |
| **Myc Tag PPM1F**             |         |                |                |
| PPM1F                         | +       | +              | NL             |
| R286A                         | −       | ND             | NL             |
| Δ13–94                        | −       | ND             | ND             |
| Δ11–148                       | −       | ND             | E              |
| Δ148–397                      | ND      | ND             | E              |
| Δ500–454                      | −       | ND             | R              |
| Δ411–454                      | −       | ND             | R              |
| Δ417–454                      | −       | ND             | ND             |
| Δ419–454                      | −       | −              | −              |
| Δ422–454                      | +       | +              | NL             |
| Δ422–454 (V174M)(R398Q)       | −       | ND             | ND             |
| Δ443–454                      | +       | +              | NL             |
| Δ441–454                      | +       | +              | NL             |
activation process of this kinase (46, 47), and the dephosphorylation of this residue by a variety of phosphatases has been demonstrated to be a critical step in regulating CaMKII activity (5–7). We therefore evaluated the ability of PPM1F to specifically dephosphorylate the Thr-286 residue of the CaMKII polypeptide by utilizing Western analysis with antibody that specifically recognizes the Thr(P)286 modified form of the protein. To eliminate the possibility that the two amino acid differences in PPM1F-A may result in aberrant phosphatase function toward CaMKII, we used a PPM1F cDNA that we had isolated earlier from the human ovarian cancer cell line SKOV3 and had found to be a 100% match to KIAA0015. When the phosphatase activity of the newly cloned PPM1F was tested along with PPM1F-A in the in vitro phosphatase assay, the activities of PPM1F and PPM1F-A were essentially the same, 9.42 and 11.7 pmol [32P]AutoCaMide3/mg total cellular protein, respectively. To facilitate detection and IP of PPM1F, an N-terminal Myc epitope-tagged PPM1F (Myc-PPM1F) was generated. Deletions in PPM1F were generated to determine the regions of the phosphatase that may be required for proper CaMKII regulation. Comparison of sequence and structural domains of PPM1F with the crystalized PP2Ca (PPM1A) indicated that amino acids within 140–454 of PPM1F were conserved. By taking this into consideration, an N-terminal deletion mutant Myc-PPM1FΔ1–148 was made by using a convenient EcoRI restriction site. This mutant corresponds to a similar deletion described above. Following the phosphatase reaction, aliquots (~40 μg of total cellular protein per reaction) of CaMKII-containing lysate were added to limiting autophosphorylation reactions followed by the addition of 50 μM KN-93. Aliquots of the activated CaM kinase were added to a phosphatase reaction mixture containing 2 mM MnCl2, 10 μM OA, and 50 μM KN-93, and the reactions were initiated by the addition of extract (~50 μg of total cellular protein per reaction) prepared from cells transfected with Myc-PPM1F or related construct. The reaction mix was incubated for 30 min at 23 °C before being terminated with the addition of SDS loading buffer. Duplicate samples were pooled and then subjected to Western blot analysis with anti-phospho-CaMKIIαβ (Thr-286/Thr-287) (upper panel), anti-CaMKIIα (middle panel), and anti-Myc epitope (lower panel) antibodies.

Because PPM1F was found to dephosphorylate CaM kinase, we assessed whether this had an effect on kinase activity. We exogenously activated CaMKIIα and subjected it to phosphatase treatment with PPM1F in vitro in a manner similar to that described above. Following the phosphatase reaction, β-glycerophosphate, a nonspecific phosphatase inhibitor shown to inhibit the rCaMKPase (33), was added to inhibit further phosphatase activity during the remainder of the assay. (The
inclusion of this inhibitor was found to be necessary based on preliminary results indicating that PPM1F was dephosphorylating the product of the CaM kinase reaction. We determined that 80 mM β-glycerophosphate (as the final concentration in the CaM kinase reaction) could inhibit nearly 100% of PPM1F activity (data not shown) and had no detrimental effect on CaM kinase activity (data not shown) as reported previously (22, 51). Aliquots of the phosphatase-treated mCaM kinase were tested for autonomous kinase activity by the in vitro CaM kinase assay in the presence of KN-93 and OA. In the absence of PPM1F (Fig. 4, CaMKII/Vec2 extract), the level of CaM kinase activity was 159 ± 10^2 pmol 32P/min/mg greater than background; however, this level was reduced to 25 ± 10^2 pmol 32P/min/mg, nearly that of background, upon treatment with PPM1F (Fig. 4, CaMKII/PPM1F extract), indicating that PPM1F was able to deactivate effectively the autophosphorylated CaMKII. The failure of R326A to affect CaM kinase activity indicates that the effect of PPM1F is not because of the phosphatase blocking CaMKII access to its substrate but rather because of its enzymatic activity.

Intracellular Binding of CaMKII by PPM1F—A variety of proteins has been shown to interact with CaMKII such as α-actinin-1 (38), syntaxin 1A (52), and the NR2A subunit of N-methyl-D-aspartate receptor (53). In particular, the catalytic subunit of protein phosphatase 2A has been found to coimmunoprecipitate with CaMKII from rat ventricular myocyte lysates (54), demonstrating a physical association of CaM kinase with one of its regulating phosphatases. The ability of PPM1F to dephosphorylate and deactivate CaMKII in vitro suggests that the two proteins may also interact. To determine whether this interaction could occur intracellularly, we performed co-IP experiments with mCaMKIIα and Myc-PPM1F (see Fig. 5).

In addition, several deletion mutants of PPM1F were assessed to identify the region responsible for binding. Because there are no amino acid sequences in PPM1F that match those identified in other proteins to be responsible for CaMKII binding (38, 52, 53), we initially utilized the mutants described earlier. Full-length as well as mutants of Myc-PPM1F were transiently cotransfected with mCaMKIIα into HEK293T, and immunoprecipitates were generated using anti-Myc antibody under stringent wash conditions. The presence of CaMKII was determined by Western blot analysis of immunoprecipitates with anti-CaMKII antibody. Exogenous CaM kinase was found to be coin-IP with full-length PPM1F (Fig. 5, lanes 3 and 6), whereas we did not detect binding of an endogenous protein to the phosphatase (Fig. 5, lane 1), indicating that PPM1F was interacting with CaMKIIα. As for identifying the binding domain of PPM1F, we began our analysis by investigating whether the N terminus of PPM1F played a role in binding to CaMKII based on the uniqueness of this region as well as on the finding that POPX2 (identical to PPM1F) bound to β1-PIX through this domain (31). To test this hypothesis, we examined the Δ1–148 mutant in co-IP experiments and found that this mutant was able to bind CaM kinase (Fig. 5, lane 4). Initial attempts to construct a truncated protein with only the N terminus of PPM1F failed to generate stable protein (data not shown). We therefore assessed the Δ148–397 mutant, which was devoid of most of the conserved phosphatase domain but contained the entire N-terminal region, to determine whether a protein consisting mainly of this domain could bind to CaMKII. Even though the Δ148–397 mutant protein was relatively unstable, it reproducibly bound CaM kinase to a markedly enhanced extent (Fig. 5, lane 5), i.e. at a level similar to that of the highly stable Δ1–148 mutant. Taken together, these results indicate that neither the N terminus nor an intact phosphatase domain is solely required for the binding of PPM1F to CaMKII.

In addition, the data suggest that the C-terminal domain, which is present in both of these mutants, may be involved. To examine this possibility specifically, we generated two mutants, Δ411–454 and Δ300–454, with relatively large deletions...
within the C terminus of PPM1F. Both mutants failed to bind mCaMKII efficiently under these conditions (Fig. 5, lanes 8 and 9); however, the result may be due, at least in part, to the low level of mutant proteins in the lysates as compared with full-length protein. Several attempts were made to use equivalent amounts of Myc-PPM1F protein by adding increasing quantities of lysate containing only the mutant protein to the co-IP reaction; however, these experiments were unsuccessful. Therefore, we could not properly assess the involvement of the C-terminal domain of PPM1F in binding to CaMKII. During these experiments, we did detect a faint band corresponding to CaMKII upon prolonged exposure of the Western blot, suggesting that a potentially weaker binding site may exist in the N terminus of PPM1F because this region was present in both of the C-terminal truncation mutants. The catalytically inactive R326A mutant (Fig. 5, lane 7) confirmed the finding made with the Δ1–148 mutant that a functional phosphatase was not required for binding. Several other mutants of PPM1F were evaluated by this method to identify the CaMKII binding domain of PPM1F, and the results of those experiments along with the results from in vitro phosphatase assays for these mutants are summarized in Table I. Overall, the results of these experiments demonstrate that full-length PPM1F is able to interact physically with CaMKIIa under stringent conditions when both proteins are overexpressed.

PPM1F Inhibits Intracellular Phosphorylation of a CaMKII-specific Substrate—Because PPM1F was found to interact with CaMKII within the cell, we hypothesized that PPM1F could regulate the activity of the endogenous kinase. Our initial attempts to evaluate the effect of transiently overexpressed PPM1F on autonomous CaMKII activity were unsuccessful; therefore, we took an indirect approach by evaluating the effect of PPM1F on CaMKII-specific phosphorylation of the known endogenous substrate vimentin. It has been reported that vimentin is phosphorylated by CaMKII in the rat fibroblast cell line 3Y1 (55) and that the Ser-82 site of this intermediate filament was found to be a unique phosphorylation site for CaMKII (56). Furthermore, the phosphorylation state of vimentin has been shown to be affected by OA indicating that phosphatases are involved in its regulation (57).

To determine whether our phosphatase could inhibit CaM kinase activity within the cell, we evaluated the effect of PPM1F on the phosphorylation state of the CaMKII-specific site Ser-82 of vimentin as detected by an antibody (MO82) that solely recognizes the phosphorylated form of this residue (43). For these experiments, we used PPM1F-B-HA, based on previous experiments showing the functionality of this construct in NIH 3T3 (data not shown), subcloned into a vector expressing GFP and containing the Pac gene. This construct (pPPM1F-B-HA/GPac) as well as parent vector were separately transfected into NIH 3T3, and 18 h post-transfection, the cells were treated with Me 2SO (−) or 1 μM ionomycin (+) for 5 min at 37 °C followed by the harvesting of the cells in a lysis buffer. The ensuing pellet was treated with a 1% SDS buffer followed by the addition of SDS loading buffer. A, replicate Western blots were generated and stained with anti-phospho-vimentin(Ser-82) antibody (MO82) (upper panel) or anti-vimentin antiserum (lower panel). B, optical density measurements of the phosphorylated and total vimentin protein bands were determined by a densitometer for three independent experiments: each experiment involved the complete process from transfection to Western analysis. The chart values represent the average ratio of the optical density values for the MO82 band to the optical density values normalized to those of the corresponding total vimentin band, and the error bars represent the range of values. *, p = 0.01 (by Student’s t test) versus Vec/Ionomycin.

Fig. 6. Reduced level of CaMKII-specific phosphorylation of vimentin in fibroblasts because of exogenous PPM1F. NIH 3T3 cells were transiently transfected with 10 μg of pPPM1F-B-HA/GPac or vector (Vec); at 18 h post-transfection, cells were refed with medium containing 4 μg/ml puromycin. Forty eight hours post-selection, the cells were treated with Me 2SO (−) or 1 μM ionomycin (+) for 5 min at 37 °C followed by the harvesting of the cells in a lysis buffer. The ensuing pellet was treated with a 1% SDS buffer followed by the addition of SDS loading buffer. A, replicate Western blots were generated and stained with anti-phospho-vimentin(Ser-82) antibody (MO82) (upper panel) or anti-vimentin antiserum (lower panel). B, optical density measurements of the phosphorylated and total vimentin protein bands were determined by a densitometer for three independent experiments: each experiment involved the complete process from transfection to Western analysis. The chart values represent the average ratio of the optical density values for the MO82 band to the optical density values normalized to those of the corresponding total vimentin band, and the error bars represent the range of values. *, p = 0.01 (by Student’s t test) versus Vec/Ionomycin.
as a quantitative assessment of the amount of phosphorylated vimentin. For this reason, the optical density ratio value for the untreated sample can be greater than or equal to 1. As seen from these results, PPM1F was consistently able to reduce the level of Ser-82 phosphorylation induced by ionomycin treatment, suggesting that PPM1F was inhibiting CaMKII. However, an alternative explanation is that PPM1F is directly dephosphorylating vimentin. To rule out this possibility, we assessed whether PPM1F would block the phosphorylation of vimentin by a cotransfected constitutively active CaMKII mutant (mCaMKIIa1–290). As seen from Fig. 7, PPM1F did not significantly affect Ser-82 phosphorylation by the constitutively active CaMKII mutant, indicating that vimentin is not being dephosphorylated by PPM1F. Taken together, these results demonstrate that exogenous PPM1F is able to regulate the intracellular activity of the endogenous CaMKII.

**DISCUSSION**

CaMKII is a multifunctional kinase that is ubiquitously expressed (58) and is able to phosphorylate a variety of substrates in vitro (1), suggesting that this kinase may contribute to the transduction of Ca^{2+}-activated signals in multiple cell types. CaMK kinase activity has been detected in human fibroblasts and has been correlated with cell cycle regulation in these cells (18, 22). However, the regulation of this kinase has not been examined previously in fibroblasts even though extensive studies have been done on neuronal tissue (7–12,15). We cloned a cDNA (PPM1F) from HF that appeared to be the human homolog of the rat CaMKPase, suggesting that this gene may be involved in the regulation of CaMK kinase in human cells. Because we were able to detect the co-expression of the two proteins in HF, we believed that CaMKII was a potential substrate for PPM1F in these cells. The dephosphorylation of 32P-AutoCaMte3 in vitro by overexpressed PPM1F provided the first indication that PPM1F was a CaM kinase phosphatase. As reported for rCaMKPase (33) as well as for hFEM-2 (30), PPM1F was found to dephosphorylate CaMKII in vitro; however, unlike the experiments with hFEM-2, the phosphorylated CaM kinase substrate used in the phosphatase reactions with PPM1F was generated under limiting autophosphorylation conditions (5 min at 5 °C as compared with 1 min at 30 °C), thereby restricting the majority of phosphorylating events to Thr-286. Furthermore, the dephosphorylation of this residue by PPM1F was monitored using the anti-Thr(P)-286 antibody.

In addition to dephosphorylating CaMKII, we found that PPM1F could deactivate this kinase in vitro. Unlike the findings made with rPP2Ca (7) and rCaMKPase (33), the reduced level of CaMKII activity could not be attributed, in part, to a concomitant reaction product dephosphorylation by PPM1F in the assay because an effective phosphatase inhibitor was included. All the in vitro analyses suggested that CaMKII was a potential substrate for PPM1F intracellularly; therefore, we proceeded to assess the interplay of these two enzymes within the cells. This is the first report of the intracellular interaction of PPM1F with CaMKII, and we were able to show that the overexpression of PPM1F in fibroblasts resulted in a reduction of the intracellular phosphorylation of a CaMKII-specific substrate (i.e. vimentin), indicating that the endogenous CaMKII can be regulated by PPM1F in fibroblasts.

In the course of identifying PPM1F as a CaM kinase phosphatase, we generated a variety of mutants within the N- and C-terminal regions of PPM1F to determine the sequences required for CaMKII regulation. The N-terminal region of PPM1F (amino acids 1–145) has been suggested to be an inhibitory domain of hFEM-2 (30). Contrary to the results reported for hFEM-2, a deletion made to the N terminus of PPM1F, such as that generated in the Δ1–148 mutant, resulted in a catalytically inactive protein even though stable protein was made. By generating a predicted protein structure of PPM1F based on a comparison of amino acid sequence and composition of PPM1F against the resolved crystal structure of human PP2C (also termed PPM1A), we believe that the failure of Δ1–148 mutant to function as a phosphatase may be explained by the fact that this truncation removes a highly conserved β-strand known to be essential in the architecture of the catalytic domain of PP2Ca (59). Because this mutant was able to bind CaMKII, the deletion of this amino acid sequence did not appear to fully disrupt the protein structure. Our result with the N-terminal deletion mutant implies that the 157–454 mutant of hFEM-2 is most likely an inactive phosphatase mutant; therefore, the apoptotic effect associated with its overexpression is not a result of its catalytic function as proposed by Tan et al. (30).

Similar to findings made with the N-terminal mutants, large deletions in the C-terminal region of PPM1F also resulted in catalytically inactive proteins. A smaller deletion in this region, such as that generated in the Δ419–454 mutant, also disrupted phosphatase activity but did not affect binding because this mutant was found to interact with the kinase, indicating that the catalytic function of the phosphatase is not required for complex formation. Based on the ability of the Δ418–397 and Δ419–454 mutants to bind CaMKII, sequential deletions between residues 397 and 419 may provide the proper mutants to study the involvement of the C-terminal end in associating with CaM kinase. However, the interpretation would be complicated by the fact that these mutations would disrupt the catalytic function of the protein in addition to the potential loss of CaMKII binding. We believe that the overall data from both types of deletion mutants suggest that CaMKII may interact intracellularly with PPM1F near its C-terminal end and to a limited extent at its N terminus. This interaction may occur directly or through a common co-factor that binds to PPM1F, because our experimental conditions do not eliminate this possibility.

The intracellular binding and regulation of CaMKII by PPM1F imply that the interaction of these two proteins plays a role in cellular processes. The importance of this interaction has been suggested by the finding that the overexpression of hFEM-2, a gene identical to PPM1F, induces apoptosis in HeLa as well as NIH 3T3 cells (30). A similar cellular response has been observed with the exogenous expression of other PPM1 phosphatases such as PP2Ca (60), PP2Cβ (61), and FIN13 (62).
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in other cell types. However, over the course of our analyses, we did not observe any detrimental effects associated with the overexpression of PPM1F in CHO-K1, HEK293T, NIH 3T3, or HeLa cells. The pro-apoptotic effect reported for hFEM-2 might therefore be specific to subtle culture conditions. Although we have not been able to identify a specific cellular phenotype, our finding that the exogenous expression of PPM1F can inhibit CaMKII-specific phosphorylation of vimentin indicates that this phosphatase can regulate components of the cellular cytoskeleton through its interaction with CaMKII. Because vimentin has been shown to play an important role in cell contractility, migration, and proliferation (63), the modulation of its activity by PPM1F, respectively. We appreciate the technical assistance of Tanya Inagaki and Dr. Koh-ichi Nagata, Aichi Cancer Center Research Institute, for providing us with the cDNA for mCaMKII and POPX2/H9251. We are grateful to Dr. Thomas Soderling, Oregon Health Sciences University, for providing us with the cDNA for mCaMKII and mCaMKIIa-290. We are also grateful to Dr. Masaki Inagaki and Dr. Koh-ichi Nagata, Aichi Cancer Center Research Institute, for the MO82 antibody. We thank Dr. Vasem Palejwala and Dr. Amy Kurland for isolating and cloning the cDNA PPM1F and PPM1F, respectively. We appreciate the technical assistance of Tanya Dasgupta and the technical services provided by Dr. Robert Donnelly, Molecular Resource Facility of UMDNJ-New Jersey Medical School. We also thank Dr. Krishna K. Jha and Dr. Hieronim Jakubowski for helpful discussions and for reviewing this manuscript.

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