Phosphorylation of Annexin I by TRPM7 Channel-Kinase*

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TRPM7 is an unusual bifunctional molecule consisting of a TRP ion channel fused to a protein kinase domain. It has been shown that TRPM7 plays a key role in the regulation of intracellular magnesium homeostasis as well as in anoxic neuronal death. TRPM7 channel has been characterized using electrophysiological techniques; however, the function of the kinase domain is not known and endogenous substrates for the kinase have not been reported previously. Here we have identified annexin 1 as a substrate for TRPM7 kinase. Phosphorylation of annexin 1 by TRPM7 kinase is stimulated by Ca²⁺ and is dramatically increased in extracts from cells overexpressing TRPM7. Phosphorylation of annexin 1 by TRPM7 kinase occurs at a conserved serine residue (Ser⁵) located within the N-terminal amphipathic α-helix of annexin 1. The N-terminal region plays a crucial role in interaction of annexin 1 with other proteins and membranes, and therefore, phosphorylation of annexin 1 at Ser⁵ by TRPM7 kinase may modulate function of annexin 1.

Recently, a new family of protein kinases has been identified, with prototypical members being mammalian eEF-2 kinase and Dictyostelium myosin heavy chain kinases, which display no homology to conventional eukaryotic protein kinases (1–3). We identified and cloned several other mammalian putative protein kinases with the catalytic domains homologous to that of eEF-2 kinase (4–6). This novel family of protein kinases was named α-kinases, since the existing evidence suggested that these kinases phosphorylate their substrates within α-helices (4), whereas conventional protein kinases phosphorylate their substrates within β-turns, loops, or irregular structures (7). Sequencing of various genomes revealed the presence of α-kinases in diverse organisms including animals, protists, and fungi (8). There are six α-kinases in mammals and more than 50 α-kinases altogether found in different organisms (α-kinases database: serine.umdnj.edu/αsorostor/alphakinase) (8).

Interestingly, after cloning the full-length cDNAs of mammalian α-kinases, it was found that two of them represent a novel signaling molecule consisting of an α-kinase catalytic domain fused to a TRP¹ ion channel (5, 10–12). This combination of an ion channel and a protein kinase is unique among known molecules. According to the recent nomenclature of TRP channels (13) these two molecules are called TRPM6 and TRPM7. To reflect the bifunctional nature of TRPM7 molecule we will refer to it as TRPM7/ChaK1 (Channel-Kinase 1).

TRPM7/ChaK1 is a member of TRPM family of the TRP superfamily of cation channels (reviewed in Refs. 14 and 15). The electrophysiological properties of the TRPM7 channel have been characterized and the channel was found to be nonselectively permeable to Ca²⁺, Mg²⁺, and other divalent metal ions (10, 11, 16). TRPM7/ChaK1 was shown to play a key role in the regulation of magnesium homeostasis in the cell (17) as well as in the regulation of anoxic neuronal cell death (18). The presence of the kinase domain is not required for TRPM7 channel activity although it may play a role in the modulation of the channel by Mg²⁺ (17) and cAMP (19). However, the exact physiological function of the kinase domain of TRPM7/ChaK1 is not known and endogenous substrates of this kinase had not been determined yet. Recently we analyzed in detail the activity of recombinant TRPM7/ChaK1 kinase domain (20), which allowed us to search for its endogenous substrates.

Here we have identified annexin 1 as the substrate for TRPM7/ChaK1. Annexin 1 is a Ca²⁺ - and phospholipid-binding protein that can promote Ca²⁺ -dependent membrane fusion (reviewed in Refs. 21 and 22). Annexin 1 was originally discovered as a mediator of the anti-inflammatory actions of glucocorticoids and was also implicated in the regulation of cell growth and differentiation (reviewed in Refs. 21 and 22) and apoptosis (23).

We found that TRPM7/ChaK1 phosphorylates annexin 1 at a conserved serine residue (Ser⁵) located within the N-terminal amphipathic α-helix. The N-terminal region plays a crucial role in interaction of annexin 1 with other proteins and membranes. Therefore, phosphorylation of annexin 1 by TRPM7 kinase may modulate function of annexin 1.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were obtained from Sigma. Radioisotopes were from PerkinElmer Life Sciences. Expression and purification of recombinant ChaK1 kinase domain was performed as described (20). Bovine annexin 1 was from Biodesign. Human annexin V was from Sigma. Human recombinant annexin II was a kind gift of Dr. Valery Alakho (Supratek Pharma, Inc.).

Fractionation of Cell Lysates and Analysis of Fractions for Phosphorylated Proteins—Mouse C2C12 cells were collected by trypsinization, washed with ice-cold phosphate-buffered saline, and lysed using Dounce homogenizer in ice-cold buffer containing 30 mM Tris-HCl (pH 8.0), 20 mM NaCl, 1 mM MgCl₂, 1 mM EDTA, 800 µL MgCl₂, 5% glycerol (w/v), complete protease inhibitor (Roche), and 1 mM phenylmethylsulfonyl fluoride. The lysates were cleared twice by centrifugation at 30,000 g for 30 min at 4 °C. The cleared lysate (containing 20 mg of total protein) was then fractionated by fast protein liquid chromatography on Mono Q HR 5/5 column (Amersham Biosciences) using 20–500 mM NaCl gradient. 40 fractions were collected (1 ml each). 10 µL of each fraction were incubated with [γ-³²P]ATP in phosphorylation mixture (as described below) with or without the addition of recombinant ChaK1.

Protein Phosphorylation Assay—Protein samples were incubated in
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FIG. 1. Annexin I phosphorylation by ChaK1. Phosphorylated proteins were analyzed by SDS-PAGE or two-dimensional electrophoresis on TLC plates and subsequent autoradiography. A, phosphorylation by ChaK1 of the following proteins: lane 1, proteins in fraction 2 after fractionation of C2C12 cell lysates; lane 3, human recombinant (recomb.) annexin I; lane 4, bovine annexin I. Lane 2, autophosphorylation of ChaK1. B, time course of annexin I phosphorylation by ChaK1. C, phosphoamino acid analysis of annexin I phosphorylated by ChaK1. Phosphoamino acid analysis was performed by hydrolysis of phosphoproteins with HCl, separation of amino acids using two-dimensional electrophoresis on TLC plates, and autoradiography. D, effect of Ca\(^{2+}\) and EGTA on annexin I phosphorylation by ChaK1. E, phosphorylation of annexin I in crude lysates from cells overexpressing TRPM7/ChaK1. HEK293 cells with tetracycline (Tet)-regulatable expression of TRPM7/ChaK1 were incubated with (lanes 2 and 4) or without (lanes 1 and 3) tetracycline. The cell lysates were incubated with [\(^{32}\)P]ATP in phosphorylation mixture with (lanes 3 and 4) or without (lanes 1 and 2) addition of recombinant (recomb.) human annexin I. The arrow indicates the position of the 210-kDa band that most likely represents the autophosphorylated TRPM7/ChaK1.

phosphorylation mixture consisting of 50 mM HEPES-KOH (pH 7.4), 10 mM MgCl\(_2\), 4 mM MnCl\(_2\), 0.5 mM CaCl\(_2\), 5 mM of pepstatin A (Sigma) in 50 mM Tris acetate (pH 4.5) (50 µl of total reaction volume) for 30 min at 37 °C. As a control the same reactions were carried out in the presence of 2 µM of peptatin A (Sigma). The reactions were stopped by boiling the samples in Laemmli sample buffer. The samples were analyzed by SDS-PAGE and autoradiography.

Preparation of Samples for MALDI-TOF Analysis—Coomassie-stained polypeptide was excised from the SDS-PAGE gel and digested with trypsin as described (24). The samples were prepared according to manufacturer's protocol (Applied Biosystems). The samples were analyzed using mass spectrometer Voyager-DE PRO Workstation (Applied Biosystems) in reflector mode. The obtained monoisotopic peptide masses were run against NCBI and Swiss-Prot data bases using MS-Fit (Protein Prospector) and PetIdent programs.

Expression and Purification of Annexin I—Human annexin I was expressed as a fusion with maltose-binding protein in Esherichia coli. A DNA fragment corresponding to annexin I was produced by PCR using HeLa animal DNA library made cDNA library (Clontech) and the following primers: GCGGATCCATGGCAATGGTATCAGAATTCCTCAAG (containing BamHI restriction site) and GCTCTAGATTAGTTTCCTCCACCAAAGGACCAC (containing XbaI restriction site).

The PCR fragment was inserted into a pMAL-c2x vector (New England Biolabs) using BamHI and XbaI restriction sites to produce the pMcAn1–4 expression construct. Expression and purification of annexin I was performed as described for the ChaK1-eat (long form) (20). The protein was cleaved with 2 µg/ml of Factor Xa (New England Biolabs) for 24 h at room temperature to remove maltose-binding protein tag from annexin I. After cleavage, annexin I contained 6 additional amino acids on its N terminus (Ile-Ser-Glu-Phe-Gly-Ser).

Phosphoamino Acid Analysis—Phosphorylation of annexin I was performed as described above. Sample preparation was performed as described (20). Phosphoamino acids were separated by two-dimensional electrophoresis on TLC plates 10 × 10 cm (cellulose on glass, Merck). First dimension was performed in pH 1.9 electrophoresis buffer containing 0.58 µM formic acid and 1.36 µM acetic acid at 1000 V for 20 min and second dimension in pH 3.5 electrophoresis buffer containing 0.87 µM acetic acid, 0.5% (v/v) pyridine, and 0.5 mM EDTA at 1000 V for 8 min. The TLC plates were stained with 0.2% ninhydrin in ethanol and exposed to x-ray film (Eastman Kodak Co.).

Phosphopeptide Mapping—Phosphorylated protein was excised from the SDS-PAGE gel. The protein was digested with trypsin as described (25). The obtained peptides were resolved by two-dimensional separations on TLC plates (Merck). In the first dimension peptides were separated by electrophoresis for 7 min at 1 kV in pH 1.9 buffer containing 0.58 µM formic acid and 1.38 µM acetic acid and in second dimension by ascending chromatography with n-butanol/pyridine/glacial acetic acid/H\(_2\)O, 75:50:15:50 (v/v). The phosphopeptides were detected by autoradiography.

Digestion of Annexin I with Trypsin and Cathepsin D—Phosphorylated protein was incubated with different amounts of sequence grade modified trypsin (Promega) for 15 min at 37 °C. The reactions were stopped by addition of 60 µg/ml of soybean trypsin inhibitor (Sigma). Samples were diluted with Laemmli sample buffer and boiled. The samples were analyzed by SDS-PAGE and autoradiography.

Phosphorylated proteins were digested with 2 µg of cathepsin D (Sigma) in 50 mM Tris acetate (pH 4.5) (50 µl of total reaction volume) for 30 min at 37 °C. As a control the same reactions were carried out in the presence of 2 µM of peptatin A (Sigma). The reactions were stopped by boiling the samples in Laemmli sample buffer. The samples were analyzed by SDS-PAGE and autoradiography.

Site-directed mutagenesis was performed with QuikChange XL mutagenesis kit (Stratagene) in accordance with manufacturer's protocol using the pMcAn1–4 expression construct as a template. The wild type and mutant annexin I were excised and purified as described above.

RESULTS

Recently we expressed TRPM7/ChaK1 kinase domain (ChaK1) in bacteria and analyzed in detail the activity of purified kinase (20). To identify substrates for TRPM7/ChaK1 we used cell lysate fractionation, phosphorylation with purified recombinant ChaK1, and subsequent peptide mass fingerprinting by MALDI-TOF mass spectrometry. By analysis with antibodies against TRPM7/ChaK1 of various cell lines we found the highest level of TRPM7/ChaK1 in C2C12 mouse myoblasts (data not shown). C2C12 cell lysate was fractionated by chromatography on Mono Q column using 20–500 mM NaCl gradient. A sample from each fraction was incubated with [\(^{32}\)P]ATP in phosphorylation mixture with or without addition of purified recombinant ChaK1. Fraction number 2 contained a polypeptide with the molecular mass of ~37 kDa that was intensively phosphorylated by ChaK1 (Fig. 1A).

The Coomassie-stained 37-kDa polypeptide was excised from the gel and subjected to digestion with trypsin. The resulting peptides were analyzed by MALDI-TOF mass spectrometry. The obtained masses of tryptic peptides were scanned against NCBI and Swiss-Prot protein data bases using MS-Fit (Protein Prospector) and PetIdent programs. The set of peptide masses matched to annexin I. To confirm that phosphorylated protein of 37 kDa was annexin I, we analyzed whether recombinant human annexin I and purified bovine annexin I can be phosphorylated by ChaK1. Indeed, we found that both recombinant human annexin I and bovine annexin I were phosphorylated by ChaK1 (Fig. 1A).

Next, we analyzed time dependence of annexin I phosphorylation by ChaK1 (Fig. 1B) and performed phosphoamino acid analysis of phosphorylated annexin I (Fig. 1C). We found that ChaK1 phosphorylates annexin I exclusively on serine residues. Since annexin I is a Ca\(^{2+}\)-regulated protein, we analyzed the effect of Ca\(^{2+}\) on phosphorylation of annexin I by ChaK1. We found that Ca\(^{2+}\) significantly stimulated phosphorylation of annexin I, while addition of 2 mM EGTA reduced this phosphorylation (Fig. 1D). We also examined whether other mem-
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resulting mutant recombinant proteins were phosphorylated by ChaK1. Samples were analyzed by SDS-PAGE and autoradiography.

with cathepsin D in the presence or absence of pepstatin A. Samples were analyzed by SDS-PAGE and autoradiography.

digested with different concentrations of trypsin as described under “Experimental Procedures.” Samples were analyzed by SDS-PAGE and autoradiography.

human recombinant annexin 1. Four mutants were produced: (i) S5A, (ii) S27A,S28A, (iii) S34A,S37A, (iv) S45A,S46A. The wild type (WT) and resulting mutant recombinant proteins were phosphorylated by ChaK1. Samples were analyzed by SDS-PAGE and autoradiography. D, digestion of phosphorylated annexin 1 with cathepsin D. Human recombinant (recomb.) and bovine annexin 1 were phosphorylated by ChaK1 and digested with cathepsin D in the presence or absence of pepstatin A. Samples were analyzed by SDS-PAGE and autoradiography (Autorad.).

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To locate the site of phosphorylation within annexin 1, partial proteolysis of phosphorylated annexin 1 was performed using different concentrations of trypsin. Annexin 1 contains a dense core and a flexible N-terminal region, which could be removed by partial proteolysis. The partial proteolysis produced a band of ~33 kDa that did not retain radioactive label (Fig. 2A), suggesting that the site of phosphorylation is located within the N-terminal region of annexin 1. The region of annexin 1, which could be cleaved off by trypsin, contains 7 serine residues that could possibly be phosphorylated by ChaK1 (Fig. 2B). Four mutants (one protein with single mutation and three proteins with two mutations) in which Ala was substituted for Ser were produced using QuickChange XL mutagenesis kit (Stratagene). The following mutants were produced: (i) S5A, (ii) S27A,S28A, (iii) S34A,S37A, and (iv) S45A,S46A. The wild type and mutant proteins were expressed in bacteria, affinity-purified, and analyzed for phosphorylation with ChaK1. We found that S5A substitution dramatically reduced phosphorylation of annexin 1 by ChaK1, while intensity of phosphorylation of other mutants were similar to wild type protein (Fig. 2C, D). Therefore, we found that ChaK1 phosphorylates annexin 1 specifically at Ser5. To confirm the location of phosphorylated residue in annexin 1, we used human recombinant annexin 1 as well as purified bovine annexin 1. Treatment of phosphorylated human recombinant or bovine annexin 1 with cathepsin D produced a 35.5-kDa band, which lost virtually all radioactive label (Fig. 2D). As a control, to account for possible phosphatase activity in the reaction, the treatment of annexin 1 with cathepsin D was carried out in the presence of pepstatin A (an inhibitor of cathepsin D). In the presence of pepstatin A, annexin 1 was not cleaved and remained radioactively labeled (Fig. 2D). Therefore, we found that ChaK1 phosphorylates annexin 1 specifically at Ser5. This serine residue is evolutionarily conserved and present in all mammalian and avian species (Fig. 3A). Ser5 is located within the N-terminal α-helix, which specifically interacts with S100A11 protein (Fig. 3B).

FIG. 2. Identification of site of phosphorylation in annexin 1. A, human recombinant annexin 1 was phosphorylated by ChaK1 and digested with different concentrations of trypsin as described under “Experimental Procedures.” Samples were analyzed by SDS-PAGE and autoradiography (Autorad.). B, the amino acid sequence of the N-terminal region of human annexin 1. C, alanines were substituted for serines in human recombinant annexin 1. Four mutants were produced: (i) S5A, (ii) S27A,S28A, (iii) S34A,S37A, (iv) S45A,S46A. The wild type (WT) and resulting mutant recombinant proteins were phosphorylated by ChaK1. Samples were analyzed by SDS-PAGE and autoradiography. D, digestion of phosphorylated annexin 1 with cathepsin D. Human recombinant (recomb.) and bovine annexin 1 were phosphorylated by ChaK1 and digested with cathepsin D in the presence or absence of pepstatin A. Samples were analyzed by SDS-PAGE and autoradiography (Autorad.).

FIG. 3. A, alignment of the N-terminal regions of annexin 1 from different species. The sequences were obtained from NCBI data bank and aligned using CLUSTAL W (1.60) and BoxShade programs. B, the location of Ser5 (indicated by arrow) in the complex between the N-terminal α-helix of annexin 1 and S100A11 (28), Protein Data Bank number 1QLS.

DISCUSSION

In this work we have identified annexin 1 as a substrate for TRPM7/ChaK1 protein kinase. Phosphorylation occurs specifically at Ser5, which is evolutionarily conserved (Fig. 3A). According to x-ray and NMR analysis Ser5 is located within the N-terminal amphipathic α-helix of annexin 1 (28–30) (Fig. 3B). This result is consistent with the hypothesis that TRPM7/ChaK1 kinase like other α-kinases can recognize and phosphorylate amino acids located within α-helices.

Annexin 1 is a member of annexin family of Ca2+-regulated
Phospholipid-binding proteins (reviewed in Refs. 21 and 22). The crystal structure of annexin 1 has been solved (30, 31) and was found to consist of a Ca\(^{2+}\) and membrane-binding compact core and N-terminal domain of 41 residues preceding the core. The N-terminal domain can interact with other proteins and also regulate membrane binding properties of annexin 1. From the x-ray studies of annexin 1, it was found that, in the absence of calcium, its N-terminal domain is buried within the core domain of the protein but gets expelled from the core upon Ca\(^{2+}\) binding (31).

A number of protein kinases were shown to phosphorylate annexin 1: annexin 1 is phosphorylated within the N-terminal domain by epidermal growth factor receptor tyrosine kinase at Tyr\(^{21}\) and by protein kinase C at Thr\(^{24}\), Ser\(^{27}\), Ser\(^{28}\), and Thr\(^{41}\) and in the core domain by PKA at Thr\(^{216}\) (reviewed in Ref. 32). It was shown that some of these phosphorylations could modulate functional properties of annexin 1 (reviewed in Ref. 32). The phosphorylation of Ser\(^{5}\) by any protein kinase has not been previously reported and it is likely to be specific for TRPM7/ChaK1 and to Valery Alakhov for providing annexin II. We thank Diana Drennan for her help in making Fig. 3.

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Annexin 1 was originally discovered as a mediator of the anti-inflammatory activity of annexin 1 is mediated, at least in part, by its interaction with formyl peptide receptor (36). This interaction is mediated via the N-terminal domain of annexin 1 (36). In fact, N-terminal peptides of annexin 1 can mimic anti-inflammatory activity of annexin 1 (reviewed in Ref. 37). Therefore, it is possible that phosphorylation of annexin 1 at Ser\(^{5}\) can also modulate its interaction with formyl peptide receptor.

The N-terminal domain of annexin 1 can also play a role in Ca\(^{2+}\)-regulated binding of annexin 1 to membranes (reviewed in Ref. 21) and therefore the phosphorylation of Ser\(^{5}\) can potentially modulate membrane binding properties of annexin 1.

What can be the physiological role of phosphorylation of annexin 1 by TRPM7/ChaK1? It has been demonstrated that TRPM7/ChaK1 is involved in the regulation of Mg\(^{2+}\) homeostasis (17), cell death (18), and proliferation (9). Considering that annexin 1 is also implicated in the regulation of cell death and proliferation, it is possible that the regulation of cell death and proliferation by TRPM7/ChaK1 involves phosphorylation of annexin 1.

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