# Insulin dependent tyrosine phosphorylation of the tyrosine internalisation motif of TGN38 creates a specific SH2 domain binding site

D.J. Stephens, G. Banting*

*Department of Biochemistry, University of Bristol, School of Medical Sciences, University Walk, Bristol BS8 1TD, UK*

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**Abstract Tyrosine-based motifs are involved in both protein targeting and, via SH2 domain binding, intracellular signalling. To date there has only been one example of such a motif acting as both an intracellular sorting signal and SH2 binding determinant, namely that of the T cell costimulation receptor, CTLA-4. We show that insulin stimulation of cultured rat hepatoma cells results in increased cell surface expression of TGN38. Furthermore, the cytosolic domain of TGN38 can be phosphorylated by the insulin receptor in vitro and tyrosine phosphorylated TGN38 can specifically bind to the SH2 domains of the spleen tyrosine kinase Syk. These data imply that tyrosine-based motifs may play a broader role than has previously been accepted and could help to integrate trafficking and signalling events.**

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*Key words:* TGN38; SH2 domain; Tyrosine phosphorylation; Insulin; Syk

## 1. Introduction

Tyrosine-based targeting signals conforming to the consensus, -YXXØ- (where Ø indicates a hydrophobic amino acid), mediate a number of intracellular protein sorting events including endocytosis and lysosomal targeting [1,2]. Internalisation at the cell surface is mediated by interaction of these motifs with the μ2 subunit of the clathrin adaptor complex, AP2 [2]. These sequences are remarkably similar to phosphotyrosine-based motifs that direct SH2 (Src-homology 2) domain binding [3,4]. SH2 domains are globular protein domains of approximately 100 amino acids that mediate a number of intracellular signalling processes via interaction with specific phosphotyrosine motifs. Despite both targeting and SH2 binding being critical events within eukaryotic cells, there have, until recently, been no examples of -YXXØ- motifs acting as both a subcellular targeting motif and, when phosphorylated, an SH2 binding determinant. During the preparation of this article, Shiratori et al. [5] published data showing that the motif -YVKM- within the cytosolic domain of the T cell costimulatory molecule, CTLA-4, was capable of interacting with both μ2 and, when phosphorylated, the tyrosine phosphatase Syp [5].

TGN38 is a type I integral membrane protein that cycles between the trans-Golgi network (TGN) and plasma membrane [6]. The sequence -SDYQRL- (amino acids 331–336) within the cytosolic domain of TGN38 has previously been shown to be required for efficient internalisation from the cell surface and targeting back to the TGN (reviewed in [7]). This motif has been employed by a number of groups to study the function of tyrosine based sorting determinants (e.g. [8–10]). TGN38 is very efficiently internalised from the plasma membrane [7] and the cytosolic domain of the protein has been shown to interact with the μ2 subunit of the AP2 clathrin adaptor complex [9,10]. Binding experiments using synthetic peptides have shown that phosphorylation of the tyrosine residue within the -SDYQRL- motif significantly reduces binding of TGN38 to μ2. However, tyrosine phosphorylation of TGN38 has never been demonstrated.

We have found that insulin simulation of cultured cells results in an increase in the amount of TGN38 at the cell surface. The cytosolic domain of TGN38 can also be phosphorylated in vitro by an enriched preparation of the insulin receptor tyrosine kinase. Furthermore, we show that tyrosine phosphorylated TGN38 can specifically bind to the SH2 domains of the spleen tyrosine kinase Syk [11]. This represents a further demonstration of the potential regulation of both trafficking and intracellular signalling through the phosphorylation and dephosphorylation of a single tyrosine residue.

## 2. Materials and methods

### 2.1. Flow cytometry

H4-II-E cells were serum starved for 2 h before stimulation for 5 min with 100 nM insulin. Cells were then washed 3 times in ice cold PBS and removed from dishes with 5 mM EDTA. All subsequent steps were performed at 4°C. Cells were incubated for 20 min in PBS+2% fetal calf serum before staining with either 2F7.1 (anti-TGN38 [12], Affinity Bioreagents, Inc.) or an isotype-matched negative control antibody (Serotec) followed by biotinylated anti-mouse antibody and streptavidin-phycoerythrin (Serotec). Cells were washed in 4 ml of PBS+2% FCS between layers. Flow cytometry was performed on a Becton-Dickinson FACSCalibur with CellQuest software.

### 2.2. In vitro phosphorylation

Thioredoxin-TGN38 fusion proteins [9] were phosphorylated as described in [13] with [γ-$^{32}$P]ATP (ICN). Briefly, insulin receptor was purified by affinity chromatography on wheat germ agglutinin sepharose [18], preactivated by incubation at 4°C in the presence of insulin and unlabelled ATP. Recombinant proteins were then phosphorylated by incubation for 30 min at 30°C in the presence of [γ-$^{32}$P]ATP. Phosphorylation reactions were separated by SDS-PAGE on 15% gels. Dried gels were imaged using a Molecular Dynamics phosphorimager.

### 2.3. SH2 domain binding

GST fusion proteins corresponding to the SH2 domains of Syk, Shb, Shp-1, p85 and PLCγ were expressed in *Escherichia coli* according to standard protocols and, following cell lysis, bound to glutathione agarose (Pharmacia). Immobilised GST fusion proteins were subsequently incubated for 2 h at room temperature with $^{32}$P-labelled TGN38 cytosolic domain, washed five times in phosphate buffered saline, pH 7.4, separated by SDS-PAGE, dried and imaged.

*Corresponding author. Fax: (44) (117) 928 8274.
E-mail: bantingg@bsa.bristol.ac.uk

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## 3. Results and discussion

We initially observed using flow cytometry that insulin stimulation of the cultured rat hepatoma cell line, H4-II-E, results in an increase in the amount of TGN38 at the cell surface (Fig. 1). Following stimulation of cells with 100 nM insulin, the mean increase in the mean fluorescence intensity was found to be 3.5 units (S.E. ±1.2 units). Increased cell surface expression following insulin stimulation has also been observed for a number of other proteins including the bifunctional mannose-6-phosphate/insulin-like growth factor receptor (M6PR/IGFIIR [14]) and the insulin responsive glucose transporter, GLUT4 [15]. These proteins, like TGN38, cycle between intracellular compartments and the cell surface [15,16]. It is possible that insulin stimulation results in active translocation of TGN38 to the plasma membrane as is seen for GLUT4 [15]. Alternatively, internalisation of the protein from the plasma membrane may be impaired. Given the intrinsic tyrosine kinase activity of the insulin receptor [17], the latter explanation would appear to be the most probable since interaction of TGN38 with the endocytic machinery (namely the μ2 subunit of the plasma membrane clathrin adaptor complex, AP2 [8–10] is blocked by phosphorylation of the -SDYQRL- motif [8].

In order to investigate the phosphorylation of TGN38 directly, insulin receptors were partially purified by wheat germ agglutinin affinity chromatography from CHO.T cells [18]. This preparation was found to be capable of the in vitro phosphorylation of the cytosolic domain of TGN38 expressed as a thioredoxin fusion protein (Fig. 2). Phosphorylation was shown to be specific to tyrosine-333 of TGN38 since mutation of this residue to alanine abolished the effect (Fig. 2A). Furthermore, the phosphorylated TGN38 fusion protein was shown to be recognised in immunoblot analysis with the phosphotyrosine-specific monoclonal antibody, 4G10. Phosphoamino acid analysis confirmed phosphorylation of tyrosine-

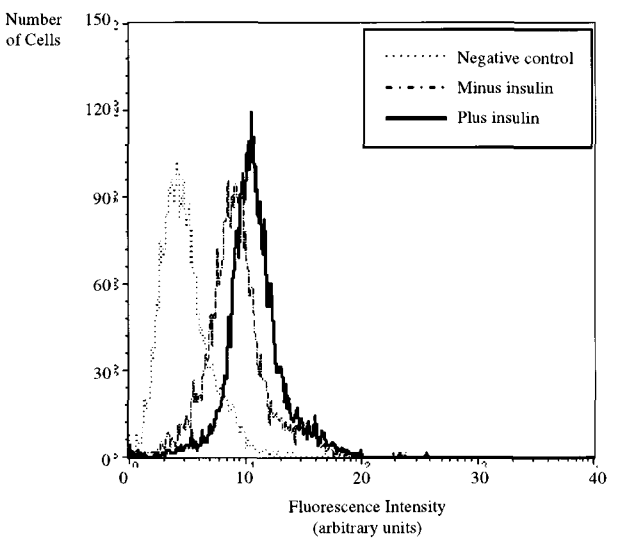


Fig. 1. Effect of insulin stimulation on cell surface TGN38. Cells were serum starved for 2 h before stimulation for 5 min with 100 nM insulin. Cells were then stained for flow cytometry at 4°C. The dotted line indicates staining with the negative control antibody, the dashed line indicates staining with 2F7.1 (anti-TGN38) without insulin treatment, the solid line indicates staining with 2F7.1 following insulin stimulation.

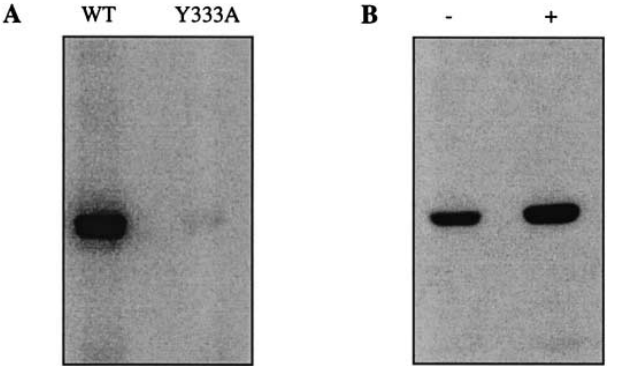


Fig. 2. In vitro phosphorylation of the cytosolic domain of TGN38. A: Phosphorylation of wild-type TGN38 (WT) and Y333A mutant (Y333A). B: Insulin responsiveness of in vitro phosphorylation.

333 (not shown). This in vitro phosphorylation event was also shown to be insulin responsive (Fig. 2B). These results, in conjunction with those from cultured cells, suggest that the insulin receptor may be capable of phosphorylating TGN38 directly resulting in an increase in TGN38 at the cell surface. However, we cannot exclude the involvement of an intermediate kinase which is activated by, and co-purifies with, the insulin receptor.

Thus the internalisation motif, -SDYQRL-, can be phosphorylated in response to insulin stimulation. Such tyrosine based internalisation motifs (conforming to the consensus -YXX∅-, where ∅ indicates a hydrophobic amino acid) are strikingly similar to phosphotyrosine motifs involved in SH2 domain binding [4]. SH2 domains are globular protein domains of approximately 100 amino acids that mediate protein tyrosine kinase signalling by binding directly to phosphotyrosine motifs [3].

Given that we had shown tyrosine phosphorylation of the consensus internalisation motif of TGN38 and that this motif also conforms to the generic SH2 domain binding motif, we decided to test binding of tyrosine phosphorylated TGN38 to a number of SH2 domains. Consensus binding motifs of a number of SH2 domains have been determined using degenerate phosphopeptide libraries and other similar approaches [4]. We tested binding of several SH2 domains to TGN38, many of which have previously been shown to bind preferentially to pYXXL sequences [4]. Tyrosine phosphorylated TGN38 was found to bind specifically to the tandem SH2 domains of the spleen tyrosine kinase Syk [19] (Fig. 3A) which is indeed selective for pYXXL motifs. No binding was detected to the SH2 domains of phospholipase Cγ [20], protein tyrosine phosphatase 1 [21], the adaptor protein Shb [22] or the p85 subunit of phosphatidylinositol-3 kinase [23] (Fig. 3A). Binding occurred to both individual SH2 domains of Syk with some selectivity for the carboxy-terminal domain (Fig. 3B, lanes 3 and 4). This is in concurrence with previously published data since the predicted phosphopeptide motif for interaction with the C-terminal SH2 domain of Syk (pY-(Q/T/E)-(E/Q/T)-L [4]) has more similarity to the sequence of TGN38 (-pY-Q-R-L) than that of the N-terminal SH2 domain of Syk (which preferentially binds to the sequence, pY-T-T-(I/L/M) [4]).

It should be noted that the insulin receptor [24], Syk [11] and TGN38 (Stephens and Banting, unpublished results) are all present in the cells of at least one tissue (spleen). This

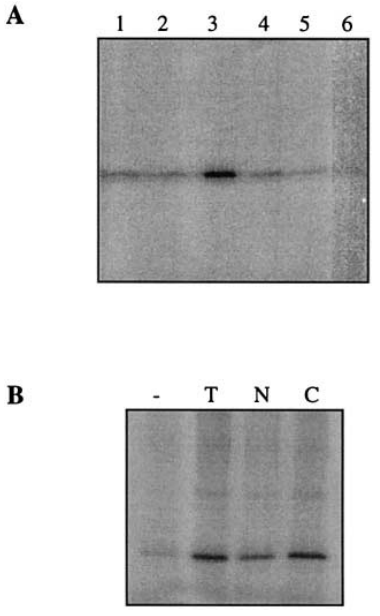


Fig. 3. Binding of SH2 domains to tyrosine phosphorylated TGN38. Immobilised SH2 domains were then incubated with in vitro phosphorylated TGN38 as Fig. 2. A: Binding to GST-SH2 domains. Lane 1, GST; lane 2, Shb; lane 3, Syk; lane 4, p85; lane 5, Shp-1; lane 6, PLCγ. B: Binding to the tandem (T), N-terminal (N) and C-terminal (C) SH2 domains of Syk.

underlines the physiological relevance of the TGN38-Syk interaction. However, the insulin receptor and TGN38 have a wider tissue distribution than Syk raising the possibility that the tyrosine phosphorylated cytosolic domain of TGN38 interacts with, as yet unidentified, SH2 domain containing proteins in other cell types. We have made considerable efforts to identify tyrosine phosphorylated TGN38 in cell lysates however, immunoprecipitation of TGN38 following insulin stimulation and subsequent immunoblotting with anti-phosphotyrosine antibodies have been unsuccessful. However, the effect of insulin stimulation of cells on the distribution of TGN38 within cells, coupled with the highly efficient tyrosine phosphorylation of TGN38 in vitro do suggest that phosphorylation may be occurring in H4-II-E cells but is below the limits of detection in our assays. It is difficult to speculate on the role of TGN38 phosphorylation since the function of TGN38 remains unknown. However, it is possible that tyrosine phosphorylation might be a mechanism for increasing cell surface expression of TGN38 or alternatively of modulating intracellular trafficking of the protein.

In conclusion, we present evidence for the insulin stimulated tyrosine phosphorylation of a defined tyrosine containing internalisation motif and its subsequent interaction with a specific SH2 domain containing protein. This has only previously been shown for the T cell surface molecule, CTLA-4 [5]. The prevalence of these motifs in transmembrane proteins [2,4] does suggest that there may be many more examples of this type and confirms that -YXXØ- motifs are relevant, not only as internalisation signals in endocytic pathways or components of tyrosine kinase signalling pathways, but also as potential sites for the integration and parallel regulation of both trafficking and signalling pathways.

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