20 Using *Xenopus* Egg Extracts to Modify Recombinant Proteins

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INTRODUCTION

General Considerations

Nearly every protein undergoes posttranslational modifications that can dramatically affect many of the protein's basic functional characteristics. Broadly speaking, posttranslational modifications fall into three categories: processing, chemical addition, and formation of complexes with binding partners. Processing encompasses proteolytic cleavage wherein a polypeptide is synthesized as a longer inactive precursor (e.g., prohormone) that is consequently cleaved to generate the mature active molecule. Chemical modifications can be quite pleiotropic, altering protein activity (disul-

Protein–Protein Interactions: A Molecular Cloning *Manual*, © 2002 by Cold Spring Harbor Laboratory Press, Chapter 20.

fide bond formation, phosphorylation), localization (fatty acid acylation, glycosylation, phosphorylation), and/or stability (acetylation, phosphorylation). Similarly, complexes formed between two or more protein-binding partners can dramatically affect protein activity (e.g., cyclin potentiation of *c*yclin *d*ependent *k*inase [cdk] activity), localization (e.g., piggyback nuclear transport), or stability (e.g., ubiquitin-mediated degradation).

Of these posttranslational modifications, protein–protein interactions are extremely important. That is, the protein-binding partner(s) with which a given protein interacts frequently governs its ultimate biology. For example, phosphorylation of the Cdc25 mitotic phosphatase by the checkpoint-activated kinase, Chk1, ultimately changes its subcellular localization, but it does so by altering the ability of Cdc25 to interact with multiple binding partners. Briefly, the Chk1 kinase phosphorylates Cdc25 on a critical serine residue, creating a binding site for 14-3-3 protein family members (Peng et al. 1997; Kumagai et al. 1998b). Binding by 14-3-3 is critical for maintaining the checkpoint-induced G_2 arrest but does not alter in vitro Cdc25 phosphatase activity. Binding of 14-3-3, however, does inhibit the association of Cdc25 with importin-α, markedly reducing the nuclear importation of Cdc25 (Kumagai and Dunphy 1999; Yang et al. 1999). Thus, chemically modifying Cdc25 (phosphorylation) ultimately down-regulates Cdc25 activity, but it does so by shifting the equilibrium from a nuclear to a cytoplasmic localization, through the action of multiple Cdc25-binding partners.

Using Cellular Extracts as Model Systems

Recombinant bacterially derived or insect cell (Sf9)-derived proteins frequently lack many of the characteristics of the native molecule, presumably due to the absence of one or more functionally significant posttranslational modifications. Because of the low cost and ease of working with bacterially expressed proteins, it is desirable to identify convenient means of conferring suitable eukaryotic modifications on such recombinant proteins. To this end, incubating bacterially derived recombinant proteins in crude cellular extracts has been a productive means through which to confer eukaryotic modifications on such recombinant proteins. For example, physiologically relevant phosphorylation and protein-binding partners can frequently be detected in vitro using crude cellular extracts. Recombinant cyclin D and cdk4 subunits produced in bacteria or insect cells do not assemble efficiently when combined in vitro but can be activated in the presence of lysates derived from proliferating mammalian cells (Kato et al. 1994). The mammalian cell lysates appear to provide two components: an assembly factor, which stabilizes the cyclin/cdk complex, and *c*dk *a*ctivating *k*inase (CAK), which activates the complex by phosphorylating a conserved threonine residue on cdk4 (Kato et al. 1994). The crystal structure of human cdk2 reveals that the analogous threonine residue resides on a structural loop that occludes the substrate-binding cleft (De Bondt et al. 1993). Thus, phosphorylation on this residue likely alters the geometry of this inhibitory loop and stabilizes an active conformation, enabling important protein–protein interactions. Similarly, crude cellular extracts have also been used to identify physiologically relevant protein-binding partners of recombinant proteins. For example, the cyclin/cdk inhibitor $p27^{Kip1}$ can be purified by cyclin E/cdk2 affinity chromatography from growth-arrested cell lysates, but not from proliferating cell lysates (Polyak et al. 1994). This differential binding pattern is due to high levels of cyclin D in proliferating cells, wherein $p27^{Kip1}$ is sequestered in cyclin D/cdk4,6 ternary complexes and is, therefore, unable to interact with cyclin E/cdk2 (Polyak et al. 1994; Reynisdottir et al. 1995).

To be of general utility, a recombinant protein salvage/modifying system must function on a wide variety of proteins from diverse cellular pathways. With the emerging results of the genome sequencing projects, it is clear that significant evolutionary conservation of protein structure and enzymatic pathways exists among all vertebrates. We have been using *Xenopus* eggs as a starting material for our salvage/modifying system. *Xenopus* oocytes and eggs have been used to study a remarkable array of pathways conserved among frogs, mice, and humans. For example, the fact that *Xenopus* oocytes injected with mammalian RNAs express, assemble, and insert functional mammalian ion channels and neurotransmitters into *Xenopus* membranes strongly argues for the

conservation of protein–protein interactions among these species (Goldin 1991). Similarly, extracts from *Xenopus* eggs have been used to study a wide variety of biological problems, including DNA metabolism, protein processing and transport, and cellular proliferation and death (Table 1). All of these pathways are rich in conserved protein–protein interactions. Thus, the *Xenopus* egg represents an excellent starting material for a recombinant protein salvage/modifying system. Frog egg extracts offer a major advantage over mammalian cell lysates because they are cost-effective, easy to prepare, highly concentrated, and have a proven track record (Table 1). Finally, in addition to their general utility as a modifying system, egg extracts are uniquely suited for cell cycle analyses (see below).

Metazoan oocyte/egg extracts have also proven invaluable for identifying cell-cycle-dependent phosphorylation and binding partners. For example, one of the first purifications of *m*aturation *p*romoting *f*actor (MPF) was accomplished using fission yeast $p13^{suc1}$ affinity chromatography of starfish oocyte extracts (Labbe et al. 1989). Similarly, starfish CAK was detected and purified on the basis of its ability to activate *Xenopus* cdc2 in a cyclin-dependent manner (Fesquet et al. 1993). More recently, activated *a*naphase *p*romoting *c*omplex (APC) or cyclosome has been purified from clam oocytes by affinity chromatography on fission yeast $p13^{suc1}$ (Sudakin et al. 1997). Finally, as detailed below, *Xenopus* egg extracts have been used to reveal physiologically relevant binding partners and/or specific phosphorylation of a large number of recombinant proteins. Indeed, the fact that specific phosphorylation events can be preserved in such crude cellular extracts, coupled with the ubiquitous nature of phosphorylation, speaks to the strength of using cell lysates to phosphorylate recombinant proteins. Moreover, because many proteins are phosphorylated in a cell-cycle-dependent manner, having a means to assess this modification in vitro in the context of the cell cycle is of enormous utility.

The *Xenopus* Egg Extract Model System

Xenopus egg extracts are a premiere system in which to evaluate cell-cycle-dependent phosphorylation of recombinant proteins. *Xenopus* egg extracts arrested specifically in mitosis or interphase can be generated, thereby facilitating study of cell-cycle-dependent modifications. Because of the conserved nature of the cell cycle machinery, physiologically meaningful modifications of cell-cycle-regulated proteins derived from any eukaryotic host can be studied in *Xenopus*. For example, cyclins from sea urchins (Murray and Kirschner 1989) and clams (Swenson et al. 1986), $p13^{suc1}$ from fission yeast (Dunphy and Newport 1989), and Cdc25 from *Drosophila* (Kumagai and Dunphy 1991) all function in a physiologically relevant manner in *Xenopus* egg extracts.

TABLE 1. Conserved Metazoan Pathways Studied in *Xenopus* Egg Extracts

Pathways	Comments
Cell cycle[a]	Cdk/cyclin-driven cell cycle regulated by known checkpoints (e.g., DNA replication/repair, mitotic spindle, chromosome cohesion)
Apoptosis[b]	Nuclear events typical of apoptosis, including Bcl2-mediated fragmentation of nuclei with resultant "laddering" of DNA
Chromatin assembly[c]	Nucleoplasmin-dependent assembly of nucleosomes around exogenous DNA template
Nuclear import/assembly[d]	Cell-cycle-regulated assembly of double-bilayer membrane, nuclear lamina, and functional nuclear pores
DNA replication[e]	Replication checkpoint-dependent single round of semiconservative DNA replication in ~1 hour
DNA recombination/repair[f]	Homologous and nonhomologous recombination, repair of mismatches, abasic sites, UV and X-ray lesions
Translocation/processing[g]	Efficient posttranslational modification (e.g., translocation, glycosylation, and signal sequence cleavage) of exogenous proteins

[a]Murray (1991); [b]Kornbluth (1997); [c]Wolffe and Schild (1991); [d]Newmeyer and Wilson (1991); [e]Madine and Coverley (1997); [f]Carroll and Lehman (1991); [g]Zhou et al. (2000).

OUTLINE OF PROCEDURE

The following protocol makes use of the *Xenopus* egg extract to phosphorylate a recombinant protein with the ultimate goal of identifying the specific phosphorylated residues. A similar approach can be used to isolate binding partners or otherwise modify a recombinant protein. Whether the goal is to identify a chemical modification or binding partner, or to attempt to activate a recombinant protein, the protocol is the same. The basic technique is to modify the recombinant protein by incubation in either a mitotic or interphase *Xenopus* egg extract, reisolate the protein by affinity chromatography, and either analyze the modification or use the modified protein as a probe for further studies (Fig. 1). An example of the latter interaction studies would be to use the unmodified and modified protein to detect differential binding in a far western (Chapter 4), co-immunoprecipitation (Chapter 5), GST-fusion pull-down (Chapter 4), or other biophysical approach (e.g., Biacore, Chapter 14).

The remainder of this protocol serves as a guide for optimizing and troubleshooting the use of *Xenopus* egg extracts to modify recombinant proteins. As an illustration of the protocol, we specifically describe analysis of the cell-cycle-dependent modifications of the *Xenopus* Cdc6 (Xcdc6) protein. The Cdc6 protein is a key regulator of the initiation of DNA replication in all eukaryotes (Diffley 1996). Cdc6 homologs bind to chromosomes in an *o*rigin *r*ecognition *c*omplex (ORC)-dependent manner and mediate the binding of other essential DNA replication machinery to DNA (Coleman et al. 1996; Tanaka et al. 1997). Recent genetic and biochemical evidence suggests that Cdc6 homologs function to load the ring-shaped *m*ini*c*hromosome *m*aintenance (MCM) complex on the DNA (Donovan et al. 1997; Perkins and Diffley 1998; Weinreich et al. 1999). MCM, in turn, appears to be one of the essential helicases involved in initiating DNA

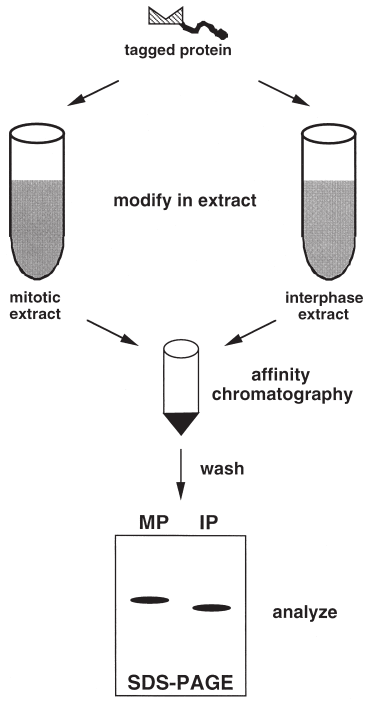

FIGURE 1. Outline showing the use of *Xenopus* egg extracts to modify proteins. Recombinant protein (which may be tagged with a fused peptide for convenient reisolation) is incubated in either an M-phase (MP) or interphase (IP) *Xenopus* egg extract. The recombinant protein is then reisolated by affinity chromatography using either the fused tag or specific antibodies, and nonspecific interactors are removed by repeated washes. Finally, the modified protein is released, and the modification is analyzed.

replication (Kelman et al. 1999; Chong et al. 2000; Shechter et al. 2000; Tye and Sawyer 2000). Both Cdc6 and the MCM family are involved in limiting DNA replication to one round per S phase. This regulatory process, called the block to rereplication, likely involves a positively acting license that can be utilized only once each S phase (Blow and Laskey 1988). In yeast, phosphorylation of Cdc6 homologs has been shown to play a role in its destruction (Jallepalli et al. 1997; Elsasser et al. 1999), whereas in metazoans phosphorylation of Cdc6 appears to cause its redistribution from the nucleus to the cytoplasm (Saha et al. 1998; Jiang et al. 1999; Petersen et al. 1999; Pelizon et al. 2000). Thus, the regulation of the Cdc6 protein and its cell-cycle-dependent phosphorylation is of general interest and also represents an excellent example wherein phosphorylation regulates the interactions and activity of a protein.

Production of *Xenopus* Egg Extracts

Xenopus egg extracts provide a biochemically tractable system that can be generated in multiple forms (Murray 1991). For the purposes of modifying recombinant proteins, we primarily use "CSF-arrested" egg extracts. The CSF-arrested extract is stalled in metaphase by the action of *c*yto*s*tatic *f*actor (CSF). CSF-arrested extracts can be induced to progress through one cell cycle following the addition of calcium. Each in vitro cell cycle consists of chromosome decondensation, nuclear envelope formation, semiconservative DNA replication, chromosome condensation, nuclear envelope breakdown, and chromosome separation. Because CSF-arrested extracts are stalled in metaphase of meiosis II, they are not in a true mitotic state. For most purposes, however, this meiotic metaphase is identical to mitosis, and this subtlety does not affect the modification of recombinant proteins. Thus, CSF-arrested extracts and CSF-arrested extracts after calcium addition are regularly used to simulate mitotic and interphase states. To ensure that interphase extracts do not return to mitosis, the protein translation inhibitor cycloheximide is added before calcium addition because in the absence of cyclin B synthesis, these extracts cannot enter mitosis. For the purposes of familiarity and brevity, we refer to CSF-arrested extracts as "M-phase" and CSF-arrested extracts after calcium addition as "interphase" throughout this procedure.

Purified recombinant protein also needs to be prepared, and this can be done using any number of schemes. Because the production of recombinant proteins is covered elsewhere in this volume (Chapter 4) and must be tailored to individual needs, we do not address this issue here. For the purposes of these experiments, it is convenient to express the protein of interest with a fused tag, for example, hexahistidine, glutathione-*S*-transferase (GST), or maltose-binding protein, to facilitate purification and reisolation. Alternatively, if specific antibodies are available, one can recover the protein by immunoprecipitation (Chapter 5). In the specific example outlined below, we expressed recombinant Xcdc6 with an amino-terminal hexahistidine tag in baculovirus-infected Sf9 insect cells and purified it by Ni-agarose chromatography (Coleman et al. 1996). Similar binding and phosphorylation studies have been performed using GST-tagged proteins expressed in bacteria (data not shown).

Modification and Reisolation of Recombinant Protein

A detailed modification protocol is provided at the end of this chapter. Briefly, recombinant protein is modified by incubating it in the *Xenopus* egg extract for 30 minutes at 23°C. Typically, we add one volume of purified recombinant protein (0.5–1 mg/ml) to nine volumes of extract. In some instances, the recombinant protein can be modified while it is bound to affinity beads, although the beads can sterically inhibit modifications in some circumstances. Thus, the utility of leaving the protein bound to beads needs to be determined empirically. We next dilute the extract containing the purified recombinant protein with four volumes of buffer, clarify it by brief centrifugation, and incubate the supernatant with affinity matrix for 30 minutes at 4°C. Finally, the matrix-bound recombinant protein is isolated by centrifugation, washed, and analyzed.

As a specific example, we observed that the endogenous Xcdc6 protein displayed a slower electrophoretic mobility during M-phase than during interphase (Fig. 2A). To characterize this modification, we wished to modify a sufficient quantity of Xcdc6 for subsequent analysis. For this purpose, full-length Xcdc6 was overproduced in Sf9 insect cells as a hexahistidine-tagged fusion protein and purified to near homogeneity by Ni-agarose chromatography (Coleman et al. 1996). To modify the eluted recombinant protein, we incubated it in either M phase or interphase egg extracts at 23°C. The histidine-tagged protein was then reisolated on Ni-agarose beads, washed, and analyzed by SDS-*p*oly*a*crylamide *g*el *e*lectrophoresis (SDS-PAGE) (Fig. 2B). In this example, the purified recombinant Xcdc6 protein migrated with the anticipated molecular mass of 61 kD (Fig. 2B, lane 3). Consistent with the endogenous protein, which displayed a retarded relative mobility when modified during M phase (Fig. 2A, lane 2), recombinant protein modified in an M-phase extract displayed a similar slower mobility (Fig. 2B, lane 4). As discussed in detail below, this M-phase modification can be attributed entirely to phosphorylation. In control experiments, the relative mobility of recombinant Xcdc6 incubated in an interphase extract was not altered significantly (Fig. 2B, lane 5). Other control experiments that did not include recombinant protein revealed a prominent Ni-agarose interactor of ~51 kD in either M-phase or interphase extracts (Fig. 2B, lanes 1,2). The identity of this 51-kD Ni-agarose interactor remains unknown (see Fig. 2B).

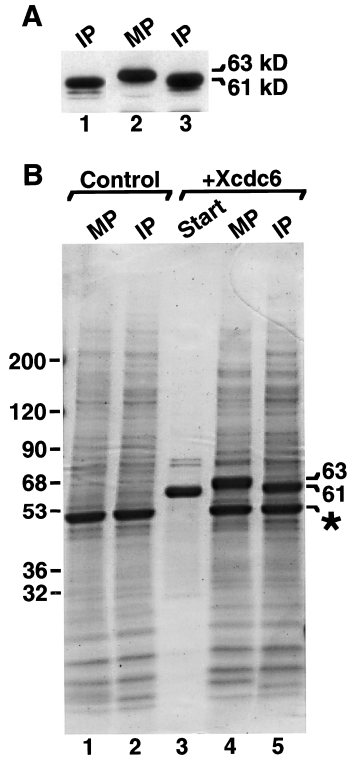

FIGURE 2. *Xenopus* Cdc6 is differentially modified during the cell cycle. (*A*) Endogenous Xcdc6 has a slower relative mobility in M phase (MP, lane *2*) than interphase (IP, lanes *1* and *3*), as detected by immunoblotting with anti-Xcdc6 antibodies. The apparent molecular weight of the M-phase and interphase forms of Xcdc6 are indicated (63 kD and 61 kD, respectively). (*B*) The M-phase modification can be recapitulated with a recombinant hexahistidine-tagged Xcdc6 (starting material, lane *3*) incubated in either an M-phase (MP, lane *4*) or interphase (IP, lane *5*) egg extract. Note: Control preparations of Ni-agarose beads treated with either M-phase (MP) or interphase (IP) extracts showed a prominent *Xenopus* protein of ~51 kD (*asterisk*) which binds Ni-agarose (lanes *1* and *2*), but is nonspecific. Following incubation in egg extracts, samples were recovered on Ni-agarose, washed, and analyzed by SDS-PAGE and Coomassie Blue staining.

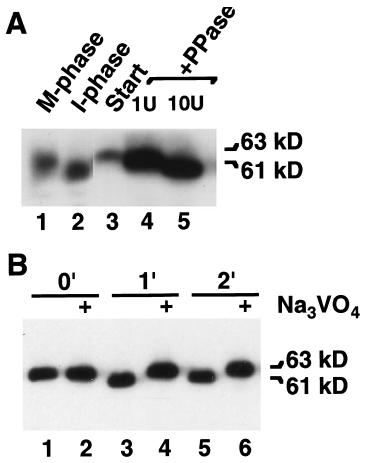

FIGURE 3. Xcdc6 is phosphorylated in a mitotic egg extract. (*A*) M-phase-modified recombinant Xcdc6 was treated with either control buffer (Start, lane *3*) or alkaline phosphatase (1 or 10 units, lanes *4* and *5*, respectively). Control lanes show the migration of endogenous M-phase (lane *1*) and interphase (I-phase, lane *2*) Xcdc6. (*B*) M-phase-modified recombinant Xcdc6 was treated with λ phosphatase for the indicated times in the absence (odd-numbered lanes) or presence (even-numbered lanes) of the phosphatase inhibitor sodium orthovanadate. All samples were processed for immunoblotting with anti-Xcdc6 antibodies.

An obvious explanation for the M-phase modification is that the Xcdc6 protein is phosphorylated in a cell-cycle-dependent manner. To confirm this, we modified recombinant Xcdc6 protein in an M-phase *Xenopus* egg extract and treated it with either alkaline (Fig. 3A) or λ (Fig. 3B) phosphatase. In either case, the electrophoretic mobility of the hyperphosphorylated form was increased upon phosphatase treatment. Significantly, the relative mobility of the phosphatase-treated protein returned to that of the endogenous interphase protein (Fig. 3A). Moreover, this increase in electrophoretic mobility was sensitive to the phosphatase inhibitor vanadate (Fig. 3B). Taken together, these results suggest that the cell-cycle-dependent shift in the molecular weight of Xcdc6 can be attributed entirely to phosphorylation during M phase and that the recombinant protein can be phosphorylated in an M-phase egg extract in a manner consistent with that seen in vivo.

Evaluation of Modification and Utilization of Modified Protein

As a validation step to ensure that a recombinant functional consequence of protein has been modified in a physiologically meaningful manner, it is important to analyze the modification. For example, Hunt and coworkers found that the catalytic subunit of CAK was inactive when isolated from bacteria, but it could be activated by "marinating" it in a *Xenopus* egg extract (Poon et al. 1993). Thus, assessing this modification required comparing the activity of the protein before and after incubation in the extract. Having established that the recombinant protein is modified in a physiologically meaningful manner, one may next define the modification. The detection of a modification depends in large part on the technique used. Detection techniques can range from assays that assess the biochemical activity of a protein to simply performing SDS-PAGE on the protein bound to washed beads. In the above example, subsequent studies revealed that the *Xenopus*-extract-mediated activation of CAK was due to phosphorylation (Poon et al. 1994).

Alternatively, if one is looking for a specific binding partner, components of protein complexes purified by affinity chromatography can be analyzed by separation on SDS-PAGE and subsequent staining. The presence of protein bands that migrate differentially from the recombinant protein of interest would indicate that a protein complex has formed. *Xenopus* crude extracts have

facilitated the identification of many such binding partners using a wide variety of "baits" in the pull-down experiments, including fission yeast $p13^{suc1}$ (Dunphy and Newport 1989), Cdc25 phosphatase (Crenshaw et al. 1998; Kumagai et al. 1998a), CRM1 nuclear export factor (Yang et al. 1998), importin (Yang et al. 1999), Pin1 propyl isomerase (Crenshaw et al. 1998), cyclin B (Yang et al. 1998), and cyclin E (Yang et al. 1999). One can increase the sensitivity for detecting a binding partner by several means. The most common is immunoblotting with an antibody directed against a known (or suspected) interactor. A more general approach involves biotinylating all proteins bound to the bait (e.g., using an Amersham biotinylation module). After transfer to a membrane, the biotinylated proteins can be detected by staining with horseradish peroxidase (HRP)-streptavidin. This approach has been used successfully to identify the *Xenopus* apoptotic regulator, Scythe, using the *Drosophila* reaper protein as bait (Thress et al. 1998). Having identified Scythe by biotinylating the proteins bound to reaper conjugated beads, these researchers expanded the purification scheme to obtain Coomassie Blue-detectable levels of Scythe for microsequencing. This approach underscores another advantage of the *Xenopus* system, in that large amounts (gram) of extract can easily be generated from a modest number of frogs.

Two approaches are generally used when analyzing phosphorylation modifications. First, one can incubate the protein in an extract in the presence of radioactive ATP and perform autoradiography to assess the extent of phosphorylation. Because one is detecting only the phosphorylated protein, this method does not require a shift in electrophoretic mobility for detection. The extract contains millimolar concentrations of endogenous ATP, and therefore this method may not produce a readily detectable product due to the limited incorporation of radioactive label. A second approach, which relies on an electrophoretic mobility shift, makes use of ^{35}S. By incubating ^{35}S-labeled recombinant protein (e.g., translated in a reticulocyte system charged with [^{35}S]methionine) in the extract, one can assess the phosphorylation (shift) over time. For example, this method allowed the rapid mapping of phosphorylated domains of Cdc25 (Kumagai and Dunphy 1992) and fission yeast Wee1 (Tang et al. 1993).

In the case of the Xcdc6 protein, we could detect the M-phase-specific modification (phosphorylation) by SDS-PAGE. To pinpoint which residues were phosphorylated during M phase, we used *m*atrix-*a*ssisted *l*aser *d*esortion/*i*onization *m*ass *s*pectrometry (MALDI-TOF-MS). This technique offers the advantage that one can obtain rapid and accurate molecular weight information on low picomole amounts of peptides present in complex mixtures, such as those that result from a proteolytic digest. Moreover, phosphorylated peptides are readily detected by MALDI-TOF-MS due to a mass shift of 80 D, or multiples of 80 D (the molecular mass of HPO_3), when comparing the phosphorylated and nonphosphorylated species.

MALDI-TOF-MS techniques are covered in more detail in Chapter 12. Briefly, following SDS-PAGE and staining, the mitotic (63 kD) and interphase (61 kD) Xcdc6 protein bands were excised, destained, and dried. Next, the gel slices containing derivatized Xcdc6 were reduced, alkylated, and digested with trypsin, and the resultant peptides were extracted and subjected to MALDI-TOF-MS analysis (Shevchenko et al. 1996). All MALDI-TOF-MS spectra were obtained on a Voyager DE time-of-flight MS (PerSpective Biosystems) operating in linear mode with delayed ion extraction. Data were analyzed using the *P*rotein *A*nalysis *W*orksheet (PAWS) program to match the observed with theoretical masses. We then assigned many of the observed mass values to the anticipated mass values, assuming first unphosphorylated and then monophosphorylated peptides. To ascertain which sites were candidate phosphorylation sites, we needed to establish stringent criteria to test our approach. First, we identified the mass peaks in the peptides derived from M-phase-treated Xcdc6 whose sizes were consistent with being monophosphorylated. Next, we verified that only the phosphorylated form of these peptides was present in M-phase-treated Xcdc6, whereas only the nonphosphorylated form was found in the peptide mix derived from interphase-treated Xcdc6. Only those shifted peaks that satisfied these stringent criteria are shown (Fig. 4). By imposing these stringent criteria, the peptides identified are likely phosphorylated stoichiometrically during mitosis and completely unphosphorylated during interphase.

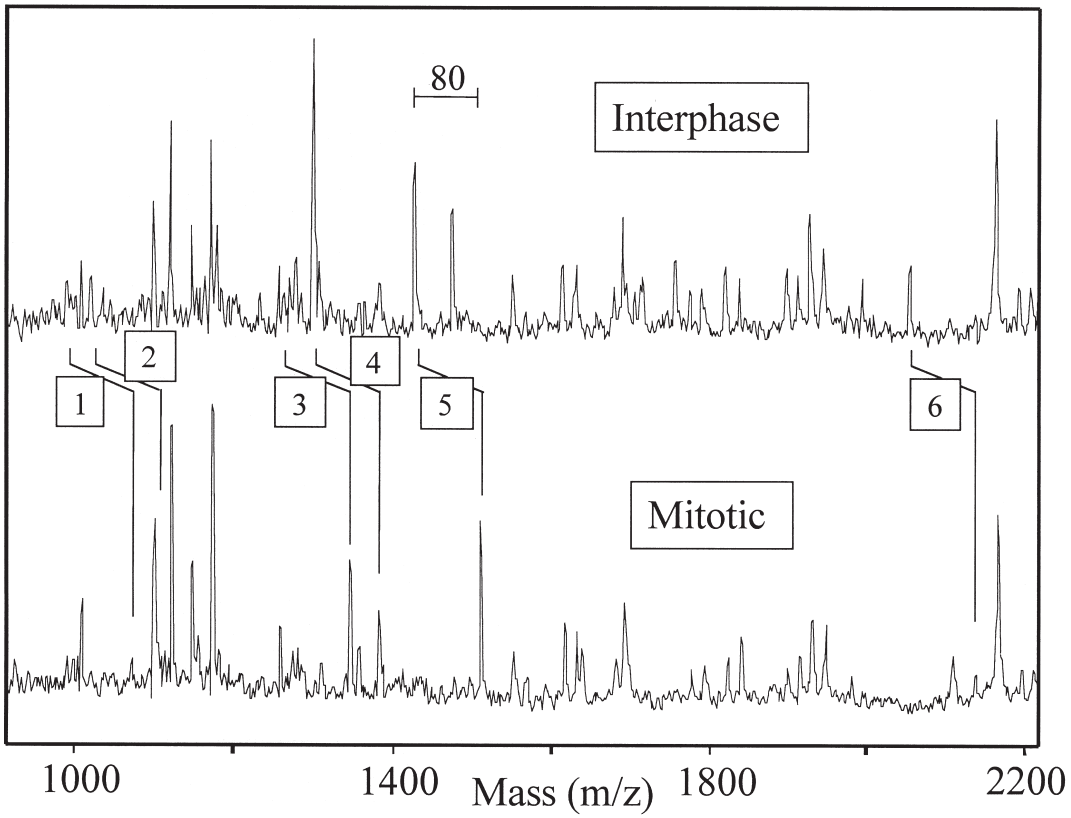

FIGURE 4. MALDI-TOF-MS peptide mass mapping of Xcdc6 phosphorylation sites. Following SDS-PAGE and staining, the M-phase- and interphase-modified Xcdc6 protein bands were excised, destained, and digested with trypsin. The extracted peptides were subjected to MALDI-TOF-MS analysis. We identified the mass peaks in the peptides derived from M-phase-treated Xcdc6 whose sizes were consistent with being monophosphorylated. Next, we verified that only the nonphosphorylated form, but not the phosphorylated form, of these peptides was found in the peptide mix derived from interphase-treated Xcdc6.

Note the presence of several peaks in the mitotic spectrum that have been displaced by 80.0 D (±0.3) relative to those of the interphase spectrum (Fig. 4, peaks 1–6). Because MALDI-TOF-MS analysis is inherently nonquantitative, it is not surprising that the relative peak heights of these interphase and mitotic peptides are not equivalent. Importantly, all of the tryptic peptides shown as M-phase-shifted peaks contain one or more serine or threonine residues (Table 2). In fact, four of the putative phosphorylation sites contain S/P or T/P residues, which are the preferred consensus recognition sites of several mitotic kinases (Table 2, peptides 4a, 4b, 5, and 6). Moreover, several of these identified phosphopeptide sequences were observed in multiple overlapping peptides (Table 2, peptides 2 and 3; 4b and 5), underscoring their strong candidacy as legitimate phosphorylation sites, a point confirmed separately by liquid chromatography electrospray ionization tandem mass spectrometry (LC-MSMS) (S. Seeholzer and T.R.Coleman, in prep.).

In summary, the *Xenopus* egg extract represents a powerful system in which to modify recombinant proteins. Most importantly, the numerous protein modifications revealed with this system are physiologically relevant. As outlined above, these modifications include phosphorylation and association with appropriate protein partners. By using recombinant proteins in such an assay, one can maximize the quantity of modified protein obtained, which can then be analyzed conveniently for biological activity, association with a particular binding partner, or presence of chemical modification. Moreover, in the specific example cited above, it is likely that the *Xenopus* egg extract phosphorylates proteins in a stoichiometric manner, greatly aiding analysis. Finally, the

TABLE 2. Potential Xcdc6 Phosphopeptide Mass Spectrometry Identification

Peptide	Interphase MH^+		Mitotic MH^+		Peptide	
number	observed	calculated	observed	calculated	site	Peptide
1	991.9	992.2	1072.4	1072.2	137–145	NSVGVQLFK
2	1021.7	1022.1	1100.9	1102.1	8–16	SQSSIQFPK
3	1264.0	1265.4	1345.4	1345.4	6–16	SRSQSSIQFPK
4a	1300.9	1300.4	1381.6	1380.4	81–92	KETGQPTTPKGR
4b	1300.9	1301.5	1381.6	1381.5	113–123	LQDPYLLSPVR
5	1429.0	1429.7	1509.7	1509.7	(112)–123 or 113–(124)	(K)LQKPYLLSPVR(K)
6	2056.6	2056.4	2137.0	2136.4	93–111	RLLFDENQAAAATPLSPK

production of egg extracts is well established, and the entire procedure takes only a half-day from production of extract to modification of protein.

Controls

The *Xenopus* egg extract is ideally suited to address cell-cycle-dependent modifications. By comparing the ability of an M-phase versus an interphase extract to modify a recombinant protein, one immediately acquires specificity. That is, the *Xenopus* egg extract system frequently has built-in internal controls for cell cycle modifications, in that parallel incubations of a desired protein can be performed in both M-phase and interphase extracts. For example, Xcdc6 is hyperphosphorylated, as revealed by a shift in relative electrophoretic mobility, when incubated in M-phase, but not interphase, egg extracts (Fig. 2B). Conversely, Cdc25 associates preferentially with 14-3-3 proteins when incubated in an interphase, but not an M-phase, egg extract (Kumagai et al. 1998a). Essential controls include treating an extract with affinity beads alone to assess the background levels of copurifying proteins. For example, in the case of Ni-agarose, we noticed a prominent 51-kD protein that bound in the presence and absence of Xcdc6 (Fig. 2B, see legend). Similarly, if one were reisolating recombinant protein from extracts via antibody immunoprecipitation, it would be critical to perform a parallel immunoprecipitation using a nonspecific control antibody.

Depending on the reagents used, the number and intensity of copurifying proteins that bind to parallel control bead preparations can confound subsequent analysis, but these difficulties can be surmounted by a number of means. A primary consideration is to limit the amount of affinity beads used in any binding reaction. That is, the binding capacity of these resins is typically quite high, and, by using a small amount (<10 μl), one can frequently favor the high-affinity interactions (i.e., tagged recombinant protein) at the expense of lower-affinity nonspecific endogenous protein interactions. One can also pretreat the extract with control beads (0.5 hour, 4°C) before actually incubating with recombinant protein. In this manner, one can clarify the extract of endogenous matrix-binding proteins before performing any modification. Similarly, one can pretreat the affinity beads with a concentrated protein mixture (e.g., fetal bovine serum; 0.5 hour, 4°C) to block nonspecific interactions (Yang et al. 1999). Finally, one can tailor specific beads to individual applications. For example, in the case of Ni-agarose, we found that it was critical to reisolate recombinant Xcdc6 in the presence of imidazole. Most hexahistidine-tagged proteins remain bound to Ni-agarose in concentrations <60 mM imidazole. We found that reisolating Xcdc6 in the presence of 50 mM imidazole significantly reduced the number of endogenous frog proteins that bound to Ni-agarose without diminishing recombinant Xcdc6 binding. Similar approaches have used low concentrations (0–10 mM) of EGTA in the binding buffer to reduce nonspecific binding without chelating nickel from the column (Kumagai and Dunphy 1995). Because hexahistidine binds Ni-agarose in the presence of denaturants (e.g., 4–8 M urea), one can

also wash the Ni-agarose with these agents and decrease nonspecific endogenous frog protein binding. Of course, this treatment can only be used in those cases where one is isolating either a very stable complex or a covalently modified fusion protein. Another elegant approach is to combine several fusion tags on one bait. For example, Claspin was recently identified as an important regulator of the Chk1 kinase using recombinant Chk1 protein fused to both GST and hexahistidine tags (Kumagai and Dunphy 2000). In this two-step approach, Chk1-binding proteins were isolated from interphase extracts on Ni-agarose beads containing bound Chk1-GST-His6 protein. These proteins were then released with imidazole and bound to glutathionine-agarose followed by SDS-PAGE. The use of two independent affinity purification steps reduces nonspecific binding. Unfortunately, no panacea exists, but rather, it is necessary to empirically determine what precautions are necessary to reduce nonspecific interactions for each application.

Protocol 1

Egg Extracts

With this protocol, CSF-arrested M-phase and interphase egg extracts are prepared.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Buffers and Solutions

Chorionic gonadotropin (CG-10, Sigma) <!>
Dissolve 10,000 IU in 10 ml of sterile H_2O; store at 4°C.

CSF-XB
- 100 mM potassium chloride (KCl) <!>
- 0.1 mM calcium chloride ($CaCl_2$) <!>
- 2 mM magnesium chloride ($MgCl_2$) <!>
- 10 mM HEPES (pH 7.7)
- 50 mM sucrose
- 5 mM EGTA (KOH)

Can be made as a 10x stock; store at –20°C; pH at 1x concentration (KOH) before use.

Cycloheximide (Sigma) <!>
Add 10 mg/ml in H_2O; store in small aliquots at –20°C.

Cysteine (2%) (Sigma) titrated to pH 7.8 with NaOH
Make up immediately before use.

Cytochalasin B (Sigma)
Dissolve in DMSO <!> (10 mg/ml); store in small aliquots at –20°C.

Dejelly solution

20x Energy mix
- 150 mM creatine phosphate (Sigma)
- 20 mM ATP
- 2 mM EGTA
- 20 mM $MgCl_2$ <!>

Store in small aliquots at –70°C.

MMR
- 5 mM HEPES (pH 7.8)
- 100 mM NaCl
- 2 mM KCl <!>
- 1 mM $MgCl_2$ <!>
- 2 mM $CaCl_2$ <!>
- 0.1 mM EDTA

Make as a 10x stock, autoclave, and store at room temperature.

Pregnant mare serum gonadotropin (Calbiochem) <!>
Dissolve 5000 IU in 1 ml of sterile H_2O; store at –20°C.

Protease inhibitor cocktail
pepstatin A (Boehringer Mannheim)
chymostatin (Boehringer Mannheim)
leupeptin (Sigma)
Add 10 mg/ml each in DMSO <!>; store in small aliquots at –20°C.

Special Equipment

Beckman TL ultracentrifuge (or equivalent) and swinging bucket rotor (TLS-55)
Although any centrifuge/swinging bucket rotor combination capable of achieving 12,000*g* is acceptable, we find that the capacity (~2 ml) of this rotor is ideal for extract production.
Clinical centrifuge
Microfuge equipped with horizontal or swinging bucket rotor (e.g., Beckman microfuge E) able to hold elongated (8 x 28 mm, 0.5 ml) microfuge tubes (Beckman)

METHOD

1. Female frogs can be obtained from a number of supply houses (e.g., Nasco). *Xenopus* is well suited to life in the laboratory. (For an excellent review of *Xenopus* husbandry, see Wu and Gerhart 1991.) Typically, maintain frogs in tap water with 30 mM NaCl at 16–22°C and feed trout chow (Purina) or *Xenopus* pellets (Nasco).
2. Prime female frogs for ovulation 3–14 days prior to use. Use 75 IU of pregnant mare serum gonadotropin per frog in 0.5 ml of sterile H_2O. Make all injections into the dorsal lymph sac using a 23-gauge needle.
3. At 12–14 hours before extract preparation, induce ovulation by injecting each frog with 800 IU of chorionic gonadotropin in 0.8 ml of sterile H_2O. Following injection, maintain each frog in a separate container containing 5 liters of H_2O with 25 g of NaCl. Typically, we inject 2–3 frogs and use the eggs of the highest quality derived from one frog.
   Unfortunately, high-quality eggs have no rigorous definition but are uniform in appearance and size and have a single white spot (the female pronucleus, or germinal vesicle) in the dark animal pole when viewed from above. Normally, >95% of the eggs from a frog are uniform, and the few substandard eggs are less dense and can be easily removed with a transfer or Pasteur pipette. Large white "snowball" eggs need to be removed. Occasionally a frog will lay "stringy" eggs, which are attached end to end and frequently of poor quality. One frog produces sufficient eggs (20–50 ml with jelly) for 1–2 ml of extract. We always use fresh extracts for our experiments because they only require about 1 hour to make, and they lose activity upon freezing.
4. Wash eggs two to three times in ~200 ml of MMR each time. Remove any debris or substandard eggs during these washes using a transfer pipette.
5. Make up ~200 ml of 2% cysteine (pH 7.8). Decant MMR and add ~50 ml of cysteine solution, swirl, decant, add ~100 ml of fresh cysteine solution, and swirl occasionally. The jelly coat is nearly invisible and roughly doubles the volume of an egg. Eggs are dejellied when they closely pack due to absence of jelly coat. Decant the cysteine solution as soon as eggs are closely packed (<5 minutes). Wash eggs with the final ~50 ml of cysteine solution and decant.
6. Wash eggs two or three times with ~200 ml of MMR each time.
7. Wash eggs four times with ~200 ml of CSF-XB each time.
8. Break off the point of a Pasteur pipette and insert a bulb over this end to produce a blunt pipette. Using this instrument, which minimizes physical damage, transfer the eggs to swing-

ing bucket elongated microfuge tubes. To minimize the transfer of buffer with eggs, tip the beaker of eggs and plunge the blunt pipette into the eggs. Fill the pipette.

9. Remove all CSF-XB from above eggs with a Pasteur pipette.
10. To each tube of eggs, add 0.5 ml of CSF-XB containing 6.6 μl of cytochalasin B stock and 2.2 μl of protease inhibitor stock.
11. Cover the top of the tube with Parafilm and gently invert several times to mix.
12. Transfer the tubes to a homemade apparatus consisting of two microfuge tubes with caps removed, one on top of the other, inside a snap-cap, 14-ml tube (e.g., Falcon 2059). The microfuge tubes form a pedestal on which the egg-containing tube conveniently rests.
13. Pack the eggs into a clinical centrifuge in three stages:

    setting 3 (~1300 rpm), 15 seconds
    setting 5 (~2000 rpm), 60 seconds
    setting 7 (~3000 rpm), 30 seconds

14. Remove all buffer from above the packed eggs with a Pasteur pipette. At this point, the eggs have not lysed.
15. Crush the eggs by centrifugation at 12,000*g* for 10 minutes at 16°C. Crushing the eggs by centrifugation, rather than homogenization, produces a more concentrated cytoplasmic extract and minimizes yolk and pigment contaminants.
16. Following centrifugation, the tubes will contain three layers: top, yellow lipid; middle, cytoplasm; bottom, yolk and packed pigment granules. Using a 21-gauge needle attached to a 3-ml syringe, slowly remove the middle (cytoplasmic) layer. Insert the needle at the interface of the cytoplasm with the yolk layer. Note the "buffy" sublayer at this interface. Because this sublayer contains many of the membranes to make nuclei, it is important to "vacuum" this sublayer with the cytoplasm.
17. Transfer the extracted layer to elongated 0.5-ml tubes and centrifuge at 14,000*g* for 2 minutes at 4°C in a swinging bucket rotor outfitted with adapters. This spin removes any contaminating yolk and lipid.
18. Using a single-edge razor, cut off top of tube just below lipid layer. In this manner, one can remove any contaminating lipid. Using a Pasteur pipette, remove the extract, taking care to avoid any yolk.
19. Weigh the cytoplasm and add energy mix (1/19 volume of 20× stock). This is the cytostatic factor (CSF)-arrested or M-phase extract.
20. Add cycloheximide (100 μg/ml) to M-phase extract. Leave on ice until ready to use (up to 6 hours).
21. Prepare the interphase extract (0.2–0.5 ml, depending on number of assays) by making 0.4 mM in $CaCl_2$ and incubating for 15 minutes at 23°C.

Protocol 2

Modification, Reisolation, and Evaluation

The recombinant protein is modified by incubation in the *Xenopus* egg extract. After binding to the matrix, the recombinant protein is isolated by centrifugation, washed, and analyzed.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Buffers and Solutions

Gel destain I
- 50% ethanol <!>
- 7% HOAc

Gel destain II
- 10% ethanol
- 5% HOAc

Gel stain
- 50% methanol <!>
- 5% HOAc
- 2.5% Coomassie Blue R250

Gel storage buffer
- 3% glycerol
- 5% HOAc

HBS
- 10 mM HEPES (pH 7.4)
- 150 mM NaCl

Chromatography Equipment

Antibody–protein A agarose (Sigma)

In our hands, we see lower nonspecific binding when we use freshly hydrated and washed protein A agarose beads.

Glutathione beads (Pharmacia)

Ni-agarose beads

We make Ni-agarose using iminodiacetic acid-agarose (Sigma). The resin is washed with H_2O in a sintered glass funnel and then incubated with 100 mM $NiCl_2$ for 15 minutes, followed by washing with H_2O and PBS with 0.02% sodium azide <!>.

Gels

Discontinuous SDS-PAGE gradient gel (Bio-Rad)

When performing mass spectrometry analysis, precast 4–15% gradient minigels offer two major advantages over freshly poured gels. First, they have minimal unpolymerized acrylamide, which can covalently modify cysteine residues and complicate any MS data. Second, minigels are preferable to full-sized gels owing to the reduced acrylamide:protein ratio within a protein band.

METHOD

1. Prepare and purify recombinant fusion protein of interest (see Chapter 5).
2. Prepare *Xenopus* CSF-arrested "M-phase" and interphase egg extract (see Egg Extracts protocol, p. 366).
3. Incubate recombinant protein in M-phase or interphase egg extract (typically 20 μl of protein in 180 μl of extract) for 30 minutes at 23°C.
4. Dilute egg extracts with 5 volumes of HBS.
5. Centrifuge at full speed (~3000 rpm) in clinical centrifuge for 2 minutes.
6. Bind recombinant protein to affinity beads (5–10 μl) for 1 hour at 4°C with rotation.

   In the case of hexahistidine-tagged recombinant protein, we found the addition of imidazole (<50 mM) considerably reduced nonspecific frog proteins from binding to the Ni-agarose (see text).
7. Wash the bound protein mixture by resuspending in 400 μl of HBS.
8. Centrifuge at full speed in clinical centrifuge for 15 seconds.
9. Repeat this wash three more times.
10. Remove as much liquid as possible, taking care to leave the bead pellet intact.
11. Add SDS loading buffer (15 μl) and boil for 4 minutes.
12. Load the sample onto a discontinuous SDS-PAGE gradient gel.
13. Stain with freshly prepared Coomassie Blue R250.

    We found that using freshly prepared stain was critical for MS analysis because it assured a more complete destain. Similarly, using a stain with high dye content (e.g., Bio-Rad) improved the subsequent MS analysis.
14. Destain with gel destain I for 1 hour at room temperature.
15. Decant and repeat step 14.
16. Destain with gel destain II overnight at room temperature. Store in gel storage buffer.

Protocol 3

Mass Spectrometry

Processing of samples for mass spectrometry analysis varies greatly depending on the individual running the machine. For a review of methods, see Chapter 12.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Buffers and Solutions

Acetonitrile (Burdick and Jackson) <!>
Alkylating buffer
  50 mM iodoacetamide <!>
  50 mM ammonium bicarbonate <!>
Ammonium bicarbonate (100 mM) <!>
Destain (50% methanol <!>, 5% formic acid <!>)
Extraction solution
  5% formic acid <!>
Reducing buffer
  10 mM dithiothreitol (DTT) <!>
  50 mM ammonium bicarbonate <!>

Biological Molecules

Trypsin solution
  20 μg/ml sequencing grade modified porcine trypsin (Promega) in 25 mM ammonium bicarbonate <!>.

Special Equipment

Mass spectrometer
Microcentrifuge
SpeedVac

METHODS

1. Closely excise the bands of interest from the gel and divide into smaller pieces (~0.5 mm^3).
2. Destain bands in 0.5 ml of destain overnight (or longer) at room temperature.
3. Discard destain and replace with 0.5 ml of destain for 2–3 hours at room temperature.

4. Discard destain and dehydrate gel slices in 0.2 ml of acetonitrile for about 5 minutes. Discard acetonitrile.
5. Repeat step 4.
6. Dehydrate the gel piece in a SpeedVac until dry.
7. Incubate the gel pieces in 100 µl of reducing buffer for 30 minutes at room temperature.
8. Centrifuge briefly to pellet the gel pieces and remove the DTT solution.
9. Incubate the gel pieces in 200 µl of alkylation buffer for 30 minutes at room temperature.
10. Centrifuge briefly to pellet the gel pieces and remove iodoacetamide solution.
11. Dehydrate gel slices in 0.2 ml of acetonitrile as in steps 4 and 5.
12. Rehydrate gel pieces in 0.2 ml of 50 mM ammonium bicarbonate.
13. Centrifuge briefly to pellet the gel pieces and remove ammonium bicarbonate.
14. Dehydrate with acetonitrile as above.
15. Dry the gel pieces in a SpeedVac.
16. Freshly prepare 20 µg of Promega trypsin in 1 ml of ice-cold 25 mM ammonium bicarbonate.
17. Add a minimal volume of trypsin (10–30 µl, depending on gel band volume) to rehydrate the dried gel pieces. Keep on ice during rehydration.
18. Centrifuge briefly to pellet the gel pieces and remove any excess trypsin solution and add a small volume of ammonium bicarbonate solution to assure complete coverage of the gel pieces.
19. Incubate overnight at 30–37°C.
20. Extract peptides by adding 1 volume of extraction solution. Collect the supernatant in a separate siliconized tube.
21. Repeat the extraction with a second volume of extraction solution. Combine the supernatants. Recovery of hydrophobic peptides may be improved by including 50% methanol or isopropanol in this second extraction.

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