A functional genomic and proteomic perspective of sea urchin calcium signaling and egg activation

Michelle M. Roux [a], Ian K. Townley [a], Michael Raisch [a], Anna Reade [a], Cynthia Bradham [b], Glen Humphreys [c], Herath Jayantha Gunaratne [d], Christopher E. Killian [e], Gary Moy [d], Yi-Hsien Su [d,1], Charles A. Ettensohn [f], Fred Wilt [e], Victor D. Vacquier [d], Robert D. Burke [g], Gary Wessel [c], Kathy R. Foltz [a,*]

[a] Department MCD Biology and Marine Science Institute, University of California, Santa Barbara, CA 93106-9610, USA
[b] DCMB Group, Duke University, Durham, NC 27705, USA
[c] Department of Molecular and Cellular Biology and Biochemistry, Brown University, Providence, RI 02912, USA
[d] Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 92093-0202, USA
[e] Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720-3200, USA
[f] Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA 15213, USA
[g] Department of Biology, Department of Biochemistry/Microbiology, University of Victoria, Victoria, BC, Canada

Abstract

The sea urchin egg has a rich history of contributions to our understanding of fundamental questions of egg activation at fertilization. Within seconds of sperm–egg interaction, calcium is released from the egg endoplasmic reticulum, launching the zygote into the mitotic cell cycle and the developmental program. The sequence of the *Strongylocentrotus purpuratus* genome offers unique opportunities to apply functional genomic and proteomic approaches to investigate the repertoire and regulation of Ca^{2+} signaling and homeostasis modules present in the egg and zygote. The sea urchin "calcium toolkit" as predicted by the genome is described. Emphasis is on the Ca^{2+} signaling modules operating during egg activation, but the Ca^{2+} signaling repertoire has ramifications for later developmental events and adult physiology as well. Presented here are the mechanisms that control the initial release of Ca^{2+} at fertilization and additional signaling components predicted by the genome and found to be expressed and operating in eggs at fertilization. The initial release of Ca^{2+} serves to coordinate egg activation, which is largely a phenomenon of post-translational modifications, especially dynamic protein phosphorylation. Functional proteomics can now be used to identify the phosphoproteome in general and specific kinase targets in particular. This approach is described along with findings to date. Key outstanding questions regarding the activation of the developmental program are framed in the context of what has been learned from the genome and how this knowledge can be applied to functional studies.
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Introduction

Fertilization initiates a series of events that coordinately launch the oocyte or egg into the developmental program. These "egg activation" events are rapid and transient, committing the formerly quiescent egg to cytoskeletal remodeling, cell cycle re-

entry and metabolic activation. In all animals studied, a rise in cytosolic free Ca^{2+} is crucial for egg activation and, in deuterostomes, the Ca^{2+} is released initially from stores in the egg endoplasmic reticulum (ER) (Runft et al., 2002; Whitaker, 2006). This Ca^{2+} release spreads as a wave across the egg from the point of sperm entry and then returns to pre-fertilization levels within a few minutes. New Ca^{2+} signals are then required for mitotic regulation and later developmental events (Ducibella et al., 2006; Whitaker, 2006). Despite the many descriptions and clear importance of Ca^{2+} dynamics at fertilization and during development (Whitaker, 2006), relatively little is known about

the molecular repertoire of Ca^{2+} signaling systems that modulate these events.

Egg activation is executed by existing egg proteins, and therefore, modulation of the localization and activity of egg proteins is achieved through post-translational modifications (Kinsey, 1997; Sato et al., 2000) and protein turnover. Thus, in order to understand the coordinated signaling events of egg activation, including the regulation of Ca^{2+} release, it is useful to have a model system that has synchronous fertilization and is biochemically tractable, as well as amenable to single cell experiments. The sea urchin egg offers these advantages and has a long history of contributions to the field of fertilization and Ca^{2+} signaling (Monroy, 1986). For example, *Lytechinus pictus* sea urchin eggs (Steinhardt et al., 1977) were the first cells in which a Ca^{2+} increase was recorded, along with the Medaka fish egg (Ridgway et al., 1977). Sea urchins are marine invertebrates, in the class Echinoidea. As Echinodermata, they are members of a phylum on the deuterostome branch of the animal phylogenetic tree and more closely related to the members of the other deuterostome phyla (the Chordata, including the vertebrates, cephaochordates, urochordates and the Hemichordata) than they are to the protostomes (Lophotrochozoans and Ecdysozoans) (Amemiya et al., 2005). The completion of the *Strongylocentrotus purpuratus* genome sequence (Consortium, in press) now adds a valuable experimental feature to the sea urchin as a model system. Analysis of the genome sequence has revealed the sea urchin's basic "calcium toolkit," based on the vertebrate model (Berridge et al., 2000, 2003), and provides context for which Ca^{2+} signaling systems are likely to be operating in eggs and early embryos. Furthermore, functional proteomic analysis has resulted in the identification of candidate proteins modulating various aspects of sea urchin egg activation as well as a global description of egg protein turnover and modification by phosphorylation at fertilization.

Materials and methods

Sequence database searches, genomic analysis and annotation

Several data sets of the *S. purpuratus* genomic sequence were analyzed: (i) the whole genome shotgun sequence; (ii) the 11/23/2004, 7/18/2005 and the 3/16/2006 genome assemblies from the Human Genome Sequencing Center at Baylor College of Medicine Human Genome Sequencing Center (BCM-HGCS, http://www.hgsc.bcm.tmc.edu/projects/seaurchin/); (iii) GLEAN3 gene models (BCM-HGSC); and (iv) the NCBI Gnomon *ab initio* gene models (http://www.ncbi.nlm.nih.gov/genome/guide/sea_urchin/). A community-accessible database of predicted cDNAs also is available (Wei et al., 2006).

Sequences encoding putative calcium toolkit components, molecules of the egg activation pathway and scaffold proteins were identified on a candidate basis by the BLAST program (www.ncbi.nlm.nih.gov/BLAST) using amino acid sequences of conserved domains and by comparing the patterns of amino acid conservation to PFAM domains (Bateman et al., 2004). Gene identification was based on the best score, and in many cases, further analysis by multiple alignment using CLUSTAL W (Thompson et al., 1994). The BLAST match in the human protein list was determined and, as corroboration, the best urchin match was also determined. In almost all cases, the best matches were bidirectional. *In silico* predictions of cDNA were confirmed or modified according to cDNA cloning and sequence when available.

Expression of the cognate mRNA of each predicted gene identified in the BLAST searches was evaluated by manual inspection of the embryonic tiling array data (Samanta et al., in press) and EST databases. In some cases, rtPCR analyses and antibody detection were also conducted (see below). If available, references detailing expression of mRNA, protein or functional activity in echinoderms were compiled and are provided on the gene list tables (Supplementary Tables 1 and 2, Tables 1 and 2).

Isolation and handling of sea urchin gametes

Adult *S. purpuratus* were collected from the Santa Barbara Channel (Goleta Pier, Goleta, CA), maintained in open system aquaria at 10–12°C and fed a diet of *Macrocystis*. Gamete collection and fertilization were as described (Foltz et al., 2004).

mRNA isolation, rtPCR and sequencing

For the predicted SFK genes, rtPCR was conducted to confirm the expression and sequence of the mRNA in eggs. Total RNA was isolated from unfertilized *S. purpuratus* eggs using Trizol (Invitrogen, Carlsbad, CA). Poly(A+) mRNA was selected by using the Micro Poly(A) Pure Kit (Ambion Inc., Austin, TX) and cDNA was synthesized (SuperScript First-strand synthesis kit, Invitrogen, Inc.). Amplification was carried out on the egg cDNA using gene specific primers that were designed based on the predicted cDNA sequences flanking the tyrosine kinase domain of the target. Amplified products were cloned into the TOPO vector (Clonetech) and sequenced (Iowa State Sequencing Facility; Ames, IA). Sequences were analyzed by alignment with the predicted *S. purpuratus* gene sequence and also subjected to a tBLASTx search to assign identity.

Egg fractionation, sample preparation and immunoblotting

For analysis of proteins for two-dimensional (2D) electrophoresis, total NP-40 soluble lysates of unfertilized eggs or eggs fertilized by addition of sperm (1:25,000 final dilution) were prepared by described methods (Kumano et al., 2001). Lysates were kept on ice and used the same day for 2D gel electrophoresis analyses. Protein concentration was determined by the BCA method (Pierce, Inc). The procedure outlined in Belton et al. (2001) was used to prepare lipid raft fractions by sucrose gradient density centrifugation from unfertilized eggs and eggs fertilized (1:50,000 final dilution) and collected 30 s post sperm addition.

To confirm expression and to localize egg proteins to membrane fractions, proteins (25 μg per lane) were separated on a 10% polyacrylamide SDS–PAGE gel. Proteins were transferred to nitrocellulose membranes and the blots were probed with affinity purified Sp-SFK1 IgY (Giusti et al., 2003) or anti-human CSK polyclonal IgG (Santa Cruz Biotech; H-75) at 0.2 μg/mL in a blocking buffer containing 3% dry milk. HRP-conjugated anti-chicken or anti-rabbit secondary antibodies were purchased from Aves Labs, Inc or Promega, Inc, respectively. Antibody detection was accomplished with EnHanced Chemiluminescence (Pierce Endogen, Inc.) followed by exposure to X-ray film.

Two-dimensional electrophoresis and proteomic analyses of unfertilized and fertilized eggs

First and second dimensional separations of total NP-40-soluble protein lysates (see above) were carried out according to published protocols (Bernard et al., 2004). Briefly, 25 μg or 35 μg soluble protein was denatured then rehydrated into isoelectric focusing (IEF) strips of various pH ranges (see Supplementary Fig. 2). Separation in the first dimension was carried out using a Multiphor II flatbed chamber (GE Healthcare) as follows: pH 3–10: 10–12 kVh, pH 4–7: 11–15 kVh, pH 6–11: 10 kVh, pH 3–5.6: 11–15 kVh, pH 5.3–6.4 and 6.2–7.5: 28–35 kVh. Proteins were then separated by size in the second dimension by SDS PAGE on Criterion 8–16% gradient gels (BioRad, Hercules, CA). All samples within a given experiment were run simultaneously and in triplicate to maximize reproducibility.

Gels were fixed and proteins were detected using multiplex staining with ProQ Diamond stain for phosphorylated proteins followed by SYPRO Ruby stain for total protein (Molecular Probes Inc, Eugene OR.) and imaged using a BioRad FX scanner with a ~600 nm band pass emission filter. Images were imported into PDQuest (version 7.4) for spot detection and excision (Bio Rad,

Hercules, CA). Selected spots within various analysis sets (see Figs. 1–6) were picked using the ProteomeWorks Robotic Spot Cutter and PDQuest Basic Excision Software (BioRad, Hercules, CA) followed by in-gel digestion with trypsin. Peptides were subjected to quadrupole time of flight tandem mass spectrometry (Q-ToF MS/MS), and peptide spectra were analyzed using ProteinLynx Global Server (PLGS) software (Waters, Milford, MA). PLGS workflows were designed to search the sea urchin genome on NCBI. Identities were assigned to proteins showing two or more non-overlapping peptide matches.

Results and discussion

Overview of the sea urchin calcium toolkit

Calcium is a crucial intracellular signal that operates over a wide temporal range to regulate many cellular processes, including egg activation and later developmental events (Ducibella et al., 2006; Whitaker, 2006). Ca^{2+} can act locally to trigger fast responses in microseconds (e.g. exocytosis at the synaptic junction), and Ca^{2+} also can control slower responses (e.g. gene transcription) over minutes to hours via global repetitive transients or waves. Berridge and colleagues (2000, 2003) provide a framework for organizing the various Ca^{2+}

signaling systems in mammalian somatic cells, referred to as the "calcium toolkit." Intracellular Ca^{2+} levels can be considered as a balance between 'on' reactions that result in Ca^{2+} influx or efflux from internal stores and 'off' reactions that remove free intracellular Ca^{2+} via a combination of buffers, pumps and exchangers (Berridge et al., 2003). Systematic, homology-based searches were conducted for *S. purpuratus* genes encoding Ca^{2+} signaling components falling into four broadly defined, functional categories: (1) stimuli that generate Ca^{2+} mobilizing signals; (2) components that mediate entry of Ca^{2+} into the cytoplasm; (3) Ca^{2+} sensors that effect changes; and (4) components that remove Ca^{2+} from the cytoplasm. The annotated genes encoding the basic calcium toolkit in the sea urchin genome are compiled in Supplementary Table 1 and diagrammed in the context of egg activation in Fig. 1. The full repertoire of predicted gene families that comprise the mammalian Ca^{2+} signaling and homeostasis systems is present in the *S. purpuratus* genome. Although likely to expand with further study, the current list (Supplementary Table 1, Fig. 1) of nearly 200 genes includes many G-protein-coupled receptors (GPCRs) and protein tyrosine kinase linked receptors (TKRs) known to modulate Ca^{2+} in signaling in mammalian cells.
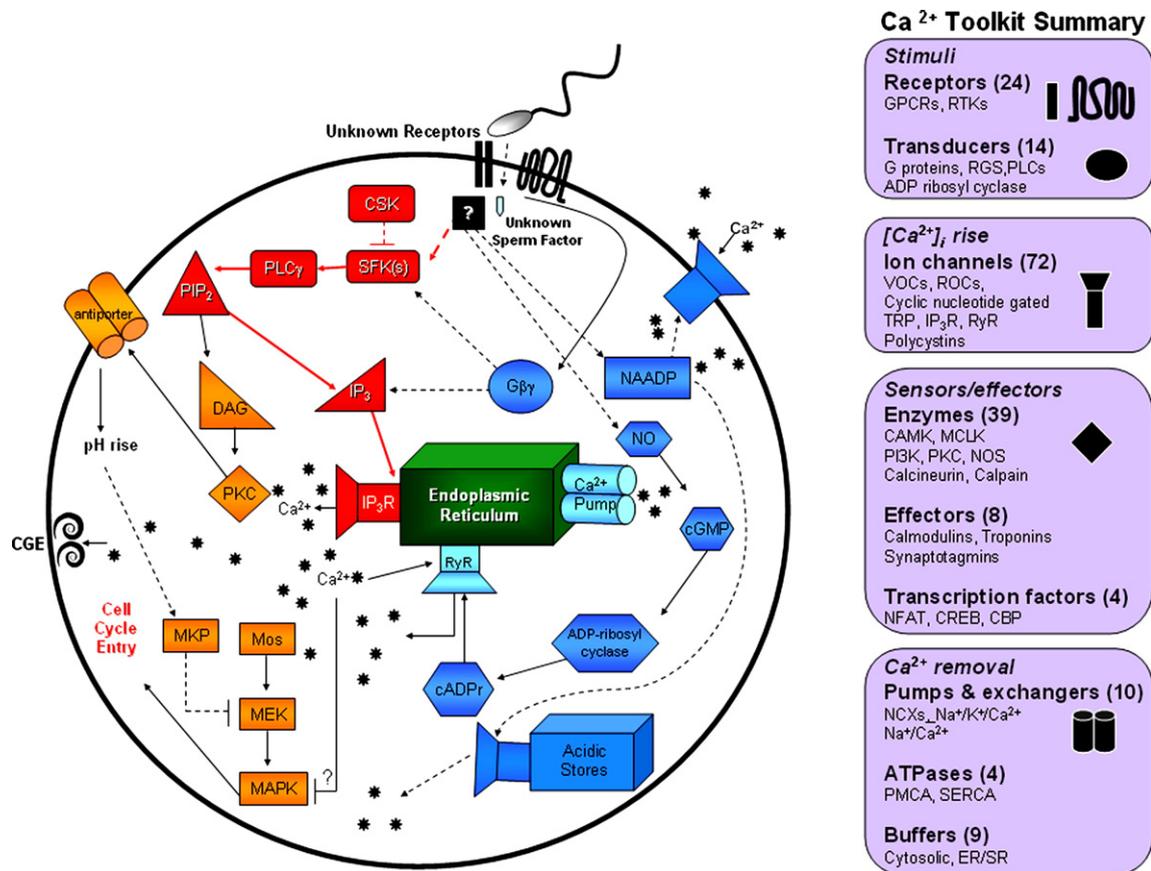

Fig. 1. A model of the major signal transduction events that occur during sea urchin egg activation. The major pathways in the sea urchin egg that mediate Ca^{2+} release and downstream responses at fertilization are shown on the left. Ca^{2+} is indicated by the starbursts. The signaling pathway shown in red (the SFK/PLC-γ/IP$_3$R pathway) is thought to be the major pathway leading to the initiation of Ca^{2+} release at fertilization. Alternative pathways for Ca^{2+} release are shown in blue. Additional proposed pathways are indicated by dashed lines. Examples of downstream egg activation events – cortical granule exocytosis (CGE), the rise in pH (through activation of a Na^+/H^+ antiporter) and the MAPK pathway – are shown in orange. A summary of the genes encoding the *S. purpuratus* Ca^{2+} toolkit is shown at the right, with the number of genes encoding the various components indicated in parentheses. See text for further discussion and Tables 1 and 2 and Supplementary Tables 1 and 2 for gene lists.

Transducers (G-proteins, regulators of G-protein signaling, ADP-ribosyl cyclase and phospholipase Cs) also are predicted. A large number of genes encoding channels (voltage-operated channels, receptor-operated channels, second messenger-operated channels, transient receptor potential ion channels, IP_3 receptor, ryanodine receptor, polycystins) and channel regulators also are present. Ca^{2+} effectors (such as synaptotagmins, troponin, calmodulin) and buffers (such as calnexin, calsequestrin, GRP78, GRP94) are present, as are a number of calcium-sensitive enzymes (NOS, PKCs, CAMKs). A substantial collection of pumps and exchangers (including NCXs, PMCA and SERCA) round out the list (Supplementary Table 1, Fig. 1). This repertoire indicates that the sea urchin Ca^{2+} toolkit is diverse and likely enables the sea urchin to modulate an array of temporally and spatially distinct Ca^{2+} signals as necessary.

The cognate mRNAs of all of the genes in the *S. purpuratus* Ca^{2+} toolkit are represented in the embryonic expression tiling array, consistent with a developmental program that relies on Ca^{2+} signaling. Many of the Ca^{2+} toolkit genes are expressed in adult tissues as well. Burke et al. (2006) discuss the various channels and channel modulators in greater detail in the context of the *S. purpuratus* nervous system. Interestingly, in almost all cases, the sea urchin genome is predicted to contain fewer members in each Ca^{2+} toolkit gene family when compared to mammals. This likely is due to a lack of duplication, which seems to be a common feature of most of the major gene families in the sea urchin (Consortium, in press; Materna et al., 2006). For example, a single *S. purpuratus* gene encodes the inositol 1,4,5-trisphosphate (IP_3) receptor, a ligand-gated Ca^{2+} channel (Supplementary Tables 1 and 2). This is the case in both *Drosophila* and *Caenorhabditis elegans*, as well as in the ascidian (*Ciona*), while there are three IP_3R genes in mammals (Patterson et al., 2004). Coupled with the ability to image Ca^{2+}, the completion of the sea urchin genome sequence positions the sea urchin egg and early embryo as an excellent deuterostome model system in which to study the modulation of Ca^{2+} signals as a biochemical and systems biology problem with minimal redundancy.

Genomic insights into Ca^{2+} signaling pathways implicated in egg activation 1. Initial release of Ca^{2+} via IP_3 production

In echinoderm (as in other deuterostome) eggs, Ca^{2+} is released from the ER via the IP_3R as a result of IP_3 produced by the hydrolysis of PIP_2 (Stricker, 1999). Other signaling pathways that are capable of regulating Ca^{2+} release clearly are present in eggs (Fig. 1) and may participate in modulating Ca^{2+} wave progression or aspects of Ca^{2+} spatio-temporal dynamics (Whitaker, 2006). However, only a few of the putative signaling components have been isolated or characterized and their precise roles in egg activation remain unknown. The completion of the sea urchin genome sequence allows for a merging of the cellular physiology data with genomic determinants that have been shown or predicted to be involved in signaling the release of Ca^{2+} during fertilization. Although much of the cellular physiology of fertilization and Ca^{2+} signaling has been conducted in *L. pictus* and *L. variegatus*, a

genus separated by about 50 Mya from *S. purpuratus*, the major egg activation events are conserved across sea urchin species (Stricker, 1999) and it is almost certain that the gene complement of *S. purpuratus* is similar to that of the other sea urchin species. The *S. purpuratus* genome sequence was mined for proteins predicted to play a role in fertilization and egg activation. Identification and annotation of these cognate genes (Fig. 1, Supplementary Table 2) fill a major gap in our knowledge of egg activation mechanisms by providing details about the genomic determinants of previously described enzymatic activities or antigens cross-reactive with antibodies generated against mammalian proteins (Stricker, 1999; Runft et al., 2002; Whitaker, 2006). Knowledge of gene structure, coupled with expression profiles, provides specific tools for studying the spatio-temporal properties of signaling at fertilization.

Ca^{2+}, which is stored in the egg ER, is released initially through IP_3Rs at fertilization, when IP_3 levels increase transiently. In sea stars (*Asterina miniata*) and sea urchins (*Lytechinus* spp.), blocking IP_3 production (Carroll et al., 1997, 1999; Shearer et al., 1999) or preventing IP_3 binding to the IP_3R (Iwasaki et al., 2002) inhibits sperm-induced Ca^{2+} release. Although the presence of the IP_3R protein, a non-voltage gated Ca^{2+} channel, has been documented in *S. purpuratus* and other echinoderm eggs based on immunological cross-reactivity with the vertebrate IP_3R (Parys et al., 1994), most studies have relied on indirect methods to assess function (reviewed in Stricker, 1999). The *S. purpuratus* genome contains a single, canonical IP_3R gene that encodes a protein with 80% identity to that of the sea star protein (Iwasaki et al., 2002). The presence of the *S. purpuratus* IP_3R cognate mRNA in the embryonic tiling assessment is consistent with the earlier work (Parys et al., 1994) indicating that this Ca^{2+} channel is expressed in *S. purpuratus* eggs. In mammalian somatic cells, there are a number of proteins that interact with and modulate activity of the IP_3R, yet none has been described in eggs. Genomic analysis identified *S. purpuratus* genes encoding orthologs of several of these mammalian IP_3R modulators (Supplementary Table 1) such as FKBP12 and a newly described protein, IRBIT (IP_3R binding protein released with inositol 1,4,5-trisphosphate) (Ando et al., 2003, 2006; Shirakabe et al., 2006). Identification of these proteins enables studies investigating the regulation of the IP_3R activity at fertilization.

The production of IP_3 leading to the activation of the IP_3R is mediated by the action of PLCs. The phospholipase C genes are highly conserved across phyla (Patterson et al., 2005). The larger family comprises six main subfamilies: PLC-β, PLC-ε, PLC-δ, PLC-γ, PLC-η and PLC-ζ (Cockroft, 2006). In mammals, each family, except for PLC-ζ (which is represented by a single gene), has at least two genes encoding isozymes (Patterson et al., 2005; Cockroft, 2006). The sea urchin genome contains a single gene for PLC-ε, PLC-δ, PLC-γ and PLC-η, and two genes encoding-β type PLCs based on sequence comparisons and phylogenetic analyses (Supplementary Table 1 and Fig. 1). Although all of the predicted *S. purpuratus* PLC genes are expressed at some point over early development as evidenced by the detection of their cognate mRNAs in the tiling data, only the PLC-δ, PLC-β4 and

PLC-γ proteins have been detected in sea urchin eggs (PLC-ε and PLC-η have not been assessed). The cDNA encoding *S. purpuratus* PLC-δ has been cloned, and the protein is expressed in gametes and embryos (Coward et al., 2004). *S. purpuratus* eggs express PLC-γ protein based on antibody cross-reactivity and enzymatic activity (De Nadai et al., 1998; Rongish et al., 1999; Shearer et al., 1999; Coward et al., 2004; Runft et al., 2004; Kulisz et al., 2005). A cDNA encoding a PLC-β has been characterized from *L. pictus* and the protein shown to be present in eggs as well (Kulisz et al., 2005). However, only PLC-γ has been demonstrated to be required for the initiation of Ca^{2+} release at fertilization in sea urchins. Inhibiting PLC-γ in *Lytechinus* spp. and *Paracentrotus lividus* (Carroll et al., 1999; Shearer et al., 1999; Runft et al., 2004), as well as sea star (*A. miniata*) eggs (Carroll et al., 1997; Runft et al., 2004) inhibits sperm-induced Ca^{2+} release, though it is possible that other isozymes play a role in amplification of the Ca^{2+} signal or later Ca^{2+}-mediated events (Coward et al., 2004; Kulisz et al., 2005).

Identification of the *S. purpuratus* PLC gene family sheds light on an outstanding question in egg activation—the nature of the triggering mechanism. What activates the pathway leading to IP_3-mediated Ca^{2+} release at fertilization? Is it receptor-mediated, require a factor delivered by sperm, or involve some combination of events? In mammals, a novel form of PLC, PLC-ζ is a sperm-borne protein that causes Ca^{2+} oscillations (Swann et al., 2006). To date, a PLC-ζ ortholog has not been detected in the sea urchin genome draft and likely is present only in mammals and some birds (Coward et al., 2005). In ascidians, frogs and teleost fish, evidence suggests that initial Ca^{2+} release involves an egg PLC-γ and tyrosine kinases (Runft et al., 2002; Sato et al., 2006; Whitaker, 2006), much the same as in echinoderms (Fig. 1). In ascidians, a sperm-borne factor (as yet unidentified) is sufficient to trigger Ca^{2+} release (Kyozuka et al., 1998; McDougall et al., 2000; Runft and Jaffe, 2000) and requires a functional SFK-PLC-γ pathway to do so (Runft and Jaffe, 2000). Extracts of sea urchin (*L. pictus*) sperm can trigger fertilization envelope elevation (and by implication, Ca^{2+} release) when injected into sea urchin eggs (Dale et al., 1985), but the existence of a sperm factor remains an outstanding question. The completion of the genome sequence allows for powerful functional proteomic approaches to identify the upstream activator (derived from sperm or egg) of the signaling pathway.

Genomic insights into Ca^{2+} signaling pathways implicated in egg activation 2. The Src Family Kinases (SFKs)

A current model (Fig. 1) for the activation of egg PLC-γ during echinoderm fertilization is via tyrosine phosphorylation by a SFK (Townley et al., 2006; Whitaker, 2006). Prior to the genome project, a single SFK (designated Sp-SFK1) had been identified in *S. purpuratus* eggs. The *S. purpuratus* SFK1 mRNA and protein are present in unfertilized eggs, and the kinase is required for normal Ca^{2+} release at fertilization (Giusti et al., 2003). However, at least 3 SFK proteins are expressed in sea star (*A. miniata*) eggs. Two of these SFKs, designated AmSFK1 (the ortholog of Sp-SFK1) and AmSFK3, are required

for initiation of Ca^{2+} release at fertilization and may be sequentially activated (O'Neill et al., 2004). Experiments designed to amplify protein tyrosine kinases by rtPCR suggested that additional SFKs were expressed in *S. purpuratus* eggs (Wessel et al., 1995; Giusti et al., 2003) as well as in eggs of the sea urchin *Anthocidaris crassispina* (Sakuma et al., 1997; Onodera et al., 1999). Genomic analyses now have revealed that at least 6 *Src*-like sequences are predicted in the *S. purpuratus* genome, along with a single member each of the other *Src* gene family members, *Csk*, *Tec* and *Abl* (Table 1). This non-receptor protein tyrosine kinase family is considered in the larger context of the sea urchin kinome in the accompanying paper by Bradham et al. (2006). While the *S. purpuratus* *Abl*, *Tec* and *Csk* genes are unambiguous orthologs to vertebrate genes, *SpFrk* is the only clear ortholog among the true *Src* members (Bradham et al., 2006). This is consistent with the proposed duplication of the *Src* genes in vertebrates (Hanks, 2003; Segawa et al., 2006). Further analyses will provide insight into functional relationships between echinoderm and vertebrate members of the *Src* gene family.

All of the *S. purpuratus* SFK genes are transcribed based on mRNA accumulation revealed in the tiling arrays, and the presence and sequence of the respective mRNAs have been confirmed by rtPCR using egg mRNA as template (Table 1). The SpAbl (Peaucellier et al., 1993; Moore and Kinsey, 1994; Belton et al., 2001), SpCSK (Fig. 2) and Sp-SFK1 (Giusti et al., 2003) proteins have been detected in eggs using specific antibodies, but the presence of the other SFK proteins in *S. purpuratus* eggs is not yet known. Even if a given kinase protein is present in eggs, functional studies are required to reveal if it plays a role in egg activation or later developmental events. For example, inhibition of tyrosine kinase activity very early in *S. purpuratus* development inhibits gastrulation nearly 24 h later (Kinsey, 1995; Livingston et al., 1998). Furthermore, the Abl protein is present, but is not required for the initiation of Ca^{2+} release at fertilization in *S. purpuratus* (Giusti et al., 2003) or sea stars (O'Neill et al., 2004). Similarly, the sea star ortholog of SpFrk (AmSFK2) is present in eggs but not active or required until later in development (O'Neill et al., 2004). The genomic identification of these various SFKs allows for development of specific tools for determining the activity profiles and functions of each. Based on comparison with the sea star orthologs, the simple prediction is that the Sp-SFK3 protein will be required at egg activation, but this must be tested directly. Other Sp-SFKs may be required as well. The potential involvement of the sea urchin Tec family kinase (SpTec) is worth investigating since this kinase has been implicated in the regulation of Ca^{2+} release (Smith et al., 2001) and actin cytoskeletal remodeling (Finkelstein and Schwartzberg, 2004) in mammalian somatic cells, which are major events of egg activation as well (Runft et al., 2002).

The identification of the *SpCsk* gene provides a tool for investigating the regulation of Sp-SFKs. In metazoans, CSK is responsible for actively repressing Src type kinase activity by phosphorylating the inhibitory tyrosine on the Src protein C terminus (Miller et al., 2000; Boggon and Eck, 2004; Segawa et al., 2006). To determine if the SpCSK protein is expressed in *S.*

Table 1
The *S. purpuratus* Src Family Kinases (SFKs)

Name	Gene ID (SPU #)	Best human match	Expression data [a]
Sp-CSK	022112	AAI06074 (HsCSK)	rtPCR in eggs, antibody detection (this work)
			Tiling data: 9/11 exons, not in 26,827–26,913 or 28,618–28,685
Sp-CSK2 (putative)	004037	BAD97346 (HsCSK variant)	(SH1 domain only)
			Tiling data: 6/7 exons, not in 185,825–185,840
Sp-SFK1	013522	CAI22300 (HsFYN)	rtPCR in eggs, embryos; mRNA blot in eggs, embryos; full length cDNA from arrayed library
	023261		Tiling data: all exons; antibody detection (Giusti et al., 2003; this work)
Sp-SFK1b	030166		SH1 domain only)
			Tiling data: 3/5 exons, not in 811–960 or 1501–1635
Sp-FRK (SFK2)	019224	NP_002022 (HsFyn-related)	(SH3, SH2, partial SH1 domains); rtPCR in eggs
	012805		Tiling data: 4/4 and 3/4, not in 3205–3220 from SPU_012805
Sp-SFK3	026766	AAH48960 (HsYes)	rtPCR in eggs, embryos
			Tiling data: all exons
Sp-SFK5	026766	AAH51270 (HsSrc)	rtPCR in eggs
			Tiling data: 10/11 exons, not 17,427–175,04
Sp-SFK6	014473	CAA26485 (Hs c-src)	(Partial SH2 and full SH1 domains)
			Tiling data: 6/7 exons, not in 14,319–14,477
Sp-SFK7	024525	CAI22300 (HsFyn)	rtPCR in eggs, embryos
			Tiling data: all exons
Sp-ABL	023952	BAD92693 (HsAbl)	rtPCR in eggs, embryos; cDNA from arrayed library
			Tiling data: 6/10 exons, not in 16,620–16,810, 18,465–18,649, 19,816–19,993, 29,831–29,977; antibody detection (Giusti et al., 2003; Belton et al., 2001; Peaucellier et al., 1993; Moore and Kinsey, 1994)
Sp-TEC (BTK)	012277	AAI01714 (HsTec)	(BTK, SH3, partial SH2, SH1 domains) Tiling data: 15/17 exons for SPU_12278, not 36,157–36,166 or 26,915–2698
	012278		1/2 exons for SPU_12277, not in 50,668–50,730

Genes predicted to encode SFKs were identified based on BLAST analysis using conserved SFK family domain sequences and evaluated by CLUSTAL W alignments. The top scoring human gene hit obtained by BLAST of the NCBI database is listed.

[a] All predicted exons were checked for representation in the egg/embryo expression tiling dataset (Samanta et al., in press). Confirmation by rtPCR is indicated and references to published expression data are provided.

purpuratus eggs and to begin to investigate the potential role of SpCSK, specific antibodies were used to localize SpCSK and Sp-SFK1 in biochemical fractions of eggs. SFK activity is associated with the detergent resistant membrane microdomain ("raft") fractions in *S. purpuratus* (Belton et al., 2001) and *Xenopus* (Sato et al., 2002, 2003) eggs. The Sp-SFK1 protein is present in the raft fraction of unfertilized eggs and enriched further in this fraction prepared from eggs 30 s post sperm addition (Fig. 2). The CSK antibody reacted specifically with a sea urchin egg protein of ca. $Mr \sim 50$ K, the predicted size for SpCSK that was present in the raft fraction of unfertilized eggs. While SpCSK protein was also present in this fraction of fertilized eggs, a shift in relative distribution out of the raft

fraction to higher density fractions was apparent (Fig. 2), consistent with the activation of Sp-SFK1 at fertilization. Further studies (such as overexpression of a constitutively active SpCSK or inhibition of SpCSK via dominant interference, as well as determining if SpCSK interacts with Sp-SFK1) are needed to determine if SpCsk plays a direct role in regulating Sp-SFK1 (or other egg SFKs) at fertilization and to investigate the regulation of subcellular localization in more detail.

In somatic mammalian cells, adaptor and scaffold proteins modulate the dynamic localization (and thus activity) of many signaling proteins, including CSK (Davidson et al., 2003; Rahmouni et al., 2005). Any signaling complex involved in initiation of Ca^{2+} release (Fig. 1) or other aspects of egg
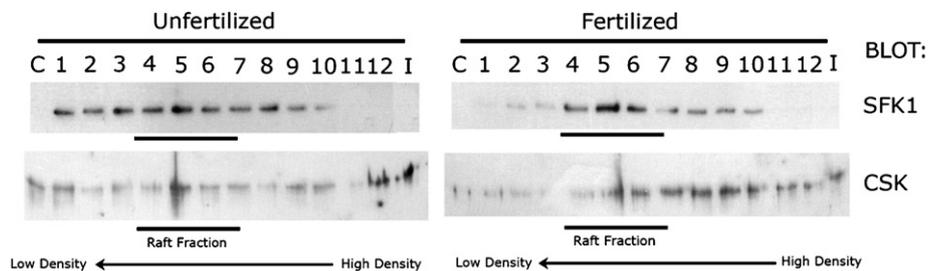

Fig. 2. Localization of Sp-SFK1 and SpCSK proteins in sea urchin egg rafts. Particulate fractions from unfertilized and fertilized (30 s post sperm addition) eggs were resuspended in 2% TX-100 buffer, adjusted to 42.5% sucrose and subjected to equilibrium density centrifugation through a 42.5/30/5% sucrose step gradient. Equal amounts of protein were separated by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose. The numbering of the gradient fractions begins with the low-density samples (fraction 1) and ends with the high-density fractions (fraction 12). The cytosolic (C) fraction and the insoluble (I) fraction are indicated. Blots were probed with the antibodies indicated followed by HRP-conjugated secondary antibodies and visualization was with EnHanced Chemiluminescence. Blots probed with Sp-SFK1 antibody were exposed for 1 min, and the blots probed with CSK antibody were exposed for 15 min.

activation is likely to include adaptor and scaffold proteins. Initial genomic analysis suggests that the sea urchin genome encodes orthologs of many known scaffolding and adaptor proteins (Table 2), including Src homology 2 (SH2) domain-containing adaptors such as NCK, CRK, GRB2 and SHC, consistent with the observed requirement for protein tyrosine kinase signaling at fertilization. In addition to these scaffold and adaptor proteins, other SH2 domain-containing proteins are present as well. A cursory assessment of the sea urchin genome using the Pfam domain search (Bateman et al., 2004) revealed 45 genes predicted to encode SH2 domain-containing proteins (Supplementary Table 3). In comparison, the human genome encodes 110 nonredundant SH2 domain-containing proteins (Liu et al., 2006). The fewer number observed in the *S. purpuratus* genome is largely due to the reduced number of members in each gene family, not lack of representation of a given gene family (Supplementary Table 3). Again, this is consistent with the observations of many gene families in the sea urchin genome (Consortium, in press).

The general model of regulation of the initial Ca^{2+} signal (Fig. 1) also invokes the activity of protein phosphatases, acting on Ca^{2+} signaling components, including the SFKs. These could include both "activating" phosphatases that would be responsible for relieving the repression on the SFKs, as well as Ca^{2+} sensitive phosphatases that would act as negative feedback modulators on the SFK activity (Supplementary Tables 1 and 2). Identification of sea urchin egg phosphatases has been difficult, due to poor cross-reactivity with antibodies against vertebrate protein phosphatases (Wessel et al., 1995; Kawamoto et al., 1999, 2000) and the lack of highly conserved domain structures. Not surprisingly, a large number of phosphatases now have been identified in the sea urchin genome (Byrum et al., 2006), complementing the kinase repertoire (Bradham et al., 2006). Of particular interest is the *S. purpuratus* ortholog of vertebrate

Table 2
S. purpuratus genes predicted to encode adaptor or scaffold proteins [a]

Name	SPU gene ID	Best human match	Accession identification
Sp-SLMAP	008120	NP_009090	Sarcolemma associated protein
Sp-CBP partial	019024	NP_004371	Creb binding protein
	019025		
	019026		
Sp-Eph	027145	P28693	Tyrosine–protein kinase receptor CEK5/CSK-binding protein-like
Sp-G3BP2 like	017827	NP_987101	Ras-GTPase activating protein SH3 domain-binding protein 2 isoform a
Sp-GRB2/DRK	003585	NP_002077	Growth factor receptor-bound protein 2 isoform 1
	003586		
Sp-GRB2	007721	NP_536739	GRB2-associated binding protein 2 isoform a
Sp-CRK	016214	NP_005198	v-crk sarcoma virus CT10 oncogene homolog
Sp-Ptpn6/11	013810	NP_002825	Protein tyrosine phosphatase, non-receptor type 6/11
Sp-ef-1	015429	NP_055382	Breast cancer anti-estrogen resistance 1
Sp-Sin	004247	NP_060589	Polymerase (RNA) III (DNA directed) polypeptide E
Sp-ITSN2	028457	NP_003015	Intersectin 1, isoform ITSN-1
	003951	NP_006268	Intersectin 2, isoform 1
	019253	NP_062541	Intersectin 2, isoform 3
Sp-Nck	014752	NP_001004722	NCK adaptor protein 2 isoform B
Sp-AKAP1	002839	X97335.1	Kinase A anchor protein
Sp-AKAP13	028249	AAL40923.1	Protein kinase A anchoring protein Ht31
Sp-SH3md2 like protein	004827	NM_020870.3	SH3 domain-containing ring finger 1
Sp-SH3D19 like	016351	NM_001009555.2	SH3 domain protein D19
	020243		
Sp-SH3gl3	023951	BC042864.1	SH3GL3 protein
Sp-Ash1	025482	AAF68983	ASH1
Sp-Ash2	018423	BAA74520	ASH2-like
Sp-Ash3-like	030149	DAA00301	TPAexp: class II basic helix–loop–helix protein
Sp-SH2-B-rs1	027685	CAB43208	Hypothetical human protein
Sp-SHC1	008698	NP_079021	SHC SH2-domain binding protein 1
Grb10 interacting G+F protein 2	025579	CAD98095	Hypothetical human protein
Sp-Cbl-r1	007862	AAH32851	Cas-Br-M (murine) ecotropic retroviral transforming sequence
Sp-Cbl	007863	AAB09293	Cbl-b truncated form 2
Sp-Clasp-2	000098	CAE45842	Hypothetical human protein
Sp-Ap3d-1	003070	BAD92041	Adapter-related protein complex 3 delta 1 subunit variant
Sp-JIP1	000237	BAD92957	MAPK 8 interacting protein 1 variant [Homo sapiens]
Sp-JIP3	001396	NP_203750	MAPK 8 interacting protein 3 isoform 2
Sp-JIP3	017187	CAB72318	c371H6.1 (KIAA0516 protein)
Sp-JIP3	019022	AAN61565	JNK-associated leucine-zipper protein
Sp-Sin-like	011145	BAA95976	KIAA1452 protein

[a] Each gene is identified by the name, determined by the annotator and the SPU number. The best human gene match was determined by a BLAST search of the nucleotide sequence for each SPU (see Materials and methods). Note that annotations are subject to change.

rPTPα (Supplementary Table 2), a phosphatase that is known to activate SFKs in mammalian immune cells (Zheng et al., 2000) and that has been implicated in activation of zebrafish eggs at fertilization via relieving repression on the Fyn kinase (Wu and Kinsey, 2002).

Genomic insights into Ca²⁺ signaling pathways implicated in egg activation 3. Additional pathways to Ca²⁺ release and modulation

Sea urchin eggs have multiple pathways that are capable of modulating Ca^{2+} levels (Fig. 1). Most of these pathways have been characterized using pharmacological agents and tools developed in vertebrate somatic cells. Genomic sequence mining now has identified the genes encoding the key components of these pathways (Supplementary Tables 1 and 2) and offers an opportunity to develop specific reagents to test their roles in activation and early development.

While IP$_3$ is the initial modulator of Ca^{2+} release in most eggs studied, other second messengers can trigger Ca^{2+} release in intact sea urchin eggs (Fig. 1). Among these are cyclic ADP-Ribose (cADPR), cGMP, NAADP and nitric oxide (NO) (Stricker, 1999; Whitaker, 2006). While none of these secondary messengers has been shown to be necessary for initiation of the Ca^{2+} rise at fertilization, live cell studies using eggs of *Lytechinus* spp. (Churchill and Galione, 2001; Galione and Churchill, 2002; Santella et al., 2004) and species from Japan such as *A. crassispina* (Kuroda et al., 2001) suggest that they may be involved in other aspects of Ca^{2+} regulation, such as the fertilization/activation potential, Ca^{2+} wave propagation or oscillations. Although recent studies have implicated the second messenger nicotinic acid adenine dinucleotide phosphate (NAADP) as a sperm-delivered activator of Ca^{2+} release in *L. pictus* eggs (Billington et al., 2002; Churchill et al., 2003), whether the sperm actually delivers a required factor at fertilization is not known in echinoderms (see above). This question is complicated further by the possibility that there may not be an NAADP "receptor" per se that acts as a Ca^{2+} channel, but rather an NAADP binding protein that modulates a Ca^{2+} channel, perhaps the ryanodine receptor (RyR) (Galione and Petersen, 2005; Galione and Ruas, 2005; Churamani et al., 2006). Although very little of the physiology surrounding these second messengers has been conducted in *S. purpuratus* eggs, the completion of the genome sequence allows for orthologs of the enzymes involved in their production and regulation to be identified in other urchin species and also opens up the possibility of conducting biochemical and physiological studies in *S. purpuratus* eggs to identify the binding target of NAADP.

Any pathway in eggs or embryos that involves Ca^{2+}-induced Ca^{2+} release via the RyR is feasible as this non-voltage gated Ca^{2+} channel is also expressed in *S. purpuratus* eggs (McPherson et al., 1992) along with the IP$_3$R (see above). These other Ca^{2+} release pathways (Fig. 1) may enhance or feed into the IP$_3$ pathway and affect initiation of Ca^{2+} release in subtle ways (Galione and Churchill, 2002). For example, the nitric oxide (NO) pathway in *S. purpuratus* (Kuo et al., 2000), *L. pictus* and *Psammechinus miliaris* eggs (Leckie et al., 2003) can trigger

cGMP-dependent protein kinase (PKG), which then activates ADP-ribosyl cyclase for production of cADPR to activate the ryanodine receptor (RyR) Ca^{2+} channel in the ER (Churchill and Galione, 2001). The *S. purpuratus* genes encoding these pathway components (Fig. 1) have been identified in the genomic analyses (Supplementary Tables 1 and 2). Because of the lack of redundancy, it should be possible to address the issue of the respective contributions and interactions of each Ca^{2+} release pathway for a given event such as initiation of Ca^{2+} release, Ca^{2+} wave propagation or Ca^{2+} oscillations during the cell cycle and development.

Finally, a number of genes encoding G-proteins have been predicted and a few have been documented as expressed in sea urchin (*Lytechinus* spp. and *S. purpuratus*) eggs (Voronina and Wessel, 2004a,b). At least one G-protein-coupled pathway is present in sea urchin eggs, and injection of antibodies against Gαs and Gαq into *L. variegatus* eggs affected sperm-induced Ca^{2+} release to some extent but did not abolish it. Rather, amplitude and duration of the lag phase were affected, suggesting a modulatory role (Voronina and Wessel, 2004a). It is possible that there is a link between G-proteins and the PLC-γ pathway via SFKs (Fig. 1) (Luttrell and Luttrell, 2004). Once the full repertoire of GPCRs and the coupled signaling machinery expressed in sea urchin eggs is delineated (Beane et al., 2006; Raible et al., 2006), it will be possible to specifically test the mechanism of GPCR signaling during egg activation.

The annotated genome now makes it feasible to use affinity chromatography coupled to mass spectrometry analysis to identify components of any of the signaling pathways shown in Fig. 1, including the initial trigger. This approach could also lead to the elucidation of the proteins involved in membrane depolarization (as part of the transient, electrical fast block to polyspermy; (Jaffe, 1976; Gould and Stephano, 2003)), membrane fusion and gamete recognition (Neill and Vacquier, 2004; Song et al., 2006). In regard to the fast block to polyspermy and fertilization potential, analysis of the sea urchin genome indicates a robust repertoire of genes predicted to encode various Na^{2+} channels and voltage gated Ca^{2+} channels which might mediate these respective events (Supplementary Tables 1 and 2). Although some channels have been characterized in sea urchin embryos (see references in Supplementary Tables 1 and 2), most have not yet been analyzed for expression or function in eggs. A discussion of many of these gene families is presented in the accompanying article by Burke et al. (2006) in the context of the *S. purpuratus* nervous system (Burke et al., 2006). The identification of these genes is a rich resource for use in characterizing the collection of events that comprise egg activation, especially given that so many are dependent on ionic changes (Whitaker and Steinhardt, 1982).

Genes encoding proteins that regulate egg activation events downstream of initial Ca²⁺ signaling

A number of changes occur in eggs wholly or in part due to the rise in cytoplasmic Ca^{2+} levels (Fig. 1). Cortical granule exocytosis, which modifies the egg surface as a permanent block to polyspermy and results in the elevation of the fertilization

envelope, is a well-studied model of coordinated cellular exocytosis. Many of the genes encoding proteins thought to be involved in the regulation and execution of this event have been described (Wong and Wessel, 2004; Song et al., 2006; Wong and Wessel, 2006). Likewise, the *S. purpuratus* genes encoding cytoskeletal proteins and their regulators now have been identified (Morris et al., 2006) and will be valuable tools for investigating the molecular mechanism of sperm nuclear entry, syngamy and mitotic spindle position, to name just a few of the critical egg activation events that involve the cytoskeleton. For example, the integrins are crucial for the actin reorganization that occurs during egg activation. They are present on *S. purpuratus* eggs and morpholino antisense oligonucleotide-mediated knockdown of the integrin beta subunit results in eggs that lack cortical actin arrays and become apoptotic (Murray et al., 2000; Burke et al., 2004). Genes encoding integrins and the signaling components that mediate integrin signaling have been identified in the sea urchin genome (Morris et al., 2006), including kinases such as FAK and Integrin-Linked Kinase (ILK) (Bradham et al., 2006). Although not thought to be involved directly in Ca^{2+} signaling, signaling through integrin complexes may also influence Src kinase activity, thus linking these two pathways in egg activation.

Another key question is how the fertilized sea urchin egg handles the dramatic changes in internal Ca^{2+} concentration. At the peak of the Ca^{2+} wave, the cytosolic Ca^{2+} concentration is 3–4 μM, but within 5–10 min, this Ca^{2+} is returned to ~ 0.1 μM (Stricker, 1999; Runft et al., 2002). The sea urchin genome contains genes encoding Ca^{2+} buffers, pumps and exchangers that may participate in this 'off' reaction (Fig. 1, Supplementary Table 1). The *S. purpuratus* Ca^{2+} ATPase gene family is discussed in more detail in an accompanying article. A cDNA representing the single SERCA pump gene in the sea urchin genome has been cloned and described in detail, and immunoblotting reveals that it is expressed in eggs (Gunaratne and Vacquier, 2006), consistent with its role in driving Ca^{2+} accumulation in the egg ER (Whitaker, 2006). The single copy gene encoding the *S. purpuratus* PMCA is highly expressed in sperm (Gunaratne et al., 2006), and in eggs and embryos as well. Several Ca^{2+}/Na^+ exchangers (NCX) are represented in the sea urchin genome (Supplementary Table 1) and a K^+-dependent exchanger has been well characterized in *S. purpuratus* sperm (Su and Vacquier, 2002). A number of genes encoding transient receptor ion channels (Trp) channels that are reported to carry Ca^{2+} are present in the genome (Supplementary Table 1), and in this light, it is interesting to note that recent mammalian somatic cell studies link tyrosine kinase (particularly Src Family Kinase) and GPCR coupled signaling to regulation of a subset of these channels, including TRPC3 (Kawasaki et al., 2006). Identification of the genes encoding the *S. purpuratus* repertoire of Ca^{2+} channels and exchangers provides a crucial bridge between egg physiology and signaling mechanisms that now can be investigated in detail.

Other examples of sea urchin egg activation events that have been well described at the biochemical and cellular physiological level include the substantial redox changes (Whitaker and Steinhardt, 1981, 1982; Schomer and Epel,

1998; Miller and Epel, 1999) and the rise in cytosolic pH that occurs after fertilization in *L. pictus* (Johnson et al., 1976; Grainger et al., 1979; Shen and Steinhardt, 1979; Whitaker and Steinhardt, 1982) and in *S. purpuratus* eggs (Johnson et al., 1976; Winkler et al., 1980). The pH change has been implicated in a number of other cellular events including: pronuclear migration in *L. pictus* and *A. punctulata* eggs (Schatten et al., 1985); initiation of translation in *L. pictus* (Epel et al., 1974; Grainger et al., 1979); and *S. purpuratus* eggs (Epel et al., 1974; Winkler et al., 1980) and DNA synthesis in both *S. purpuratus* and *L. pictus* (Mazia and Ruby, 1974; Miller and Epel, 1999). Pharmacological studies in *S. purpuratus* (Johnson et al., 1976) and *L. pictus* eggs (Shen, 1989) indicate that the pH rise is mediated through a Na^+/H^+ exchanger, but detailed mechanistic studies require more specific tools and knowledge of the precise exchanger(s) expressed in eggs. Analysis of the sea urchin genome sequence reveals genes that are predicted to encode redox effectors and Na^+/H^+ exchangers, as well as a number of other proton exchangers and receptors that mediate ionic exchange (Fig. 1, Supplementary Table 2), setting the stage for further investigation.

Another important group of egg activation effectors is the canonical Protein Kinase C (PKC) enzymes and other Ca^{2+} sensitive kinases such as the Ca^{2+}/calmodulin-dependent protein kinases (CaMKs). These protein families are described in more detail in the companion paper by Bradham et al. (2006), but it is worth noting that a number of these kinases are expressed in *S. purpuratus* eggs (Supplementary Tables 1 and 2) and past functional studies in *L. pictus* strongly support their role in later egg activation events (Baitinger et al., 1990; Shen and Buck, 1990).

Finally, entry into the mitotic cell cycle is perhaps the most obvious downstream egg activation event. Sea urchins are one of only a few groups of animals that produce eggs that are naturally matured and arrested in a haploid, quiescent state. Fertilization directly triggers mitotic cell cycle entry while most other animals produce oocytes that are arrested in meiosis and fertilization triggers the completion of meiosis followed by mitotic entry. Because the same signaling pathways modulate meiotic arrest, meiotic completion and mitotic cell cycle entry (Kishimoto, 2004), it has been difficult to tease apart specific molecular mechanisms for each. The sea urchin, therefore, offers the opportunity to study mitotic cell cycle entry directly, without this complication. The genes encoding the *S. purpuratus* cell cycle regulators and machinery now have been documented (Fernandez-Guerra et al., 2006). Among the most important signaling pathways governing cell cycle re-entry downstream of the intracellular Ca^{2+} rise is the Mitogen Activated Protein Kinase (MAP kinase) pathway. Genes encoding many members of this signaling pathway, including MAP kinases, MAPK kinases and MAPKK kinases, as well as the phosphatases implicated in their regulation, now have been documented in the sea urchin genome (Supplementary Table 2; Byrum et al., 2006; Bradham et al., 2006).

In several species of sea urchin (*P. lividus*, *L. pictus* and *S. purpuratus*), MAPK activity has been implicated in regulating

cell cycle events, but the data differ regarding the precise role of MAPK (Chiri et al., 1998; Philipova and Whitaker, 1998; Carroll et al., 2000; Kumano et al., 2001; Kumano and Foltz, 2003; Philipova et al., 2005a,b; Philipova and Whitaker, 2005; Zhang et al., 2005, 2006). An outstanding question is whether there are multiple MAPK signaling pathways, which might explain the conflicting results reported (Philipova et al., 2005a; Zhang et al., 2005, 2006). In the S. purpuratus genome, a single gene has been identified to date that encodes an ERK-like MAP kinase (Bradham et al., 2006). The cognate mRNA is present in unfertilized eggs (Kumano and Foltz, 2003), and monoclonal antibodies directed against vertebrate ERKs detect a protein of the correct size in S. purpuratus eggs and embryos (Carroll et al., 2000; Kumano et al., 2001; Kumano and Foltz, 2003). In contrast, there are at least five genes encoding MAPK kinases (Bradham et al., 2006) and commercial antibodies specifically detect an MEK-like protein in eggs (Kumano et al., 2001). The identification of these pathway components and development of specific tools allows for investigation of an outstanding question—the mechanism of the unique haploid arrest of the egg and subsequent mitotic re-entry at fertilization. More detailed investigations of downstream targets, the effectors of the cell cycle re-entry, are now also possible. The first several cell cycles do not require transcription, so these MAP kinase signaling targets are unlikely to be the typical ones (transcription factors) commonly found in somatic cells (Lewis et al., 1998; Garrington and Johnson, 1999; Pearson et al., 2001). Rather, these and other effectors of egg activation will be maternal proteins that are regulated post-translationally. Thus, the information provided by the genome is only the first step in understanding the mechanism of egg activation—identification of the maternal proteins is crucial.

The significance of the egg proteome—genes involved in the post-translational modification of maternal proteins that mediate egg activation

The egg is a complex cell that contains many mRNAs and proteins that will not be used until later in development. In addition, no transcription and very little translation occurs in the first 10–15 min after sea urchin fertilization, yet many of these dramatic cellular events are initiated – and many are completed – during that time. Thus, it is imperative to identify proteins that are already present in the unfertilized eggs and the post-translational protein modifications that must be occurring to regulate their interactions and activities. The availability of the annotated sea urchin genome sequence enables the use of functional proteomics to identify these maternal proteins.

Two of the better-studied post-translational events are protein turnover via targeting to the proteasome and protein phosphorylation. In L. pictus, proteasome-mediated protein turnover begins within 15–20 min after fertilization, and a peak in proteasome activity occurs at ~ 100 min post fertilization, right before nuclear envelope breakdown (Kawahara et al., 2000). Inhibition of proteasome activity results in over-replication of DNA and blocks mitotic entry, extending the role of protein degradation as a cell cycle regulator beyond that of mitotic exit

(Kawahara et al., 2000). Genomic analyses have revealed a number of components of the S. purpuratus translational control apparatus, proteasome and ubiquitination pathways, which will allow for a more detailed study of structure, function and regulation (Fernandez-Guerra et al., 2006; Morales et al., 2006). An emerging model for the egg to embryo transition is the required degradation of key proteins, regulated by conserved kinases (Greenstein and Lee, 2006). These studies have been carried out mainly in C. elegans, allowing for precise genetic models to be constructed. The sea urchin zygote is a good model for the complementary biochemical experiments. Genes encoding a number of the components identified in the C. elegans egg to embryo transition, including DYRK, CDK-1, GSK-3 and KIN-19 (Shirayama et al., 2006; Stitzel et al., 2006), now have been identified in the sea urchin (Supplementary Table 2).

The transition from egg to embryo has been described as a series of translational regulatory events (Vasudevan et al., 2006). The dramatic rise in protein synthesis that occurs in S. purpuratus eggs is independent of transcription (Epel, 1967; Brandhorst, 1976) and likely requires the degradation of 4E-BP proteins, which sequester the 5′ cap binding subunit of the eIF4F initiation factor (eIF4E). In eggs of Sphaerechinus granularis, fertilization-induced phosphorylation of 4E-BP is concomitant with its dissociation from eIF4E and subsequent degradation (Cormier et al., 2001), linking protein synthesis and turnover with phosphorylation.

Protein phosphorylation, especially on tyrosine, is another major regulatory event that occurs during egg activation in all metazoans studied (Kinsey, 1997; Sato et al., 2006; Whitaker, 2006), but the identities of the phosphoproteins, kinases and phosphatases are largely unknown. Candidate protein tyrosine kinases activated in S. purpuratus eggs at fertilization have included members of the receptor tyrosine kinases as well as non-receptor tyrosine kinases, especially the SFKs (Fig. 1, Supplementary Table 2). It is also likely that protein serine/ threonine phosphorylation is important. The PKC and the MAP kinase pathways, for example, are implicated in later egg activation events (see above). The completion of the S. purpuratus genome sequence enables the coupling of the powerful biochemical and cellular approaches available in the sea urchin to functional proteomic methods in order to identify key maternal proteins that regulate egg activation.

Baseline characterization of the egg proteome and phosphoproteome

Historically, the biochemical tractability and synchronicity of the sea urchin zygote have made it an attractive model for studying global protein synthesis. Brandhorst (1976) used two-dimensional gel electrophoresis to visualize differences between unfertilized and fertilized sea urchin (L. pictus and S. purpuratus) egg proteins, providing the first glimpse of the egg proteome and changes derived from initiation of maternal mRNA translation that had been described earlier by Epel (1967). Grainger et al. (1986) then took advantage of the biochemical tractability and synchronous development of the early sea urchin embryo to analyze temporal differences in total protein complement at

various stages by comparative 1- and 2-dimensional gels. The sequence of the sea urchin genome together with improvements in 2D gel electrophoresis and protein detection methods sets the stage for a thorough proteomic investigation of egg activation and early development.

Studies of protein turnover and dynamic phosphorylation events that occur in unfertilized eggs and in zygotes at various times after fertilization were initiated. Following 2D gel electrophoresis, proteins were visualized with SYPRO Ruby stain. Optimal, reproducible resolution of as little as 25 µg soluble protein was achieved using all available pH gradients (Supplementary Fig. 2). Analysis of the broadest pH range separation (pH 3–10) data set reveals that there are ~740 individual protein spots that are reproducibly visualized and easily resolved in unfertilized eggs (Fig. 3A). This represents the detectable baseline *S. purpuratus* egg proteome. The same type of analysis can be conducted for any time point after fertilization (Fig. 3B). Separation on pH 4–7 gradients (Supplementary Fig. 2B) resulted in optimal resolution of the majority of proteins. Table 3 (first column) summarizes the number of reproducibly detectable proteins in unfertilized eggs and at various times post fertilization using the pH 4–7 gradient. For comparison, previous studies reported the presence of ~400 proteins in eggs (Brandhorst, 1976) or ~100 proteins (Grainger et al., 1986) in *L. pictus* and *S. purpuratus* eggs using ³⁵S-methionine labeling and silver staining and pH ranges of 4.5–7.3 and 5–7, respectively. The increased number of detectable proteins (Table 3) likely is the result of improved resolution and detection sensitivity.

After visualization of the total protein complement, the same gels were then stained with ProQ Diamond, which detects serine-, threonine- and tyrosine-phosphorylated proteins (Steinberg et al., 2003). Analysis of the phosphorylated proteins revealed that ~30% of the detected proteins in unfertilized eggs are phosphorylated (Fig. 3A′, Table 3). This represents the reproducibly detectable baseline egg phosphoproteome and is consistent with observations in mammalian systems indicating that one-third of all cellular proteins are phosphorylated (Ahn and Resing, 2001). The exquisite synchronicity of *S. purpuratus* fertilization allows for assessment of rapid time points. At 2 min post sperm addition, the number of detectable phosphorylated proteins doubles, and by 30 min, the percentage of phosphorylated proteins has returned to pre-fertilization levels (Table 3). These quantitative results complement and extend previous studies of bulk protein phosphorylation changes in eggs at fertilization (reviewed in Kinsey, 1997).

Fertilization-responsive changes in the proteome and phosphoproteome

Temporal differences in the total and phosphoproteome were analyzed in more detail. Soluble protein lysates from a single batch of unfertilized eggs and at various times after fertilization were analyzed by 2D gel electrophoresis in triplicate and stained with SYPRO Ruby to analyze total proteins (Fig. 4). First, individual protein spots were tracked over a time course in triplicate to assess reproducibility. As expected, some proteins remained relatively stable over a 30 min time course while others disappeared reproducibly. For example, three proteins readily detected in unfertilized eggs (Fig. 4, arrows) were barely detectable by 2 min post fertilization and entirely gone by 15 min. The loss of a protein spot could be due to a post-translational modification that shifts its position on the gel, degradation or perhaps a subcellular relocalization event. For example, proteins present in the cortical granules would be lost after exocytosis at fertilization. It is also possible that a protein
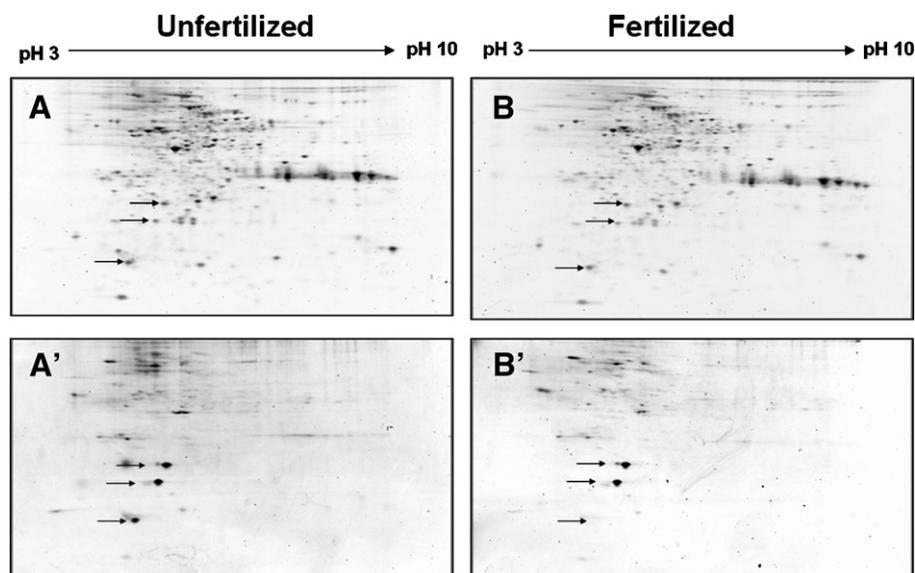

Fig. 3. Multiplex detection of the sea urchin proteome. Total NP-40 soluble lysates (35 µg) from unfertilized (A, A′) and fertilized eggs 30 min post sperm addition (B, B′) were separated by isoelectric focusing using IEF 11 cm pH 3–10 strips then separated by Mr on an 8–16% gradient SDS gel. Panels A and B were stained with SYPRO Ruby which detects total protein. These same gels were then stained with ProQ Diamond, which detects phosphorylated tyrosine, serine and threonine residues (A′, B′). Arrows indicate examples of spots detected with both SYPRO and ProQ Diamond stains for multiplexed reference between gels and correspond to areas enlarged for analysis in Fig. 5.

Table 3
Detection and comparison of total and phosphorylated *S. purpuratus* proteome in unfertilized eggs and zygotes

Time point	Total detectable proteins	Total phosphorylated proteins	% proteins phosphorylated	Representative egg activation events [a]
Egg	604	182	30	Maintenance of haploid arrest; metabolic housekeeping (no Ca^{2+} signaling)
2 min	464	307	66	Src family tyrosine kinase activity peaks; Ca^{2+} wave completed, cortical granule exocytosis; initiation of cytoskeletal remodeling; PKC activation; initiation of pH rise; activation of NAD kinase, increase in $NADP^+$ and NADPH; increase in O_2 consumption
15 min	587	248	42	Changes in MAPK activity; pronuclear entry and syngamy; mRNA translation and amino acid transport begins; mitotic cell cycle events initiate; Ca^{2+} levels return to near pre-fertilization values
30 min	450	110	25	DNA synthesis nearly complete; protein synthesis and turnover steady

Total soluble protein lysates (25 µg) from unfertilized sea urchin eggs and eggs at various times post sperm addition were separated by isoelectric focusing using IEF 11 cm pH 4–7 strips then by Mr on a 8–16% gradient SDS gel. Triplicate gels were stained for total protein detection (SYPRO dye), and then the same gel was stained to detect phosphorylated proteins (ProQ Diamond dye). Gels were imaged and analyzed as described in Materials and methods. Numbers listed represent the average value of triplicate sample runs within the same experiment.

[a] Only a subset of egg activation events are listed and times are approximate, based on *S. purpuratus* eggs fertilized at 15–17°C (see Whitaker and Steinhardt, 1982; Townley et al., 2006). Note that the fast block to polyspermy (and fertilization potential rise) as well as sperm–egg membrane fusion is complete within 1 s. An increase in Ca^{2+} is detected within 10 s. Many of the other events listed in the 2 min time point initiate within the first 1–5 min (see Fig. 1, also).

could re-localize to a microdomain in the egg and become insoluble under the conditions used. All of these possibilities can be assessed using variations of stains, solubilization and fractionation procedures, depending on the question being addressed.

Specific, temporal differences in the phosphoproteome also were assessed. Soluble protein lysates from a single batch of unfertilized and eggs at various times after fertilization were analyzed by 2D gel electrophoresis and stained with ProQ Diamond to detect phosphoproteins. Three different regions of the same gel are shown as examples of the kinds of changes that are detected over an expanded time course (Fig. 5). Several proteins that are phosphorylated in unfertilized eggs remain stably phosphorylated over time (Fig. 5A). However, some proteins are phosphorylated in unfertilized eggs and are dephosphorylated after fertilization (Fig. 5B); in this example, dephosphorylation has occurred by 5 min post insemination (Fig. 5B, arrow). Other proteins become phosphorylated (Fig. 5C) or their phosphorylation state oscillates. For example, two proteins (Fig. 5C, circled) are phosphorylated by 30 min post insemination and remain phosphorylated while a third protein (Fig. 5C, arrow) is phosphorylated by 5 min and then decreases in phosphorylation at 30 min only to increase phosphorylation again at 45 min post insemination. Several other proteins visible in this region of the gel appear to have increased in phosphorylation following fertilization but are left unlabeled for simplicity. The ability to resolve changes in the phosphorylation state of a large number of proteins over a period of time sets the stage for a comprehensive analysis of the role of phosphorylation in egg activation at fertilization.
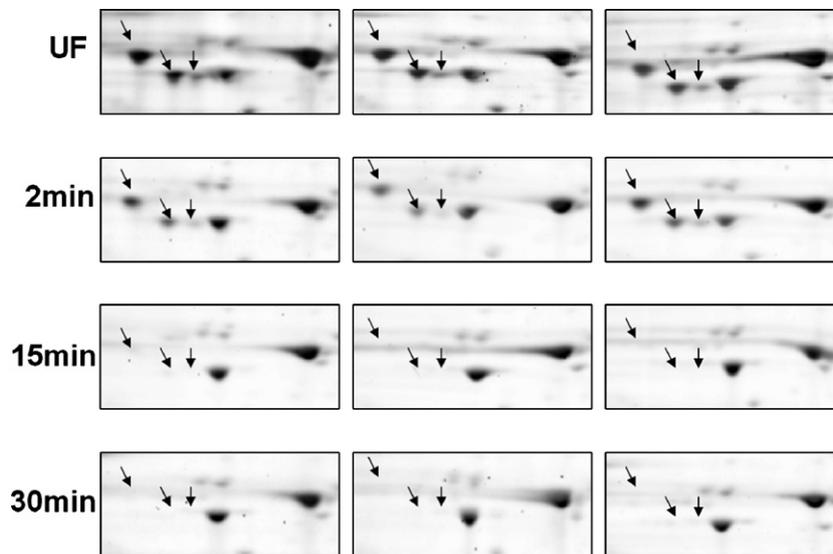

Fig. 4. Temporal analysis of the total proteome. Eggs from a single sea urchin were collected as unfertilized and at various times post sperm addition as labeled. Total soluble proteins (25 µg) were separated by isoelectric focusing using IEF 11 cm pH 4–7 strips then separated by Mr on a 8–16% gradient SDS gel. Individual samples were run in triplicate and stained with SYPRO Ruby to detect total protein. The same area of the triplicate gels is shown at the different time points. Arrows indicate position of representative proteins over the first 30 min post sperm addition.
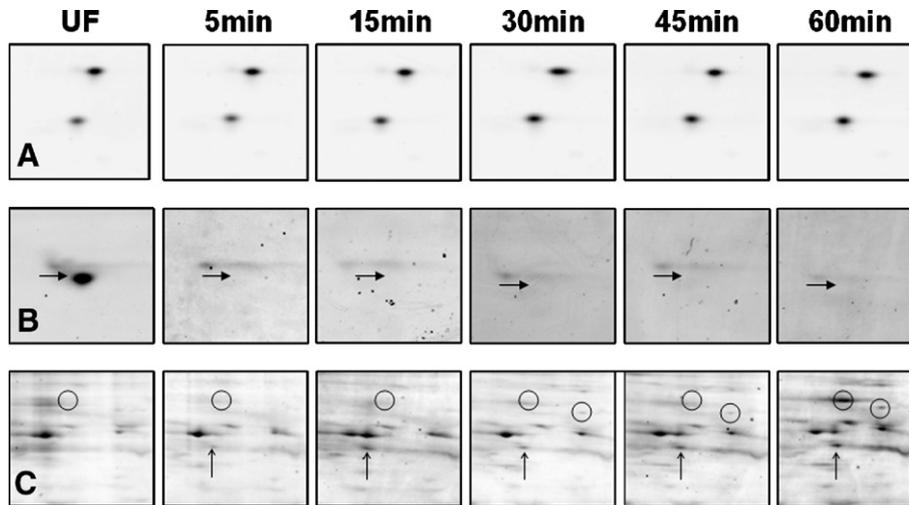
Fig. 5. Temporal detection of the sea urchin egg phosphoproteome. Eggs from a single adult urchin were collected as unfertilized and at various times post sperm addition as indicated. Total soluble protein lysates (35 μg) were separated by isoelectric focusing using IEF 11 cm pH 3–10 strips then separated by Mr on an 8–16% gradient SDS gel. Gels were stained with ProQ Diamond to visualize proteins phosphorylated at tyrosine, threonine and serine residues. Three areas of the same gels (A–C) are shown from the time course. Series A is an example of two phosphoproteins that remain stable over the time course. Series B (arrow) is an example of a protein that is phosphorylated in unfertilized eggs and becomes rapidly dephosphorylated. Series C is a region of the gel in which it is possible to visualize proteins that are changing multiple phosphorylation stages over the 60 min time course. The circles identify proteins that become phosphorylated gradually over time. The thin arrows denote an example of a protein that becomes rapidly phosphorylated following insemination.

Identification of proteome components by mass spectrometry

The goal of this study is to move beyond the global proteome and phosphoproteome dynamics to identification and characterization of the individual proteins. Analyses of the proteome and phosphoproteome at various times after fertilization (Table 3) reveal dynamic changes that relate to the egg activation events occurring at given time points (Fig. 1). To begin to compare the unfertilized egg proteome with that of a fully activated egg (30 min post fertilization), spots representing proteins that fell into two distinct classes were chosen: those that were shared by both the unfertilized egg and 30 min post insemination zygote proteomes and those that were differentially represented in the two proteomes. Twelve individual spots were excised from the same coordinates on each of the gels (Fig. 6) and subjected to in-gel trypsin digestion. The digested samples were analyzed by Q-ToF tandem mass spectrometry, and the resulting peptide spectra were analyzed and assessed for matches against the predicted sea urchin genome. In all 12 cases, at least two unique (non-overlapping) peptide matches were discerned for each spot picked (Table 4) and identities were assigned with a high confidence level. Eight of the twelve proteins yielded peptide spectra that matched already-annotated *S. purpuratus* genes. The remaining four matched GLEAN predictions were then assessed and annotated. Not surprisingly, two of the most abundant proteins present in both unfertilized and 30 min zygote
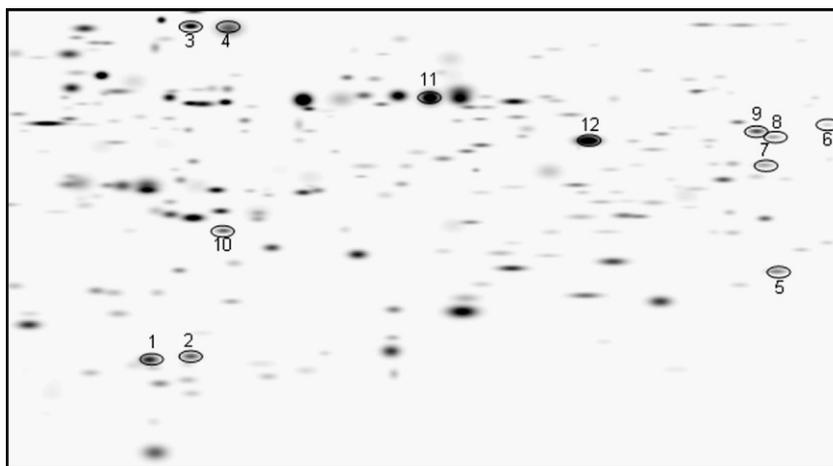

Fig. 6. Protein identification by quadrupole time of flight tandem mass spectrometry. Total soluble protein samples (25 μg) from unfertilized eggs or at 30 min post fertilization of a single sea urchin were separated by 2D gel electrophoresis using IEF 11 cm pH 3–10 strips followed by separation on an 8–16% gradient SDS gel. Triplicate gels were stained with SYPRO Ruby total protein stain. Shown is the synthetic image of the matched sets created using the PDQuest software using all six gels. Circled spots indicate the proteins excised from the gels. Following in-gel trypsin digestion, peptides were analyzed by Q-ToF MS/MS. Spot identification numbers refer to the assigned identities shown in Table 4.

Table 4
Identification of *S. purpuratus* egg proteins using Q-ToF MS/MS

Protein identity by Q-ToF MS/MS [a]	*S. purpuratus* gene name	GENE ID (SPU #)	Analysis Set (PDQuest) [b]	Sequence coverage (%)	Unique peptide matches	Unique peptide sequences
1. 18 kDa egg cortical vesicle protein	Sp-Cvp18	007216	Undetected in fertilized	4.3	2	(R)TIAFTVER(T)
2. 18 kDa egg cortical vesicle protein	Sp-Cvp18	007216	Undetected in fertilized	4.3	2	(R)TIAFTVER(T) (R)DDEVFLR(L)
3. GTP binding protein	Sp-rendezvin	027023	Undetected in fertilized	11	5	(N)IGEALDWTGR(F) (R)LAEGLLDADADR(P) (R)IPVFSYISVG(N) (R)RGNEILVQC(K) (R)TPNDWELMYR(V)
4. GTP binding protein	Sp-rendezvin	027023	Undetected in fertilized	11	4	(T)EALDKFLNYDR(G) (K)SGSIIATFR(F) (R)IPVFSYISR(G) (R)GNEILVQFK(T) (R)TPNDWELVYV(V) (K)SGSIIATFR(F)
5. Peroxiredoxin	Sp-Peroxiredoxin1	014869	Undetected in fertilized	28	5	(K)IPLLSDMCGK(I) (R)DYGIMIEK(E) (R)GLFIIDDKGTLR(Q) (R)QITINDLPVGR(S) (R)LVQAFQFTDK(F)
6. Fumarylacetoacetase	Sp-Fumarylacetoacetase	020609	Undetected in fertilized	9.1	2	(R)LLAADEPTLRDNADLR(E) (R)ASSWISGTPVR(R)
7. Tubulin alpha 2 chain	Sp-alpha-tubulin2	004143	Undetected in unfertilized	5.7	3	(K)ELIDQVLDR(V) (A)VAEALARL(D) (R)NLDIERPTYTNLNR(L)
8. Acyl-CoA dehydrogenase	Sp-Acadsb	005150	Undetected in unfertilized	11.8	2	(I)KEATMCKLLSAEVASLT(T) (R)GITAFLVDR(D)
9. CG1106-PB isoform B	Sp-Gelsolin	003985	Detected in both	5	3	(R)VLPEQYEPR(L) (R)ISDADGSLDMEAMDGEISK(D) (K)NALSYASNYLNK(T)
10. Synaptotagmin	Sp-Syt1/2	002292	Detected in both	21.6	7	(R)LFQVEYAIEAIK(L) (R)TNEGIVLAVEK(R) (R)VTSPLMEATSIEK(I) (D)PSGTFLQFDAK(A) (K)AIGSGSEGAQSQLQEVFDK(S) (K)LSSTNVEVATITPQNK(F) (R)VTLVELAR(L)
11. Tubulin	Sp-alpha-tubulin5	019990	Detected in both	13.2	6	(K)TIGGGDDSFNTFFSETGAGK(H) (K)ELIDQVLDR(I) (R)NLDIERPTYTNLNR(L) (H)FPLATYAPVISAEK(A) (K)DVNAAIATIK(T) (A)VAEALARL(D)
12. Actin homolog 2	Sp-actin (predicted)	009481	Detected in both	20.5	8	(R)AVFPSIVGR(P) (R)VAPEEHPVLLTEAPLNPK(A) (R)DLTDYLMK(A) (R)GYSFTTTAER(E) (K)SYELPDGQTITIGNER(F) (K)DLYANTVLSGGSTMFPGIADR(M) (K)IRIIAPPER(K) (K)QEYDECGPGIVER(K)

[a] Numbers refer to protein spots identified in Fig. 6.
[b] Analysis sets were user-defined during analysis in PDQuest and are based on qualitative differences between replicate groups. Sequence coverage and unique peptide matches are reported from ProteinLynx Global Server (PLGS) software. Unique peptide sequences are also predicted from analysis of mass spectra using PLGS. See Materials and methods.

proteomes were α-tubulin and actin, known to be present and relatively stable during early sea urchin development (Bibring and Baxandall, 1977; Alexandraki and Ruderman, 1985). Gelsolin, an actin remodeling protein (Silacci et al., 2005),

also fell into this class. Interestingly, two proteins that are present in unfertilized eggs, but absent in the 30 min fertilized proteome are cortical vesicle proteins (Sp-Cvp18 and Sp-rendezvin; Fig. 6, Table 4), which likely are lost during cortical granule exocytosis.

Again, however, absence of a detectable signal at any given coordinate could be due to either a degradation event or to a post-translational modification that causes a shift from the coordinates. For example, in unfertilized eggs, both Sp-Cvp18 and Sp-rendezvin were identified from two adjacent spots and likely represent differential phosphorylation states. In the case of tubulin, this could be due to glutamylation or acetylation (Luduena, 1998), and consistent with this, one form of alpha tubulin was observed to present in unfertilized eggs but absent at 30 min post fertilization (Table 4).

These results indicate that the annotated sea urchin genome is a valuable resource for proteomic analyses. Current efforts are focused on identifying proteins that change phosphorylation state at fertilization or in response to modulation of specific signaling pathways involved in egg activation and cell cycle regulation. This approach also is amenable to analysis of proteins that form temporally dynamic complexes isolated by co-immunoprecipitation or protein affinity chromatography. The completion of the sea urchin genome now allows the promise offered by the biochemistry and synchronicity of the sea urchin egg to be realized by providing the missing link needed to identify the proteins.

Conclusions

In summary, the completion of the sea urchin genome sequence propels the field of fertilization biology forward. It provides the ability to develop specific probes to test the function of known components and to identify new components involved in regulating Ca^{2+} and the myriad of cellular changes that occur during egg activation. The strength of the sea urchin egg as a synchronous, easily handled, biochemical model will allow for continued functional proteomic approaches to outstanding questions of fertilization biology and basic cell biology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.09.006.

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