The 47-kD Fragment of Talin Is a Substrate for Protein Kinase P

By Peter C. Simons and Laurence Elias

This laboratory has been characterizing protein serine/threonine kinase reactions of hematopoietic tissues, whose most distinguishing characteristics in vitro are stimulation with vesicular phosphatidyl glycerol, and the ability to function using Mn\(^{2+}\) as the sole divalent cation. The major protein substrates are a 73-kD protein and a protein migrating near ovalbumin on sodium dodecyl sulfate polyacrylamide gel electrophoresis. The 47-kD protein was partially purified from cells harvested by leukapheresis from a patient with acute myelogenous leukemia, using ammonium sulfate precipitation and ion exchange chromatography. This partially purified ion-exchange fraction contained an endogenous kinase activity with characteristics similar to those we previously described of protein kinase P (protein kinase, phospholipid-stimulable: PK-P), but not typical of any form of protein kinase C (PK-C). With longer phosphorylation, the 47-kD band showed increasingly lower mobility demonstrable both by Coomassie blue staining and autoradiography, suggesting both that it was multiply phosphorylated, and that the excisable band was pure. The protein was thus eluted from preparative gel slices and digested with endoproteinase lys C. Sequence data from the fragments identified the protein as the 47-kD calpain fragment of talin, a protein found in focal adhesion plaques and some cell-cell contacts. PK-C phosphorylated the 47-kD protein, as has been reported previously, and phosphopeptide mapping disclosed a similar pattern of phosphorylation using either PK-C or the endogenous activity. The 47-kD protein labeled with the endogenous kinase contained predominantly phosphoserine, with some phosphothreonine and a trace of phosphotyrosine. Intact, purified talin was also phosphorylated by PK-P in a phospholipid-stimulable manner, but at 1/20 the rate of the 47-kD fragment.

© 1993 by The American Society of Hematology.
Table 1. Partial Purification of the 47-kD Substrate

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Vol (mL)</th>
<th>mg/mL</th>
<th>mg Total</th>
<th>Purification*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>50</td>
<td>30</td>
<td>1,500</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>10</td>
<td>44</td>
<td>440</td>
<td>3.4</td>
</tr>
<tr>
<td>CM cellulose, 0.05 mol/L to 0.1 mol/L NaCl</td>
<td>3</td>
<td>4.5</td>
<td>13</td>
<td>110</td>
</tr>
</tbody>
</table>

* Based on total protein only. Because of possible losses, we estimate a 50-fold actual purification.

MeOH/10% HOAc, then destained for 1 to 16 hours in 5% MeOH/7% HOAc. They were finally reduced in size by incubation in 50% MeOH for 30 minutes to 2 hours before drying between cellophane sheets overnight on an open rack. Autoradiographs were obtained by exposing Kodak X-AR film (Eastman-Kodak, Rochester, NY) to the gels for 2 hours at room temperature. Protein determinations were done by the method of Bradford.9

Purification of the 47-kD substrate. Forty milliliters of frozen leukocytes from a patient with acute myelogenous leukemia (AML) was thawed and mixed with an equal volume of 2× basic substrate buffer (BSB; 1× BSB is 10 mmol/L sodium phosphate, pH 6.5, 2 mmol/L ethylenediamine tetracetic acid, 1 mmol/L [ethylenebis(oxyethylenenitrilo)] tetracetic acid, 1 mmol/L dithiothreitol), to which the following protease inhibitors had been added, reaching the indicated concentrations: 10 μg/mL each of aprotinin, pepstatin (in dimethyl sulfoxide) and leupeptin, plus 0.05 mmol/L phenylmethylsulfonyl fluoride (in isopropanol). This mixture was homogenized with a Brinkman Polytron (Sybron Corp., Westbury, NY) at a setting of 8 for 30 seconds, then centrifuged at 27,000g for 15 minutes. The supernatant was brought to 30% saturation of ammonium sulfate by addition of saturated ammonium sulfate, allowed to incubate on ice for 20 minutes, then centrifuged at 10,000g for 10 minutes. The pellets were collected by centrifugation at 10,000g for 15 minutes and resuspended in 2× BSB buffered to pH 6.5 before application to the CM cellulose.
minutes. The supernatant was removed, brought to 70% saturation, and centrifuged as before. The 30% to 70% pellet was dissolved in a minimal volume of BSB, and desalted on a 200 mL column of Biogel P6DG (BioRad Laboratories) in BSB. The protein was then applied to an 18-mL column of carboxymethyl cellulose in BSB. The column was eluted with 20 mL each of BSB, BSB + 0.05 mol/L NaCl, BSB + 0.1 mol/L NaCl, BSB + 0.15 mol/L NaCl, and BSB + 0.3 mol/L NaCl. These fractions were concentrated with Centriprep 30 units (Amicon Corp, Beverly, MA) to about 2 mL, glycerol was added to a final concentration of 10%, and the samples were frozen at -70°C. Repeated freeze/thaw cycles did not affect the phosphorylation measurably.

Electroelution and desalting. The appropriate blue band was cut from a Coomassie-stained gel and shaken gently in Laemmli Biogel P6DG (BioRad Laboratories) in BSB. The protein was then centrifuged and added to a final concentration of 10%, and the samples were frozen in a Speed Vac (Savant Instruments, Hicksville, NY), and divided quickly with a micropipet (to avoid dilution), concentrated twofold in a Speed Vac (Savant Instruments, Hicksville, NY), and divided into two 0.1-mL portions in microfuge tubes. Twenty microliters of the appropriate blue band was electroeluted at 200 V into an adjacent small chamber; the blue color collected in 10 minutes and the volume fortuitously decreased in the collection chamber. The blue, protein-containing solution was removed quickly with a micropipet (to avoid dilution), concentrated twofold in a Speed Vac (Savant Instruments, Hicksville, NY), and divided into two 0.1-mL portions in microfuge tubes. Twenty microliters of 50% acetic acid was added to each tube to visualize the protein during the subsequent 15-minute methanol/chloroform/water precipitation. The resulting light blue pellet was dissolved without drying in 8 mol/L urea at 37°C for 1 hour. This was used for SDS-PAGE, when mixed 1:1 with 2X Laemmli sample buffer, which showed 50% yield for electroelution and precipitation combined. The 8 mol/L urea solution was also used for enzymatic cleavage and subsequent peptide separation and sequencing; this latter work was performed at the W.M. Keck Foundation Biotechnology Resource Laboratory in New Haven, CT, using endoproteinase lys C for cleavage.

Other methods. SDS-PAGE of protein fragments phosphoamino acid analysis of protein were performed as described. Immunodetection was performed as described, except that a second blocking step using 5% nonfat dry milk solids was done at 37°C for 30 minutes before adding the antititin ascites fluid (Sigma) diluted 1 to 200. The ECL system (Amersham, Arlington Heights, IL) was used to visualize the protein according to the manufacturer's protocol at a dilution of 1 to 10,000 of the secondary antibody.

RESULTS

Cells from a patient with AML were used as a source of protein substrates for protein kinase P (PK-P) in a scheme based upon earlier work. Ammonium sulfate fractionation followed by a stepwise elution from carboxymethyl (CM) cellulose resulted in a fraction (0.05 mol/L to 0.1 mol/L NaCl eluate) which was purified about 50-fold compared with the initial crude supernatant (Table 1). Because of the many protein kinases and protein phosphatases present in the initial supernatant, neither enzyme nor substrate quantification was possible, thus the fold purification is estimated from the total protein reduction. The fractions taken were relatively large, chosen so that less than 50% of the substrate was in side fractions. We shall refer to the 0.05 mol/L to 0.1 mol/L NaCl eluate as the partially purified preparation from now on.

We discovered that at this stage of purification, the substrate preparation had an endogenous protein kinase activity with the PK-P properties noted previously in cell lines and in whole human spleen extracts. Purification of substrates from HL-60 cells by this method showed endogenous kinase activity in the same partially purified fractions (data not shown). Figure 1 shows the results of in vitro protein kinase assays using 5 µg of the preparation under various conditions and with two added enzymes. The prominent autoradiographic band migrated with ovalbumin described as 43 kD and 45 kD from two suppliers, and with the 43-kD form of actin (data not shown). Lanes 1, 2, 3, and 5 show the phospholipid preference of the endogenous protein kinase; phosphatidyl glycerol is better than phosphatidyl inositol, which is better than phosphatidyl serine, which is better than no lipid, as has been found previously. Addition of purified protein kinase C (PK-C) in lane 4, in the presence of phosphatidyl glycerol and Mn2+, indicates that PK-C can exhibit activity under these conditions and raises the question of whether the phosphorylation observed is caused by endogenous PK-C. Lanes 5 and 6 show the results of addition of diacyl-glycerol and substitution of Mg2+ plus Ca2+ for Mn2+, ie, change to protein kinase C conditions. They show minimal phosphorylation, indicating first that the endogenous enzyme is minimally active under these conditions. Second, because calcium does not inhibit the noncalcium dependent forms of PK-C, the minimal phosphorylation in lane 6 shows that no significant amount of any form of PK-C is present in this partially purified preparation. The addition of PK-C in lane 7 is a positive control for PK-C conditions, and simultaneously shows that a 47-kD protein is an efficient substrate for PK-C. We emphasize that the phosphorylations leading to lanes 6 and 7 were performed under the same conditions, and that any endogenous PK-C should have resulted in autoradiographic bands in lane 6 as are found in lane 7. The addition of cAMP-stimulated protein kinase catalytic subunit, PK-A, in lanes 8 and 9 (PK-P and PK-A conditions, respectively) shows that there is minimal classical PK-A present in the preparation, and that the 47-kD protein is at best a poor substrate for PK-A (lane 9). The substantial phosphorylation of the 47-kD band in Fig 1, lane 3, is thus proven not to be caused by any form of PK-C or PK-A.

The time course of phosphorylation of the 47-kD band was investigated, and the results are shown in Fig 2. In lanes 1 and 2, the phosphorylation was performed for the standard 30 minutes, and the autoradiogram shows that in the presence of phospholipid vesicles, a major band of phosphorylation is apparent at 45 kD, with a minor band at 47 kD. In lanes 3 and 4 the same conditions were continued for 30 minutes more, resulting in three radioactive bands at about 45, 47, and 49 kD. In lanes 5 and 6 a reaction performed as in lanes 1 and 2 was brought to 1 mmol/L with unlabeled ATP and allowed to continue for 30 minutes more, ie, a pulse-chase experiment. The autoradiogram shows that in the presence of phospholipid vesicles, the two radioactive bands of lane 2 develop into the three bands seen in lane 6, indicating a precursor to product relationship.

The 47-kD Coomassie blue band in the above experiment...
shifted to a slightly slower mobility when highly phosphorylated, and this effect was investigated further with a larger amount of protein for visualization. Figure 3 shows the position of the ~47-kD band of a heat inactivated sample (75°C, 5 minutes) in lane 1, while samples phosphorylated for 0.5, 1, and 2 hours were electrophoresed in lanes 2, 3, and 4, respectively. The two major, slower-migrating bands constitute the bulk of the protein. They could represent multiple phosphorylations of a single protein, or at least one phosphorylation per molecule of two distinct proteins, each of which is a major part of the original 47-kD band.

The 47-kD band was accordingly isolated from Coomassie-stained gels by electroelution, and the protein was purified away from residual salts and SDS using an acid methanol/chloroform/water precipitation (see Materials and Methods). The resulting protein was dissolved in 8 mol/L urea, reduced and carboxamidomethylated, diluted to 2 mol/L urea, and digested with endoproteinase lys C as described. The protein fragments were separated by reverse phase high pressure liquid chromatography, and two of them were subjected to automated Edman degradation. The sequences found were searched for homology in the Protein Identification Resource, Swiss-Protein, and Genbank data sources using the FASTA algorithm. Table 2 displays the two peptide sequences with the corresponding segments of mouse talin, a cytoskeletal protein found in focal adhesion plaques. Of the 24 defined residues and two implied lysine residues found in the two peptides, only one difference was noted, a conservative human N to murine S replacement. The complete sequence of human talin has not been reported. Analysis of the amount of protein subjected to protease digestion and the amount of the peptides sequenced indicated that the talin fragment must have been a major, if not the predominant, protein in the 47-kD band. Because the talin fragment was a major constituent of the 47-kD band, and the major constituents of the 47-kD band were phosphorylated by PK-P in Fig 3, we conclude that the talin fragment was phosphorylated by PK-P.

PK-C is known to phosphorylate the 47-kD calpain fragment of talin, and this fact was used to test further the hypothesis that PK-P was merely phosphorylating some other protein that was cohabiting the 47-kD position. We allowed samples of the partially purified substrate preparation to react with both the endogenous kinase under PK-P conditions, and with added PK-C under PK-C conditions, and the 47-kD band was excised from gels of these reactions. The proteins were each divided in half, one of which was treated with 3-bromo-3-methyl-2-(2-nitrophenylmercapto)-3H-indole (BNPS-skatole) (which cleaves after tryptophan residues), the other with cyanogen bromide (which cleaves after methionine residues). The resulting peptides were separated by SDS-PAGE, and the radioactive peptides from corresponding digests were of the same molecular weights and same relative radioactive intensities, Fig 4. These data suggest that PK-P and PK-C both phosphorylate the 47-kD fragment of talin. Phosphoamino acid analysis showed the presence of phosphoserine, phosphothreonine, and phosphotyrosine in the ratio of 14:1:trace, respectively, on the 47-kD protein that had been radiolabeled under PK-P conditions (data not shown).

We investigated the ability of PK-P to phosphorylate intact talin. Talin was purified from human platelets with slight modification from the previously described method. We chromatographed the platelet extract on diethyl aminoethyl cellulose, then Biogel A-5m (BioRad Laboratories), and finally on phosphocellulose. When the preparation was subjected to SDS-PAGE and stained with Coomassie blue, no other protein band was visible at 10% the level of the talin band. The partially purified kinase preparation was used to phosphorylate the talin, and reaction products were separated by SDS-PAGE. The proteins in the gel were transferred to nitrocellulose, and the talin band was excised from gels of these reactions. The talin fragment was separated by SDS-PAGE, and the radioactive peptides from corresponding digests were of the same molecular weights and same relative radioactive intensities, Fig 4. These data suggest that PK-P and PK-C both phosphorylate the 47-kD fragment of talin.
phosphorylation of an ~240-kD band only when both PK-P and talin were present, with a fourfold stimulation in the presence of phosphatidyl glycerol. Figure 5B shows the presence of immunoreactive talin at both ~240 kD and ~200 kD, expected of intact talin and the large calpain fragment of talin, respectively. Only the intact talin is phosphorylated, suggesting that phosphorylation is restricted to the 47-kD domain. Intact talin was phosphorylated at approximately 1/20 the level of the 47-kD fragment in this experiment, which implies that part of the intact talin covers the phosphorylation sites.

Radiolabeled talin and pp47 were electroeluted from gel slices, digested with BNPS-skatole, and the resulting fragments were separated by SDS-PAGE as before. Figure 6 shows an autoradiogram of this gel. Four of the prominent bands from intact talin (apparent relative molecular masses of 32, 23, 21, and 7 kD) have counterparts from pp47. The fifth prominent band from intact talin (apparent relative molecular mass of 22 kD) does not have a counterpart from pp47; this may represent the junction of pp47 and the rest of the talin molecule. The pp47 15-kD band has no counterpart in the talin digest: this phosphorylation may be strongly disfavored in the intact molecule. This fragmentation study corroborates the assignment of pp47 as a fragment of talin.

**DISCUSSION**

Talin is an ~235 kD protein widely distributed in cell types, that was first found concentrated at points of focal adhesion of cultured cells, discrete patches where cytoskeletal actin stress fibers terminate at the plasma membrane and integrins bind to the fibronectin or vitronectin on the cell surface.7 Talin has subsequently been found in the cytoplasm of many cells, in cell-cell contacts of lymphocytes,20,21 and is the prominent 235-kD platelet protein that was known to be hydrolyzed into polypeptides of ~200 kD and ~47 kD by calpains I and II in vitro and after platelet stimulation by thrombin.17,22 Talin has been shown to bind to
talin. Radioactively phosphorylated pp47 and intact talin were electroeluted, cleaved with NBPS-skatole, and the resulting fragments were separated by SDS-PAGE as before. The figure is an autoradiogram of the final gel. Lane 1 contains pp47 fragments, lane 2 contains talin fragments.

Fig 6. Chemical fragmentation of phosphorylated pp47 and intact talin. Radioactively phosphorylated pp47 and intact talin were electroeluted, cleaved with NBPS-skatole, and the resulting fragments were separated by SDS-PAGE as before. The figure is an autoradiogram of the final gel. Lane 1 contains pp47 fragments, lane 2 contains talin fragments.

integrins\(^{23}\) and to vinculin,\(^{24}\) another cytoskeletal protein found in focal adhesions. It is phosphorylated in cells by a tyrosine kinase and by a phorbol ester-stimulated kinase, presumably PK-C.\(^{7}\) Talin has been phosphorylated by PK-C in vitro,\(^{25}\) and immunoprecipitation has shown changes in levels of talin phosphorylation upon stimulation of cultured cells which varied according to cell type.\(^{26}\) Talin that was radioactively phosphorylated by PK-C in vitro was then cleaved by calpain II in vitro; the majority of the radioactivity was found in the 47-kD fragment.\(^{16}\) Mouse talin has been cloned and sequenced, and the 47-kD fragment has been hypothesized to correspond to the membrane attachment domain.\(^{27}\) Among various blood cell types, talin is situated to play many roles: in adhesion or movement during normal hematopoiesis, diapedesis and lymphocyte homing; in cell-cell interactions; and in platelet activation. Control of talin’s movement from cytoplasm to membrane, the decision to make or break adhesions, the cleavage and phosphorylation of talin, and any possible feedback effects of its fragments will probably be found to differ from cell type to cell type.

The SDS-PAGE ‘47-kD’ position of phosphorylated proteins from WBCs and platelets has engendered lively discussions. Molecular weight assignments of proteins in this range are likely to vary between investigators because the usual SDS-PAGE marker near the position is ovalbumin, which is described as 43 kD or 45 kD by various suppliers, and the molecule can be isolated so as to contain various amounts of sugars and phosphates on it that can affect migration.\(^{28,29}\) Cell type-specific and species-specific isoforms of proteins complicate identification further, such as the isoforms of vinculin.\(^{30}\) At least four candidate proteins of ~47 kD have been proposed as the “one” that was phosphorylated during degranulation of human platelets, including lipocortin,\(^{31}\) inositol 1,4,5-triphosphate 5-phosphomonoesterase,\(^{32}\) the pyruvate dehydrogenase \(\alpha\) subunit,\(^{33}\) and a regulatory protein for actin.\(^{34}\) More recently a novel phosphoprotein of this molecular weight has been purified,\(^{35}\) cloned and sequenced,\(^{36}\) and named “pleckstrin.” There are thus multiple important protein kinase substrates of about 47-kD in whole cell extracts including the 47-kD talin fragment, which was known to be a substrate for PK-C.

In this report, we show that this talin fragment is also a substrate for PK-P. First, we showed that the phospholipid-dependent phosphorylation of the 47-kD band in a partially purified preparation was not caused by any form of PK-C or PK-A (Fig 1, lanes 6 and 9) although PK-C can clearly also phosphorylate this moiety (Fig 1, lane 7; Fig 4). We showed that the majority of the protein in the 47-kD band in our partially purified human leukemic cell extract was phosphorylatable, thereupon exhibiting decreased mobility on SDS-PAGE (Fig 3, lane 4). The band was electroeluted, proteolytically digested, and the two peptide fragments that were sequenced showed 96% identity with the 47-kD domain of mouse talin (Table 2). Finally, purified talin was phosphorylated by our enzyme on the 47-kD portion (Fig 5), and phosphopeptide mapping showed the correspondence of the endogenous protein with the purified talin (Fig 6). This talin fragment can probably be distinguished from pleckstrin by isoelectric focusing (the calculated pl of the talin fragment is 8.43, versus a pl for pleckstrin of \(-6.935\), but investigators should be cautious in identifying proteins in this molecular weight range by one-dimensional SDS-PAGE alone. This caution is especially cogent in view of the variable electrophoretic mobility we observed with hyperphosphorylation.

The physiologic relevance of this phosphorylation reaction of a talin fragment remains to be elucidated. An intriguing possibility is that when talin is in contact with vinculin and/or integrins, such as in a focal adhesion, its conformation is changed so that PK-P can phosphorylate it. This part of talin is proposed to be the membrane attachment region\(^{27}\) and the stimulation of its phosphorylation by phospholipids is consistent with that proposal. It might be the 48-kD cytoskeletal protein that was found to be phosphorylated and solubilized during neutrophil activation.\(^{37}\) Phosphorylation of a 20-kD cytoskeletal protein by PK-C has been shown to enhance its susceptibility to calpain digestion\(^{38}\); perhaps further proteolysis of the 47-kD talin fragment is similarly enhanced. However, the fragment appears to be fairly abundant and stable; thus, it may link focal adhesion change to other signal transduction pathways. For example, the
mouse sequence contains a putative nuclear localization motif starting at residue 402: KKKSK. Phosphorylation of this serine residue could strongly affect the cellular location of pp47. Thus focal adhesion change could result in different nuclear signals depending on the state of activation of PK-P. Such possible relationships will need further research to be elucidated.

REFERENCES

25. Litchfield DW, Ball EH: Phosphorylation of the cytoskeletal protein talin by protein kinase C. Biochem Biophys Res Commun 134:1276, 1986
The 47-kD fragment of talin is a substrate for protein kinase P

PC Simons and L Elias