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Phosphorylation of threonine residues on Shc promotes ligand binding and mediates crosstalk between MAPK and Akt pathways in breast cancer cells

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ABSTRACT

Scaffold proteins play important roles in regulating signalling network fidelity, the absence of which is often the basis for diseases such as cancer. In the present work, we show that the prototypical scaffold protein Shc is phosphorylated by the extracellular signal-regulated kinase, Erk. In addition, Shc threonine phosphorylation is specifically up-regulated in two selected triple-negative breast cancer (TNBC) cell lines. To explore how Erk-mediated threonine phosphorylation on Shc might play a role in the dysregulation of signalling events, we investigated how Shc affects pathways downstream of EGF receptor. Using an *in vitro* model and biophysical analysis, we show that Shc threonine phosphorylation is responsible for elevated Akt and Erk signalling, potentially through the recruitment of the 14-3-3 ζ and Pin-1 proteins.

1. Introduction

Scaffold proteins play an important role in propagation of signals from cell surface receptors. Bringing signalling proteins into proximity on a common structure provides increased opportunity for regulation of downstream response through signal specification and amplification. Initiation of signal transduction from receptor tyrosine kinases (RTKs) is dominated by tyrosine phosphorylation, however this is often superseded by serine/threonine kinase up-regulation. Juxtaposing of tyrosine and serine/threonine cognate binding sites on a scaffold protein provides the opportunity for fine-tuning of RTK signal transduction through modulating protein–protein interactions (Deribe et al., 2010; Johnson and Lewis, 2001; Seet et al., 2006).

The prototypical scaffold protein ShcA (henceforth Shc) has been extensively studied in regard to its ability to recruit proteins dynamically in RTK-mediated signalling. It plays a complex role as a hub for binding of numerous signalling proteins. Despite having no intrinsic enzyme activity, Shc is involved in signal transduction associated with poor prognosis in breast cancer patients (Ursini-Siegel and Muller, 2008). The *ShcA* gene encodes three isoforms of 46, 52 and 66 kDa (p46, p52 and p66 respectively) which all possess an N-terminal PTB domain and a C-terminal SH2 domain which sequentially sandwich a CH1 domain. Shc binds through its phosphotyrosine binding (PTB) domain to tyrosyl phosphates on activated RTKs and undergoes a conformational change which exposes its SH2 domain (George et al., 2008). Within the sequence of Shc are a number of tyrosine, serine and threonine phosphorylation sites which enable Shc to recruit a large, multi-functional array of proteins which appear to be temporally controlled subsequent to RTK activation (Zheng et al., 2013). Shc therefore forms a dynamic hub for protein recruitment.

It has been demonstrated that under non-stimulated conditions Shc (p52Shc) binds directly to the proline-directed serine/threonine kinase, Erk (extracellular signal-regulated kinase, aka. mitogen-activated protein kinase (MAPK)) (Suen et al., 2013). In so doing Erk is restricted from engaging in MAPK signalling. Erk is released from Shc and becomes up-regulated in response to RTK-activated MAPK signalling. On activation Erk phosphorylates a wide range of downstream effector protein substrates (Arur et al., 2009; Carlson et al., 2011), including

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several scaffold proteins, *e.g.* Grb2-associated binding protein 1 (Gab1) (Gu and Neel, 2003; Lehr et al., 2004; Roshan et al., 1999; Yu et al., 2002), fibroblast growth factor receptor substrate 2 alpha (Frs2α) (Lax et al., 2002; Wu et al., 2003) and insulin receptor substrate 1 (IRS1) (Arur et al., 2009; De Fea and Roth, 1997). While Shc has recently been established to be a substrate for Erk, the significance of these phosphorylation events has not been investigated (Zheng et al., 2013). Erk-mediated phosphorylation of Shc could regulate its ligand binding capability allowing modulation of downstream signalling.

Serine/threonine and tyrosine phosphorylation are considered to be inversely correlated events, for example phosphorylation on serine/threonine residues of a scaffold protein reduces the level of tyrosine phosphorylation. Since phosphorylated tyrosine residues are required to propagate downstream signalling from a RTK, reduction in signalling through subsequent serine/threonine phosphorylation results in a negative feedback mechanism for pathway control (Lax et al., 2002; Yu et al., 2002). Interestingly, a positive feedback role for serine/threonine phosphorylation on scaffold proteins has also been shown. For example, Erk phosphorylation of Gab1 enhances the recruitment of the p85 subunit of phosphoinositide 3-kinase (PI3K) to Gab1, resulting in an increase in PI3K and Erk signalling (Yu et al., 2001).

Here we show that three threonine residues on Shc are selectively phosphorylated by Erk *in vitro* and in breast cancer cell lines post-EGF stimulation. Phosphorylation of these residues leads to elevated Erk and Akt phosphorylation post-EGF stimulation. We demonstrate how phosphorylation on one of these residues appears in two selected triple-negative breast cancer cell lines and investigate the structural implications of these phosphorylation events. We identify two proteins whose phosphothreonine-dependent association with Shc provides evidence for additional downstream signal mediation.

2. Results

2.1. Three threonine residues on Shc are substrates for Erk

Three threonine residues on Shc (T214, T276 and T407) are contained within the Erk consensus substrate sequence (S/T-P). Incubation of purified Shc with active Erk2 in the presence of ATP and $MgCl_2$ revealed that Shc is threonine phosphorylated on the putative Erk substrate sites (Fig. 1A). Since the commercially available pan-anti-T-P antibody cannot distinguish between the three substrate sites for Erk on Shc, we analyzed Erk-phosphorylated, trypsin-digested Shc by mass spectrometry. This showed that three T-P motifs on p52Shc were phosphorylated after incubation with Erk (Supplemental Fig. 1). Of the three sites only T214 has previously been shown to be a substrate for Erk (Zheng et al., 2013). Phosphorylation of T276 on p66Shc by an unknown kinase (Khanday et al., 2006; Rajendran et al., 2010) has previously been reported. T214 and T276 are found in the CH1 domain, whilst T407 is in the SH2 domain (Fig. 1B). Both T214 and T407 are highly conserved across various species, whereas T276 is less so (Fig. 1C).

Since phosphorylation of Shc has previously been shown to exert regulatory control of ligand binding (George et al., 2008), we investigated whether the phosphorylation of threonine residues affected the ability of Shc to bind to a RTK. We expressed and purified a triple mutant form of Shc in which the three threonine residues were replaced by the phosphorylation charge mimetic of threonine; glutamate (T214/276/407E or ^{TE}Shc) and measured its binding to a known Shc-binding peptide corresponding to the TrkA receptor. The mutation of the threonine residues to glutamate mimics the triple phosphorylated state of Shc. Isothermal titration calorimetric (ITC) data show that the phosphorylation state of the threonine residues has no effect on recruitment to the receptor demonstrated by the comparable dissociation constants for the binding wild type, ^{WT}Shc, or ^{TE}Shc to the RTK-derived peptide (K_d ~54 nM and 38 nM, respectively; Fig. 1D).

We previously reported a gating mechanism driven by tyrosine phosphorylation on Shc, whereby the SH2 domain is only available for ligand-binding when the CH1 domain is phosphorylated on its tyrosine residues (George et al., 2008). It has also been proposed that phosphorylation on Y317 introduces rigidity to the protein and limits the dynamic motions of the PTB and SH2 domains in molecular dynamics simulation studies (Suenaga et al., 2009, 2004). Hence, there is a precedent for regulation imposed by inter-domain interactions. We therefore explored the possibility that threonine phosphorylation induces a conformational change in Shc. The structural model of the full length Shc protein suggests that T214 and T276 reside on helices (PDB 1WCP) (Suenaga et al., 2004). We therefore employed circular dichroism spectroscopy (CD) to investigate the effect of threonine phosphorylation on the secondary structure of Shc. We compared the CD signals from the full length unphosphorylated and threonine-phosphorylated Shc. As expected the CD signal is commensurate with the full length Shc protein (ShcFL)containing α-helical, β-strand and disordered structure (Supplemental Table 1). However, no significant changes in the structural composition between unphosphorylated and threonine-phosphorylated forms were observed as shown by the overlapping signals from the wild type and mutant Shc proteins (Fig. 1E). A further lack of impact on the protein structure was reflected in the thermostability of Shc which was unaffected by threonine phosphorylation Shc (Fig. 1F). Finally, the similar pattern of protease digestion observed for the ^{WT}Shc and ^{TE}Shc proteins (Fontana et al., 2004) confirmed that no structural variation was imparted by threonine phosphorylation (Supplemental Fig. 2).

2.2. Phosphorylation of T214 is prevalent in triple-negative breast cancer cells

To investigate the potential pathological relevance of the threonine phosphorylation events we screened for the presence of phosphorylation on T214 (pT214) in a number of transformed/cancer cell lines with a specific antibody against pT214 (efficiency of antibody shown in Supplemental Fig. 3). We initially focused on breast cancer due to the previously reported critical role of Shc in this disease (Ursini-Siegel and Muller, 2008) (Fig. 2 and Supplemental Fig. 4). Non-transformed MCF10A cells (Soule et al., 1990) were used for comparison with the selected cancer cell lines (Fig. 2C). Cells were stimulated with EGF for between 2 and 30 min to investigate the temporal pattern of Shc threonine phosphorylation. Cells were also separately pre-incubated with the MAPK/Erk kinase, Mek, inhibitor U0126 to abolish Erk activity (Mek is upstream of, and responsible for phosphorylation and activation of Erk), in order to confirm that the phosphorylation event is mediated by Erk. We first immunoprecipitated total Shc and then probed for pT214. Although all cell lines exhibited Erk activity upon EGF stimulation, only the triple negative cell lines MDA-MB-468 and MDA-MB-231 exhibited a significant level of phosphorylation on T214 (Fig. 2A and B). None of the other cell lines tested showed evidence of phosphorylated T214, (*i.e.* non-transformed MCF10A cells (Fig. 2C) and other non-triple negative cell lines; MCF7 (Fig. 2D), MDA-MB361, A431, HEK293T (Supplemental Fig. 4)). Two of the known binding partners for Shc in EGF signalling, epidermal growth factor receptor, EGFR, and growth factor receptor protein-binding protein 2, Grb2, were probed as controls for Shc function (Fig. 2A–D and Supplemental Fig. 4). To confirm that Shc is threonine phosphorylated in the triple-negative cell lines, we immunoprecipitated Shc using the pT214 antibody and then probed for Shc (Fig. 2E and F). In agreement with the previous immunoprecipitation experiments, Shc is phosphorylated on T214 in both MDA-MB-468 and MDA-MB-231 cells.

It has been reported that serine/threonine phosphorylation affects tyrosine phosphorylation in scaffold proteins, so we examined the phosphorylation levels on the three known Shc tyrosine sites, 239, 240 and 317 in response to threonine phosphorylation. If Shc threonine phosphorylation affects the concomitant tyrosine phosphorylation level, we should observe a consistent change in Shc tyrosine