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Research paper

Purification and biochemical analysis of catalytically active human cdc25C dual specificity phosphatase

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ABSTRACT

We describe a reliable and efficient method for the purification of catalytically active and mutant inactive full-length forms of the human dual specificity phosphatase cdc25C from bacteria. The protocol involves isolating insoluble cdc25C protein in inclusion bodies, solubilization in guanidine HCL, and renaturation through rapid dilution into low salt buffer. After binding renatured proteins to an ion exchange resin, cdc25C elutes in two peaks at 350 and 450 mM NaCl. Analysis by gel exclusion chromatography and enzymatic assays reveals the highest phosphatase activity is associated with the 350 mM NaCl with little or no activity present in the 450 mM peak. Furthermore, active cdc25C has a native molecular mass of 220 kDa consistent with a potential tetrameric complex of the 55-kDa cdc25C protein. Assaying phosphatase activity against artificial substrates pNPP and 3-OMFP reveals a 220 kDa form of the phosphatase is active in a non-phosphorylated state. The protein effectively activates cdk1/cyclin B prokinase complexes in vitro in the absence of cdk1 kinase activity in an orthovanadate sensitive manner but is inactivated by A-kinase phosphorylation. In vitro phosphorylation of purified cdc25C by cdk1/cyclin B1, cdk2/cyclin A2 and cdk2/cyclin E shows that distinct TP/SP mitotic phosphorylation sites on cdc25C are differentially phosphorylated by these 3 cdk/cyclin complexes associated with different levels of cdc25C activation. Finally, we show that endogenous native cdc25C from human cells is present in high molecular weight complexes with other proteins and resolves mostly above 200-kDa. These data show that untagged cdc25C can be purified with a simple protocol as an active dual specificity phosphatase with a native molecular mass consistent with a homo-tetrameric configuration.

1. Introduction

Dephosphorylation of both threonine 14 and tyrosine 15, prerequisite for cdk1 activation [1–3], is brought about by dual specificity phosphatases from the cdc25 family of proteins ([4], reviewed in Refs. [5–8]). In human cells, three classes of cdc25 dual specificity phosphatases have been described, cdc25A, B and C. Each class is subdivided again as a result of alternative splicing such that to date at least 2 isoforms of cdc25A, 3 isoforms of cdc25B and 5 isoforms of cdc25C have been described (cf. [5,7] and references therein). These three phosphatase classes contain a highly conserved C-terminal catalytic domain but diverge significantly in their amino terminal regions [9–12]. Initial analyses suggested that cdc25A played a role in G1/S-phase while cdc25B and C played roles at mitosis ([13,14], reviewed in Refs. [3,7]). Recent data have implied that this separation of function is incomplete since cdc25A and B also appear to be essential for mitotic entry and we have recently shown that cdc25C plays a role in S-phase [15,16], while data from mice models and RNA interference has shown that at least partial functional redundancy exists (reviewed in Refs. [17–19]).

While cdc25C is one of the unique protein phosphatases to have been identified via its specific substrate, cdk1, relatively little is known of its biochemical properties. A number of reports have described that its phosphatase activity is modulated in a manner dependent on its phosphorylation state [20–24], and it can be isomerized in a phosphorylation dependent manner again affecting its activity [25,26]. Phosphorylation of S216 in human cdc25 creates a binding site for the 14.3.3 chaperon family of proteins [27], which in turn modulates the cytolocalization of cdc25C [28] in coordination with both nuclear export and nuclear import mechanisms [29] and

Abbreviations: 3-OMFP, 3-*O*-methylfluorescein phosphate; BME, β-mercaptoethanol; C377S, human cdc25C mutated cysteine 377 serine; cdk, cyclin dependent kinase; DTT, dithiothreitol; IPTG, isopropyl β-D-1-thiogalactopyranoside; orthovanadate, sodium orthovanadate; (sodium tetraoxovanadate (V)), Na_3VO_4; A-kinase, cAMP dependent protein kinase; pNPP, para-nitrophenyl phosphate; Plk1, polo like kinase 1; WT, wild type; MAPK, mitogen activated kinase; ERK, extracellular-signal-regulated kinases; BRCA1, breast cancer 1.

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the differential protein–protein interactions of cdc25C including association with protein phosphatases type-1 [30] and type-2A ([31,32] and references therein). The principal difficulty in obtaining robust biochemical data for cdc25C relates essentially to the problems in purifying the full-length protein. From, mammalian and amphibian sources or insect cells, the protein associates tightly with 14.3.3 and via this interaction, cdc25C co-purifies and co-immunoprecipitates with a wide variety of proteins including cdk/cyclin complexes making biochemical analysis difficult [30,31,33]. In bacteria, the protein is insoluble and when re-solubilized by dialysis against decreasing concentrations of guanidine hydrochloride frequently re-precipitates (CF, NL unpublished observations). Here, we present biochemical data to show that GST-tagged cdc25C, although used in a number of publications [14,20–22,24,30,31,33], is mostly a very high molecular weight complex while displaying some in vitro phosphatase activity. We describe a method to isolate soluble cdc25C from bacteria by rapid dilution that produces stable and active cdc25C. Partial biochemical characterization shows the protein to have high activity when measured against pNPP or 3-OMFP and a native molecular mass of 220 kDa in the presence or absence of reducing agents, consistent with a potential tetrameric conformation. Furthermore, purified cdc25C is fully functional in activating inactive cdk1/cyclin B1 pro-kinase but is inactivated by phosphorylation by A-kinase. Purified cdc25C is differentially phosphorylated and activated by cdk1 and cdk2 cyclin complexes. Lastly, we provide data confirming that in human cell extracts endogenous native cdc25C is present in high molecular weight complexes and physically associated with Plk1 but not with cyclin B1 nor cdk1. In native mammalian cell extracts, most cdc25C resolves above 200-kDa and is not present in a pool at 60 kDa.

2. Results

2.1. Renaturation of full-length active human cdc25C by rapid dilution of its insoluble form in inclusion bodies

The dual specificity phosphatase cdc25C is subject to multiple levels of modulation through post-translational modification and isomerization. A number of reports have previously described the use of GST-tagged cdc25C [14,20–22,24,29,30] or tagged with 6-His for subsequent purification using metal-affinity resins such as Ni-NTA or Talon [34]. Our method involved isolation of insoluble cdc25C in bacterial inclusion bodies through repetitive rounds of extraction in high salt, solubilization in 6 M guanidine hydrochloride, rapid re-solubilization/renaturation of the protein by dilution in low salt buffer, purification and concentration by ion exchange chromatography as outlined in Fig. 1A. As shown in Fig. 1B, at 37 °C, wild type (Wt) cdc25C is expressed robustly after IPTG induction as determined by 1D PAGE. The protein is essentially insoluble since it is detectable only in the bacterial pellet after sonication and treatment with lysozyme (Fig. 1C, lanes S and P1). We exploited this to purify the protein by pelleting and washing inclusion bodies at increasing ionic strength up to 1.5 M NaCl (Fig. 1C, lanes P2–P4). After solubilization of the inclusion bodies from pellet 4 in 6 M guanidine hydrochloride, the protein was renatured by rapid dilution 1:100 in 100 mM Tris–HCl pH 7.5, 10 mM DTT. The solubilized proteins were concentrated and further purified by anion exchange chromatography on Sepharose-mono Q. Analysis of the column elution profile by 1D-SDS-PAGE (Fig. 1D), shows that most of the renatured proteins bind to the column under these conditions with relatively little protein detectable in the flow through fractions (Ft). Application of an increasing salt gradient (graphically outlined above Fig. 1D), is accompanied by three peaks as detected by UV monitoring (marked 1–3 above Fig. 1D) and proteins with an apparent molecular mass around 60 kDa (the calculated mass for cdc25C when analyzed in SDS-denatured conditions), are eluted in fractions under peaks 2 and 3 as shown by coomassie blue staining (Fig. 1D, arrow represents the relative mobility of the 60 kDa marker). Fractions (500 μl) corresponding to the maximum UV absorbance for peaks 1, 2 and 3 eluting at 250, 350 and 450 mM NaCl were further analyzed by western blot for cdc25C and phosphatase activity. When compared to the material loaded (Fig. 1E, lane L) only peaks 2 and 3 contain cdc25C when probed by immunoblotting. Similar results were also obtained for the C377S inactive mutants, although the majority of cdc25C–C377S protein elutes at 450 mM NaCl (data not shown). In vitro phosphatase assays against pNPP (Fig. 1E, lower panel) reveals the major peak of phosphatase activity is associated with the proteins eluting at 350 mM NaCl (peak 2), a small fraction associating with the 450 mM peak (peak 3) and no activity associated with the UV peak 1 at 250 mM (Fig. 1E, lower panel).

We next examined the native molecular mass of the cdc25C-containing fractions to determine if the protein was active as a monomer. Native cdc25C proteins from the two Mono-Q peaks which displayed pNPP activity were subject to molecular sizing on a Superose 6HR 10/30 column in the presence of 150 mM NaCl. As shown in Fig. 1F, proteins present in the 350 mM peak (peak 2) elute with a molecular mass around 220 kDa which is associated with the majority of the pNPP activity. A small proportion of this fraction elutes with high molecular weight complexes in the void volume of the gel-filtration resin (see below). In contrast, cdc25C present in the 450 mM fraction (peak 3) contains little or no pNPP activity and while there is some enrichment of the protein at around 220 kDa (Fig. 1G), cdc25C proteins elute across the entire molecular mass range of the column (data not shown). This would suggest that the 450 mM peak comprises a combination of mostly inactive and poorly refolded cdc25C. The apparent molecular mass of renatured, active cdc25C is consistent with that anticipated for a tetrameric complex of cdc25C. From these data it is clear that after the renaturation protocol used here, cdc25C refolds in two forms, a major peak of inactive protein in the form of both high molecular weight complexes and denatured proteins and a peak comprising about 25% of the renatured protein as an active tetrameric complex. From a typical experiment this protocol yields 0.9–12 ng/ml bacterial culture of active cdc25C and the specific activity ranges against pNPP are 45–50 nmol min^{-1} mg^{-1} for peak 2 and 4–5 nmol min^{-1} mg^{-1} for proteins in peak 3. Similar resolution of the complexes was also obtained with the C377S protein suggesting that the formation of the tetrameric complex is not dependent on phosphatase activity (data not shown).

2.2. Active cdc25C is a tetramer while GST–cdc25C fusion proteins are high molecular mass oligomers

To examine if other forms of bacterially expressed cdc25C have a similar mass, we cloned the ORF of human cdc25C in frame with an N-terminal GST tag and expressed the protein in bacteria. As shown in Fig. S1, an 80-kDa protein (the expected mass for the GST–cdc25C fusion) is specifically over-expressed when bacteria are induced with IPTG (Fig. S1, panel A) and immunoreactive with anti-cdc25C. After affinity chromatography on glutathione Sepharose and elution with 10 mM glutathione, the peak fraction (by protein concentration) was subject to gel filtration chromatography on a Superose 6HR 10/30 FPLC column and the resulting fractions analyzed for cdc25C by western blot and for pNPP activity. When the elution profiles and activity curves for Wt-untagged cdc25C and GST-tagged cdc25C were compared (Fig. 2), we observed that the bulk of the GST-tagged protein eluted with a molecular mass between 30 and 40 MDa (Fig. 2, panel B) in the column void volume, whereas only a clearly separated fraction of the untagged refolded protein resolves in the column void (Fig. 2, panel A). Comparing the phosphatase activity profiles reveals