Kinetworks™ Analysis of the Phosphoproteome During Frog Oocyte Maturation

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Abstract

A large burst in total protein phosphorylation occurs during *Xenopus laevis* oocyte meiotic maturation near the time of germinal vesicle breakdown (GVBD) due to the activation of a gamut of protein kinases. We undertook the systematic analysis of over 200 human phosphorylation sites in the frog oocyte system using the Kinetworks™ KPSS phospho-site profiling services available from Kinexus (www.kinexus.ca). These services employ immunoblotting screens with highly validated antibodies against conserved phosphorylation sites. Immunoreactive signals were successfully detected for 87 phosphorylation sites in 63 known phosphoproteins. Following GVBD, 50 of these phosphorylation sites exhibited 2-fold or more enhanced phosphorylation signals, whereas 6 other phosphorylation sites underwent greater than 50% reductions when compared to immature oocytes. The simultaneous tracking of large numbers of phosphoproteins in the frog oocyte system should reveal novel inter-relationships between these proteins and facilitate the mapping of signal transduction networks that regulate cell cycle transition through M-phase.

Introduction

With completion of the sequencing of the genomes of human and other species, the most challenging and fruitful biomedical research endeavor of this decade will be the mapping of cell signalling systems and establishing their linkages to normal and disease related processes. Over 518 human protein kinases are responsible for the phosphorylation of as many as 100,000 sites in most proteins. But only a few thousand phosphorylation sites have been identified to date. Therefore, our knowledge of the architecture of protein kinase signalling networks is extremely rudimentary, but the application of systems biology approaches should uncover the true complexity of these networks. To this end, Kinexus has tested more than 2500 commercial antibodies for the accurate and quantitative detection of protein kinases, phosphatases and their phosphoprotein substrates in our commercial Kinetworks™ immunoblotting services. The conversion of an oocyte into a fertilizable eggs is one of the first developmental steps in sexual reproducing organisms. Like most animals, oocytes from frogs are blocked at the beginning of prophase of the first meiotic cell division. Progesterone or insulin treatment can initiate resumption of meiotic maturation of frog oocytes with the breakdown of the nuclear envelope [also known as germinal vesicle breakdown (GVBD)] occurring about 4-6 h later. Near the time of GVBD, there is a burst of phosphorylation of hundreds of proteins. However, very few of these proteins have been characterized. There are many parallels with the regulation of meiosis and mitosis, including the activation of cyclin-dependent kinase (CDK1). Therefore, knowledge of M phase regulation in the frog oocyte is likely to be extremely relevant to our understanding of mitosis in human tumour cells. In this preliminary study, we used 346 human phospho-site antibodies to discover phosphorylation changes that accompany *Xenopus laevis* oocyte maturation.

Materials & Methods

Total cell lysates were prepared from African clawed frog *Xenopus laevis* oocytes treated with and without 10 µM progesterone for up to 7 hours. Oocytes were washed with ice-cold PBS, scrapped in lysis buffer [20 mM Tris, 20 mM β-glycerophosphate, 150 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM Na_3VO_4, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM PMSF, 2 µg/ml leupeptin, 4 µg/ml aprotinin and 1 µg/ml pepstatin A] and sonicated for 15 sec. Cell debris was removed by centrifugation at 100,000 rpm for 30 min at 4°C. Protein concentration was determined by the Bradford assay. For each Kinetwork™ analysis, 350 µg of total protein were resolved on a 13% single-lane mini-SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. Using a 20-lane multiblotter, the membrane was incubated with different mixtures of up to 3 antibodies per lane that react with specific phospho-sites in proteins of distinct molecular masses. After further incubation with a mixture of relevant HRP conjugated secondary antibodies, the blots were developed using ECL and signals were quantified using Quantity One software (Bio-Rad, Hercules, CA). Detailed information and protocols of the Kinetworks™ analysis have been published [Pelech et al. (2003) Methods in Molecular Medicine Series: Cancer Cell Signalling Kinetworks™ protein kinase multiblot analysis. (D. Terrian, ed.) vol. 218, 99-111 Humana Press] and can also be found at the Kinexus Bioinformatics Corp. website (www.kinexus.ca).

Results

Treatment of Stage VI oocytes individually dissected from the frog ovaries with progesterone or insulin results in GVBD approximately 4 to 6 hours later. GVBD is evident by the appearance of a light coloured spot in the dark animal hemisphere of the oocyte as depicted in Figure 1.

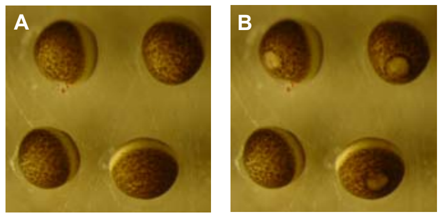


FIGURE 1. Germinal vesicle breakdown during *Xenopus laevis* oocyte maturation. Light microscopic images of frog oocytes about 4.5 h (Panel A) and 5 h (Panel B) after addition of 10 µM progesterone.

Extracts from immature and GVBD positive frog oocytes were separately fractionated and analyzed using 5 standard Kinetworks™ phospho-site screens (i.e. KPSS 1.3, 2.0. 3.0, 4.0 and 5.0) as well as several Kinetworks™ custom screens. Figure 2 shows examples of the Kinetworks™ immunoblots of the immature and GVBD positive frog oocyte lysates. In addition to the detection of at least 15 known phosphoproteins, the KPSS 1.3 screen also revealed reduced phosphorylation of 11 unknown cross-reactive phosphoproteins and increases in the phosphorylation of another 9 unknown proteins associated with oocyte maturation.

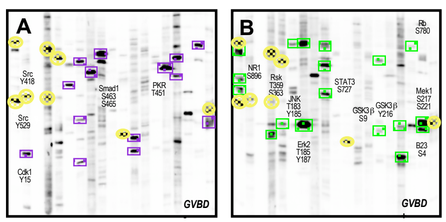
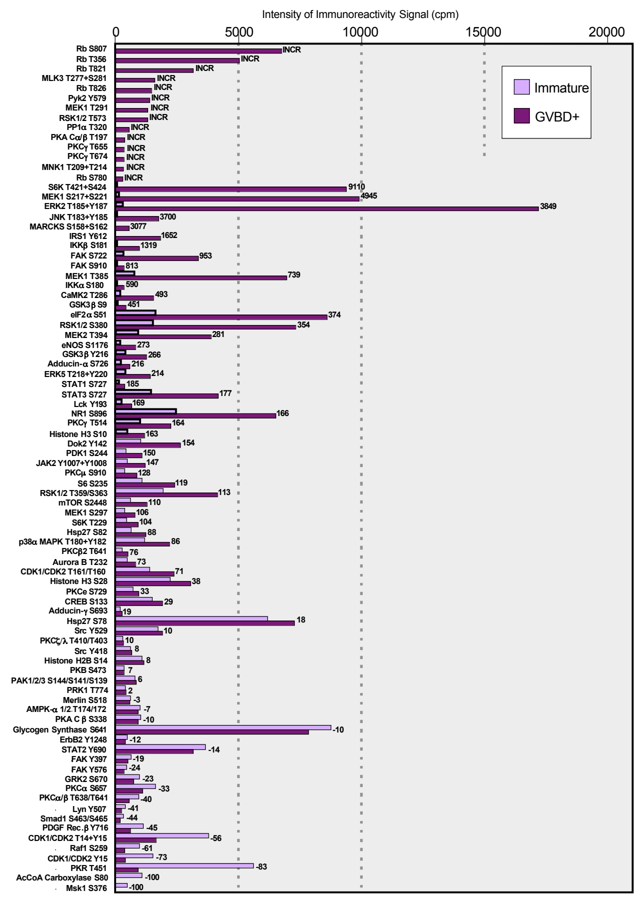


FIGURE 2. Kinetworks™ KPSS 1.3 phospho-site analysis of lysates from immature (Panel A) and GVBD positive (Panel B) frog oocytes. Phosphoproteins that revealed either a reduction or increase in immunostaining in the GVBD positive oocytes are boxed with purple or green, respectively. Some of the phosphoproteins that showed no change in immunostaining are circled in yellow.

The results from the various Kinetworks standard and custom screens with a panel of 346 commercial phospho-site antibodies sourced from 8 different suppliers are summarized in Table 1.

TABLE 1 - Quantification of immunoreactivity signals for known target phosphoproteins with appropriate molecular masses following SDS-polyacrylamide gel electrophoresis. The human amino acid number designations for the phosphorylation sites are provided in Column 2, and the number of different phospho-site antibodies tested for each site is shown in Column 3. The intensity of the immunoreactive signals for the target protein bands have been presented as counts per minute (cpm). Column 6 shows the percent change in immunoreactive signal intensity associated with induction of oocyte maturation within 1 hour after GVBD.

Phosphoprotein	Phospho-Site in Humans	# Ab Tested	Immature (cpm)	GVBD+ (cpm)	Percent Change
4E-BP1	S65	1	0	0	
AcCoA Carboxylase	S80	1	1094	0	-100
Adducin-α	S726	2	188	594	216
Adducin-γ	S693	2	196	270	19
AMPK-α 1/2	T174/172	1	1017	943	-7
ASK-1	S83	1	0	0	
ATF2	T51/T53	1	0	0	
ATM	S1981	3	0	0	
Aurora B	T232	1	473	819	73
Bad	S75	1	193	17	-91
Bad	S99	3	0	0	
BMX (Etk)	Y40	1	0	0	
BRCA1	S1497	1	0	0	
Btk	Y223	1	0	0	
Caldesmon	S789	1	0	0	
CaMK2	T286	3	264	1565	493
CaMK2	T305	1	0	0	
Caveolin-2	S23	1	0	0	
Caveolin-2	S36	1	0	0	
CDK1/CDK2	T14+Y15	5	3778	1662	-56
CDK1/CDK2	Y15	3	1520	410	-73
CDK1/CDK2	T161/T160	2	1399	2392	71
Cofilin 2	S3	1	0	0	
Cortactin	Y421	1	0	0	
CREB	S133	6	1496	1929	29
Crystallin αB	S19	1	0	0	
Crystallin αB	S45	1	0	0	
Crystallin αB	S59	1	193	0	-100
DNA Topoisomerase IIα	T1342	2	0	0	
Dok2 Mouse	Y142	1	1047	2662	154
EGF Recept.	Y1068	1	0	0	
EGF Recept.	Y1086	1	0	0	
EGF Recept.	Y1148	1	0	0	
eIF2α	S51	2	1811	8588	374
eIF2Bε	S540	2	0	0	
eIF-4E	S209	2	0	0	
Elk1	S383	3	0	0	
eNOS	T494	1	0	0	
eNOS	S1176	1	220	822	273
ErbB2 (HER2)	Y1139	2	0	0	
ErbB2 (HER2)	Y1248	2	488	429	-12
Erk1	T202+Y204	6	144	161	12
Erk2	T185+Y187	6	435	17180	3849
Erk5	T218+Y220	1	450	1411	214
ERα	S167	1	0	50	INCR
FAK	Y397	2	637	516	-19
FAK	Y576	2	458	355	-23
FAK	Y577	1	248	0	-100
FAK	S722	1	319	3360	953
FAK	S910	1	40	365	813
FKHR (FOXO1A)	S22	2	0	0	
FKHR (FOXO1A)	S256	2	0	0	
Fos	T232	2	0	0	
FosB	S284	1	0	0	
FosB	S302+S303	1	0	0	
GABA(A)R2	S893	1	0	0	
GAP-43	S41	1	0	0	
GFAP	?	1	0	0	
GluR1	S831	1	0	0	
Glycogen Synthase	S641	1	8762	7842	-10
GRK2	S670	1	985	751	-24
GSK3α	S21	3	29	188	548
GSK3α	Y279	4	0	0	
GSK3β	S9	3	80	441	451
GSK3β	Y216	4	348	1273	266
Histone H1	CDK1 sites	1	0	0	
Histone H2A.X	S139	1	0	0	
Histone H2B	S14	1	1090	1176	8
Histone H3	T3	1	33	178	433
Histone H3	S10	3	450	1183	163
Histone H3	T11	1	33	152	355
Histone H3	S28	2	2222	3066	38
Hsp27	S15	2	0	0	
Hsp27	S78	1	6187	7284	18
Hsp27	S82	2	655	1231	88
IkBα	S32+S36	2	0	0	
IKKα	S180	2	54	373	590
IKKβ	S181	1	70	993	1319
IKKε	T501	1	0	0	
Insulin Recept.	Y999	1	155	265	71
Insulin Recept.	Y1185	1	0	0	
Insulin Recept.	Y1189/Y1190	1	0	0	
IRS1	S307	1	0	0	
IRS1	S527	1	0	0	
IRS1	Y612	1	105	1840	1652
IRS1	Y1179	1	0	0	
JAK2	Y1007+Y1008	1	487	1205	147
JNK (SAPK) p40	T183+Y185	4	46	1748	3700
JNK (SAPK) p48	T183+Y185	4	168	201	20
Jun	S63	1	0	0	
Kit	Y703	2	0	0	
Kit	Y721	1	0	0	
Kit	Y730	1	0	0	
Kit	Y936	2	0	0	
Lck	S157	1	0	0	
Lck	Y193	1	248	668	169
Lck	Y504	2	0	0	
Lyn	Y507	2	415	247	-41
MAPKAPK-2	T222	1	0	0	
MAPKAPK-2	T334	2	0	0	
MARCKS	S158+S162	2	18	572	3077
MDM2	S186	1	0	0	
MEK1	S217+S221	2	196	9889	4945
MEK1	S297	3	391	805	106
MEK1	T291	4	0	1338	INCR
MEK1	T385	3	830	6964	739
MEK2	T394	3	1020	3895	281
MEK3/6	S189/S207	2	240	161	-33
Merlin NF2	S518	1	616	600	-3
Met	Y1003	2	0	0	
Met	Y1230/Y1234/Y1235	2	0	0	
MLK3	T277+S281	1	0	1605	INCR
Mnk1	T209+T214	1	0	328	INCR
Msk1	S376	1	474	0	-100
mTOR	S2448	2	612	1285	110
Myosin Light Chain	S180	1	0	0	
MYPT1	T696	1	0	0	
NFκB p65	S276	1	0	0	
NFκB p65	S529	1	0	0	
Nibrin/Nbs1	S343	1	0	0	
NMDA NR2B	Y1474	1	0	0	
NR1	S896	2	2450	6515	166
p27 Kip1	S10	3	0	0	
p38α MAPK	T180+Y182	5	1180	2197	86
p53	S6	1	0	0	
p53	S9	2	0	0	
p53	S315	1	0	0	
p53	S392	6	0	0	
S6K p70/p85	T229	1	460	939	104
S6K p70/p85	T389	4	127	98	-23
S6K p70/p85	T421+S424	1	102	9394	9110
PAK1/2/3	S144/S141/S139	1	810	856	6
PAK1/2/3	T423	1	0	0	
Paxilin	Y31	2	0	0	
Paxilin	Y118	1	0	0	
Paxilin2	S393	1	0	154	INCR
PDGF Recept. β	Y716	1	1142	633	-45
PDK1	S244	1	436	1089	150
PKA Cα/β	T197	1	0	377	INCR
PKA C β	S338	1	1033	930	-10
PKA RII	S98	1	0	0	
PKB (Akt)	T308	4	53	69	29
PKB (Akt)	S473	5	350	376	7
PKCα	S657	2	1651	1104	-33
PKCα/β	T638/T641	2	952	575	-40
PKCβII	T641	1	286	503	76
PKCδ	Y313	2	148	180	22
PKCδ	T507	2	58	166	186
PKCδ	S664	1	48	100	107
PKCε	S729	2	727	969	33
PKCγ	T514	2	859	2267	164
PKCγ	T655	1	0	365	INCR
PKCγ	T674	1	0	360	INCR
PKCµ (PKD)	S910	2	387	883	128
PKCθ	T538	1	0	0	
PKCζ/λ	T410/T403	1	304	334	10
PKR	T451	3	5614	932	-83
PLK1	T210	2	0	0	
PP1α	T320	1	0	568	INCR
PRK1	T774	1	423	433	2
PRK2	T816	1	0	0	
Progesterone Recept.	S190	1	0	0	
Progesterone Recept.	S294	1	0	0	
Pyk2	Y579	1	0	1401	INCR
Rad9 Yeast	S122	1	0	0	
Raf1	S259	7	988	385	-61
Rb	T356	1	0	5048	INCR
Rb	S612	2	97	132	37
Rb	S780	1	0	298	INCR
Rb	S807	1	0	6763	INCR
Rb	S807/S811	3	18	250	INCR
Rb	T821	1	0	3163	INCR
Rb	T826	1	0	1478	INCR
RFA2 Yeast	S1260	1	0	0	
Ribosomal S6	S235	2	1090	2395	119
RSK1/2	T359/S363	1	1952	4159	113
RSK1/2	S380	1	1616	7335	354
RSK1/2	T573	1	0	1325	INCR
SH3BP2	S427	1	0	0	
Shc	Y239/Y240	2	194	183	-6
Smad1	S463/S465	3	339	190	-44
SMC1	S957	2	0	0	
Src	Y418	4	635	686	8
Src	Y529	3	1742	1934	10
STAT1	Y701	2	117	0	-100
STAT1	S727	1	134	382	185
STAT2	Y690	1	3660	3154	-14
STAT3	S727	2	1505	4172	177
STAT4	Y693	1	0	0	
STAT5A	Y694	3	0	0	
Syk	Y352	1	0	0	
Synapsin 1	S9	2	0	0	
Synapsin 1	S605	1	0	0	
Tau	T547	1	0	0	
Tau	S712	1	0	0	
Tyrosine Hydroxylase	S19	1	0	0	
Tyrosine Hydroxylase	S62	1	0	0	
Tyrosine Hydroxylase	S71	2	0	0	
Vimentin	S6	1	0	0	
Vimentin	S33	1	0	0	
Vimentin	S55	1	0	0	
ZAP70	Y319	1	0	0	


Intensity of Immunoreactivity Signal (cpm)
0
5000
10000
15000
20000
Immature
GVBD+
Rb S807 INCR
Rb T356 INCR
Rb T821 INCR
MLK3 T277+S281 INCR
Rb T826 INCR
Pyk2 Y579 INCR
MEK1 T291 INCR
RSK1/2 T573 INCR
PP1α T320 INCR
PKA Cα/β T197 INCR
PKCγ T655 INCR
PKCγ T674 INCR
MNK1 T209+T214 INCR
Rb S780 INCR
S6K T421+S424 9110
MEK1 S217+S221 4945
ERK2 T185+Y187 3849
JNK T183+Y185 3700
MARCKS S158+S162 3077
IRS1 Y612 1652
IKKβ S181 1319
FAK S722 953
FAK S910 813
MEK1 T385 739
IKKα S180 590
CaMK2 T286 493
GSK3β S9 451
eIF2α S51 374
RSK1/2 S380 354
MEK2 T394 281
eNOS S1176 273
GSK3β Y216 266
Adducin-α S726 216
ERK5 T218+Y220 214
STAT1 S727 185
STAT3 S727 177
Lck Y193 169
NR1 S896 166
PKCγ T514 164
Histone H3 S10 163
Dok2 Y142 154
PDK1 S244 150
JAK2 Y1007+Y1008 147
PKCµ S910 128
S6 S235 119
RSK1/2 T359/S363 113
mTOR S2448 110
MEK1 S297 106
S6K T229 104
Hsp27 S82 88
p38α MAPK T180+Y182 86
PKCβ2 T641 76
Aurora B T232 73
CDK1/CDK2 T161/T160 71
Histone H3 S28 38
PKCe S729 33
CREB S133 29
Adducin-γ S693 19
Hsp27 S78 18
Src Y529 10
PKCζ/λ T410/T403 10
Src Y418 8
Histone H2B S14 8
PKB S473 7
PAK1/2/3 S144/S141/S139 6
PRK1 T774 2
Merlin S518 -3
AMPK-α 1/2 T174/172 -7
PKA C β S338 -10
Glycogen Synthase S641 -10
ErbB2 Y1248 -12
STAT2 Y690 -14
FAK Y397 -19
FAK Y576 -24
GRK2 S670 -23
PKCα S657 -33
PKCα/β T638/T641 -40
Lyn Y507 -41
Smad1 S463/S465 -44
PDGF Rec.β Y716 -45
CDK1/CDK2 T14+Y15 -56
Raf1 S259 -61
CDK1/CDK2 Y15 -73
PKR T451 -83
AcCoA Carboxylase S80 -100
Msk1 S376 -100

Conclusions and Future Directions

1. Many of the known human phosphorylation sites are present in the frog, and they are regulated during oocyte maturation.
2. The phospho-site antibodies cross-reacted with over two hundred as yet unidentified phosphoproteins in the frog oocyte, and the phosphorylation states of many of these were also altered during oocyte maturation. The availability of phospho-site antibodies against these phosphoproteins could facilitate their purification and identification by mass spectrometry.
3. The ability of Kinetworks™ analyses to track large panels of phosphoproteins will permit elucidation of possible relationships between these phosphoproteins in the future with oocyte maturation time course experiments in the absence and presence of diverse protein kinase inhibitors.

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