The Second Messenger Binding Site of Inositol 1,4,5-Trisphosphate 3-Kinase Is Centered in the Catalytic Domain and Related to the Inositol Trisphosphate Receptor Site*

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A segment of inositol 1,4,5-trisphosphate 3-kinase responsible for inositol 1,4,5-trisphosphate (InsP₃) binding was characterized and confirmed by three different approaches employing the fully active expressed catalytic domain of the enzyme. Part of this moiety was protected from limited tryptic proteolysis by InsP₃. Sequencing of this region, inhibited enzyme activity and interfered with inositol phosphate binding. Detailed enzyme kinetic parameters of 32 site-directed mutants revealed residues Arg-276 and Lys-303 in this segment and Arg-322, located nearby, as directly involved in InsP₃ binding. Part of this region is similar in sequence to an InsP₃ binding domain characterized on the cDNA level (4–6) (for review, see Ref. 7). For the IP3R the InsP₃ binding domain has been confined to the amino-terminal 580 amino acids, with a minimal binding moiety of 353 amino acids (amino acids 226–578), displaying somewhat reduced affinity for InsP₃ (8). Particularly, three conserved basic amino acids have been point mutated by site-directed mutagenesis as being involved in ligand binding (8). Furthermore, photoaffinity labeling with InsP₃ analogs identified a peptide comprising amino acids 476–501 of the IP3R as being part of the InsP₃ binding site (9).

Besides the IP3R some members of a family of inositol polyphosphate/inositol phospholipid 5-phosphatases are also capable of binding to InsP₃ with high affinity (for review, see Refs. 10 and 11). An InsP₃ binding segment has been located in the catalytic domain of type II inositol polyphosphate 5-phosphatase, with two short, highly conserved amino acid motifs (WXGDXINXR and PXWCDXR), identified by mutational analysis as being involved in catalysis and/or substrate binding (12). Particularly the arginine residue (Arg-480; bold above) was found to increase the enzyme’s $K_m$ for InsP₃, reflecting a reduced affinity for InsP₃ (12).

Two further proteins, which do not metabolize InsP₃, have been found to bind to its photoaffinity analogs and specific affinity purification (13). One of these proteins was identified by peptide sequencing as phospholipase Cδ1, and the other one was found to be closely related to phospholipase Cδ1, although lacking any detectable phospholipase C activity (14). Only for phospholipase Cδ1 the structural requirements for InsP₃ binding are already fairly well understood because the pleckstrin homology domain of this enzyme (amino acids 17–132) has been identified as the InsP₃-binding element, and a crystal structure of this domain bound to InsP₃ has been obtained (15). Within the NH₂-terminal part of the pleckstrin homology domain (amino acids 17–60) five basic, one acidic, and one aromatic amino acid were identified as being directly involved in InsP₃ binding (15). Two further basic amino acids within this protein segment were found to be indirectly involved in binding by site-directed mutation. IP3K is known as a highly selective InsP₃-binding protein (16) with high affinity for this substrate, as illustrated by $K_m$ values ranging from 0.7 to 3.1 μM (17–21) depending on the

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§ The abbreviations used are: InsP₃, inositol 1,4,5-trisphosphate; InsP₅, inositol 1,3,4,5-tetakisphosphate; IP3K, inositol 1,4,5-trisphosphate 3-kinase; IP3R, inositol 1,4,5-trisphosphate receptor; GST, glutathione S-transferase; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; CaM, calmodulin; IP3K_WCDR; COOH-terminal fragment (amino acids 147–452) of chicken IP3K.

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source and isoform examined. Several cDNAs for this enzyme have been cloned, e.g. three different isoforms, designated A–C, from human (Refs. 22, 23, and GenBank accession no. D38169), two isoforms from rat (24–26), and one isoform from chicken (21). In the nematode worm Caenorhabditis elegans one gene for IP3K has been identified, which is alternatively spliced into three different mRNAs (27).

The catalytic domain of the enzyme has been mapped to the C terminus with the last 275 amino acids being indispensable for enzyme activity (28). This domain also shows the highest degree of amino acid conservation (30% identities) between isoforms and between species. Within this region Lys-197 and Asp-414 have been found to be involved in ATP binding in rat isoform A by site-directed mutagenesis (29). A recent study has gathered evidence for the involvement of one lysine residue (Lys-262) in rat isoform A (30) in InsP_3 binding.

Here we demonstrate that in the chicken IP3K A-isoform a 35-amino acid region (amino acids 271–305) is protected against tryptic cleavage after InsP_3 binding and that monoclonal antibodies recognizing epitopes within this region have inhibitory effects on the enzyme activity and interfere with InsP_3 binding to the enzyme. Moreover, the enzymatic properties of recombinantly expressed mutant proteins clearly indicate the direct involvement in InsP_3 binding of three residues (Arg-276 and Lys-303) within or close to this region (Arg-322) and a participation in binding of five neighbored residues, all lying within a 73-amino acid segment, centered in the catalytic domain.

**EXPERIMENTAL PROCEDURES**

Cloning, Expression, and Purification of a GST-IP3K Fusion Protein—A bacterial expression vector, designated pGEX-SE1, was constructed by ligation of a 570-base pair fragment from a polymerase chain reaction product (polymerase chain reactions 5 and 4 (21)), comprising amino acids 164–351 of chicken IP3K, into vector pGEX2T (Amersham Pharmacia Biotech, Freiburg). XL1 Blue bacteria transformed with pGEX-SE1 according to Ref. 31 were used for overexpression of the GST-IP3 fusion protein (Pharmacia GST fusion kit). After 2 h of induction of protein expression by 0.1 mM isopropyl-1-thio-

α-D-galactoside, the cultures were centrifuged for several minutes. The bacterial pellet was resuspended in 1/50 of the culture volume of phosphate-buffered saline and frozen at −20 °C. The subsequently thawed bacterial suspension was subjected to lysis by addition of the following components at the respective final concentrations: 0.5 mg/ml lysozyme, 0.1 mg/ml DNase I, 0.1% Triton X-100, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM PMSF, and by incubation at 35 °C for 20 min on ice. Fusion protein aggregated in inclusion bodies was pelleted by centrifugation at 10,000 × g for 10 min. The pellet was extracted once with 1/3 of the resuspension volume of detergent buffer (200 mM NaCl, 20 mM Tris-Cl, 2 mM EDTA, 1 mM DTT, 1% deoxycholate, 1% Nonidet P-40 (at pH 7.5)) and twice with the same volume of Triton buffer (20 mM Tris-Cl, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100 at pH 7.5) by vigorous vortexing for several minutes. The extracted pellet was solubilized in 1/3 of the resuspension volume of 8 M urea in water, centrifuged at 10,000 × g, and the supernatant dialyzed exhaustively against dialysis buffer (1 × phosphate-buffered saline, 0.5 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.1% Triton X-100). Most of the fusion protein precipitated again during dialysis and was collected by centrifugation at 10,000 × g. The pellet was solubilized again in 4 ml of 8 M urea and dialyzed 1:50 with dialysis buffer. The again dialyzed protein remained soluble and was supplemented with 0.1 mM phenylmethylsulfonyl fluoride, generally mixed with 0.5 ml of glutathione-Sepharose resin and incubated for 40 min at room temperature under repeated mixing. The resin was washed on a column with 3 × 10 ml of dialysis buffer. The bound GST-IP3 fusion protein was eluted in three 1-ml fractions using elution buffer (50 mM Tris-Cl, 10 mM glutathione, 100 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 1 mM EDTA, 0.1% Triton X-100, pH 7.5). For these steps the resin was kept for 40, 15, and 15 min at room temperature in elution buffer, respectively.

Generation of Monoclonal Antibodies against a GST-IP3K Fusion Protein—Monoclonal antibodies were produced according to the technique in Ref. 32. A BALB/c mouse was injected on day 0 and day 15 intraperitoneally and subcutaneously with 10 μg of affinity-purified GST-IP3K fusion protein in complete and incomplete Freund’s adjuvant, respectively. 53 days later the mouse was boosted with 100 μg of GST-IP3K fusion protein and 200 μg of double-stranded RNA intravenously and intraperitoneally. 3 days after the boost the splenocytes were fused with SP2/0 myeloma cells.

**Generation of COOH-terminal Truncated GST-IP3K Fusion Proteins for Epitope Mapping of Monoclonal Antibodies—**Plasmid pGEX-SE1 was modified for nested deletion of the IP3K coding fragment from its 3 ’-end by cleavage with AccI and ThII and insertion of a double-stranded DNA fragment created from the complementary oligonucleotides (5′-TCATTCAAGGCTCACTGCAGTACCTGCAGTCGT-3′ and 5′-ND2 (5′-TCATTCAAGGCTCACTGCCATTCTTTGATCT-3′) into the gap. The resulting plasmid pGEX-SE570 was cleaved with KpnI and XbaI and then subjected to a nested deletion reaction using the nested deletion kit of Amersham Pharmacia Biotech. The reaction products were used to transform competent XL1 Blue cells (Strategene), and the resulting transformants were screened by plasmid minipreparation and restriction enzyme digest for the length of the IP3K coding region. Several truncated plasmids were selected andideoxynucleotide sequenced (33) using an Applied Biosystems 377 automated Sequenator. Bacterial colonies harboring plasmids of interest were then cultured and induced for the expression of GST fusion proteins as described above. Lysates, prepared as described above, were directly used for SDS-PAGE and Western blotting.

Production of the Recombinant IP3K and Mutants in Escherichia coli—A 306-amino acid fragment of chicken comprising CaM binding and catalytic domain, designated IP3KΔN, as well as the derived mutant proteins were overexpressed in E. coli BL21(DE3) bacteria transformed with plasmid pEGIK or mutated derivatives thereof and purified on phosphocellulose and CaM-Sepharose as described (21).

**Partial Proteolytic Cleavage of Recombinant IP3K**—For a time course of proteolysis, affinity-purified IP3KΔN at a concentration of 0.48 mg/ml was digested in a 50-μl volume at 30 °C in 50 mM sodium phosphate buffer, pH 7.8, with 0.01 mg/ml trypsin (sequencing grade) for up to 20 min. Trypsin was added from a 0.5 mg/ml stock solution in 1 mM HCl, and 10-μl samples were withdrawn from the reaction and stopped by mixing with 3.3 μl × 4 × SDS-PAGE sample buffer (0.2 mM Tris-Cl, pH 6.8, 15% glycerol, 8% SDS, 0.8 mM mercaptoethanol, 0.4 mg/ml bromophenol blue) and immediate heating to 95 °C for 5 min. For experiments regarding the effects of substrates on proteolysis, either 1 mM ATP or 250 μM InsP_3 or both were added to the same reaction buffer. The dependence of proteolysis on the InsP_3 concentration was determined in parallel reactions (10 μl) containing 50 mM Tris-Cl, pH 7.5, 0.1 mM InsP_3, 0.01 mg/ml trypsin, and InsP_3 varying from 0.5 to 100 μM. After 20 min the reaction was stopped as above. For the determination of NH_2-terminal peptide sequences of proteolysis intermediates, samples separated by SDS-PAGE were blotted to polyvinylidenefluoride membranes (34), and immobilized protein bands were sequenced on an Applied Biosystems 470 A Sequanator.

**Site-directed Mutagenesis of IP3K Catalytic Domain**—The mutagenic oligonucleotide primers were essentially as described in Ref. 35. Either plasmid pEGIK (21) or plasmid pGEM-IP3K Xba/Sph, based on the vector pGEM3Z (Amersham Pharmacia Biotech) and containing a 460-base pair insert of chicken IP3K representing amino acids 147–299, was used as template. For the pGEM-IP3K Xba/Sph template, primers NP24 (5′-GACGTTGTAAAACGACGGCCAGTG-3′) and RP24 (5′-AACGCTATGACCATGATGTACCCCC-3′) were used as flanking primers, whereas for the pEGIK template primers UB24 (5′-GAAGGCTCAGCTAGGCAAGCCTGGAGTTTAC-3′) and UB40 (5′-GTCGATGACCTCTCTGAGAGAAGATGGTTTA-3′) were used as flanking primers. All oligonucleotides were from MWG Biotech (Ebersberg, Germany). The resulting polymerase chain reaction products were ligated into appropriately digested plasmid vectors. E. coli XLI Blue cells transformed with the ligation products were selected for positive transformants by plasmid minipreparation and restriction digests. Insert sequences were verified by dideoxynucleotide sequencing. Appropriate restriction fragments of mutated polymerase chain reaction products were ligated into plasmid pEGIK. For the expression of mutant proteins BL21(DE3) bacteria were transformed with these vectors.

**Optical Enzyme Assay for Determination of Specific IP3K Activity and Km Values for Substrates of Recombinant IP3K and Mutants—**IP3K activity and the Km values for InsP_3 were determined as described previously (21). The Km values for ATP were derived from initial rates with a saturating initial InsP_3 concentration of 25 μM and varying initial concentrations of ATP (0.025–2 mM). Statistical testing was with t test for unpaired samples.
**RESULTS**

**InsP₃ Protects a 35-Amino Acid Fragment of IP3K from Limited Proteolysis**—We subjected the recombinantly expressed catalytic domain of chicken IP3K₁₄₇₋₃₀₆ to a limited protease treatment in the absence or presence of ATP and InsP₃ and analyzed the resulting proteolytic fragments on SDS-PAGE. In the absence of substrates, cleavage with trypsin resulted in the immediate formation of at least five distinct bands with slightly reduced apparent molecular masses (36–32 kDa) down to a more protease-resistant fragment of 31 kDa (Fig. 1A). The most prominent stable cleavage intermediate of lower molecular mass was a 16-kDa fragment that was formed shortly after the 31-kDa fragment. The same cleavage pattern was found if ATP was present in the reaction mix at a concentration of 1 mM (not shown). In contrast, the presence of 250 μM InsP₃ led to the formation of a quite stable, prominent 21-kDa digestion intermediate, whereas the formation of the 16-kDa fragment was suppressed (Fig. 1B). Besides, a stabilization of a 31-kDa fragment occurred also. The same pattern of proteolytic fragments was observed when both substrates ATP (1 mM) and InsP₃ (250 μM) were present (not shown). Amino-terminal peptide sequences of isolated proteolytic fragments were determined (Fig. 2). For the 31-kDa band two superimposed sequences with initial yields of 70 and 49 pmol, respectively, were found, which could be easily assigned to two fragments starting at Tyr-178 and Ala-191, respectively. The 21-kDa band yielded a single peptide sequence (initial yield 159 pmol) corresponding to a fragment starting at Glu-271. Two superimposed sequences were also found for the 16-kDa band, which could be attributed to two fragments starting at Tyr-306 and Glu-311 with initial yields of 78 and 63 pmol, respectively. The observed Mₑ values imply that all fragments are not or only minimally truncated at the COOH terminus of the catalytic domain. Therefore, a segment protected from cleavage by trypsin in the InsP₃-bound state (i.e. the NH₂-terminal part of the 21-kDa fragment which is not present in the 16-kDa band) comprises 35 amino acids ranging from Glu-271 to Arg-305 (Fig. 3). Using varying InsP₃ concentrations during tryptic digestion, an EC₅₀ value of 36 μM was derived for the inhibition of tryptic cleavage after residues Arg-305 and Arg-310 as detected by the percentage of 21-kDa fragment formation. An even lower EC₅₀ value (2.6 μM) was found for the inhibition of cleavage after residue Arg-270 related to the percentage of 31-kDa band formation (Fig. 4).

**Monoclonal Antibodies Specific for Chicken IP3K Bind to Epitopes within the “InsP₃-protected” Region and Show Partial Inhibitory Effect on IP3K Activity**—For 15 monoclonal mouse antibodies, generated against an IP3K fragment ranging from Thr-164 to Val-341 and designated Kin3–Kin17, the epitope location and their effect on enzyme activity were investigated. Western blot experiments with a set of NH₂-terminally or COOH-terminally truncated IP3K fragments, generated either by tryptic digestion of IP3K₁₄₇₋₃₀₆ or by nested deletion of the coding sequence for a GST-IP3K fusion protein (see “Experimental Procedures”), revealed for all monoclonal antibodies only epitopes within the 35-amino-acid segment protected by InsP₃ against tryptic cleavage. All antibodies recognized the NH₂-terminally truncated 21-kDa fragment starting at Glu-271 (Fig. 5A) and none the 16 kDa fragment starting at Thr-306 (Fig. 5B). On the other hand, the COOH-terminally truncated fragment from Thr-164 to Glu-285 was recognized extremely weakly only by antibodies Kin4, -6, -9, and -17 (Fig. 5E). The fragment from Thr-164 to Leu-289, four amino acids longer, was recognized strongly by six antibodies (Kin4, -6, -9, -13, -15, -17).
and -17; Fig. 5D), whereas all antibodies reacted with the fragment from Thr-164 to Ala-317 (Fig. 5C). The weak lower M₅₇ bands appearing in Fig. 5E in the lanes of the antibodies Kin8, -11, -12, and -13 appear to be caused by cross-reactivity toward unrelated E. coli proteins, which are more prominent on this particular blot, since the staining sensitivity was enhanced in order to detect the weak specific reactivity toward the IP3K fusion protein. From this pattern of immunoreactivity we can conclude that the motif “VDPL” (position 286–289) is part of the epitopes recognized by six of the monoclonal antibodies and that their complete epitopes (epitope A) lie between Glu-271 and Leu-289. The epitope recognized by all other antibodies (epitope B) is contained within the segment from Ala-290 to Arg-305. A schematic representation is shown in Fig. 3.

In enzyme assays performed with IP3K₄₋₁₋₋₋ under Vₘ₉₉ conditions for both substrates in the presence and absence of antibodies, all antibodies inhibited IP3K activity by at least 30% and up to 70% when they were present at a molar ratio of 0.7 to 3, antibody binding sites to IP3K (Fig. 6). In all cases the observed inhibition clearly exceeded the inhibition of 11% measured in control reactions with a nonspecific antibody. To clarify the mechanism by which the antibodies exert their inhibitory effects on IP3K, two of the strongest binding antibodies (Kin4 binding to epitope A and Kin8 binding to epitope B) were selected for a determination of the effect on the Kₘ values of IP3K. No direct effects on the Kₘ values for ATP (data not shown) were found but only Vₘ₉₉ effects. However, in experiments in which InsP₃, acting as a competitive product inhibitor for InsP₃, was included from the beginning at a concentration of 5 μM, Kin4 showed a synergistic effect with InsP₃ in increasing the Kₘ value for InsP₃ (Fig. 7A), whereas Kin8 partially antagonized the effect of InsP₃ on the Kₘ value for InsP₃ (Fig. 7B).

Amino Acids within the Region Protected from Proteolysis by InsP₃ Contribute to InsP₃ Binding—To elucidate the role of individual amino acids in the cleavage protected region more thoroughly, we introduced several point mutations into this part and the neighboring segments of the protein. Most of these mutated residues are shown in Fig. 3. The mutant enzyme forms were bacterially expressed as IP3K₄₋₁₋₋₋ fragments, purified to about 70% homogeneity on phosphocellulose. Most were additionally affinity purified by CaM-Sepharose affinity chromatography (to >98% homogeneity) and then subjected to a detailed analysis of specific activity and Kₘ values for both substrates. From 32 mutations generated, three (double mutant D240A/D241A, C254S, and R259N) obviously displayed aberrant folding behavior because the mutant proteins became entirely insoluble and therefore could not be studied further. Among the remaining 29 mutants, 12 turned out to have no significantly altered enzymatic properties compared with the wild-type enzyme. Three mutants, designed to reproduce previously described enzymatic knock-out mutants of rat IP3K isoform A (29, 30), also resulted in completely inactive enzymes (K255A, K255N, and D407S). Therefore, also in avian IP3K-A, Lys-255 (corresponding to Lys-262 in rat isoform A) and Asp-407 (corresponds to Asp-414 in rat isoform A) are indispensable for catalysis. However, a conservative substitution at the critical residue Lys-255 (K255R) displayed no enzymatic alterations against the wild-type (Fig. 8A–D), demonstrating that only a positively charged amino acid is essential at this particular position.

With respect to ATP binding, five novel mutants (K176L, D245A, E247L, K327Q, and the double mutant D245A/E247L) showed significantly elevated values of the Kₘ for ATP ranging from 1.6- to 2.1-fold (Fig. 8D), whereas their Kₘ values for InsP₃ remained nearly unchanged. These residues either lie between 23 and 94 amino acids upstream or 22 residues downstream of the protein region that is protected by InsP₃ from proteolysis. InsP₃ binding was altered significantly in 10 mutants regarding eight residues (Fig. 8A), four of which (Lys-274, Arg-276, Lys-277, and Lys-303) lie within the InsP₃-protected region, whereas two (Thr-267 and Arg-270) lie only four and one residue, respectively, upstream of the tryptic cleavage site in

![Fig. 3. Schematic representation of the sequence of the chicken IP3K fragments used in this study. Horizontal bars represent (from top): recombinantly expressed IP3K containing CaM binding and catalytic domain (first bar), three GST-IP3K fusion proteins generated by nested deletion (second through fourth bars), 21- and 16-kDa proteolytic fragments (fifth and sixth bars), and the full-length IP3K from chicken (seventh bar). The box hatched with vertical lines represents the NH₂-terminal domain in the full-length protein; horizontal lines are the corresponding segment in the recombinant protein. The CaM binding domain is represented by a black box and the catalytic domain by a box with diagonal shading. The amino acid segment recognized by most monoclonal antibodies is represented by a continuous black bar below, designated Epitope A; the segment recognized by six of the antibodies is represented by a discontinuous bar, designated Epitope B; the segment in the recombinant protein. The CaM binding domain is represented by a black box and the catalytic domain by a box with diagonal shading. The amino acid segment recognized by most monoclonal antibodies is represented by a continuous black bar below, designated Epitope A; the segment recognized by six of the antibodies is represented by a discontinuous bar, designated Epitope B; the segment in the recombinant protein.](image-url)
the InsP₃-bound state of IP3K. The remaining two residues Arg-322 and Lys-327, on the other hand, lie 17 and 22 amino acids downstream of the InsP₃-protected region.

The $K_m$ values for InsP₃ of the mutants R276L, K303Q, and R322L display highly significant 11-, 16-, and 15-fold increases, respectively, compared with wild-type IP3K. Even the R276K and R322K mutations, which retain a positive charge at the respective positions, still have significantly (2.3-fold and 8.4-fold) increased respective positions, still have significantly (2.3-fold and 8.4-fold) increased $K_m$ values for InsP₃. Although the maximal catalytic activities of mutants R276L and R276K equal or rather exceed that of the wild-type protein (Fig. 8C), mutants K303Q, as well as R322K, and R322L, display strongly impaired catalytic activity, with 3.8–33-fold lower $V_{\text{max}}$ values (all exhibiting $p < 0.001$) than wild-type (Fig. 8C). In particular, the conservative substitution in R322K, resulting in a 28-fold lower specific activity, indicates a strict requirement for arginine at this particular position for InsP₃ binding and efficient catalysis.

Although not as evident as the above loss of function mutations, the $K_m$ values for InsP₃ of five other mutants turned out to be equally interesting because they unexpectedly exhibited lower $K_m$ values for InsP₃ (indicative of better substrate binding) than the wild-type. The mean apparent $K_m$ of these mutants for InsP₃ is in all cases significantly ($p < 0.005$) reduced about 2-fold to between 0.3 and 0.35 μM compared with 0.69 μM for the wild-type. The increased apparent affinity of these mutants for InsP₃ is also reflected (with the exception of K327Q) in their increased specific activities (Fig. 8C) and in their increased catalytic processivities ($k_{\text{cat}}$), derived from $V_{\text{max}}/K_m$ (Fig. 8B).

**DISCUSSION**

A 35-amino acid region almost in the middle of the 275-mino acid catalytic domain of chicken IP3K is protected from proteolytic attack, derived from Ala-191 to Arg-270 must also participate in InsP₃ binding. The affinities of the proteolytic fragments for InsP₃ is in all cases significantly ($p < 0.005$) reduced about 2-fold to between 0.3 and 0.35 μM compared with 0.69 μM for the wild-type. The increased apparent affinity of these mutants for InsP₃ is also reflected (with the exception of K327Q) in their increased specific activities (Fig. 8C) and in their increased catalytic processivities ($k_{\text{cat}}$), derived from $V_{\text{max}}/K_m$ (Fig. 8B).

**FIG. 4.** Dependence of the protection against proteolysis on InsP₃ concentration. In panel A the Coomassie-stained SDS-PAGE analyses of samples from identical tryptic digests of IP3K at varying InsP₃ concentrations are compared. InsP₃ concentrations in the digests are indicated above each lane in μM. Lane M contains molecular mass marker proteins with molecular masses indicated in the right margin. Lane C shows the undigested starting material. In panel B the percentages of the protected 21- and 31-kDa fragments after digest, as measured by densitometry, are plotted against the InsP₃ concentration. The positions of the expected fragments are indicated in the right margin; positions and molecular masses of marker proteins are indicated in the left margin.

**FIG. 5.** Epitope characterization of monoclonal antibodies against chicken IP3K. Proteolytic fragments (panels A and B) of chicken IP3K and bacterially produced GST-IP3K fusion proteins in bacterial lysates (panels C–E) were probed simultaneously with 15 monoclonal antibodies against chicken IP3K in parallel lanes (see “Experimental Procedures”). In panel A the immunoreactivity of the monoclonal antibodies toward NH2-terminally truncated 31- and 16-kDa fragments of chicken IP3K, resulting from a tryptic digest in the absence of InsP₃, is shown; in panel B a tryptic digest in the presence of 250 μM InsP₃ with defined, NH2-terminally truncated 31- and 21-kDa fragments was used. In panels C–E the immunoreactivity toward COOH-terminally truncated GST-IP3K fusion proteins is shown, using crude bacterial lysates containing IP3K fragments comprising amino acids 164–317 in panel C, 164–289 in panel D, and 164–285 in panel E. The positions of the expected fragments are indicated in the right margin; positions and molecular masses of marker proteins are indicated in the left margin. Lane numbering corresponds to the number of the respective antibody used to incubate the membrane strip. FF, GST-IP3K fusion protein. In panel F, identical amounts of the lysates used in panels C–E were blotted and incubated with an anti-GST antibody to ensure the expression of the truncated fusion proteins. Lane A contains the fragment 164–289; lane B, fragment 164–317; and lane C, fragment 164–285.
stream of Lys-255 was made unlikely by previous studies (30).

The significant inhibitory effects of all antibodies binding to epitopes directly downstream of Arg-270 on the catalytic activity of IP3K could be the result of (i) a direct blocking or displacement of residues involved in catalysis, (ii) steric hindrance of the entry of substrates, or (iii) a general conformational inactivation of the enzyme. In the first two cases this would imply the direct participation of epitope-forming residues in InsP3 binding or their close proximity to the binding pocket. However, the inhibitory effect of the antibodies could not be attributed directly to an interference with InsP3 binding because no significant changes in the $K_m$ value for InsP3 were found in presence of selected strong inhibitory antibodies. Still, the observed interference of both subtypes of antibodies with binding of InsP4, exemplified with Kin4 and Kin8 indicates that their epitopes in either case may overlap with the InsP3/InsP4 substrate/product binding site and may constitute that part of the enzyme which interacts with the additional phosphate group of InsP4.

By mutational substitution of positively charged residues within the protease-protected region and the antibody epitopes we confirmed that Arg-276 and Lys-303 participate directly in InsP3 binding but not in ATP binding. How these basic residues interact with individual phosphates of substrate or product is clarified in ongoing substrate selectivity studies. A further indication that this segment of IP3K is mainly involved in inositol phosphate binding is the fact that seven other mutations within or close to this region also show effects on $K_m$ for InsP3 and therefore on InsP3 binding to the active site. The intriguing observation that five of these replacements promote rather than inhibit InsP3 binding needs to be clarified by three-dimensional structure determinations. As indicated by their increased $k_{cat}$ values, these mutants indeed represent artificially optimized versions of IP3K concerning catalytic processivity. It will be interesting to learn what has prevented the natural enzyme from evolving toward these forms.

Our results also support and complement previous findings that showed that Lys-262 in rat IP3K isoform A, corresponding to Lys-255 in our IP3K, is essential for catalysis and InsP3 binding (30). Our nonconservative mutations in this position did also abolish catalytic activity in chicken IP3K, whereas the conservative one did not (see “Results”). All of our additional mutations affecting InsP4 binding lie closely downstream of this apparent starting point of the InsP3 binding segment, identified by an NH2-terminal deletion of the rat isoform, corresponding to Leu-252 in our enzyme. We have now identified a total of eight amino acids involved in InsP3 binding, within a 73-amino acid segment from Lys-255 to Lys-327, covering also the segment protected by InsP3 binding against tryptic cleavage and the antibody epitopes (see Fig. 3).

Our studies also discovered the hitherto unknown participation in ATP binding of four residues (Lys-176, Asp-245, Glu-247, and Lys-327), which are all located outside this segment. Taken together with the residues described previously as being essential in ATP binding (29) it thus appears that two ATP binding segments, making up the NH2- and COOH-terminal ends of the catalytic domain, are flanking a central segment involved in InsP3 binding. In a previous study (30) short COOH-terminal truncations by up to nine amino acids led to an almost complete (1/3800) loss of enzyme activity, whereas InsP3 binding was only reduced by about 1 order of magnitude. This indicates that either enzyme activity or ATP binding depends strongly on the presence of a most likely helical intact
COOH terminus. The much weaker influence of these truncations on InsP₆ binding than on catalytic activity may be indicative of a more indirect facilitative, e.g. conformational, involvement of the COOH terminus in InsP₆ binding and thus does not contradict the model of an InsP₆ binding core segment.

The part of this segment encompassing residues 259–284 shows a distant similarity to a segment of the IP3R (24) containing four residues involved in InsP₆ binding (8). In fact, the exchange of Arg-276 in IP3K and of the corresponding arginine residue in the IP3R has the same consequence: the reduction of InsP₆ affinity (Fig. 9). Also, the mutation of Lys-268 in IP3K and of corresponding Lys-501 in IP3R has the same effect, namely no alteration of InsP₆ affinity. The main differences regarding the effects of mutations are found for residues Arg-270, Lys-272, and Lys-274 of IP3K, which correspond to residues Arg-504, Arg-506, and Lys-508 of IP3R. An intriguing recent observation even allowing further molecular dissection of this kinase into functional segments comes from a comparison of the recently identified sequences of InsP₆ kinases before publication. We are grateful to Sigrid Poll for excellent technical assistance as well as to Dr. Gerhard Müller-Newen (RWTH Aachen, Germany) for help in constructing vector pGEX-SE1. We thank all students of the molecular biology courses of the Study Course Biochemistry/Molecular Biology of the University of Hamburg, who have contributed continuously to mutagenesis and expression of mutant proteins. In particular we thank students Anne Buschmann, Christian Buschmann, Ines Krohn, Dennis Poppau, and Tonio Wilcek for substantial contributions. We are indebted to Robin F. Irvine (University of Cambridge, U.K.) and Adolfo Saiardi (Johns Hopkins University, Baltimore, MD) for providing us with manuscripts on InsP₆ kinases before publication.

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FIG. 8. Enzymatic parameters of mutant IP3K proteins. In panel A the Kₘ values for InsP₆ determined for either phosphocellulose-purified or CaM-Sepharose-purified mutant IP3K proteins are presented. Results are means of at least three independent measurements, with S.D. indicated by error bars. Hatched bars indicate mutants with significantly increased and black bars mutants with decreased Kₘ values for InsP₆. In panel B the apparent rate constants (Vₘₐₓ) derived from Vₘₐₓ/Kₘ for InsP₆ are depicted with their respective error bars. In panels C and D the specific activities and the Kₘ values for ATP for CaM-Sepharose-purified mutant proteins are shown. Mutant proteins only purified by phosphocellulose chromatography are marked (*). Uninterrupted vertical lines indicate ± 1 S.D. or error range of the respective wild-type (WT36kDa) value, respectively. WT32kDa is a proteolytically shortened form of the recombinant protein, which was also purified by phosphocellulose chromatography.

FIG. 9. Sequence comparison of segments in IP3K and IP3R containing amino acids involved in InsP₆ binding. Homologous residues within the segments are boxed. Residues that have been mutated are given as bold characters. The arrows are pointing to the amino acids that have been used for replacement. The effects of these mutations are indicated either by bold characters when InsP₆ affinity was reduced or by normal characters when InsP₆ binding was not influenced.

The positioning of the ATP γ-phosphate close to the acceptor hydroxyl of the inositol ring.
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