myo-Inositol Monophosphatase Is an Activated Target of Calbindin D28k*

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Calbindin D28k (calbindin) is a member of the calmodulin superfamily of Ca2+-binding proteins. An intracellular target of calbindin was discovered using bacteriophage display. Human recombinant calbindin was immobilized on magnetic beads and used in affinity purification of phage-displayed peptides from a random 12-mer peptide library. One sequence, SYSSIAYPKSHS, was strongly selected both in the presence of Mg2+ and in the presence of Ca2+. Homology search against the protein sequence data base identified a closely similar sequence, ISSIKEKYPSHS, at residues 55–66 in myo-inositol-1(or 4)-monophosphatase (IMPase, EC 3.1.3.25), which constitutes a strongly conserved and exposed region in the three-dimensional structure. IMPase is a key enzyme in the regulation of the activity of the phosphatidylinositol-signaling pathway. It catalyzes the hydrolysis of myo-inositol-1(or 4)-monophosphate to form free myo-inositol, maintaining a supply that represents the precursor for inositol phospholipid second messenger signaling systems. Fluorescence spectroscopy showed that isolated calbindin and IMPase interact with an apparent equilibrium dissociation constant, KD, of 0.9 μM. Both apo and Ca2+-bound calbindin was found to activate IMPase up to 250-fold, depending on the pH and substrate concentration. The activation is most pronounced at conditions that otherwise lead to a very low activity of IMPase, i.e. at reduced pH and at low substrate concentration.

Calbindin D28k (calbindin) is a highly conserved member of the calmodulin super family of EF-hand proteins, containing 261 residues and 6 EF-hand motifs. It is expressed in many tissues including brain, intestine, kidney, and pancreas. Many reports have focused on the presence of calbindin in specific tissues including brain, intestine, kidney, and pancreas. Many properties are relatively well characterized, surprisingly little is known about the regulation of the enzyme. Here we show that calbindin and IMPase bind to each other with a KD of ~1 μM and that calbindin increases the phosphatase activity of IMPase.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant calbindin was expressed in Escherichia coli and purified as described (17). The apo form of calbindin was prepared using gel filtration of protein/EGTA mixtures (15). IMPase was purified from bovine brain using ammonium sulfate precipitation, heat treatment, and phenyl-Sephrose chromatography, as described in detail elsewhere.‡ Rat recombinant calretinin and human recombinant secretagogin were gifts from Patrick Groves and Malgorzata Palczewska (Nencki Institute, Warsaw, Poland). myo-Inositol-1-monophosphate from soybean was purchased from Sigma and Tween 20 from Riedel de Hahn. The Ph.D.-12 Phage Display Peptide Library kit was used in a homology search against the protein sequence data base.

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§ The abbreviations used are: calbindin, calbindin D28k; IMP, myo-inositol-1-phosphate; IMPase, myo-inositol-1 or 4-monophosphatase; PI, phosphatidylinositol; IPn, myo-inositol-1,4,5-triphosphate; PEG, polyethylene glycol.
was obtained from New England Biolabs. The kit contains a random peptide 12-mer phage display library, *E. coli* ER2738 host strain for phage amplification, and a sequencing primer (5'-CCCTCATAGTTAGCGTAACG). Additional primer of the same sequence was purchased from DNA Technologies (Aarhus, Denmark). Dynabeads M-280 were from Dynal Biotech (Oslo, Norway). Sulfoisocyanimidyl-6-(biotinoamido)hexanoate (NHS-LC-biotin), biotin (immuno pure), bovine serum albumin (immuno pure, fraction V), 2-(4-hydroxyazo)-benzoic acid (HABA), and avidin were from Pierce. All buffers and other chemicals were of analytical grade. Synthetic peptides with acetylated N terminus and amidated C terminus were purchased from Malmo General Hospital, department of Clinical Chemistry (ISSIKEKYPHS), and Medprobe (Oslo, Norway) (SYSSIAKYPHS).

**Phage Display**—Phage display studies were carried out as described in detail in the manual provided with the Ph.D.-12 Phage Display Peptide Library kit. In short, calbindin was conjugated to NHS-LC-biotin and bound to magnetic beads covered with streptavidin (Dynabeads M-280). The beads were then equilibrated with the phage display library for 60 min. Parallel experiments were carried out in 50 mM Tris/HCl, pH 7.5, 150 mM KCl, 0.05% Tween 20 with either 1 mM CaCl$_2$ or 1 mM MgCl$_2$ plus 100 µM EGTA. The beads were then washed ten times with the respective buffer to remove weakly bound phages, and the more strongly bound phages were eluted by washing the beads with 0.2 M glycine buffer at pH 2.2 containing 1 mg/ml bovine serum albumin. The eluates were neutralized by adding 1 M Tris/HCl, pH 9.1, and used to infect *E. coli* ER2738 at mid log-phase (OD = 0.5). After 5 h of cultivating, phages were purified from the culture supernatant using PEG precipitation.

**DNA Sequencing**—Phage eluate from the third round of affinity purification was used to infect *E. coli* ER2738 and spread on LB plates with isopropyl β-D-thiogalactoside and 5-bromo-4-chloro-3-indolyl-β-D-galactoside to obtain plaques deriving from single phages. Sequencing templates were prepared from individual plaques as described in the manual for the Ph.D.-12 Phage Display Peptide Library kit. DNA sequencing reactions were performed in an Eppendorf Gradient Mastercycler using Big Dyes Cycle Sequencing kit from Applied Biosystems and the sequencing primer. The reaction products were separated on an ABI Prism 310 genetic analyzer from Applied Biosystems.

**Interaction between Calbindin and IMPase**—The interaction between calbindin and IMPase was studied in a quartz cuvette with 3 mm path length using fluorescence spectroscopy. Excitation spectra were recorded on a Perkin Elmer LS50B fluorescence spectrometer between 200 and 300 nm (bandwidth 2.5 nm) with emission at 331 nm (bandwidth 3 nm), thus monitoring mainly tryptophan fluorescence. Two stock solutions containing a defined calbindin concentration, [CB], were mixed in different proportions to obtain calbindin concentrations between 0.5 and 8 M. A second series of samples contained 100 µM IMPase and one with 100 µM IMPase and one with 10 µM IMPase, respectively. The fluorescence excitation spectra were recorded for each sample, and the spectrum of each IMPase-calbindin mixture was subtracted from the sum of spectra recorded separately for IMPase and calbindin at the same concentration. The resulting fluorescence difference, $\Delta F$, was plotted as a function of total calbindin concentration in Fig. 1 for excitation at 296 nm, and the data was fitted using the following equation:

$$\Delta F = \Delta F_0 + \Delta F_{\text{max}} \left(\frac{[\text{CB}]}{[\text{CB}] + K_D}\right)$$

with the free calbindin concentration, [CB], calculated as follows:

$$[\text{CB}] = \frac{0.5[C_{\text{IMP}}] + K_D - C_{\text{CB}}}{0.25[C_{\text{IMP}}] + C_{\text{CB}} + K_D + \sqrt{(0.25[C_{\text{IMP}}] + C_{\text{CB}} + K_D)^2 - 4[C_{\text{IMP}}] + C_{\text{CB}}}}$$

$C_{\text{IMP}}$ is the total IMPase concentration and $C_{\text{CB}}$ the total calbindin concentration.

**RESULTS**

**Phage Display Studies**—A random 12-mer peptide library displayed on bacteriophage surface was used to search for sequences with high affinity for calbindin. Peptides binding to human recombinant calbindin were enriched in three rounds of affinity purification, with intervening amplification of high affinity binding phages in *E. coli*. Parallel experiments were carried out in the presence of 1 mM Ca$^{2+}$ or 1 mM Mg$^{2+}$. *E. coli* infected with the eluate from the third round were spread on plates to obtain individual clones for DNA sequencing. In the presence of Ca$^{2+}$, the sequence SYSSIAKYPHS was strongly enriched and found in 29 of 30 sequenced clones. In the presence of Mg$^{2+}$, the same sequence was dominating and found in 11 of 12 sequenced clones.

**Homology Search**—The sequence SYSSIAKYPHS was used...
A KOH, 150 mM KCl, 2 mM MgCl₂, 0.1 mM EGTA at pH 7.1, and 37 °C.

B. The fluorescence difference reaches a maximum at close to 10 μM calbindin. The data at one wavelength and computer fit is shown in Fig. 1. The fluorescence difference reaches a maximum at close to 10 μM calbindin, indicating a 1:1 (or 2:2) stoichiometry. The best fit was obtained using $K_D = 0.9 \mu M$.

Interaction between Calbindin and Synthetic Peptides—The interaction between calbindin and synthetic peptides with the sequence SYSSIAKYPSHS or ISSIKEKYPSHS was studied using tryptophan fluorescence excitation spectroscopy. Similar spectral changes (sum versus mix) were obtained as with intact IMPase but requiring larger peptide concentrations, indicating that the binding was weaker (data not shown). Optimal fits to the data were obtained with $K_D = 160 \mu M$ for the SYSSIAKYPSHS peptide and $K_D = 330 \mu M$ for the ISSIKEKYPSHS peptide. It may seem surprising to have purified a peptide with such a low affinity for calbindin D₂₅K. However, each phage particle displays five identical copies of the peptide. This allows the phage to interact with more than one immobilized calbindin molecule to yield an apparent high affinity (avidity).

Effect of Calbindin on IMPase Activity—The dephosphorylation of IMP to produce inositol and phosphate was monitored by $^1H$ NMR spectroscopy. $^1H$ NMR spectra were acquired for 2 mM IMP at different time points after adding catalytic amounts of IMPase in the absence and presence of 50 μM calbindin. The dephosphorylation of IMP into inositol was 4-fold faster in the presence of calbindin than in its absence (data not shown). This indicates that the phosphate activity of IMPase is enhanced by calbindin. The cellular concentration of IMP is, however, around 20 μM, which yields poor NMR signals. Further activity measurements were therefore performed using a chromophoric method to quantitate released phosphate (18).

Using the chromophoric assay, the IMPase activity was measured in the presence of 2 mM Mg²⁺ and 100 μM EGTA to avoid perturbations from Ca²⁺ ions. Assays were performed in the absence and presence of calbindin (0.01–600 μM) at substrate concentrations ranging between 2.5 and 300 μM (Fig. 2). These data show that the activity of IMPase is enhanced by the presence of calbindin. Half-maximal activation is reached at calbindin concentrations of 1–10 μM. It also appears that the less substrate used the larger the effect of calbindin (Fig. 2B).

Control Experiments—No hydrolysis of IMP was observed in control experiments with 100 μM calbindin but no IMPase. No free phosphate was detected in reaction mixtures with 100 μM calbindin and 0.04 μM IMPase but no IMP. The activity of IMPase was not enhanced in the presence of calbindin digested with trypsin or pepsin (not shown). Calbindin digested with trypsin or pepsin did not bind to IMPase (not shown). The activating effect of calbindin on IMPase activity could be displaced by the ISSIKEKYPSHS peptide (not shown), whereas the unrelated peptide SIGIAQPHIDYNNVS⁵ had no effect. The activity of IMPase was also measured in the presence of the proteins calretinin and secretagogin (19, 20), both of which contain six EF-hands and are homologous of calbindin, and in the presence of a few other macromolecules (HIDYNNVS, SIGIAQPHIDYNNVS, poly-γ-glutamic acid, poly-L-lysine, PEG 4000, PEG 8000, cytochrome c, and calmodulin). At comparable conditions, the IMPase activity in the presence of 25 μM macromolecule divided by the activity of IMPase alone (the relative activity) was 1.01 for calretinin, 0.99 for secretagogin, 1.06 for HIDYNNVS, 0.99 for SIGIAQPHIDYNNVS, 1.13 for poly-γ-Glu, 0.94 for poly-L-Lys, 1.00 for PEG 4000, 0.99 for PEG 8000, 1.20 for cytochrome c, 1.41 for calmodulin, and 3.22 for calbindin. Small stimulatory effects on IMPase activity are hence observed in the presence of calmodulin, cytochrome c, and poly-glutamic acid. All the other proteins and substances, including calretinin and secretagogin, show no or negligible activation of IMPase. The experiments were performed three times using double samples, and the deviations were less than 5%.

pH Effect on IMPase Activity—Using the chromophoric assay, the IMPase activity was measured in the presence of 2 mM...
Mg\textsuperscript{2+} and 100 \textmu M EGTA at pH values ranging from 6.0 to 8.0 (Fig. 3). The activity of IMPase is steeply pH-dependent between pH 7 and 6, and the activity is practically lost at pH 6 (Fig. 3A). No released phosphate could be detected at pH 6 even after a 4-h reaction time. In the presence of calbindin, the IMPase activity is only slightly reduced below pH 7, and a significant level of activity remains at pH 6. Therefore, the relative activation by calbindin is larger the lower the pH (Fig. 3B). At pH 6 the relative activation cannot be calculated because no activity is observed in the absence of calbindin.

Ca\textsuperscript{2+} Effects on IMPase Activity—Using the chromophoric assay, the IMPase activity was measured in the presence of 2 mM Mg\textsuperscript{2+} and variable Ca\textsuperscript{2+} concentrations (Fig. 4). The activity of IMPase is reduced at Ca\textsuperscript{2+} concentrations above ~10 \textmu M, and at 1 mM Ca\textsuperscript{2+} the activity is lost. However, calbindin activates the enzyme significantly also in the presence of Ca\textsuperscript{2+}. The IMPase activity observed at 400 \textmu M Ca\textsuperscript{2+} and 10 \textmu M calbindin is comparable with that observed in the absence of both Ca\textsuperscript{2+} and calbindin.
DISCUSSION

In this report we show that calbindin D_{28k} interacts with (Fig. 1) and activates IMPase (Fig. 2–4). The activation is highly specific because calretinin and secretagogin, two hexa EF-hand proteins that are homologous to calbindin D_{28k} (58 and 37% sequence identity, respectively), do not have any observable effect on the activity of the enzyme. The calbindin-binding peptide enriched from the phage display library (SYSSIAKYPSHS) displays 75% identity with residues 55–66 in IMPase (ISSIKEKYPSHS). Sequence identity is obtained for residues 56–58 (SSI) and 61–66 (KYPHS). The three-dimensional structure of IMPase (21) is a symmetric homodimer in which the side chains of residues 61–66 are surface-exposed in an extended loop (Fig. 5). Residues 55–61 are at the end of an α-helix that may function to stabilize the loop. The two identical 55–66 sites are maximally separated in the structure such that the homodimer may bind two calbindin molecules without steric hindrance (calbindin, 30 kDa, is slightly smaller than the IMPase monomer, 32 kDa). These structural features make residues 55–66 an ideal site for protein-protein interaction. The 12-residue segment is strongly conserved. It is identical in IMPase of human, porcine, rat, and African clawed frog origin, whereas the bovine sequence has a Thr in stead of Ser at position 57.

IMPase is a key enzyme in the regulation of the activity of the PI signaling pathway (Fig. 6), which is of major importance in all eukaryotes. IMPase hydrolyzes IMP to produce phosphate and myo-inositol, the starting material for synthesis of PI, as well as phosphatidylinositol mono- and di-phosphates (PIP and PIP_2), and subsequently IP_3. IMPase hence acts in the PI signaling pathway (Fig. 6), which is of major importance during cell stress (24–26). It is striking that the relative activation of IMPase by calbindin is largest during stress conditions such as low substrate concentration (Fig. 2) and during acidosis (Fig. 4). The latter condition often occurs as a side effect of Ca^{2+} entry. During cell stress conditions, calbindin may act as a survival molecule, which ensures the activity of IMPase under conditions at which the activity would otherwise be lost.

Lithium, carbamazepine, and valproic acid are effective mood-stabilizing treatments for bipolar affective disorder. Recently, it has been shown that all these drugs act by causing cellular inositol depletion (27). Based on these findings it was suggested that the development of new therapies for bipolar disorder should focus on inositol phosphate metabolism (27). Both IMPase and the IP_3 receptor are considered attractive therapeutic targets for conditions in which the amount of Ca^{2+}/inositol signaling needs to be controlled. It has been proposed that lithium inhibits IMPase, thereby causing a depletion of inositol, reduced synthesis of PIs, and attenuated PI-linked signal transduction (28). A serious drawback with Li^+ therapy is the narrow margin between the therapeutic (0.5–1 mM) and toxic (>2 mM) doses (28). Li^+ may interfere with the homeostasis of different electrolytes and essential ions as well as ion transport. There is considerable interest in finding novel regulators of IMPase with a larger therapeutic concentration window and fewer side effects. The discovery of calbindin as a potential cellular activator of IMPase could be of interest in the development of novel pharmacological treatments for conditions due to an imbalance of the Ca^{2+}/IP_3 pathways. Substances may be constructed that enhance or attenuate the activity of IMPase based on its interaction with calbindin, for example by competing with either protein for binding to the other one, by altering the affinity of calbindin for IMPase, by altering rates of association and dissociation of the two proteins, by altering the Ca^{2+}-binding properties of calbindin, by affecting the expression levels of calbindin, or by otherwise affecting its ability to regulate IMPase.

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