A Dictyostelium homologue of the metazoan Cbl proteins regulates STAT signalling

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Summary
Cbl proteins downregulate metazoan signalling pathways by ubiquitylating receptor tyrosine kinases, thereby targeting them for degradation. They contain a phosphotyrosine-binding region, comprising an EF-hand and an SH2 domain, linked to an E3 ubiquitin-ligase domain. CblA, a Dictyostelium homologue of the Cbl proteins, contains all three conserved domains. In a cblA– strain early development occurs normally but migrating CblA slugs frequently fragment and the basal disc of the culminants that are formed are absent or much reduced. These are characteristic features of mutants in signalling by DIF-1, the low-molecular-mass prestalk and stalk cell inducer. Tyrosine phosphorylation of STATc is induced by DIF-1 but in the cblA– strain this response is attenuated relative to parental cells. We present evidence that CblA fulfils this function, as a positive regulator of STATc tyrosine phosphorylation, by downregulating PTP3, the protein tyrosine phosphatase responsible for dephosphorylating STATc. Thus Cbl proteins have an ancient origin but, whereas metazoan Cbl proteins regulate tyrosine kinases, the Dictyostelium Cbl regulates via a tyrosine phosphatase.

Key words: Cbl, SH2 domain, Dictyostelium, STAT, DIF, PTP

Introduction
Binding of extracellular factors to receptor tyrosine kinases (RTKs) triggers signal transduction pathways that are used in animals to regulate processes such as cell proliferation, differentiation, motility and survival. In order to guarantee the temporal and spatial control of RTK-mediated signalling, RTK activity has to be tightly regulated. One such regulatory mechanism, which serves to attenuate RTK signalling, is the removal of receptor complexes from the membrane by endocytosis and subsequent degradation. Cbl (Casitas B-lineage lymphoma) proteins play a major role in these processes (Dikic and Giordano, 2003; Dikic and Schmidt, 2007).

Cbl proteins contain a phosphotyrosine binding domain [also known as a tyrosine kinase binding (TKB) domain] at their N terminus, which comprises a four helix-bundle, a Ca2+ binding EF-hand and a highly variant SH2 domain (Meng et al., 1999; Thien and Langdon, 2001). The TKB domain is connected, via a conserved linker region, to a C4H4 type RING finger. The RING finger has E3 ligase activity (Joazeiro et al., 1999; Levkovitz et al., 1999) and directs the mono-ubiquitylation of activated RTKs. This promotes RTK endocytosis and endosomal sorting of RTKs for lysosomal degradation (de Melker et al., 2001; Dikic and Giordano, 2003; Levkovitz et al., 1998; Longva et al., 2002). In addition to their role in RTK signal termination, Cbl proteins are also involved in the mediation of positive RTK signalling to downstream targets by acting as multi-domain adaptors (Swaminathan and Tsygankov, 2006).

Cbl proteins in which the RING finger and/or linker region have been mutated or deleted, which results in altered Cbl function, are oncogenic in animal models (Andoniou et al., 1994; Bonita et al., 1997; Langdon et al., 1989; Thien and Langdon, 1997b; Thien and Langdon, 2001). It is believed that they act as dominant-negatives by competing with the wild-type protein for binding sites (Bonita et al., 1997; Thien and Langdon, 1997a). Recently the first human oncogenic CBL form has been identified, in an acute myeloid leukaemia patient, underlining the attractiveness of CBL proteins as potential therapeutic targets (Sargin et al., 2007). There are Cbl orthologues in C. elegans (Yoon et al., 1995) and in D. melanogaster (Meisner et al., 1997), where they play important roles in developmental signalling. We have identified a Cbl-like protein in Dictyostelium, a facultative multicellular organism that possesses a small but diverse set of SH2 domain proteins.

Dictyostelium cells exist during vegetative growth as solitary amoebae but when their bacterial food source is exhausted they aggregate together in response to pulses of cAMP emitted from the centre of the aggregation territory. Some of the cells within the aggregate differentiate into prespore cells and synthesise and secrete a chlorinated hexaphenone, DIF-1 (henceforth termed DIF), that directs uncommitted cells in the population to become prestalk cells (Kay and Thompson, 2001). Addition of DIF to cells directs nuclear translocation of a GATA and two bZIP transcription factors (Huang et al., 2006; Keller and Thompson, 2008; Thompson et al., 2004; Zhukovskaya et al., 2006). DIF also induces tyrosine phosphorylation of a signal transducer and activator of transcription (STAT) protein, STATc, at a site near its C terminus (Fukuzawa et al., 2001). This causes STATc to homodimerise, via reciprocal SH2 domain-tyrosinephosphotyrosine interactions, and to accumulate in the nucleus. The tyrosine kinase that phosphorylates STATc is unknown but the rise in tyrosine phosphorylation of STATc is, in part at least, caused by DIF-induced inhibition of PTP3, the protein tyrosine phosphatase that dephosphorylates STATc (Araki et al., 2008). Understanding the regulation of PTP3 activity is therefore key to understanding STATc activation and we present evidence that the Dictyostelium Cbl homologue, CblA, upregulates STATc tyrosine phosphorylation via an inhibitory effect on PTP3 accumulation.

Results
CblA is a homologue of the metazoan Cbl proteins
CblA contains long tracts of simple repeat sequence, a feature common to many Dictyostelium proteins (Fig. 1). Scattered amongst...
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Three domains that score highly in a BLAST search: an EF-hand, an SH2 domain and a RING finger (E3 ubiquitin ligase) domain. The RING finger contains the conserved cysteine and histidine residues that constitute its structural framework, and most of the other conserved residues are also present. The BLAST program designates the EF-hand as a Cbl type EF-hand sequence. Also, the highly conserved SH2 domain sequence GTF (overlined in Fig. 1) is substituted by the sequence GSY in the metazoan Cbl proteins and by the related sequence GYY in CblA. Metazoan Cbl proteins contain a four-helix bundle near their N termini. Hence CblA was analysed using JPRED (Cuff and Barton, 2000): a program that identifies regions of secondary structure based upon neural network analysis. This predicts four alpha helical regions near the N terminus (Fig. 1). Most Cbl proteins contain a proline-rich region near the N terminus but this is absent from CblA. However, the shorter of the two Drosophila Cbl splice variants also lacks a proline-rich region (Thien and Langdon, 2001). It is therefore the metazoan Cbl protein that most closely resembles CblA. Thus all the universally conserved Cbl family features are present within CblA and they lie in the same order along the protein as in metazoan Cbl proteins.

**Analysis of cblA expression and intracellular localisation**

RT-PCR shows that the cblA mRNA is present at a relatively low level in growing cells and accumulates during the first few hours of development, to reach a plateau during aggregation (Fig. 2A). An antibody was generated and purified using a C terminus proximal CblA peptide sequence. Although the antibody cannot be employed for immunostaining or immunoprecipitation it is usable in western transfer. At least up to the slug stage, the latest stage analysed, the CblA protein accumulation pattern is quite similar to the RNA accumulation pattern (Fig. 2B).

The antibody was used in western transfer of crude cellular subfractions to determine the intracellular localisation of the CblA protein (Fig. 3). The great majority of the protein is in the cytosolic fraction. A small proportion appears to be nuclear but this may reflect cytoplasmic contamination of the nuclei.

**Developmental phenotype of cblA− strains**

A cblA-null (cblA−) strain was generated by homologous recombination. The disruption construct was created using an in vitro transposition method (Abe et al., 2003) and an insertion point

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**Fig. 1.** Comparison of CblA to metazoan Cbl protein family members. The top part of the figure is the output from a BLAST search, showing the predicted RING finger, EF-hand and SH2 domains. It also shows the positions of simple repeat sequence (blue) and the positions of four alpha helical segments (black arrowheads) and their length, as predicted by the program JPRED (Cuff and Barton, 2000). The lower three panels show alignments of CblA with Cbl family EF-hand domains, with a Cbl type and two orthodox SH2 domains and with Cbl family RING fingers. The red lines over the SH2 domains indicate the universally conserved R residue and the divergent adjacent residues that initially confused identification of the Cbl SH2 domain (Meng et al., 1999).

**Fig. 2.** Developmental time course of CblA expression. Cells were allowed to develop for the indicated times (in hours) and stages (s, slug; MC, mid-culminants; C, culminants) on filters. The times in B are equivalent to the following stages: 6 hours, streaming; 10 hours, tight aggregates; 12 hours, tipped aggregates and first fingers; and 14 hours, slugs. Cells were analysed for (A) CblA RNA by RT-PCR and (B) CblA protein by western transfer. The loading control in A is Ig7, a constitutively expressed RNA. Loading in the western transfer was monitored by total protein staining and was approximately equal in all lanes.
was selected in which the drug resistance cassette interrupts the EF-hand. Homologous recombinants, which occurred at a frequency of approximately 10%, were identified by PCR and confirmed by Southern transfer (data not shown). Expression of cblA in the putative null strains was investigated by RT-PCR and western transfer. Both analysis methods confirm that, for the selected strains, disruption is complete (Fig. 4A,B).

cblA cells grow at a normal rate and develop to the first finger stage correctly. The migratory cblA slugs that are formed are, however, very variable in size and often fragment along their length (Fig. 5A). Also, cblA culminants produced when migrating slugs enter terminal differentiation have either a much reduced or no basal disc. This is apparent from phase-contrast images (Fig. 5A) and is confirmed by the absence and/or reduction of cells expressing ecmB:lacZ (a stalk and basal disc marker) in the position normally occupied by the basal disc (Fig. 5B). Presumably as a consequence of this defect, the fruiting bodies that are formed frequently collapse on to the substratum to form a tangled mass. These two phenotypic features, slugs that fragment along their length and a much-reduced basal disc, are also characteristic of mutants in DIF biosynthesis and DIF signalling (Keller and Thompson, 2008; Saito et al., 2008).

(NB For the cblA clone used throughout this study, strain JGW-JL-1, there is an apparent difference with the previous studies; the basal disc formation defect is seen only if slugs are allowed to migrate away from the site of aggregation. However, there is clonal variation and a second disruptant strain, JGW-JL-2 behaves in the same way as the published mutants, i.e. there is little or no outer basal disc in culminants whether or not they derive from newly formed or migratory slugs.)

DIF-inducible STATc tyrosine phosphorylation is reduced in the cblA- strain

We next analysed the CblA-dependence of prospective target proteins, basing our choice of candidates on the implied link to DIF signalling in the cblA- strain. When assayed in a monolayer system dimB-null strains are profoundly defective in the DIF-mediated induction of ecmA gene expression (Huang et al., 2006; Zhukovskaya et al., 2006). By contrast, we find that DIF induction of ecmA gene expression occurs normally in the cblA- strain and DIF induces the nuclear accumulation of DimB (Fig. 6A,B). Having failed to detect a defect in the DIF induction of ecmA expression, we analysed the other molecularly characterised DIF signalling pathway: the STATc activation pathway.

The activation of STATc by DIF was assayed in cells starved for 4 hours then separated into cytosolic, nuclear (Strmecki et al., 2007) and crude membrane fractions (Theibert and Devreotes, 1986). Aliquots of the fractions were analysed by western transfer using the CblA antibody.

70% reduction in the inducible tyrosine phosphorylation of STATc

We did not discern a decrease in the amount of nuclear-localised STATc in the cblA- (data not shown) but this was only monitored visually; we did not attempt to quantify fluorescence intensity. Also, it is of course possible that the correlation between tyrosine phosphorylation and nuclear accumulation is non-linear.

Stress-inducible STATc tyrosine phosphorylation is normal in the cblA- strain

STATc is activated by hyper-osmotic stress with similar initial kinetics as with DIF but to a higher peak level and for a much longer period of time (Araki et al., 2003). It was therefore of interest to determine whether CblA also regulates the stress response. Cells, at 4 hours of development were either left untreated or incubated with 200 mM sorbitol for 15 minutes. Sorbitol treatment induced an equivalent level of STATc tyrosine phosphorylation in control and cblA- cells (Fig. 7C). Thus CblA does not regulate the stress-responsiveness of STATc.

CblA regulates STATc tyrosine phosphorylation via an effect on PTP3

The tyrosine phosphorylation status of STATc is determined by the balance of activity between an unidentified tyrosine kinase and the PTP3 tyrosine phosphatase (Araki et al., 2008). Thus one potential
explanation for the reduced DIF-induced tyrosine phosphorylation of STATc in the cblA– strain is that CblA acts as a negative regulator of PTP3. This possibility was investigated using the cblA– strain. The concentration of PTP3 was assessed using an antibody directed against an internal PTP3 peptide. When used in western transfer of either PTP3:myc or PTP3ΔCS:myc transformant lysates, the antibody detects bands of the expected sizes of the myc-tagged proteins as well as a band of 40 kDa (Fig. 8A). The higher mobility bands are detected when the blot is re-probed with a myc antibody but the 40 kDa protein is not (data not shown).

PTP3 seems to be highly susceptible to proteolytic degradation and the 40 kDa species is we believe an internal fragment derived from PTP3. The evidence for this comes from comparing the amount of 40 kDa protein in control, untransformed cells and in cells over-expressing PTP3:myc (Fig. 8B). Over-expressing clones have a much higher level of 40 kDa protein than the non-transformed parental strain; averaging three independent experiments there was an approximate 20-fold excess of 40 kDa protein in PTP3:myc-expressing cells. Indeed, in non-transformed cells, there is no detectable full-length PTP3 protein, presumably indicating that, in the case of the endogenous PTP3 protein, there is complete proteolysis. This conclusion is supported by similar observations using an antibody raised against recombinant PTP3 (Gamper et al., 1996) and our unpublished studies using two internal peptides as immunogens in which all three antibodies failed to detect full-length endogenous PTP3.

In the cblA– strain the 40 kDa PTP3-derived fragment is present at a much higher relative level and this holds true whether or not DIF is present (Fig. 8C). In four such independent western transfer analyses there was on average eight times more 40 kDa protein in cblA– cells than in the control cells. Given the paradigmatic mechanism of action of Cbl proteins, this result suggested that CblA may constitutively downregulate PTP3 by a direct interaction. In order to determine whether there is a physical linkage between CblA and PTP3, their differently tagged forms were assayed for co-immunoprecipitation. No interaction was detectable (data not shown). This negative result is, however, difficult to interpret as it could simply indicate that binding of CblA to PTP3 leads to very rapid degradation of PTP3 via the proteasome. Unfortunately, concentrations as high as 100 μM of the commonly used proteasome inhibitor clasto-lactacystin β-lactone had no discernible effect on PTP3. This is in agreement with a study by Mohanty et al. (Mohanty et al., 2001), who also tested two other commonly used proteasome inhibitors: MG132 and MG262. They concluded that the lack of effect was due to restricted permeability of the drugs. This technical impasse precluded further pharmacological investigation of this issue. We were also unable to use a genetic approach to the problem, by expressing intact and RING finger deleted forms of CblA in cblA-null cells, because over-expression of full-length CblA proved deleterious to the cells.

**Discussion**

The importance of SH2 domain-mediated interactions in the metazoa is attested by the presence of 110 SH2 domains in the mouse and human ‘SH2omes’ (Liu et al., 2006). Dictyostelium presents a much simpler situation with just 13 SH2 domains (Eichinger et al., 2005). The functions of most of the 13 are known or can be inferred and we have now shown that one of them, CblA, has a domain organisation that is similar to metazoan Cbl proteins. Furthermore, the EF-hand and the SH2 domain of CblA each have
higher relative sequence homology to their metazoan Cbl counterparts than to the EF-hands and SH2 domains of other, non-Cbl proteins. Cbl proteins are multi-functional adaptors but we have not been able to demonstrate direct binding of CblA to a specific substrate. We have, however, shown that CblA functions as a negative regulator of the concentration of PTP3.

Regulation of multicellular development by CblA

Overt phenotypes caused by the cblA mutation first become manifest at the slug stage. The slugs fragment along their length and basal disc formation is defective. These are also characteristic features of mutants that are involved in DIF signalling but, unlike the dimA-, dimB- and mybE-null mutants (Fukuzawa et al., 2006; Huang et al., 2006; Thompson et al., 2004; Zhukovskaya et al., 2006) ecmA remains DIF inducible in the cblA strain. Thus it is possible to genetically uncouple the morphological phenotypes, slug fragmentation and aberrant basal disc formation, from the defective ecmA induction phenotype.

In a cblA strain DIF induces STATc tyrosine phosphorylation with normal kinetics but the magnitude of the response is approximately three times lower than in control cells. The proto-oncogene Cbl has been shown to be involved in regulating STAT tyrosine phosphorylation in metazoan cells (Blesofsky et al., 2001; Goh et al., 2002; Rathinam et al., 2008; Ueno et al., 1997; Wang et al., 2002). These effects are mediated by modulation of tyrosine kinase activity but our evidence suggests that CblA exerts its effect by acting upon the accumulation of PTP3 protein tyrosine phosphatase. In the cblA strain there is an increase in concentration of a 40 kDa protein fragment that is derived from PTP3. This could, in principle, be due to the direct binding of CblA to PTP3, but it was not possible to detect an interaction by co-immunoprecipitation. Also, PTP3 is not detectably tyrosine phosphorylated, when assayed by either isotopic labelling (Gamper et al., 1996) or using a phosphotyrosine antibody (our unpublished results). There could, therefore, be an intermediary adaptor protein. Alternatively, proteolytic cleavage of PTP3 to liberate the 40 kDa sub-fragment may reveal a cryptic tyrosine phosphorylation site but the fact that the PTP3 antibody does not immunoprecipitate the 40 kDa species (our unpublished data) prevented testing of this possibility.

A signalling relationship between the STATc and DimB DIF induction pathways?

Combining the information for both the known DIF signalling pathways leads us to propose the scheme presented in Fig. 9. The two pathways have separate effects on, respectively, ecmA gene transcription and STATc activation but they exert a common effect
on the two morphogenetic processes: the maintenance of slug integrity and basal disc formation. In the case of DimB we propose a positive effect on the transcription of ecmA and genes involved in the two morphogenetic processes. In the case of PTP3 we propose a negative effect on STATc and on the two morphogenetic processes, similar to those of 'DIF-less' mutants, such as the dimB-null strains. We suggest, therefore that both PTP3 and the DIF synthesis and response genes impinge at a common point to regulated morphogenesis.

However, CblA is not necessary for DIF responsiveness of ecmA or for DimB activation, hence the proposed dichotomy.

CblA and the stress induced STATc activation pathway
CblA appears not to be involved in the STATc stress pathway, because this response is unaffected in the cblA mutant. Thus the stress and DIF activation pathways can be uncoupled genetically. We believe that the insensitivity of the stress response to CblA mutation reflects the fact that, upon stress treatment, serine-threonine phosphorylation of PTP3 is much increased relative to DIF treatment, and the inhibition of PTP3 enzymatic activity is completely inhibited by DIF-induced phosphorylation but is partially inhibited by stress-induced phosphorylation (Fig. 9).

Materials and Methods
Cell culture, transformation and development
The Ax2 axenic derivative of NC4 (a gift from G. Gerisch) was cultured at 22°C in HL5 medium (Watts and Ashworth, 1970) and transformed by electroporation (Pang et al., 1999). Potential knockout clones were selected at 10 μg/ml blasticidin S and over-expressors at 20 μg/ml G418. Clones were isolated by growth on a lawn of bacteria or by plating on 96-well plates. Exponentially growing cells were harvested and washed twice in the phosphate buffer KK2 (16.5 mM KH2PO4, 3.8 mM K2HPO4, pH 6.2). For suspension development, cells were resuspended in KK2 at a concentration of 1×107 cells/ml and were shaken for 4 hours at 200 r.p.m. For late developmental stages, cells were plated on 1.5% non-nutrient agar plates or agar plates bearing 45 μm pore filters (Millipore). β-Galactosidase staining was performed as described previously (Dengermann et al., 1989).

Reverse transcriptase PCR
Total cell RNA was prepared using the Mini RNAeasy kit (Qiagen) with on-column DNA digestion. RT–PCR was performed with the Titanium-One-Step RT–PCR kit (Clontech). The cblA primers were: fwd960 5’-AAA CTC AAA GAT ATT CAG TTT CAT A-3’ and rev1926 5’-TGA ACA TAA TGAACA ACA ACT TAA ATG A-3’. IGF7 is present at a relatively constant level throughout Dictyostelium development, so reverse transcriptase PCR with IGF7 primers was used as loading control. The IGF7 primers were: rev5’-TTC CCT TTA GAC CTA TGG ACC TTA GCG-3’ and fwd5’-TTA CAT TAT GAG AAA CCA AGC G-3’. ecmA reverse transcriptase PCR was performed as described previously (Zhuokovskaya et al., 2006). For quantitative PCR (qPCR) analysis, RNA was made as described above. cDNA synthesis was performed with the ImProm-II™ Reverse Transcription System (Promega).

Gene disruption and generation of CblA and PTP3 expression constructs
The cblA gene was cloned and disrupted in E. coli by random insertion of a transposon containing a blasticidin resistance cassette (Abe et al., 2003). Clones were screened by PCR and a disrupant in the EF-hand sequence was selected (position 880 bp). It was used to generate disrupants of the cblA gene in Dictyostelium and these occurred at a frequency of about 10%. All expression constructs were generated using the semi-constitutive actin 15 promoter to direct transcription, and cells were selected to high copy number using a G418 resistance cassette contained within the vector. A GFP expression construct was generated by cloning GFP at the N terminus of CblA. All the PTP3 expression constructs used are described by Araki et al. (Araki et al., 2008).

Fig. 8. Western transfer analysis of the PTP3 tyrosine phosphatase. (A) Cells transformed with PTP3:myc or PTP3ΔCS:myc and selected with 20 μg/ml G418 were developed as in Fig. 7A, lysed and analysed by western transfer using affinity purified PTP3 antibody. The loading control was GSK3. (B) Ax2 cells or Ax2 cells transformed with PTP3:myc were developed as in Fig. 7A and analysed by western transfer as in A. The loading control was GSK3. (C) Ax2 cells or cblA– cells were developed as in Fig. 7A and then treated with 100 nM DIF. Samples removed at the indicated times were analysed by western transfer as in A. The loading control was total STATc protein.

Fig. 9. CblA, PTP3 and cellular signalling. This scheme suggests that the apparent downregulation of PTP3 by CblA leads to a stimulation in the DIF-induced tyrosine phosphorylation of STATc. In the case of stress the strong negative effect engendered by osmotic stress, probably mediated by increased serine threonine phosphorylation, is not relieved by CblA. The cblA– has morphological phenotypes, similar to those of 'DIF-less' mutants, such as the dimB-null strains. We suggest, therefore that both PTP3 and the DIF synthesis and response genes impinge at a common point to regulated morphogenesis.
Immunostaining

Polyclonal rabbit antisera were generated against the CblA peptide RSRITRIVINIFKS and against the PTP3 peptide GIRSLSSP5KR. These peptides contained a non-coding cysteine residue at, respectively, their N and C termini, and they were coupled to Affi-gel (Bio-Rad) in order to purify the antibody. Other antibodies used were: GS3K antibody (Millipore), total anti-SAP (TH3) and anti-phospho-Tyr307–312–STAT (C9A). For western transfer, proteins were separated on pre-cast 4-12% polyacrylamide gels (Novex) and electro-transferred to nitrocellulose membranes. Membranes were blocked with 5% skimmed milk in TBS-Tween (Tris-buffered saline, 0.05% Tween 20) for 30 minutes, then incubated with primary antibody overnight at 4°C. Signals were detected using HRP-a-conjugated goat anti-mouse and goat anti-rabbit antibodies (Bio-Rad) with a chemiluminescent detection system (Pierce). To quantify changes in loading were normalised using the general STATc antibody, 7H3. References

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