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Chlamydia trachomatis tarp is phosphorylated by src family tyrosine kinases

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ABSTRACT

The translocated actin recruiting phosphoprotein (Tarp) is injected into the cytosol shortly after *Chlamydia trachomatis* attachment to a target cell and subsequently phosphorylated by an unidentified tyrosine kinase. A role for Tarp phosphorylation in bacterial entry is unknown. In this study, recombinant *C. trachomatis* Tarp was employed to identify the host cell kinase(s) required for phosphorylation. Each tyrosine rich repeat of L2 Tarp harbors a sequence similar to a Src and Abl kinase consensus target. Furthermore, purified p60-src, Yes, Fyn, and Abl kinases were able to phosphorylate Tarp. Mutagenesis of potential tyrosines within a single tyrosine rich repeat peptide indicated that both Src and Abl kinases phosphorylate the same residues suggesting that *C. trachomatis* Tarp may serve as a substrate for multiple host cell kinases. Surprisingly, chemical inhibition of Src and Abl kinases prevented Tarp phosphorylation in culture and had no measurable effect on bacterial entry into host cells.

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A Gram-negative obligate intracellular bacterium, *Chlamydia trachomatis*, is the leading cause of preventable blindness worldwide and the most prevalent bacterial pathogen causing sexually transmitted disease in the western world [1]. Chlamydiae initiate their intracellular developmental cycle by actively gaining entry into host cells. The extracellular infectious form of the developmental cycle is referred to as an elementary body (EB). Once engulfed by the host cell, the EB differentiates into the replicative reticulate body (RB) within the protective confines of a membrane bound parasitophorous vacuole termed an inclusion [2].

EB invasion of non-phagocytic cells is the product of coordinated cytoskeletal remodeling characterized by the formation of pedestal like structures and hypertrophic microvilli that are directly triggered by the invading Chlamydiae [3,4]. A recently identified *C. trachomatis* type III secreted protein called Tarp, for translocated actin recruiting phosphoprotein, is tyrosine phosphorylated by an unidentified host cell kinase and is spatially and temporally associated with the recruitment of actin at the site of EB invasion [5]. Subsequent *in vitro* analysis revealed that the C-terminal domain of Tarp nucleated actin filaments independently of tyrosine phosphorylation or additional host factors such as Arp2/3 [6]. Despite all pathogenic *Chlamydia* species harboring a Tarp ortholog, only *C. trachomatis* Tarp contains the tyrosine rich repeat domain and is phosphorylated upon cellular contact [7]. The function of Tarp phosphorylation and the signaling molecules that associate with phosphorylated Tarp are unknown.

We demonstrate here that the tyrosine rich repeat region of *C. trachomatis* L2 Tarp harbors multiple Src kinase target sequences, and is phosporylated by recombinant p60-src. Tyrosine residues 136 and 140 (and the corresponding amino acids in repeats 2–6) are likely the sites of phosphorylation of this kinase. Interestingly, EBs were able to invade cells lacking Src kinases and these cells were capable of phosphorylating Tarp. Our study suggests multiple kinases phosphorylate specific Tarp residues ensuring phosphorylation of this secreted effector irrespective of host kinase repertoires. Furthermore, we demonstrate that EB entry is not dependent on Tarp phosphorylation, as host cells treated with PP2, a Src and Abl family tyrosine kinase inhibitor that effectively blocked Tarp phosphorylation had no effect on bacterial invasion.

Materials and methods

Cloning and protein expression. Previously described clones of L2 Tarp served as the template for PCR mutagenesis designed to identify the tyrosine residues phosphorylated by host cell kinases [6]. Individual point mutations of a single L2 Tarp repeat were introduced by site directed mutagenesis.

pGEX-6P-1 plasmids encoding the Tarp fusion proteins were transformed into the BL21 strain of *Escherichia coli* (Novagen, Madison, WI). Protein expression and purification were performed according to the procedures outlined in the Bulk GST Purification Module (GE Healthcare: Amersham Biosciences AB, Piscataway, NJ).

GST fusion protein pull-down and kinase experiments. Recombinant L2 Tarp proteins were phosphorylated by host cellular

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extracts in pull down assays. HeLa 229 cells were suspended in 100 mM KCl, 10 mM HEPES (pH 7.7), 2 mM $MgCl_2$, and 2 mM ATP (buffer A) and disrupted by sonication. Insoluble material was removed by centrifugation (12,000 rcf, 25 min, 4 °C). Glutathione–Sepharose beads were incubated with 10 μg of GST fusion proteins or GST for 1 h at 4 °C in PBS (Amersham Biosciences). GST- fusion protein coated sepharose beads were washed twice with PBS and once with buffer A prior to the addition of approximately 100 μg of HeLa extracts. Extracts and beads were incubated together for 2 h at 4 °C, room-temp or 37 °C, washed three times with fresh buffer A, and bound proteins were eluted using sample buffer [8]. Kinase assays were performed similarly with HeLa extracts replaced with purified recombinant kinases Src, Yes, Fyn c-Abl, and Zap 70 (Millipore, Temecula, CA).

SDS–PAGE, immunoblotting, and antibodies. Proteins were separated on SDS–10% polyacrylamide gels [8] and immunoblotted as previously described [5–7]. The anti-phosphotyrosine 4G10 monoclonal antibody and anti-Src, clone GD11 was purchased from Upstate (Lake Placid, NY). The anti-actin C4 monoclonal antibody was purchased from Chemicon International. The Anti-c-Abl (Ab1) rabbit polyclonal sera was purchased from Calbiochem, EMD Biosciences, (La Jolla, CA) Polyclonal rabbit antibodies directed towards the *Chlamydia* antigens, Tarp was developed at Rocky Mountain Laboratories (Hamilton, MT) and has been described previously [5].

Kinase inhibitors. L929 fibroblasts plated into 24-well plates were treated with the kinase inhibitor, PP2 [4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo-[3,4-*d*]pyrimidine] at media concentrations of 25–200 μM for 2 h (A.G. Scientific, Inc., San Diego, CA and EMD Biosciences La Jolla, CA). *C. trachomatis* L2 was used to infect drug treated fibroblasts at an MOI of 50 for indirect immunofluorescence microscopy and an MOI of 1000 for Western blot analysis. A complete list of tyrosine kinase inhibitors tested in culture with indicated concentrations is summarized in Supplemental Table 1. Percent of Tarp phosphorylation was determined by densitometry (Image J) of immunoblot signal compared to DMSO control.

Results

Recombinant Tarp proteins are tyrosine phosphorylated by HeLa extracts and purified p60-Src. C. trachomatis

Tarp is rapidly tyrosine phosphorylated upon exposure to the host cell cytosol at the site of EB attachment and thought to play a role in the cytoskeletal rearrangements accompanying internalization. Presumably, phosphorylation of Tarp, as with the phosphorylation of other known bacterial secreted effectors from other organisms, initiates signal transduction cascades important for the establishment of intracellular residence [9]. To better ascertain the significance of Tarp phosphorylation, we sought to identify the host cell kinase(s) responsible for this *in vivo* modification. Glutathione-S-transferase (GST) fusions to the tyrosine rich N-terminal repeat region as well as other domains of the Tarp protein were previously constructed (Fig. 1A) [6]. The addition of soluble HeLa extracts to Tarp proteins harboring the tyrosine rich domain resulted in phosphorylation as shown by immunoblotting with phosphotyrosine specific monoclonal antibody [6]. *C. trachomatis* L2 Tarp harbors six tandem tyrosine rich repeats of similar sequence (Fig. 1B). Sequence analysis of the six individual repeats has revealed that each contains at least one predicted Src-like substrate site (Fig. 1C) [9]. Interestingly, three of the six repeats appear to contain two overlapping Src-like substrate sites (Fig. 1C). To determine if a Src family member was capable of phosphorylating Tarp, recombinant Tarp proteins were incubated with purified p60-

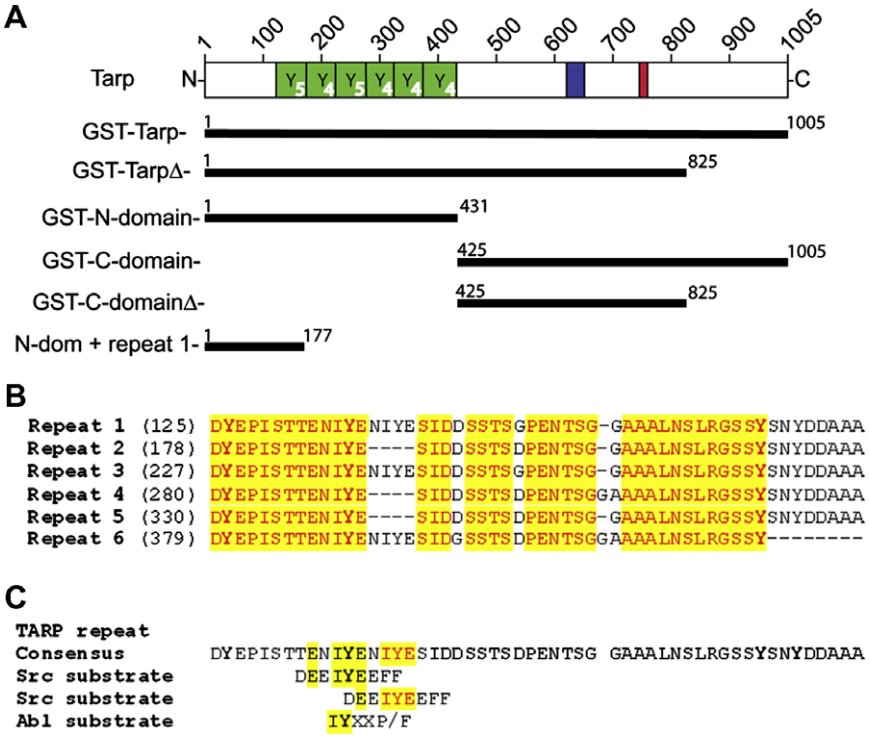

Fig. 1. Schematic of GST-TARP fusions employed in this study and sequence of the tyrosine rich repeats harboring Src-like kinase substrate sites. (A) L2 Tarp harbors an N-terminal tyrosine rich repeat region (YYYYY, green boxes), which are contained within the GST fusions; GST-Tarp, GST-TarpΔ, and GST-N-domain. Each repeat contains either 4 or 5 tyrosines indicated in the lower right corner of each green box. N-dom + repeat 1 harbor the first tyrosine rich repeat. A proline dense domain (blue box) and actin binding domain (red box) are contained within the C-terminal half of Tarp located in the GST fusions; GST-C-domain and GST-C-domainΔ. Numbers on each bar indicate L2 Tarp amino acids contained within the GST fusion protein. (B) An alignment of the 6 tyrosine rich repeats of *C. trachomatis* L2 Tarp. Each repeat contains at least 4 tyrosine residues that are indicated in bold type. Identical residues are highlighted in yellow. (C) The consensus Tarp repeat harbors two overlapping peptide sequences similar to a Src and Abl substrate.