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Structural insights into phosphoinositide 3-kinase catalysis and signalling

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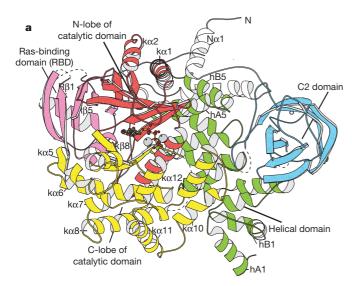
Phosphoinositide 3-kinases (PI3Ks) are ubiquitous lipid kinases that function both as signal transducers downstream of cellsurface receptors and in constitutive intracellular membrane and protein trafficking pathways. All PI3Ks are dual-specificity enzymes with a lipid kinase activity which phosphorylates phosphoinositides at the 3-hydroxyl, and a protein kinase activity. The products of PI3K-catalysed reactions, phosphatidylinositol 3,4,5trisphosphate (PtdIns(3,4,5)P₃), PtdIns(3,4)P₂ and PtdIns(3)P, are letters to nature

The mammalian PI3Ks can be divided into three classes on the basis of their structure and substrate specificity². The class I PI3Ks are receptor-regulated heterodimeric enzymes that preferentially phosphorylate PtdIns(4,5)P₂ *in vivo*. The class IA PI3Ks (consisting of p110 α , p110 β or p110 δ catalytic subunits) associate with a p85 adaptor protein that is essential for interaction of these PI3Ks with receptor tyrosine kinases. The class IB PI3K (PI3K γ) is activated by heterotrimeric G-protein subunits and associates with a p101 adaptor that is required for full responsiveness to G $\beta\gamma$ heterodimers^{3,4}. Class I PI3Ks are also activated by Ras. Class II PI3Ks are distinguished by a carboxy-terminal C2 domain and preferentially use PtdIns and PtdIns(4)P as substrates. Class III enzymes phosphorylate only PtdIns and lack the Ras-binding domain.

We have determined the structure of the catalytic subunit (residues 144–1,102) of porcine PI3K γ . This construct contains all of the homology regions (HR) found in class I PI3Ks (HR1, HR2, HR3 and HR4) and has a catalytic activity similar to that of the full-length enzyme. The amino-terminal region missing from our construct of PI3K γ is important for interaction with the p101 adaptor⁵, and the analogous region of PI3K α interacts with the p85 adaptor. The enzyme has a modular structure consisting of four domains: a Ras-binding domain (RBD), a C2 domain, a helical domain and a catalytic domain (Fig. 1). The RBD, C2 and catalytic domains have folds similar to these modules in other proteins involved in signal transduction. The helical domain has a fold akin to HEAT repeat containing structures involved in protein–protein interactions.

The catalytic domain of the enzyme consists of a smaller Nterminal lobe (residues 726-883) and a larger C-terminal lobe (884–1092). The N-terminal lobe from k β 3 to k α 3 and the first part of the C-terminal lobe (up to the end of $k\beta 10$) have a fold similar to protein kinases (reviewed in ref. 6), and this similarity extends to many of the details of the ATP-binding site (Fig. 2). This region is among the most conserved regions of the PI3Ks (Fig. 3). The structural similarity of PI3K to protein kinases is consistent with PI3Ks having protein kinase activity in addition to their lipid kinase activities^{7,8}. The sequence alignment in Fig. 3 shows the regions of PI3K that structurally superimpose with tyrosine protein kinase c-Src. The N-terminal lobe comprises a five-stranded antiparallel β -sheet flanked on one side by a helical hairpin (k α 1-k α 2) and a small two-stranded β -sheet ($\beta 1-\beta 2$), and on the other side by the k α 3 helix and the C-terminal lobe. Strands k β 3-k β 7 correspond to the five-stranded β -sheet found in the protein kinases. The k β 3-k β 4 loop corresponds to the protien kinase β 1- β 2 loop (also known as the glycine-rich or P-loop). This loop interacts closely with the phosphates of the bound ATP, but unlike the protein kinases, it contains no glycine. Instead, the side chain of Ser 806, a residue that is conserved in all PI3Ks, interacts with the β -phosphate (Fig. 2). Lys 833 at the end of $k\beta 5$, corresponding to Lys 72 of c-AMP-dependent protein kinase, interacts with the α -phosphate of ATP. This residue is conserved in all PI3Ks and is covalently modified by wortmannin⁹. There are two metal-binding sites (Me; Fig. 2). Me I interacts with the conserved Asn 951, whereas Me II interacts with Asp 836 and Asp 964.

N- and C-terminal lobes are linked through a loop between strands $k\beta7$ and $k\beta8$. This loop forms the deepest wall of the ATP-



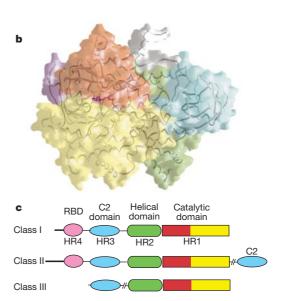


Figure 1 Overall structure of PI3K_Y. **a**, Ribbon diagram of PI3K_Y (prepared with MOLSCRIPT³¹) showing the four domains: RBD (magenta), C2 (blue), helical (green) and catalytic with N-lobe (red) and C-lobe (yellow). The N-terminal region preceding the RBD

and the ordered portion between the RBD and C2 domain are white. **b**, The solventaccessible surface of the enzyme in the same orientation as in **a** (prepared with GRASP²⁹). **c**, Domain organization of the PI3K classes.

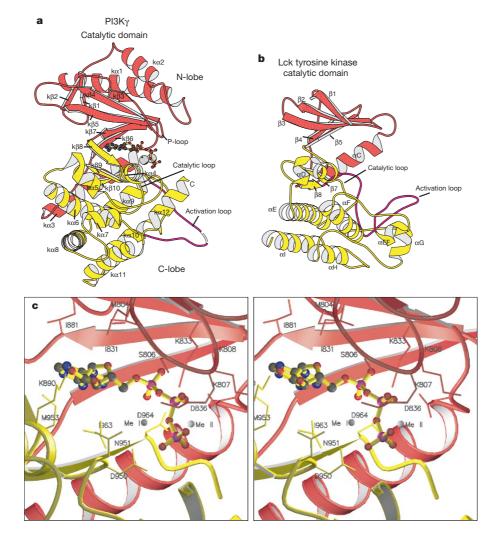


Figure 2 The catalytic domain of PI3K γ . **a**, Ribbon diagram of the PI3K γ catalytic domain with bound ATP. The two disordered residues in the middle of the activation loop (magenta) are represented by dotted lines. **b**, The active conformation of the Src family

protein kinase Lck³⁰ (PDB entry 3lck). **c**, Stereo diagram of Pl3K_Y active site with bound ATP and two Lu³⁺ ions (Me I and Me II).

binding pocket and provides two hydrophobic contacts with the adenine moiety of the ATP. The C-terminal lobe forms part of the ATP-binding site, as well as the binding site for phospholipid substrates. The region between k α 6 and k β 9 (residues 943–951) corresponds to the catalytic loop of the protein kinases; mutations of residues in this loop corresponding to Asp 946, Arg 947, Asp 950 and Asn 951 of PI3Ky abolish kinase activity of PI3Ks^{7,8}.

The C-terminal lobe contains a segment (964–988) analogous to the activation loop in the protein kinases; this loop is essential for the substrate specificity of the PI3Ks¹⁰. In the ATP-Lu³⁺ complex, much of this loop (968-982) is disordered. In the structure of an enzyme/chloramine T complex, all but two residues (Phe 975 and Leu 976) are visible, although high *B*-factors suggest that this loop is flexible. The activation loop is on the surface of the enzyme between the C-terminal helix k α 12 on one side and k α 10 on the other. We attempted to soak phospholipid analogues into PI3Ky crystals, but no substrate was evident in the electron density. Consequently, we modelled phospholipid headgroup binding, but because conformational changes probably occur in the activation loop and possibly in the C-terminal helix upon substrate binding, our model is only approximate. In the model, the headgroup is positioned in a cavity lined by the C-terminal helix k α 12, the activation loop and the catalytic loop (Fig. 4). This places the 5-phosphate of a PtdIns(4,5)P2 adjacent to Lys 973 and the I-phosphate near Lys 807 and Lys 808. Lys 973 acting as a ligand of the 5-phosphate may explain why this residue is not found in the class II PI3Ks that do not phosphorylate phosphoinositides with a 5-phosphate. The basic residues nearest the 4-phosphate are Arg 947 and Lys 973. The specificity of the class III PI3Ks for phosphatidylinositol may be explained by their shorter activation loop, which may not leave sufficient space to accommodate a 4-phosphate at the bottom of the headgroup-binding pocket. PI3Kô autophosphorylates in a region just beyond the C-terminal helix ka1211, which results in enzyme inhibition probably by sterically preventing substrate binding. The proximity of the C-terminal segment to the substrate-binding site is consistent with autophosphorylation of this region.

The mechanism originally proposed for the enzymatic activity of protein kinases involved a residue acting as a general base to deprotonate the hydroxyl of the substrate and generate a nucleophile that would attack the γ -phosphate of ATP. In cAMP-dependent protein kinase (cAPK), this base has been proposed to be Asp 166, which corresponds to Asp 946 of the PI3K γ -946-DRH-948 sequence that is conserved in all PI3Ks. In the structure of PI3K γ , however, Asp 946 is not in a location where it could function as a general base catalyst. The constellation of residues in the active site in the presence of ATP-metal suggests that Asp 946 may simply have a structural role in maintaining the ATP-binding pocket. Therefore, either PI3K has no general base catalyst, in which case the mechanism could be primarily dissociative, involving a metaphosphate transition state¹², or a different residue assumes this function. One possible candidate is His 948; although its side chain is not near the γ -phosphate of ATP, a rotation around $\chi 1$ would place it in a location such that it could interact with the 3-hydroxyl of the lipid headgroup.

PI3Ks are one of the effectors for Ras proteins (reviewed in ref. 13). Binding of PI3K to Ras is affected by mutations in both switch I and switch II regions of Ras (residues 30-38 and 60-76, respectively)^{14,15}. These two regions change conformation upon GTP binding and are binding sites for a diverse array of downstream effectors; however, mutations in these switch regions differentially affect the binding of various effectors.

The RBD of PI3K γ (residues 220–311) has the same fold as the RBD of Raf¹⁶ and RalGDS¹⁷, two other well-characterized effectors of Ras (Fig. 5). The RBD of PI3K consists of a five-stranded mixed β -sheet (R β 1–R β 5) flanked by two α -helices (R α 1 and R α 2). Residues 228–230 (in the RB1–RB2 loop) and 257–265 (in the $R\alpha 1 - R\beta 3$ loop) are disordered.

The crystal structure of Ras-related protein Rap1A in complex with the RBD of protein kinase c-Raf¹⁶ and the structure of Ras in complex with the RBD of RalGDS¹⁷ suggest a structural basis for effector specificity. The structures of both of these complexes were determined using the isolated RBD, without the catalytic portions

Data collection and r	nultiple isomorphou	s replacement phasing sta	atistics					
Data set	Resolution (Å)	Observations/ unique reflections	Completeness (last shell) (%)	R _{merge} ‡‡	⟨I/σ⟩ (last shell)	No. of sites	Phasing powerlll	R _{iso} §§
Native*††	2.4	144,973/37,485	97.2 (90.6)	8.5	16.0 (3.1)	-	-	-
LuCl ₃ -1† [☆]	2.2	191,292/49,599	95.5 (93.3)	9.5	14.3 (1.1)	7	1.7	0.23
LuCl ₃ -2ࠠ	3.5	43,038/12,484	99.7 (98.2)	8.5	11.3 (3.4)	3	1.9	0.18
Lanthanides§++	3.0	71,426/19,180	97.9 (97.1)	4.5	15.6 (2.3)	8	1.9	0.24
ATM ++	2.7	94,900/25,688	92.6 (60.2)	4.8	17.0 (5.7)	5	0.8	0.22
lodine¶††	2.6	102,511/28,856	93.2 (67.1)	6.0	13.6 (1.4)	3	0.1	0.21
Refinement statistics								
Data set	Resolution (Å)	Protein atoms	Waters	R _{crystal} ¶¶	R _{free} ¶¶ (% data)	R.m	.s.d. from ideality	(##
					. ,	Bonds	Angles	Dihedrals
LuCl ₃ -1	25.0-2.2	6,813	89	0.25	0.30 (5.4)	0.013Å	1.7°	23°
lodine¶	25.0-2.6	6,954	14	0.26	0.33 (5.0)	0.005 Å	1.1°	21°
Mn#	25.0-2.6	6.837	26	0.26	0.32 (5.6)	0.005 Å	1.2°	21°

* The native crystal was soaked in 2.5 mM InsP₃, 1.0 mM ATP and 10 mM MgCl₂ for 1 h. Although this was the native crystal for heavy-atom phasing, the final high-resolution structure refinement used data from LuCl₃-1.

+ LuCl₃-1 crystal was soaked in 20 mM LuCl₃ and 1.25 mM ATP for 1 h 40 min.

‡ LuCl₃-2 crystal was soaked in 20 mM LuCl₃ and 1.3 mM ATP for 4 h. S Lanthanides crystal was soaked for 4 h in a mixture of 3.3 mM each of GdCl₃, TbCl₃, HoCl₃, ErCl₃, TmCl₃, and LuCl₃ with 1.26 mM ATP and 1 mM EMTS

ATM crystal was soaked for 22 h in 10 mM sodium aurothiomalate.

n lodine crystal was soaked for 75 min in 1 mM Nal3 and 1 mM chloramine T. This crystal was originally prepared in an attempt to iodinate tyrosine residues as a heavy atom derivative, but no evidence of tyrosine iodination was seen in the resulting structure. #Mn crystal contained 1.4 mM ATP and 14 mM MnCl₂

* Data were collected at ESRF beamline ID2b

†† Data were collected at ESRF beamline ID14-4

 $\begin{aligned} & || \mathbf{D} = \mathbf{L}_{int} \mathbf{L}_$

 $\P R_{\text{cryst}}$ and $R_{\text{free}} = \Sigma |F_{\text{obs}} - F_{\text{calc}}|/\Sigma F_{\text{obs}}$; R_{free} calculated with the percentage of the data shown in parentheses.

R.m.s. deviations for bond angles and lengths in regard to Engh and Huber parameters

				1α2 1/1/1/1/1/1 -								
		150 160			.90	200		210	220	2	30 24	
ΙЗКγ		SEETLAFORQLNALIGYDVIDVSN										
13Kα 13Kβ		REEKI.LNREIGFAIGMPVCEFDM PGEKLDSKIGVLIGKGLHEFDS										
ізкδ	108	RVKKL.INSQISLLIGKGLHEFDS	LCDPEVNDF.RAK	MCQFCEEAA	ARRQQL.G	WEAWLQYSFP	LQLEPSAQTW	GPGTLRL	PNRALLVNVKFT	GSEES	FTFQVSTKDVP	LALMACALR
sC2 alGDS	319 17	VANGHELFEVSE	ERDEEVAAFCH									DLLIYQILC D. <u>KAPIVIRKAMD</u>
	1,											20.1011101100110
		Rβ3	Rβ4	Ra	2	RB5		RBD/	C2 domain li	nker		
			∕			- ````						
ΙЗКγ	261	270 .IPESQNERDFVLRVCG	280	290	300	310	320	330	340			350 36
I3Kα	233	LLSSEQLKLCVLEYQGKYILKVOG	CDEYFLEKY	PLSQYKYIRSC	IMLGRMPNLK	MMAKESLYSQ	LPMDCFIMPS	YSRRISTAT	PYMNG			.TSTKSLWVINRAL
13Kβ 13Kδ	236	GKEDEVSPYDYVLQVSG ROPLVEOPEDYTLOVNG	RVEYVFGDH	PLIQFQYIRNC	VMNRALPHFI	LVECCKIKKM	YEQEMIAIEA	AINRNSSNL	PLPLPPKK	• • • • • • • • • •	•••••	RIISHVWENNNPF
SC2		RWDVGDFVLKPCG										
ps34	1											
alGDS LCδ	58 626	DEPE <u>DYELLQI</u> I										
						c0 -1						
	С2β2	C2β3		C2β4		omain <u> </u>			C2	2β6	C2β7	
		370 380	⇒>	400	410	=>		440	450	460		
ε3κγ	368	IDIPVLPRTADLTVFVEAN		•	•		KGALINIOTY	•				•
сзкά	341	ATYVNLNIRDIDKIYVRTG	IYHGGEPLCDNVN	TQRVP.CS	NPRWNEWL	NYDIYIPDLP	RAARLCLSIC	SVKGRKG	AKEEHCPLAWG	IINLFDYTDT	LVSGKMALNLW	PVPHGLEDLLNPIG
13Kβ 13Kδ		VKGNKLNTEETVKVHVRAG IQGSKVNADERMKLVVQAG										
3C2		THRIPIIWATSYEDFYLSCS										
ps34 LCδ		LEDPMLKFSGLYQETCSDLYVTCQ GQQLPKVNKNKNSIVDPKVIVE										
LCO	030	GOOLER VINC INTRASIVDER VIVE	<u>IH</u> GV.GRD1 <u>G5RQ</u>	<u>1AV11</u> .NNG	. FINPRWLMEP	<u>EF</u> EVIVP		<u></u>		<u>DF1G</u>	<u>VOTTEM</u> INOL	<u>KOGIKHVHLLSK</u>
			C2β8 C2,	/helical do	main linker	r 🔶	hA1		hB1	hB1'	hA2	hB2
						0		Δ0				
		500 510	520	•	540		550	560	570	580	590	600
13Κγ 13Κα	494 467	QGSFNADKLTSATNPDKENSMS NKETPCLELEFDWFSSVVKFPDMS										
гзкβ	465	FPDELEEMLNPMGTVQINPYTENA	TALHVKFPENKKQ	PYYYPPFDKII	EKAAEIASSD	SANVSSR.GG	KKFLPVLKEI	LDRDPLSQL	CENEMDLIWIL	QDCREIFPQ	SLPKLLLS	IKWINKLEDVAQLQA
13Kδ sC2	454 769	NPIGIVRSNPNIDSA SAPNFHQPDS										
ps34		MSRLAKLTKAHRQGHMVKVDWLDR										
ĽCδ	774		LFVKISIQD									
ĽЗKά	616 591	620 630 WWDQSALDVGLIMQLLDCNFSDEN PPIKPEQAMELLDCNYPDPM	VRGFAVRCLEKYL	EDDDVLH TDDKLSQ	YLIQLVQVLK	670 FEPYHDSA YEQYLDNL	lvrflikkal	690 RNKRIG INQRIG	.HFFFWHLKSE	71 AQSRH.YQQ 1.HNKT.VSQ	RFAVILEAYLRO RFGLLLESYCR	GTAMLHDFIQQ AC.GMYLKHLNRQV
13Kγ 13Kα 13Kβ 13Kδ sC2 ps34 P2A	616	WAU G20 630 WWDQSALDVGLIMQLLDCNFSDEN	UXIXIXXXX 640 650 VRALAVQKLES.L VRGFAVRCLEKYL VRCFAVRCLEKYL VRCFAVRCLEKYL VRCFAVRCLEKYL VRCFAVRSLEK.L VRCFAVRSLEX.L VRCFAVARLRQ.A	EDDDVIH TDDKLSQ SDEELSQ TDDELFQ SDAELLD DDEDLIM	YIYYYYYYYY 660 YYLQUVQAVK YYLQUVQVLK YYLQUVQVLK YYLQUVQVLK YYLQUVQALK YYLQUVQALK YYLQUVQALK	670 FE PYHDSA YE QYLDNL YE PFLDCA YE SYLDCE YE CYLDSP YE. / ENLEQD	hB4 680 LARFLLKRGL LVRFLLKRAL LSRFLLERAL LIKFLLDRAL PLVRFLLKRAV DLCTFLLSRAS	690 RNKRIG JINQRIG GNRRIG ANRKIG SDLRVT KNSTLA	TATATATATATATATATATATATATATATATATATATA	71 AQSRH.YQQ I.HNKT.VSQ I.HNPA.VSV I.HVPS.VAL J.KDSQ.FSI C.EDQD.TQQ	NAVITATION 0 720 RFAVILEAYLR QFGULLESYCR QFGVILEAYCR RFGLILEAYCR RYQYLLAALLC RDPKTHEMYLM	TANANA 730 2C.GTAMLHDFTQQ AC.GMYLKHLNRQV 2S.VGHMKVLSKQV 2R.THHMKVLMKQG 2CGKGLREEFNRQC M.RRFSQALLKGD
13Kα 13Kβ 13Kδ sC2 ps34	616 591 599 572 879	VIII VIII 620 620 630 WDQSALUGITMQLLCNFSDEN PEIREQAMELLCONPDEM PELPUSALELLOFNPDQY PELPUSALELLDFSPPDQF THMHQDALGLLHATFPDQE NEMDUEGSLELISSHTINFT	UXIXIXXXX 640 650 VRALAVQKLES.L VRGFAVRCLEKYL VRCFAVRCLEKYL VRCFAVRCLEKYL VRCFAVRCLEKYL VRCFAVRSLEK.L VRCFAVRSLEX.L VRCFAVARLRQ.A	EDDDVIH TDDKLSQ SDEELSQ TDDELFQ SDAELLD DDEDLIM	TATA A A A A A A A A A A A A A A A A A	1 670 FEPYHDSA YEQYIDNI YEPFIDCA YESYIDCE YECYIDSP YE/ENLEQD NDHVYAIR se domain 1	hB4 680 LARFILKGEL LVRFILKGL LVRFILKAL LVRFILKAL LVRFILKRAV LCTFLISRAS ZEAATSNIKKI N-terminal	690 RNKRIG INQRIG GNRRIG ANRKIG SOLRVT SKNSTLA WEKFGKEWA	TATATATATATATATATATATATATATATATATATATA	71 AQSRH.YQQ I.HNKT.VSQ I.HNPA.VSV I.HVPS.VAL J.KDSQ.FSI C.EDQD.TQQ	NAVITATION 0 720 RFAVILEAYLR QFGULLESYCR QFGVILEAYCR RFGLILEAYCR RYQYLLAALLC RDPKTHEMYLM	TANANA 730 2C.GTAMLHDFTQQ AC.GMYLKHLNRQV 2S.VGHMKVLSKQV 2R.THHMKVLMKQG 2CGKGLREEFNRQC M.RRFSQALLKGD
13Kα 13Kβ 13Kδ 5C2 25534	616 591 599 572 879	VIII UNIVII UNIVII UNI 620 630 WDQSALDVGLIMQLLDCNF5DEN PPIKPEQAMELLDCNYPDM MELPPRALELLDFNYPDQY PELPVLSALELLDFSFPDCH THWNKQDALGLLHATFPDQE NFMDVEDSLELISSHYINFT LIPATVELAEDAKWR	CALL CALL CALL CALL CALL CALL CALL CALL	EDDVIH TDKLSC SDEELSC TDDELFC SDELFC DDEDLIM AGQLGVEFFDE	660 WILQLVQAVK YILQLVQVIK YILQLVQVIK YILQLVQVIK YILQLVQVIK YILQLVQVIK KINSLCMWI KINSLCMWI KINSL	U 670 FEPYHDSA YEQYLDNL YEQYLDNL YESYLDCE YECYLDSP VE./ENLEQD MDHVYAIR se domain 1 kβ2	$ \frac{hB4}{MMNAVA} $	690 RNKRIG INQRIG CARRIG SDLRVT SKNSTLA VEKFGKEWP lobe kβ4	YNTYTYTY 700 .HFLFWFIRSE .HFFFWHRSE .HFLFWHRSE .HFLFWHRSE .NYLYWYVIVE MATTI IFKVLAM	71 AQSRH.YQQ A.HNKT.VSQ V.HIPA.VSV A.HVPS.VAL KDSQ.FSI C.EDQD.TQQ GGDPNYLHRM kβ5	NATATALAN 0 720 REAVILEAY REGLILESYCR REGLILESYCR REGLILEAYCR RYGYLLAALCO RDPKTHEMYLM ITTLFCINVLSE 	730 730 2C.GTAMLHDFTQQ AC.GMILHINROV SS.VGHMKVLSKQV R.THHMKVLSKQV R.THHMKVLSKQV M.RRFSQALLKGD VC.GODI
13Kα 13Kβ 13Kδ 5C2 0534 P2A	616 591 599 572 879 356 403	VINI- ONIVITALIONESDEN PERFECTIONED PERFECTION PER	UNITATATA 640 650 VRALAVCKLES.L VRGFAVRCLES.L VRGFAVRCLES.L VRSFANKSLRX.L VRSFANKSLRX.L VRRVAVARLRQ.A VRLAITEYMPL.L kozz 760 770	EDDVIH TDKLSQ SDEELSQ TDDELFQ SDAELLE DDEDLIM AGQLGVEFFDE	G60 YILQLVQAVK YILQLVQVIK YILQLVQVIK YILQLVQVIK YILQLVQVIK YILQLVQVIK YILQLVQVIK YILQLVQVIK KINSLCMWI KINSLCMWI KINSL KINSL KINSL KINSL	II 670 FE PYHDSA YE QYLDNL YE PFIDCA YE SYLDCE YE CYLDSP YE./ENLEQD MD HVYALR se domain 1 kB2 790	hB4 (80) LARFLLKRGL LVRFLLKRGL LVRFLLKRAL LVRFLLERAL LVRFLLRAV LCTFLLSRAS ZEAATSNLKKL N-terminal kB3 800	690 RNKRIG INQRIG GNRRIG ANRKIG SOLRVT KINSTIA VEKFGKEWP lobe kβ4 810	YUTYYYYYYY 700 HFLFWFIRSE: HFLFWFIRSE QFLFWFIRSE HFLFWFIRSE HFLFWFIRSE NYFFWIRKE NYFYWYVIVEX HATTIFKVLAM 820	71 AQSRH. YQQ A. HAKT. VSQ V. HIPA. VSV A. HVPS. VALI KDSQ. FSI C. EDQD. TQQ SEDPNVLHRM kβ5 830	UNIXIAAAA 0 720 RFAVILEAYLRA RFGLILEAYCR RFGLILEAYCR RFGLILEAYCR RTGLILEAYCR RTGLILEAYCR RDPKTHEMYLM ITTLFCINVLSE UNIXIAALCO 840	
13Kα 13Kβ 13Kδ =C2 0=s34 P2A 13Kγ 13Kα	616 591 572 879 356 403 742 713	UNI CONTRACT	ATTATATATA 640 650 VRAIAVQKLES, L VRGPAVRCLEK/L VREYAVGCLE, M. VRSPAIKSLRK, L VRRVAVARLAQ, A VRIATIEYMPL, L KO2 VRIATIEYMPL, L KO2 VRVAVIATIEN KO2 KO2 KO2 KO2 KO2 KO2 KO2 KO2	EDDVLH TDKLSQ SDEELSQ TDAELLD DDEELLD AGQLGVEFFDE	660 WILQINQAVK WILQINQVIK WILQINQVIK WILQINQVIK WILQINQVIK WILQINQVIK KINSLOWAVI	$\begin{array}{c} \hline \\ \hline $	hB4 680 LARFILKRGL LNRFILKRAL LSRFILERAL LINRFILBRAL VINFILKRAV VINFILKRAV VINFILFRAN V	690 RNKRIG GNRRIG SOLRVT SOLRV	XUIVIVIVI 700 HFLFWFLKSE HFLFWFLKSE OFLFWFLKSE HFLFWFLKSE HYFFWLIKDE NYLYWYVVE HATTIPKVLAM 820 KCADPT. ALSE ENPDINS SLI	71 AQSRH.YQQ 4. HNKT.VSQ 4. HVPS.VAL .KDSQ.FSI .EDQD.TQQ XEDDNVIHEM kβ5 830 ETIGIIFKH QUNELIFKN	UNIXIANA 0 720 REAVILEAYLRA REGLILEAYCR REGLILEAYCR ROPKIHEMYLM ITTLFCINVLSE UNIXIAL GEDLRQDMLIL GEDLRQDMLIL	
13Kα 13Kβ 13Kδ 5C2 5S34 52A 13Kγ 13Kα 13Kα	616 591 572 879 356 403 742 713 720	VINI- ONDORNALIVGLINGLIDORFSDEN PPIKPEQAMELLICORFSDEN PPIKPEQAMELLICORFSDEN PPIKPEQAMELLICORFSDEN PRICENTROLOGICALINATPPODE NEMDVEDSLELISSHYNFT VINIVALAVINAL 750 LQKVTDIKSLSAEKYDV NLTSLIKLNAV.KIN	UTATIANA 640 650 WRGFAVRCLEKYL WREYAVCLEKYL WREYAVCLEKYL WREYAVCLEKYL WREYAVCLEKYL WREYAVCLEX WREYAVCLEX WREYAVCHIST WREYAVCH	EDDVIH TDELSC SDELSC SDELFC SDELFC DDELFC DDEDLM AGQLGVEFFDF DDEDLM AGQLGVEFFDF	G60 YILQUVQAVK YILQUVQVLK YILQUVQVLK YILQUVQVLK YILQUVQALK YILQUVQALK KINSLCWWWI KINSLCWWWI KINSLCWWWI KINSLCWWWI KINSLCWWWI ISDIQSFIV 1.GDIQSFIN	$\begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} $	hB4 680 LARFILKRGL LINFILKRGL LINFILKRGL LINFILKRAL LINFILKRAL LINFILKRAV N-terminal kB3 800 EKCKVMASK. KECRIMSSA.	690 RNKRIG INQRIG INQRIG SDLRVT KNSTLA VEKFGKEWP lobe kβ4 810 .KKPLWLEF .KRPLWLEM .KKPLWLEM	INTANANA 700 .HELFWEISE .HEFFWEISE .HEFFWEISE .NYLYWVIVE .NYLYWVIVE	71 AQSRH.YQQ 4. HINKT.VSQ 4. HIVPS.VAL KDSQ.FSI C. EDQD.TQQ XCDPNVI.HRM 8.30 KB5 8.30 RETIGLIFKN GVIFKN	UAINIAAAAA 0 720 REPAULLEAYLR REPAULLEAYLR REPAULLEAYCR REPAULLEAYCR RUPULLEAYCR RUPULLAALCC RUPKTHEMYLN RUPKTHEMY	
13Kα 13Kβ 13Kδ 5C2 5c34 52A 13Kα 13Kα 13Kα	616 591 572 879 356 403 742 713 720 693	UNI CONTRACT	ATTATATATA 640 650 VRAIAVQKLES, L VREYAVCLEQ, U VREYAVACLEQ, U VREYAVARLEQ, A VREYAVARLEQ, A VREYAVARLEQ, A VREYAVARLEQ, MC KOZ KOZ KOZ KOZ KOZ KOZ KOZ KOZ	EDDVIH TDEKLSC SDEELSC TDELLY SDAELLD DDEDLIM AGQLGVEFFDF	GG0 GG0 VILQLVQAVK VILQLVQVLK VILQLVQVLK VILQLVQVLK VILQLVQVLK KINSLCVWH KINSLCVWH KINSLCVWH KINSL KINS	$\begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \begin{array}{c} \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} $	hB4 680 LARFILKRGI LVRFILKRGI LVRFILKRAU LORFILKRAU LORFILKRAU LORFILKRAU LORFILKRAU LORFILKRAU LORFILKRAU B00 B00 EKCKWASK. KECRIMSSA. EKCKVMASK.	690 RNKRIG INQRIG CMRRIG ANRKIG NEKFGKEWP 10be kβ4 810 .KKPLWLEF .KKPLWLWY .MKPLWLWY	INITYIYYYYY 700 HELFYFHSE: HELFYHLSE HELFYHLSE HYFWLLKDE HYFWLLKDE HATI IPKVLAM 820 KCADPT. ALSE EENPDINS.ELLI NNKVFGE.DSV SNEEASOGSV	71 IAQSRH.YQQ /.HIPA.VSV A.HNKT.VSQ /.HIPA.VSV A.HOSQ.FSI .EDQD.TQQ SEDDNYLHEM kβ5 830 RETIGIIFKH 'QNNEIIFKN S VIFKN	UNINIAAAAA 0 720 REPAULEAYLR REPGLILESYCR REGLILESYCR REGLILEAYCR RYQYLLAALLO ROPKTHEMYLN ITTLFCINVLSE 	VIIIIIIIII 730 7
13Kα 13Kβ 13Kδ 5C2 52A 13Kγ 13Kα 13Kβ 13Kβ 13Kδ 5C2 5334	616 591 599 572 879 356 403 742 713 720 693 1001 539	UNIUNIUNIU 620 630 WDQSALDVGIIMQLLOORSDEN PPIKPEQAMELLOONSDEN PPIKPEQAMELLOONSDEN PPIKPEQAMELLOONSDEN PPIKPEQAMELLOONSDEN PPIKPEQAMELLOONSDEN PPIKPEQAMELLOONSDEN 	UTITUTUTU 640 650 WRGTAVQKLES, L WRGTAVQKLES, L WRGTAVQKLES, L WRTAVAQKLES, L WRTAVARLEQ, A WRLAI IEYMPL, L KO2 VRTAVARLEQ, A KO2 KO2 KO2 KO2 KO2 KO2 KO2 KO2	EDDVIH TDKLSC SDELSC SDELSC DDEDLM AGQLGVEFFDE NLQNINL RPDFMDA QSAYREA QEAYLEA	G60 G60 WILQLVQQVK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILQLVQVIK KINSLCMQVI KINSLCMQVI KINSLCMQVI KINSLCMQVI KINSLCMQVI 780 .PQSFRVPYD IQGILSPIN .LSHLQSPID .LSHLQSPID CRLPLS	$\begin{array}{c} \begin{array}{c} \begin{array}{c} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} $	hB4 (680 LARFILKRGL LINFILKRGL LINFILKRGL LINFILKRAL LINFILKRAL LINFILKRAL M-terminal kB3 800 EKCKVMASK. KECRINSSA. KECRINSSA. KECRINSSA. RDCSYFNSN. RDCSYFNSN.	690 RNKRIG INDRIG GNRRIG GNRRIG SULRVT KINSTLA VEKFEKEWE lobe kKPLMLEF .KRPLWLM KRPLWLM MKPLMLME .AVPLKLSF .LMPAQLFF	INTANANA 700 HELFVFLSE HELFVFLSE HELFVFLSE HYFFVLKDE NYLYWYVIE 820 KCADPT. ALSP ENPDINS ELL NKVFCE DSV SNEEASGSSV QNVDPL	71 VAQSRH. YQQ A. HNKT. VSQ 7. HIPA. VSV 4. HVPS. VAL 4. KNSQ. FSL 2. EDQD. TQQ XEDENYI HEM 830 WETIGI IFKH QNNEI IFKN 5 VIFKN 5 VIFKN SENIRVIFKC XIPVIFKC	ALALANAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	VIVINI 730 2C. GTPMLHDFTQQ AC. GMYLKHLNRQV SS. VSHMKVLSRQV M. THHMKVLSRQV M. RRFSQALLKGD VC. GODI 850 21. LRIMESIWETE 1. I.RIMESIWETE 1. I.RIMESIWETE 21. LRIMESIWETE 21. LRIMESIWETE 21. I.RIMENIWQP 21. I.RIMENIWQP 21. I.SIMOKLIKKE
13Kα 13Kβ 13Kδ 13Kδ 13Kδ 13Kα 13Kα 13Kβ 13Kδ 13Kδ 13Kδ 13Kδ 13Kδ 13Kδ 13Kδ 13Kδ 13Kδ 13Kδ 13Kδ 13Kδ 13Kδ 13Kβ 13Kβ 13Kβ 13Kδ 13Kb	616 591 572 879 356 403 742 713 720 693 1001	VIVI OVI OVI OVI OVI OVI OVI OVI OVI OVI	UTITUTUTU 640 650 WRGTAVQKLES, L WRGTAVQKLES, L WRGTAVQKLES, L WRTAVAQKLES, L WRTAVARLEQ, A WRLAI IEYMPL, L KO2 VRTAVARLEQ, A KO2 KO2 KO2 KO2 KO2 KO2 KO2 KO2	EDDVIH TDKLSC SDELSC SDELSC DDEDLM AGQLGVEFFDE NLQNINL RPDFMDA QSAYREA QEAYLEA	G60 G60 WILQLVQQVK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILQLVQVIK KINSLCMQVI KINSLCMQVI KINSLCMQVI KINSLCMQVI KINSLCMQVI 780 .PQSFRVPYD IQGILSPIN .LSHLQSPID .LSHLQSPID CRLPLS	$\begin{array}{c} \begin{array}{c} \begin{array}{c} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} $	hB4 (680 LARFILKRGL LINFILKRGL LINFILKRGL LINFILKRAL LINFILKRAL LINFILKRAL M-terminal kB3 800 EKCKVMASK. KECRINSSA. KECRINSSA. KECRINSSA. RDCSYFNSN. RDCSYFNSN.	690 RNKRIG INDRIG GNRRIG GNRRIG SULRVT KINSTLA VEKFEKEWE lobe kKPLMLEF .KRPLWLM KRPLWLM MKPLMLME .AVPLKLSF .LMPAQLFF	INTANANA 700 HELFVFLSE HELFVFLSE HELFVFLSE HYFFVLKDE NYLYWYVIE 820 KCADPT. ALSP ENPDINS ELL NKVFCE DSV SNEEASGSSV QNVDPL	71 VAQSRH. YQQ A. HNKT. VSQ 7. HIPA. VSV 4. HVPS. VAL KNSQ. FSL 2. EDQD. TQQ XEDENYI HEM 830 WETIGI IFKH QNNEI IFKN 5 VIFKN 5 VIFKN SENIRVIFKC KYPVIFKH	ALALANAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	
13Kα 13Kβ 13Kδ 5C2 52A 13Kγ 13Kα 13Kβ 13Kβ 13Kδ 5C2 5334	616 591 599 572 879 356 403 742 713 720 693 1001 539	VINI-UNIVINI-UNI GOO 630 WDQSALDVGI.TMQLI.LOCNFSDEN PPIKPEQAMELL.LOCNFSDEN PPIKPEQAMELL.LOCNFDDM PRILPPRALELLDFNYPDQF PRILPVERALELLDFNYPDQE PRILPVERALELLDFNYPDQE INVIVINICALELLATFPDQE VINIVINICALELATFPDQE VINIVINIVINIVINICALELATFPDQE VINIVINICALELATFPDQE VINIV	UNIXIANA 640 650 VRETAVQCLES, L VREFAVRCLEKYL VREYAVCLEKYL VREYAVARCLEKYL VREYAVARCLEKYL VREYAVARCLEKYL KRZ VREYAVARCLEY KRZ KRZ KRZ KRZ KRZ KRZ KRZ KRZ	EDDVIH TDKLSC SDELSC SDELSC DDEDLM AGQLGVEFFDE NLQNINL RPDFMDA QSAYREA QEAYLEA	660 660 WILQLVQAVK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILQLVQVIK KINSLCWWI 	$\begin{array}{c} \begin{array}{c} \begin{array}{c} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \end{array} \\ \begin{array}{c} \end{array} \\ \end{array} $	hB4 680 1ARPILKRGI LISRFILKRGI LISRFILKRAI LISRFILFAL LISRFILFAL INRFILKRAV ICTFLISRAS EEAATSNIKKI N-terminal μβ3 	690 RNKRIG GRRRIG GRRRIG GRRRIG GRRRIG KRYLIA KKYLKA 810 KKPLMLEF KRPLNLV MKPLMLEF KRPLNLV MKPLMIM MKPLMIM FGEVMGTW	INTANANA 700 HELFYFLASE HELFYFLASE HELFYFLASE HYFFYLLDG NULYWYUVE HATTIFKVLAM 820 KCADPT.ALSE RENEDINS.ELL NNKVFGE.DSW SINEFAGSGSSW QNDPLC	71 2AQSRH. YQQ A. HNKT. YSQ A. HNYDS. VAL. KISQ. FSI KISQ. FSI KISQ. FSI SQDD. TQQ SQDDWYI.HRM KISQ 	UNINIAAAAA 0 720 REPAULEAYLR REPAULEAYLR REPAULEAYCR REPAULEAYCR RYQYLLAALLO ROPKHEMIN MITLECINVISE 	VIVIUM 730 2C. GTPMLIDFTQQ AC. GMYLKHLNRQV SS. VSHMKVLSKQV XS. VSHMKVLSKQV M. RRFSQALIKGD CCGGQL CCGQDI kX3 VIVIVIVI 850 21. LRIMESIWETE 21. LRIMESIWETE 21. LRIMESIWETE 21. LRIMESIWETE 21. LRIMESIWETE 21. LRIMESIWETE 21. LRIMESIWETE 21. LRIMESIWETE 21. LRIMESIWETE 21. LSIMEVLIKKQB
13Kα 13Kβ 13Kδ 5C2 52A 13Kγ 13Kα 13Kβ 13Kβ 13Kδ 5C2 5334	616 591 599 572 879 356 403 742 713 720 693 1001 539	VINI- VINI- CO CO CO CO CO CO CO CO CO CO	UNIXIAAAA 640 650 VRAIAVQKLES. L VREYAVCLEQU VREYAVCLEQU VREYAVCLEQU VREYAVCLEQU VREYAVCLEQU VREYAVCALQU VREYAVCAUCA VREYAVCAUCA VREYAVCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUCAUC	EDDVLH TDEKLSC SDEELSC TDDELLSC SDEELLSC SDEELLM AGOLGVEFFDF DDEDLIM AGOLGVEFFDF D REDFMDA QEAYLEA QEAYLEA LLCENEKMNIS	G60 G60 WILQLVQAVK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILQLVQVIK KINSLCMWH KINSL 780 .PQSFRVPYD .CRLPSPIN CRLP	G70 FEPYHDSA YEQYLDNL YESYLDCE YESYLDCE YECYLDSP YE./ENLEQD MDHVYAIR se domain 1 k22 790 POLKAQALVI PAHQLGNLFLI PAHQLGNLFLI PAHQLGNLFLI PEXLIXGIVP SELLIXKGIVP SELLIXKGIVP SELLIXKGIVP Se domain (UXVXVXI	hB4 (80) 1ARFILKRGL UNFILKRGL 1NRFILKRAL USRFILERAL UNFILKRAL UNFILKRAL VITFLISRAS EEATSNIKKI N-terminal k3 800 EKCKVMASK. KECRIMSSA. EKCKVMASK. EKCKVMASK. EKCKVMASK. EKCKVMASK. EKCKVMASK. CCTPMDSK. 20059FINSN. 20059F	690 RNKRIG CNRRIG CNRRIG CNRRIG SOLRVI KNSTIA VEKPEKEMP lobe KRPLMLEF KRPLMLEF KRPLMLEF TGEVMKIF TGEVMKIF lobe	XIII XIXIII 700 .HELFWEISEE .HEFFWEISEE .HEFFWEISEE .NYLYWYVIVE .NYLYWYVIVE .NYLYWYVIVE .NYLYWYVIVE .NYLYWYVIVE .NYLXXX .NYLXXX .NYLXXX .NYLXXX .NYLXXX .NYLXXX .NYLXXX .NYLXXX .NYLXXX .NYLXXX .NYLXXX .NYLXXXX .NYLXXXX .NYLXXXX .NYLXXXX .NYLXXXX .NYLXXXX .NYLXXXX .NYLXXXX .NYLXXXXX .NYLXXXXX .NYLXXXXX .NYLXXXXXX .NYLXXXXXXX .NYLXXXXXXXXX .NYLXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	71 2AQSRH. YQQ A. HNKT. YQQ A. HNYS. VAL HVPS. VAL LODO. TQQ SQDPWI.HRM SIFKN IIFKN IIFKN IIFKN IIFKN IIFKN IIFKN IIFKN IIFKN IIFKN IIFKN IIFKN IIFKN 	ALALANAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	VIVINIVA 730 730 730 730 730 730 730 730 730 730
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 ΙΞΚά ΙΞΚά ΙΞΚά ΞΞΚά <!--</td--><td>616 591 599 572 879 356 403 742 713 720 693 1001 539 270 8855 834 837 811 1115 668</td><td>UNITALI UNITALI 620 630 WDQSALDVGI:MQLLCONFSDEN PPIKPEQAMELLCONFSDEN </td><td>CHININAL 640 650 VRGTAVKCLES, L VRGTAVKCLES, L VRGTAVKCLES, L VRGTAVKCLES, L VREYAVKCLES, L VREYAVKCLES, L VREYAVKCLES, L VREYAVKCLES, L VREYAVCLES, L VREYAVCLE</td><td>EDDVIH TDEKLSC SDEELSC TDELLY SDAELLI DDEDLIM AGQLGVEFFDF U U NILONINI QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA NICAFKDEVI.S GALQFNSHTIL AJAAAFNKOALL ATAAFNKOALL ATAAFNKOALL</td><td>G60 G60 WILQLVQAVK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILSQLVQAIK KINSLCMAWI KINSLCMAWI KINSLCMAWI KINSLCMAWI CONTRACTOR RANGE CONTRACTOR KINA</td><td>$\begin{array}{c} U \\ \hline & 0 \\ \hline \hline \hline & 0 \\ \hline \hline \hline & 0 \\ \hline \hline \hline \hline & 0 \\ \hline \hline \hline \hline \hline \hline & 0 \\ \hline \hline$</td><td></td><td>690 RNKRIG CRIRTIG CRIRTIG CRIRTIG CRIRTIG CRIRTIG CRIRTIG CRIRTIG CRIPTICA BIO KRPLMLA MKPLMLA MKPLMLA MKPLMLA MKPLMLA CRIPTICA CRIPA CRIPTICA CRIPTICA CRIPTICA CRIPTICA CRIPA CR</td><td>XUXYXXXX 700 .HELFWFLKSE .HEFWHLKSE .HEFWHLKSE .HEFWHLKSE .HEFWHLKSE .HEFWHLKSE .HEFWLKSE .HEFWLKSE .HEFWLKSE</td><td>71 24,05RH. YQQ 1. HNRT. YSQ 1. HNRT. YSQ 1. HVPS. VAL KSQ. FSI KSQ. FSI SQD TQQ GDPWIHPM SQD TQQ SQD IFAN IFAN IFAN IFAN IFAN IFAN IFAN IFAN IFAN IFAN IFAN </td><td>ALANAAAA 0 720 720 720 720 720 720 720 720</td><td>VIVINI 730 730 2C.GTPMLHDFTQQ AC.GMYLKHLNRQV SS.VSHMKVLSRQV XS.VSHMKVLSRQV M.RRFSQALIKGD XC.GODI 850 21.LRIMESIWETE 1.IRIMESIWETE 1.IRIMESIWETE 1.ISLMKEA M.IRIMLIKKEA M.</td>	616 591 599 572 879 356 403 742 713 720 693 1001 539 270 8855 834 837 811 1115 668	UNITALI UNITALI 620 630 WDQSALDVGI:MQLLCONFSDEN PPIKPEQAMELLCONFSDEN	CHININAL 640 650 VRGTAVKCLES, L VRGTAVKCLES, L VRGTAVKCLES, L VRGTAVKCLES, L VREYAVKCLES, L VREYAVKCLES, L VREYAVKCLES, L VREYAVKCLES, L VREYAVCLES, L VREYAVCLE	EDDVIH TDEKLSC SDEELSC TDELLY SDAELLI DDEDLIM AGQLGVEFFDF U U NILONINI QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA QEAYLEA NICAFKDEVI.S GALQFNSHTIL AJAAAFNKOALL ATAAFNKOALL ATAAFNKOALL	G60 G60 WILQLVQAVK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILQLVQVIK WILSQLVQAIK KINSLCMAWI KINSLCMAWI KINSLCMAWI KINSLCMAWI CONTRACTOR RANGE CONTRACTOR KINA	$\begin{array}{c} U \\ \hline & 0 \\ \hline \hline \hline & 0 \\ \hline \hline \hline & 0 \\ \hline \hline \hline \hline & 0 \\ \hline \hline \hline \hline \hline \hline & 0 \\ \hline \hline$		690 RNKRIG CRIRTIG CRIRTIG CRIRTIG CRIRTIG CRIRTIG CRIRTIG CRIRTIG CRIPTICA BIO KRPLMLA MKPLMLA MKPLMLA MKPLMLA MKPLMLA CRIPTICA CRIPA CRIPTICA CRIPTICA CRIPTICA CRIPTICA CRIPA CR	XUXYXXXX 700 .HELFWFLKSE .HEFWHLKSE .HEFWHLKSE .HEFWHLKSE .HEFWHLKSE .HEFWHLKSE .HEFWLKSE .HEFWLKSE .HEFWLKSE	71 24,05RH. YQQ 1. HNRT. YSQ 1. HNRT. YSQ 1. HVPS. VAL KSQ. FSI KSQ. FSI SQD TQQ GDPWIHPM SQD TQQ SQD IFAN IFAN IFAN IFAN IFAN IFAN IFAN IFAN IFAN IFAN IFAN 	ALANAAAA 0 720 720 720 720 720 720 720 720	VIVINI 730 730 2C.GTPMLHDFTQQ AC.GMYLKHLNRQV SS.VSHMKVLSRQV XS.VSHMKVLSRQV M.RRFSQALIKGD XC.GODI 850 21.LRIMESIWETE 1.IRIMESIWETE 1.IRIMESIWETE 1.ISLMKEA M.IRIMLIKKEA M.
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other PI3K family members. Alignments are based on the Pfam database. The last line of the alignment illustrates proteins with structurally similar domains. Residues that superimpose with PI3K γ are underlined. Sequences are porcine PI3K γ (o02697), human PI3K α (p42336), human PI3K β (p42338), human PI3K δ (o00329), human class II PI3K

(HsC2-PI3K, o00750), human Vps34 (s57219), RalGDS (1lfd), phospholipase C8 (1djx), PR65/A regulatory subunit (1b3u) and tyrosine kinase c-Src (1fmk). Dashed lines in the secondary structure indicate disordered residues and // indicates a large gap in the sequence.

of the effector molecules. The PI3K γ structure shows how the RBD interacts with the rest of the enzyme. The RBD of PI3K γ contacts the N-lobe and to a lesser degree the C-lobe of the catalytic domain. Residues in R α 2 and the R β 3–R β 4 loop interact with the catalytic domain, mainly with the k β 1–k β 2 and k β 4–k β 5 loops and helix k α 6. The relationship of the RBD to the rest of the enzyme suggests two possible mechanisms by which Ras binding may cause effector activation: a recruitment mechanism, in which Ras increases PI3K activity by translocating the enzyme to the plasma membrane; and an allosteric mechanism, in which Ras binding to the RBD causes a conformational change that would be propagated through the RBD/ catalytic domain interface to affect substrate or cofactor binding.

By superimposing the RBDs of RalGDS and PI3K γ , we constructed a model of Ras interaction with PI3K γ (Fig. 5) from which we can rationalize the differential effects of various switch I and switch II mutants on PI3K binding compared with other effectors. T35S and D38E mutations in Ras switch I eliminate PI3K binding, but do not affect Raf binding¹⁵. The E37G mutation abolishes binding to PI3K and Raf but not to RalGDS. The Y40C mutation does not affect PI3K binding, but abrogates Raf and RalGDS binding. In the switch II region, the Y64G mutation eliminates PI3K and neurofibromin binding but has no effect on Raf binding¹⁴.

In our model of the PI3K–Ras interaction, residues Glu 37, Asp 38, Tyr 40 and Tyr 64 are at the PI3K–Ras interface. Lys 234 of PI3K could form a salt bridge to Glu 37 of Ras and Lys 255 at the C-

terminal end of R α 1 could form a salt bridge with Asp 38 of Ras. Lys 255 at the C-terminal end of R α 1 could form a salt bridge with Asp 38 of Ras. Lys 255 in PI3K γ is probably analogous to Lys 227 in PI3K α , in which mutation K227E blocks PI3K α binding to Ras¹⁵. Tyr 40 interacts with Lys 32 in RalGDS (numbering as in ref. 17); however, the very different orientation of the Lys 32 equivalent in PI3K γ (Lys 234) may not allow this interaction, which may account for the insensitivity of PI3K to the Y40C mutation. On the other hand, Tyr 64 in switch II is in a position to form a hydrogen bond with PI3K Asp 238, but this residue has no specific interaction with RalGDS, which may explain the sensitivity of PI3K to the Ras Y64G mutation.

The PI3K γ C2 domain (residues 357–522) is an eight-stranded antiparallel β -sandwich consisting of two four-stranded β -sheets (Fig. 6). The fold of this domain is the same as the type II C2 domain found in PLC δ 1¹⁸. The N-terminal regions of all three PI3K classes have C2 domains, whereas the class II enzymes have an additional C2 domain at the C terminus (Fig. 1). The segments leading from the RBD into the C2 domain and from the C2 domain to the helical domain are not ordered.

C2 domains are often involved in Ca²⁺-dependent or Ca²⁺independent phospholipid membrane binding using three loops known as CBRs located at one end of the domain. The CBRs for PI3K γ are the loops connecting β 1 with β 2 (CBR1), β 3 with β 4 (CBR2), and β 5 with β 6 (CBR3). The CBR3 of PI3K γ is quite long compared with other C2 domains and is disordered in our structure.

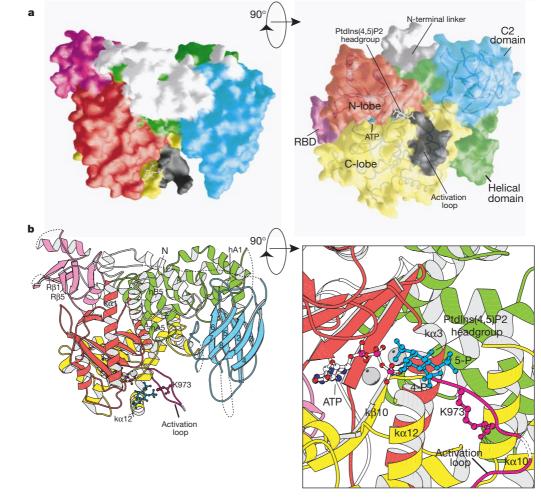


Figure 4 Model of phospholipid headgroup interactions with PI3K_Y. **a**, Orthogonal views of the solvent-accessible surface. The activation loop is black. An inositol 1,4,5- trisphosphate (InsP₃) molecule (white ball-and-stick) is modelled in the active site with the

3-OH near the γ -phosphate of the bound ATP. **b**, The same views in ribbon representation showing the activation loop (magenta) and InsP₃ (blue). The right panel is expanded to show features of the putative headgroup interaction.

The C2 domain interacts primarily with the helical domain, but it also interacts with the linker segment before the RBD and with the C-terminal lobe of the catalytic domain. The surface of the C2 domain contacting the rest of PI3K γ is nearly identical to the surface of the PLC δ 1 C2 domain that contacts the catalytic domain of PLC δ 1.

PI3K can bind phospholipid membranes in the absence of other protein components in a Ca²⁺-independent manner and can carry out processive catalysis at the membrane surface. By analogy with enzymes such as protein kinase C and cytosolic phospholipase A2, the C2 domain of PI3K may participate in membrane interaction. Consistent with this, we found that the isolated C2 domain from PI3Kγ binds multilamellar phospholipid vesicles similarly to the full-length enzyme (data not shown). In PI3Kβ and PI3Kδ, CBR3 (residues 395-417 of PI3K δ) is particularly rich in basic residues that may be important for membrane binding.

The structure of a type II β phosphatidylinositol phosphate kinase (PIPK) has been reported¹⁹. This dimeric enzyme, which phosphorylates phosphoinisitides at the 4-hydroxyl, consists of a single, catalytic domain. The dimer has an extensive flat, positively charged surface which was proposed to be the membrane-binding interface of the enzyme. Although the N-lobe of PIPK is structurally related to the catalytic domain of PI3K γ , the location of PI3K γ C2 domain with respect to the catalytic domain sterically precludes membrane interactions using the surface of PI3K γ analogous to the putative PIPK membrane-binding surface. Given the location of the membrane-binding loops from the C2 domain and the cavity in the catalytic domain that must accommodate the PtdIns(4,5)P₂

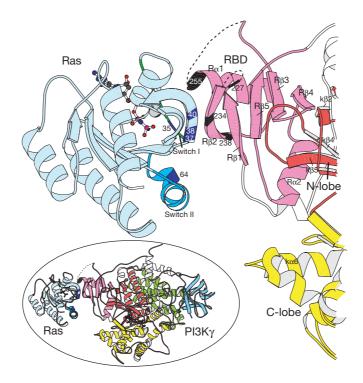


Figure 5 Model of the Ras–PI3K γ interaction based on the structure of the RalGDS–Ras complex. Inset, overall view of Ras–PI3K γ interaction. Residues in Switch I (green) and Switch II (cyan) regions of Ras that influence effector binding are highlighted with dark

blue stripes, whereas residues in the RBD of PI3K (purple) that are likely to be involved in Ras binding are shown as black stripes. Note the proximity of the RBD to the two lobes of the catalytic domain.

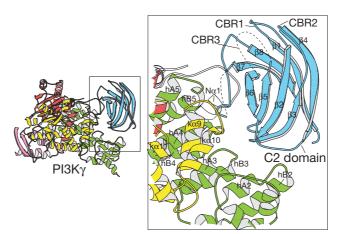


Figure 6 Ribbon diagram of the PI3K_Y C2 domain, and the interactions it makes with the rest of the enzyme. The elements of the helical and catalytic domains interacting with the C2 domain are shown. The domain in relation to the whole enzyme is shown to the left.

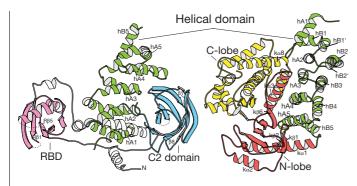


Figure 7 The helical domain (for colours see Fig. 1). The A/B antiparallel helical pairs characteristic of the HEAT motif topology consist of hA1/hB1, hA2/hB2, hA3/hB3, hA4/hB4 and hA5/hB5. Left, interaction between the helical domain and the RBD and C2 domain (the rest of the protein is removed for clarity). This interaction involves principally the A-helix surfaces. Right, interactions between the helical domain and the catalytic domain.

headgroup, the membrane-binding surface of PI3K γ could consist of the CBRs, the crevice between the N- and C-lobes of the catalytic domain, and the tip of the activation loop (Fig. 4a, right panel represents a view from the membrane surface).

The helical domain of PI3K (545-725) consists of five A/B pairs of anti-parallel helices (Fig. 7). The first two pairs have one kinked helix each, hB1/hB1' and hB2/hB2'. This region has been variously referred to as HR2, the PI3K accessory domain and the PIK domain, but its function is not known. The paired arrangement of a series of helices connected into a right-handed superhelix is reminiscent of the PR65/A regulatory subunit of protein phosphatase 2A (PP2A)²⁰. PR65/A is a member of a diverse group of proteins that contain 3-25 tandem repeats of a short sequence, called the HEAT motif. The HEAT motif consists of paired helices A and B arranged so that the A and B helices within a pair are antiparallel, and the A and B helices from one motif are parallel to the A and B helices of the next motif in the sequence. Although no HEAT sequence motif is apparent in the helical domain of PI3K, its structure is quite similar to that of PR65/ A in terms of the arrangement of helices, the length of the A/B units, and the angle between the A/B pairs.

The function of HEAT repeats is to form protein–protein interactions. In importin- β , interactions with the small GTPase Ran involve the surfaces of the B helices²¹. In PR65/A, mutagenesis has implicated the loops connecting the A/B pairs as the region responsible for interaction with PP2A²⁰. In PI3K γ , the helical domain is central to the interdomain packing: the surface formed by the A helices interacts with the catalytic domain; the loops connecting A and B helices within a pair pack against the C2 domain; and the loops between helical pairs pack against the RBD (Fig. 7). Much of the 'B' surface is solvent exposed and may interact with other proteins that bind PI3K γ , such as the p101 adaptor or G $\beta\gamma$ subunits.

The helical domain is common to both PI3K and PI4K families and serves as a spine on which the other domains are fastened. One of the proteins in which the HEAT sequence motif was first noted is the target of rapamycin, TOR, a yeast homologue of human FRAP (reviewed in ref. 22). FRAP has a C-terminal domain with clear sequence homology to the catalytic domain of PI3Ks. The secondary-structure prediction for the remainder of FRAP suggests that most of FRAP, apart from the catalytic domain, may consist of helical repeats folded into a right-handed superhelix as in the helical domain of PI3K γ .

This first structure of a PI3K provides a framework within which mutagenesis and detailed kinetic studies can be carried out to establish the enzymatic mechanism and the mode of activation by Ras and heterotrimeric G-protein subunits.

Methods

Protein expression, purification and crystallization

The cell-free extract of baculovirus-infected Sf9 cells expressing the His-tagged catalytic subunit of porcine PI3K γ (residues 1–143 deleted) was used for protein purification using Talon resin, followed by thrombin cleavage, anion and cation exchange, and gel filtration chromatography. Crystals were grown by mixing 1 µl of PI3K (3.5–4.0 mg ml⁻¹, in a buffer containing 20 mM Tris-HCl pH7.2, 1% v/v ethylene glycol, 1% w/v betaine, 0.02% w/v CHAPS and 5 mM dithiothreitol) with 1 µl of a reservoir solution containing 150–200 mM Li₂SO₄, 100 mM Tris-HCl pH 7.25 and 14–15% PEG 4000.

Data collection and structure determination

Crystals have C2 symmetry with unit-cell dimensions of a = 143.3 Å, b = 67.6 Å, c = 107.0 Å, $\beta = 95.9^\circ$, and contain one protein molecule in the asymmetric unit. Diffraction data were collected at ESRF beamlines ID2 and ID14-4 at 100K after freezing crystals in a cryoprotectant consisting of 150–200 mM Li₂SO₄, 100 mM Tris-HCl pH 7.25, 12% glycerol and 20% PEG 4000. Data were processed using MOSFLM²³ and CCP4 programs²⁴. The structure was determined by multiple isomorphous replacement methods. Heavy-atom positions were located using Solve²⁵ and refined with Sharp²⁶ (Table 1). A model was built into the electron-density maps using the program O²⁷ and refined using CNS²⁸. The average *B*-factor for all atoms is 60 Å². The structure has no residues in disallowed regions of the Ramachandran plot.

The highest resolution data obtained were for the complex containing ATP and lutetium; refinement resulted in a model with a free *R*-factor of 0.30 to a resolution of 2.2 Å. This complex has 854 residues visible in the electron-density map. Crystals with and without ATP had only minor differences in side-chain conformations in the active-site residues. PI3Ks require a Mg^{2+} or Mn^{2+} cofactor for enzymatic activity. In complexes with Lu^{3+} , Mg^{2+} or Mn^{2+} , each of the metals binds at the same two sites.

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Correspondence and requests for materials should be addressed to R.L.W. (e-mail: rlw@mrc-lmb.cam.ac.uk). The accession code for the atomic coordinates of the structure at the Protein Data Bank is 1qmm.

Sustained oscillations in living cells

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Glycolytic oscillations in yeast have been studied for many years simply by adding a glucose pulse to a suspension of cells and measuring the resulting transient oscillations of NADH¹⁻¹². Here we show, using a suspension of yeast cells, that living cells can be kept in a well defined oscillating state indefinitely when starved cells, glucose and cyanide are pumped into a cuvette with outflow of surplus liquid. Our results show that the transitions between stationary and oscillatory behaviour are uniquely described mathematically by the Hopf bifurcation¹³. This result characterizes the dynamical properties close to the transition point. Our perturbation experiments show that the cells remain strongly coupled very close to the transition. Therefore, the transition takes place in each of the cells and is not a desynchronization phenomenon. With these two observations, a study of the kinetic details of glycolysis, as it actually takes place in a living cell, is possible using experiments designed in the framework of nonlinear dynamics. Acetaldehyde is known to synchronize the oscillations¹⁰. Our results show that glucose is another messenger substance, as long as the glucose transporter is not saturated.

The ubiquitous glycolytic pathway is the first step in sugar catabolism, producing ATP, NADH and pyruvate. Under anaerobic conditions, the NADH is reused in the subsequent fermentation of the pyruvate. Transient glycolytic oscillations are known to occur in suspensions of yeast cells^{1,2}; see ref. 12 for measurements of most of the relevant metabolites during oscillatory glycolysis. The observed bulk oscillations of intracellular NADH depend on the ability of individual cells to synchronize their oscillations. This capability was proven by mixing two suspensions oscillating 180° out of phase: the bulk oscillations of NADH disappear immediately after the mixing, but reappear spontaneously after some time^{3,4,10}.

Synchronized bulk oscillations depend on a sufficiently high cell density⁶. They are promoted by anaerobiosis, especially when induced by cyanide^{4,9}, which is a potent inhibitor of cytochrome c oxidase in the respiratory chain.

Glycolytic oscillations are normally induced by a pulse of glucose, and the resulting oscillations change gradually as the excess of extracellular glucose is used up. An attempt was made to extend the duration of the oscillations using an infusion system where glucose was slowly injected into the cell suspension¹⁴. However, owing to cell ageing, accumulation of waste products or dilution of the suspension, the observed oscillations were still transient. In both the infusion experiment and the glucose-pulse experiment, the duration of the oscillating transient depends on the enzymatic composition of the yeast cells^{3,7,8,14}. In the glucose-pulse experiments, the longest transient trains of oscillations are found when a saturated, high-affinity glucose transporter provides the cell with an almost constant flow of glucose throughout the transient¹¹. In our experimental setup, a continuous-flow stirred tank reactor (CSTR), we control the glycolytic flow by means of the inflows, to give truly sustained oscillations.

To obtain these oscillations, we prepare *Saccharomyces cerevisiae* as described in ref. 8. The yeast is grown in batch culture at 30 $^{\circ}$ C to the point of glucose depletion. The cells are then removed from the growth medium and starved for a couple of hours, before being resuspended in phosphate buffer and kept below 5 $^{\circ}$ C. The resulting suspension flows into a stirred and thermostat-regulated reactor through a peristaltic pump. Solutions of glucose and cyanide flow into the reactor through two stepper–motor controlled piston burettes (Fig. 1). We monitor the oscillations by measuring NADH fluorescence.

Figure 2 shows a typical recording of stable undamped oscillations in the cell suspension. In principle the oscillations could continue forever with constant amplitude and period. This is possible because our setup is a truly open system. In practice, the amount of yeast cells available from the batch growth limits the duration of the run to 14 h in our experiments.

The environmental conditions of the cells can be changed by altering the flow rates of the inflows. Depending on flow rate we observe either a stationary state or continuous oscillations. The flow rate where the stationary state becomes unstable is known as the bifurcation point. As the flow rate is changed from this critical value, the amplitude of the oscillations grows in proportion to the square root of the deviation from the critical value. For a dynamic system such behaviour is characteristic of a supercritical Hopf bifurcation (SHB). This description is, of course, only valid sufficiently close to the bifurcation point. In Fig. 3 we show how the amplitude varies with the glucose flow rate. A similar agreement with bifurcation theory is seen when the flow rate of cyanide is

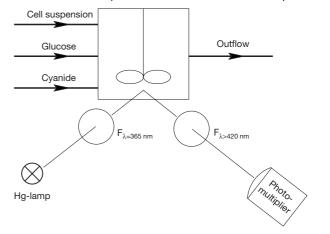


Figure 1 The experimental setup. F designates optical filters. See details in the Methods section.