Fyn is required for oxidative- and hyperosmotic-stress-induced tyrosine phosphorylation of caveolin-1

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Caveolin-1 is phosphorylated on Tyr^{14} in response to both oxidative and hyperosmotic stress. In the present paper, we show that this phosphorylation requires activation of the Src family kinase Fyn. Stress-induced caveolin phosphorylation was abolished by three Src kinase inhibitors, SU6656, PP2 and PD180970, and was not observed in fibroblasts derived from a Src, Yes and Fyn triple-knockout mouse ($SYF^{-/-}$). Using cell lines derived from single-kinase-knockout mice ($Src^{-/-}$, $Yes^{-/-}$ and $Fyn^{-/-}$), we show that expression of Fyn, but not Src or Yes, is required for stress-induced caveolin phosphorylation. Heterologous expression of Fyn in the $SYF^{-/-}$ and $Fyn^{-/-}$ cells was sufficient to reconstitute stress-induced caveolin phosphorylation, and overexpression of Fyn in wild-type cells induced hyperphosphorylation of caveolin. Fyn was autophosphorylated following oxidative stress, verifying activation of this kinase. Interestingly, there was a concomitant increase in the phosphorylation of Fyn on its Csk (C-terminal Src kinase) site, indicating feedback inhibition. Csk binds to phosphocaveolin [Cao, Courchesne and Mastick (2002) J. Biol. Chem. **277**, 8771–8774] and should phosphorylate any co-localized Src-family kinases. Oxidative-stress-induced phosphorylation of caveolin-1 also requires expression of Abl [Sanguinetti and Mastick (2003) Cell Signal. **15**, 289–298]. Using inhibitors and cells derived from knockout mice, we verified a requirement for both Abl and Fyn in stress-induced caveolin phosphorylation in a single cell type. Our data suggest a novel mechanism for attenuation of Src-kinase activity by Abl: stable tyrosine phosphorylation of a scaffolding protein, caveolin, and recruitment of Csk. Paxillin, a substrate of both Abl and Src, organizes a similar regulatory complex.

Key words: Abl, caveolin, Fyn, oxidative stress, Src, stress-induced tyrosine phosphorylation.

INTRODUCTION

Activation of signalling pathways in response to stress allows cells to react rapidly either through initiation of cellular survival pathways or, if too extreme, through programmed cell death (for reviews see [1,2]). The exact mechanisms by which stresses such as changes in cell volume or oxidation damage are sensed and then transmitted into the nucleus have not been fully elucidated, but are known to involve protein-kinase cascades. The Src family of non-receptor tyrosine kinases are among the kinases activated by cellular stress [3–6]. Src-family kinases are necessary for activation of MAPKs (mitogen-activated protein kinases), such as ERK (extracellular-signal-regulated kinase), JNK (c-Jun N-terminal kinase) and BMK1 (big MAPK 1), major players in the transcriptional regulation of stress-induced genes [7–9]. They are also required for the activation of JAK1 (Janus kinase 1), Ras, p90 ribosomal S6 kinase and phosphoplipase D [10,11], which play roles in cell survival. Abl (Abelson tyrosine kinase) is also activated in response to cellular stress [12,13]. However, in contrast with the Src kinases, activation of Abl is involved in stress-induced cell death [14,15].

The Src kinases comprise a family with nine members: Src, Fyn, Yes, Lyn, Lck, Blk, Fgr, Hck and Yrk. Three members, Src, Fyn and Yes, are nearly ubiquitously expressed. Regulation of Src-family-kinase activity occurs through phosphorylation on two tyrosine residues [16]. Autophosphorylation in the activation loop (Tyr^{416} in Src) increases kinase activity. Conversely, phosphorylation of a tyrosine residue in the C-terminal tail (Tyr^{527} in Src) inhibits the kinase through an intramolecular SH2 (Src homology 2)–phosphotyrosine interaction. This interaction stabilizes a 'closed' or inactive conformation of the kinases. Phosphorylation of the inhibitory C-terminal site of the Src-family kinases is catalysed by Csk. Src-family kinases are activated either by dephosphorylation of the Csk site or by displacement of the C-terminal tail by another phosphoprotein.

Recently we described a novel mechanism for the regulation of Csk in cells [17]. Csk binds via an SH2-domain interaction to the caveolar coat protein, caveolin-1, when caveolin is phosphorylated at Tyr^{14}. Caveolin-1 is phosphorylated in response to cellular stress, as well as in response to insulin, endothelin I and angiotensin II [19–24]. The binding of Csk to tyrosine-phosphorylated proteins is required for both the proper targeting of Csk (a soluble protein) to its membrane-associated substrates, as well as for the activation of the kinase [25,26]. Therefore, when phosphorylated on Tyr^{14}, caveolin-1 serves as a docking site for SH2-domain-containing proteins, such as Csk, for regulated recruitment to and activation of signalling proteins in caveolae membranes.

Src-family kinases are highly enriched in caveolae. Src-family members, such as Lck and Fyn, that are tandemly acylated with myristate and palmitate are targeted to cholesterol-rich membrane domains, such as caveolae/lipid rafts [27,28]. Src kinases also

Abbreviations used: Abl, Abelson tyrosine kinase; MAPK, mitogen-activated protein kinase; BMK1, big MAPK 1; Csk, C-terminal Src kinase; ERK, extracellular-signal-regulated kinase; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; HF, human fibroblast; JAK, Janus kinase; JNK, c-Jun N-terminal kinase; SH2, Src homology 2; $SYF^{-/-}$, Src, Yes and Fyn triple-knockout mouse; WT, wild-type.

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bind directly to caveolin through a second protein-interaction domain termed the 'scaffolding domain' (amino acids 81–101 in caveolin-1; [29]). Therefore phosphorylation of caveolin-1 on tyrosine localizes Csk in proximity to its Src kinase substrates in caveolae. In this manner, stress-induced activation of Src kinases is attenuated through the concomitant recruitment and activation of Csk in caveolae. Numerous additional signalling proteins bind to the caveolin scaffolding domain [29]. Accordingly, caveolin functions to assemble lipid–protein complexes that comprise highly organized cellular signalling centres.

Caveolin-1 was originally identified as a major tyrosine-phosphorylated protein in v-Src (viral Src)-transformed cells [30]. Caveolin phosphorylation is elevated in cells that overexpress either Fyn [20] or Src [31], and both kinases can phosphorylate caveolin *in vitro* [32]. Previous work using a dominant-negative Src construct demonstrated a requirement for Src-family kinases in hyperosmotic-shock-induced caveolin phosphorylation [23]. However, caveolin is also highly phosphorylated in v-Abl (viral Abl)-expressing cells [33] and in cells overexpressing Abl [34], and Abl can directly phosphorylate caveolin-1 on Tyr^{14} *in vitro* [33] and in cells [17]. Additionally, we have shown, using cells derived from Abl-knockout mice, that Abl is required for oxidative-stress-induced phosphorylation of caveolin-1 on Tyr^{14} [34]. In contrast, expression of Src was not required for this phosphorylation. Caveolin-1 was phosphorylated normally in cells derived from Src-knockout mice.

Based on these observations, we hypothesized that Abl was the oxidative-stress-induced caveolin kinase (oxidative stress → Abl → phosphocaveolin), while a Src-family member was the hyperosmotic-shock-induced caveolin kinase (osmotic shock → Src-family kinase → phosphocaveolin). To test this hypothesis, we used Src-kinase inhibitors, as well as cells from Src-family-kinase-deficient mice. Unexpectedly, we found that Src-kinase activity is required for both oxidative-stress- and osmotic-shock-induced caveolin phosphorylation, indicating a complex relationship between Src-kinases and Abl in caveolin phosphorylation. Furthermore, we found that the Src-family-kinase members activate distinct signalling pathways in cells. Our major findings of the present study are: (i) Fyn expression is necessary for both oxidative- and osmotic-stress-induced caveolin phosphorylation, whereas expression of Src and Yes are not; (ii) oxidative stress activates both Fyn and Src. Concomitantly, oxidative stress increases the phosphorylation of Fyn on its inhibitory Csk site. In contrast, there is no increase in the phosphorylation of Src on its Csk site, perhaps due to differential localization in cells; (iii) as observed previously with Abl, overexpression of Fyn was sufficient to induce high levels of caveolin phosphorylation in primary human fibroblasts. Overexpression of Fyn also recruited Csk to caveolin-enriched low-density complexes in these cells; (iv) using Src-kinase inhibitors and cells derived from Abl-knockout mice, we demonstrate that both Src-family kinases and Abl are required for oxidative-stress-induced caveolin phosphorylation in a single cell type. These results provide evidence for a novel signalling complex containing both Abl and Fyn that is required for stress-induced caveolin phosphorylation, and suggest a novel mechanism through which Abl can attenuate the activity of Src-family kinases.

EXPERIMENTAL

Materials

All tissue culture reagents were purchased from Atlanta Biologicals (Norcross, GA, U.S.A.). Sugen 6656, PP2 and PP3 were purchased from Calbiochem. PD180970 was a gift from Dr Alan Kraker (Pfizer Global Research and Development, Ann Arbor, MI, U.S.A.). Antibodies were purchased as described: rabbit polyclonal anti-caveolin-1 antibodies from Transduction Laboratories (Lexington, KY, U.S.A.) and Santa Cruz Biotechnology (N20, Santa Cruz, CA, U.S.A.); monoclonal antibodies specific for phosphocaveolin (PY14) and phosphotyrosine (PY20 and 4G10) from Transduction Laboratories and Upstate Biotechnology Inc. (Lake Placid, NY, U.S.A.); rabbit polyclonal (Fyn 3) and mouse monoclonal (Fyn 15) antibodies against Fyn from Santa Cruz Biotechnology; rabbit polyclonal antibodies specific for Src (GD11) from Santa Cruz Biotechnology; horseradish-peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG antibodies from Bio-Rad Laboratories (Hercules, CA, U.S.A.).

Cell lines and growth conditions

Normal primary human foreskin fibroblast (HF) cells were a gift from Dr Greg Pari (University of Nevada, Reno, NV, U.S.A.). $Fyn^{-/-}$, $Yes^{-/-}$, $Src^{-/-}$ and control mouse fibroblasts were a gift from Dr Keith Burridge (University of North Carolina, Chapel Hill, NC, U.S.A.) [35]. $SYF^{-/-}$ (triple knockout) cells and control fibroblasts were a gift from Dr Jonathon Cooper (Fred Hutchinson Cancer Research Center, Seattle, WA, U.S.A.) [36]. $Abl^{-/-}$ fibroblasts and Abl^{+} fibroblasts were a gift from Dr Jean Wang (University of California, San Diego, CA, U.S.A.; [37]). Cells were maintained at 37 °C in DMEM (Dulbecco's modified Eagle's medium) (high-glucose), supplemented with 10 % (v/v) FBS (foetal bovine serum), 2 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin.

To express proteins in these cell lines, a recombinant adenoviral expression system was used. Recombinant adenovirus was generated essentially as described by He et al. [38], with modifications to improve the efficiency of recombination as described in [39]. The adenoviral vector that was used in these experiments expresses GFP (green fluorescent protein) in addition to the gene of interest from tandem CMV (cytomegalovirus) promoters. Therefore GFP fluorescence was used as a marker for infection, and multiplicity of infection and optimal viral-incubation times were determined using GFP fluorescence. Cells were infected at a multiplicity of infection of 1 (defined as the amount of virus needed for 100 % infection of cells) and were incubated for 48 h before experiments to allow maximal protein expression. Control adenovirus was generated from an empty adenoviral vector. Virus expressing Fyn was constructed using a coding sequence obtained from Dr Sara Courtneidge (Van Ardel Research Institute, Grand Rapids, MI, U.S.A.). Virus expressing Abl was constructed using a coding sequence from Dr Jean Wang.

Cellular-stress experiments

To induce oxidative stress, cells were treated with the indicated concentrations of H_2O_2 for 5 min. Cells were then washed twice with ice-cold PBS before harvesting. For hyperosmotic-stress experiments, cells were washed twice with warm PBS then were treated with iso-osmotic buffer (20 mM Hepes, pH 7.4, 80 mM NaCl, 470 μM KCl, 130 μM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 95 mM sorbitol) or hyperosmotic buffer (20 mM Hepes, pH 7.4, 80 mM NaCl, 470 μM KCl, 130 μM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose and 695 mM sorbitol) for 15 min. Cells were then washed twice with ice-cold PBS and harvested.

Cell lysates and immunoprecipitation experiments

Whole-cell lysates were prepared by resuspending the cells in solubilization buffer [10 mM Tris, pH 8.0, 150 mM NaCl, 1 %

(v/v) Triton X-100, 30 mM octylthioglucoside, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 1 mM benzamidine and 0.1 mM PMSF] and incubating on ice for 1–2 h. Triton X-100- and octylthioglucoside-insoluble material was removed by centrifugation for 10 min at 16 000 ***g*** in a microcentrifuge and the supernatants were retained. Immunoprecipitation of proteins and analysis of samples by immunoblotting was performed as described previously [33].

Sucrose density-gradient centrifugation

Cells infected with adenovirus were scraped into PBS and spun at 800 ***g*** to pellet. Cell pellets were resuspended in 25 mM Mes, pH 6.0, 150 mM NaCl and 0.2 % (v/v) Triton X-100, subjected to ten strokes with a Dounce homogenizer, then sonicated for three 10 s bursts at maximal output for a microtip. Lysates were mixed 1:1 with 80 % sucrose in 25 mM Mes, pH 6.0, and 150 mM NaCl, then layered with 2 ml each of 35 % sucrose, 25 % sucrose, 15 % sucrose and 5 % sucrose in 25 mM Mes, pH 6.0, and 150 mM NaCl. Gradients were centrifuged in an SW41 rotor for 20 h at 4 °C at 35 000 rev./min in a Beckman ultracentrifuge. After centrifugation, gradients were fractionated into 12 fractions of 1 ml from top to bottom. Fractions were analysed by Western blotting.

Two-dimensional gel electrophoresis

To enrich for phosphoproteins, anti-phosphotyrosine immunoprecipitations were performed as described [33]. Immunoprecipitated proteins were eluted from the beads with 5 mM phenyl phosphate. Proteins in the eluates were boiled in 1 % (w/v) SDS to disrupt caveolin oligomers, then were precipitated with chloroform and methanol. Samples were resuspended and analysed by two-dimensional gel electrophoresis following the manufacturer's instructions using 11 cm immobilized pH gradient (IPG) strips, pH 4–7 (Amersham Biosciences) for the first dimension and SDS/PAGE on 12 % gels for the second dimension.

RESULTS

Both osmotic-shock and oxidative-stress-induced phosphorylation of caveolin-1 require Src-family kinase activity

Hyperosmolarity and oxidants such as H_2O_2 induce phosphorylation of caveolin on Tyr^{14} [21,23]. We had previously observed that oxidative-stress-induced phosphorylation of caveolin required expression of the non-receptor tyrosine kinase Abl [34]. Volonte et al. [23] had previously implicated Src-family kinases in osmotic-shock-induced caveolin phosphorylation. Both Abl and Src are activated in response to cellular stresses [4,5,12,13] and both can phosphorylate caveolin-1 *in vitro* [32,33]. To examine whether or not there was a differential requirement for Src-family kinase activity in hyperosmotic- versus oxidative-stress-induced caveolin phosphorylation, we employed a small-molecule inhibitor of Src-family kinases, Sugen 6656 (Figure 1A). We pre-treated primary HFs with 10 μM Sugen 6656 or DMSO for 30 min and subsequently incubated the cells in hyperosmotic buffer for 15 min or 2 mM H_2O_2 for 5 min. Control cells were pre-treated with DMSO and were then incubated in an iso-osmotic buffer or in medium alone. Cell lysates were analysed by Western blotting using an antibody specific for caveolin-1 phosphorylated on Tyr^{14} (PY14). As anticipated, cells incubated in 2 mM H_2O_2 or in a hyperosmotic buffer in the presence of DMSO alone demonstrated a marked increase in phosphocaveolin as compared with non-stressed cells (Figure 1A). Unexpectedly, however, pre-treatment with Sugen 6656 completely blocked stress-induced caveolin phosphorylation in cells exposed to either hyperosmotic buffer or H_2O_2. To verify this result, we employed two other inhibitors of Src family kinases, PP2 and PD180970, which are structurally unrelated to Sugen 6656. Both inhibited caveolin phosphorylation in response to either oxidative stress or osmotic shock (results not shown).

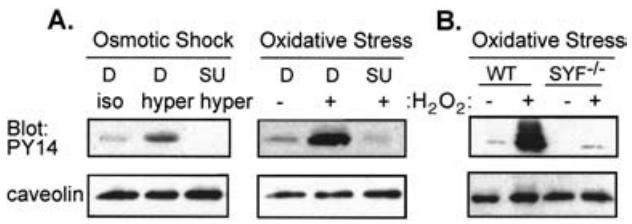


Figure 1 Src-family-kinase activity is required for both osmotic-shock- and oxidative-stress-induced phosphorylation of caveolin

(**A**) Primary HFs pre-treated with 10 μM Sugen 6656 (SU) or DMSO (D) for 30 min. (**B**) WT mouse fibroblasts or fibroblasts derived from a $SYF^{-/-}$ mouse. Cells were incubated in hyperosmotic (hyp) or iso-osmotic (iso) buffer for 15 min or in media with (+) or without (−) 2 mM H_2O_2 for 5 min. After incubation, cells were washed and lysed in solubilization buffer and were analysed by Western blotting with antibodies against phosphocaveolin (PY14) and caveolin.

To examine this in another way, we utilized a cell line derived from a knockout mouse deficient in three nearly ubiquitously expressed Src-family kinases, Src, Yes, and Fyn ($SYF^{-/-}$). $SYF^{-/-}$ cells and fibroblasts derived from a wild-type (WT) mouse were incubated in 2 mM H_2O_2 or medium alone (Figure 1B). Cellular lysates were prepared and analysed by Western blotting. Exposure of WT cells to oxidative stress stimulated caveolin phosphorylation. In contrast, $SYF^{-/-}$ cells failed to catalyse caveolin phosphorylation in response to stress. These results established that at least one of the three deficient Src-family kinases is required for stress-induced caveolin phosphorylation.

Fyn expression is necessary for stress-induced caveolin phosphorylation

Based on our result that $SYF^{-/-}$ cells fail to catalyse stress-induced caveolin phosphorylation, we examined each kinase independently using cell lines derived from single kinase knockout mice ($Src^{-/-}$, $Fyn^{-/-}$ and $Yes^{-/-}$). WT, $Fyn^{-/-}$ and $Src^{-/-}$ cells were incubated in hyperosmotic buffer or 2 mM H_2O_2 to generate cellular stress (Figure 2A). Lysates were prepared and analysed by Western blotting with antibodies against phosphocaveolin (PY14), caveolin and phospho-ERK. We found that both WT and $Src^{-/-}$ cells exhibited a marked increase in caveolin phosphorylation after treatment with H_2O_2 or hyperosmotic buffer. In fact, stress-induced caveolin phosphorylation was higher in the $Src^{-/-}$ cells than in the WT cells. In contrast, cells lacking Fyn kinase failed to catalyse caveolin phosphorylation after exposure to either hyperosmotic or oxidative stress. To investigate this further, WT, $Fyn^{-/-}$ and $Src^{-/-}$ cells were treated with increasing doses of H_2O_2 and analysed for phosphocaveolin content (Figure 2B). We found that caveolin was phosphorylated in a dose-dependent manner in WT and $Src^{-/-}$ cells in response to concentrations of H_2O_2 from 1 to 5 mM. In contrast, there was little to no phosphocaveolin detected in $Fyn^{-/-}$ cells, even at the highest dose of H_2O_2. Together, these results establish that

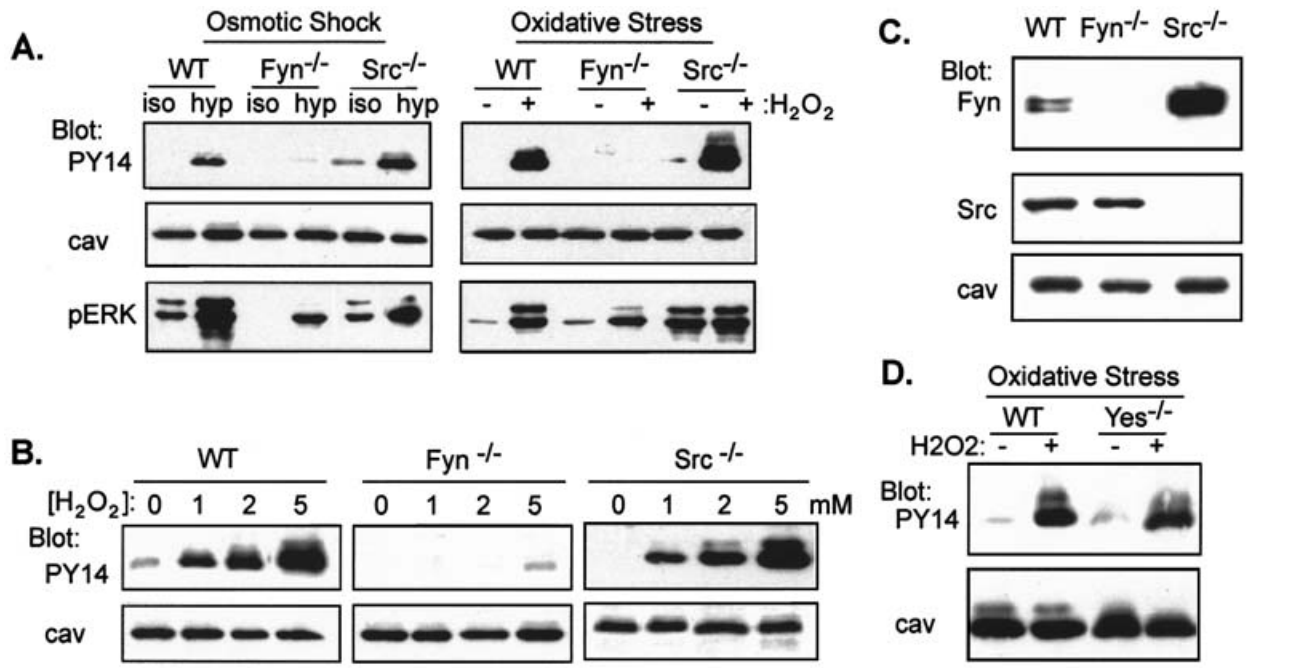


Figure 2 Expression of Fyn, but not Src or Yes, is required for induction of caveolin phosphorylation by both osmotic shock and oxidative stress

(**A**) Fibroblasts derived from WT mice or from Fyn$^{-/-}$ or Src$^{-/-}$ mice incubated in an iso-osmotic (iso) or a hyperosmotic (hyp) buffer or in medium with (+) or without (−) H_2O_2. (**B**) WT, Fyn$^{-/-}$ or Src$^{-/-}$ cells incubated in increasing concentrations of H_2O_2 ranging from 0 to 5 mM. (**C**) Levels of Fyn detected in anti-Fyn immunoprecipitates (top panel) and Src and caveolin in cell lysates (middle and bottom panels) from WT, Fyn$^{-/-}$ and Src$^{-/-}$ cells. (**D**) WT and Yes$^{-/-}$ cells incubated with (+) or without (−) H_2O_2. Cell lysates were analysed by Western blotting with antibodies against phosphocaveolin (PY14), caveolin (cav), activated ERK (pERK), Fyn or Src.

Fyn and not Src phosphorylates caveolin in response to cellular stress. The levels of expression of Fyn and Src in the WT, Fyn$^{-/-}$ and Src$^{-/-}$ cellular lysates were determined by Western blotting. We detected no Fyn in the Fyn$^{-/-}$ cells (Figure 2C, top panel). Interestingly, we found that Src$^{-/-}$ cells overexpress Fyn compared with WT cells. This overexpression of Fyn correlates with the increase in caveolin phosphorylation observed in the Src$^{-/-}$ cells compared with WT cells. We confirmed that Src$^{-/-}$ cells do not express Src (Figure 2C, middle panel). Src expression was not elevated in the Fyn$^{-/-}$ cells compared with WT cells. Finally, we examined the role of Yes kinase in stress-induced caveolin phosphorylation (Figure 2D). Yes$^{-/-}$ cells have a normal caveolin stress response as shown by an increase in phosphocaveolin in response to oxidative stress. These data indicate that Fyn may be the only Src-family kinase that phosphorylates caveolin-1 in response to cellular stress.

Cellular stress is known to initiate MAPK signalling cascades; ERK, JNK and p38 have all been implicated in stress signalling [1,2]. Specifically, ERK has been shown to be a major player in the activation of survival-signalling pathways in response to transient, moderate cellular stress and apoptotic-signalling pathways in response to long-term stress. Lack of phosphorylation of caveolin could either indicate a loss of the caveolin kinase specifically, or a complete inability of the cells to respond to cellular stress. To assess whether or not the Fyn$^{-/-}$ cells could still respond to stress, we examined phospho-ERK levels in each cell line (Figure 2A, bottom panel). We detected phosphorylated ERK in cells exposed to hyperosmotic stress, as well as oxidative stress, in WT cells, while phospho-ERK was elevated under basal conditions in the Src$^{-/-}$ cells. ERK was also phosphorylated in response to stress in Fyn$^{-/-}$ cells, although to a lesser extent. Therefore cells lacking Fyn expression are still responding to stress, but lack of Fyn specifically blocks stress-induced caveolin phosphorylation.

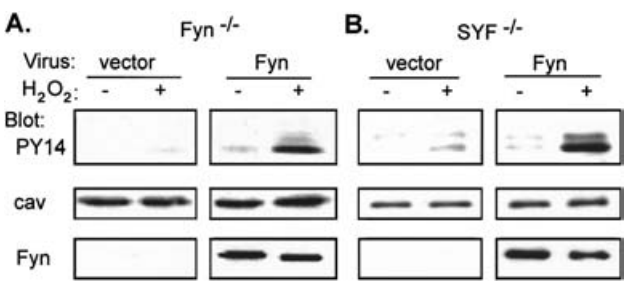


Figure 3 Expression of Fyn reconstitutes oxidative-stress-induced caveolin phosphorylation in both Fyn$^{-/-}$ and SYF$^{-/-}$ cells

Fyn$^{-/-}$ (**A**) or SYF$^{-/-}$ (**B**) cells were infected with adenovirus expressing GFP alone (vector) or GFP and Fyn (Fyn). At 2 days post-infection, cells were incubated in medium alone (−) or medium with H_2O_2 (+). Cells were lysed and lysates analysed by Western blotting with antibodies against phosphocaveolin (PY14), caveolin (cav) and Fyn.

Reconstitution of Fyn$^{-/-}$ and SYF$^{-/-}$ cells with Fyn is sufficient to restore stress-induced caveolin phosphorylation

If Fyn$^{-/-}$ and SYF$^{-/-}$ cells fail to catalyse phosphorylation of caveolin in response to stress due specifically to a loss of Fyn expression, then reconstitution of these cells with Fyn should restore stress-induced caveolin phosphorylation (Figure 3). We reconstituted Fyn$^{-/-}$ cells with Fyn using an adenoviral expression system (Figure 3A). Control cells were infected with an empty adenoviral vector. At 2 days post-infection, cells were left in a basal state or subjected to oxidative stress, and lysates were analysed by Western blotting. Vector-infected Fyn$^{-/-}$ cells remained non-responsive to cellular stress with no detectable phosphocaveolin, verifying that viral infection alone does not induce caveolin phosphorylation in these cells. Importantly,

reconstitution of cells with Fyn effectively restored stress-induced caveolin phosphorylation in $Fyn^{-/-}$ cells. To determine if Fyn could reconstitute caveolin phosphorylation in the absence of other Src kinases, Fyn expression was reconstituted in the $SYF^{-/-}$ cells. $SYF^{-/-}$ cells were infected with vector or Fyn expressing adenovirus and were then incubated in H_2O_2 to induce oxidative stress (Figure 3B). Infection of $SYF^{-/-}$ cells with empty-vector virus had no effect on caveolin phosphorylation in response to treatment with H_2O_2. However, reconstitution of $SYF^{-/-}$ cells with Fyn rescued stress-induced caveolin phosphorylation (Figure 3B). This result indicates that Fyn expression is sufficient for stress-induced caveolin phosphorylation in the absence of Src and Yes.

Fyn is activated, and is then rapidly inactivated, in response to oxidative stress

Overexpression of Fyn was sufficient to induce caveolin phosphorylation. When Fyn was overexpressed in normal (WT) mouse embryonic fibroblasts by infection with the Fyn adenovirus, the basal levels of phosphocaveolin increased to roughly the same level of phosphorylation as observed in response to cellular stress in vector-infected cells (Figure 4A, lanes 2 and 3). Significantly, oxidative stress led to hyperphosphorylation of caveolin in the Fyn-overexpressing cells (Figure 4A, lane 4), indicating that the activity of the expressed Fyn was stimulated further by stress. As we previously observed with Abl [34], overexpression of Fyn in primary HF cells was sufficient to induce very high levels of caveolin phosphorylation (Figure 4B, lysate, lane 2). To verify that the multiple bands observed in the lysate with the PY14 antibody were all due to phosphocaveolin, sequential immunoprecipitations were performed. First, anti-phosphotyrosine immunoprecipitations were performed to enrich for phosphoproteins. Incubation of lysates with anti-phosphotyrosine antibody completely immunodepleted the phosphocaveolin signal from the supernatants (Figure 4B, αpTyr super, lane 4), which was recovered in the phenyl phosphate eluates (Figure 4B, lane 6). The phosphocaveolin bands in the eluates could be completely immunodepleted by immunoprecipitation with a caveolin-1-specific antibody (Figure 4B, αCav super, lane 8) and were recovered in the immunoprecipitates (Figure 4B, αCav ippt, lane 10), demonstrating that they were all phosphorylated caveolin-1. The gel-shifted forms of caveolin are due to phosphorylation, and can be completely collapsed by treatment with phosphatase [40]. Two-dimensional gel analysis indicates that there are six sites phosphorylated on caveolin-1 in the Fyn-overexpressing cells (Figure 4C). Only one of these sites is on a tyrosine residue, Tyr^{14} [32,34,40].

Activation and inactivation of Src-family kinases occurs through tyrosine phosphorylation on either stimulatory or inhibitory tyrosine residues. Autophosphorylation on Tyr^{416} in Src leads to full activation of the kinase. We confirmed that oxidative stress activates Fyn by analysis of Fyn immunoprecipitates with phospho-specific antibodies against the active site of Src-family kinases (Figure 5A, pY416). Under basal conditions, no active-site phosphorylation was detected in the anti-Fyn immunoprecipitates, indicating that Fyn is largely inactive. This site was phosphorylated after incubation in H_2O_2 to induce oxidative stress. We observed no signal in immunoprecipitates from $Fyn^{-/-}$ cells demonstrating the specificity of the pY416 antibody. A non-phospho-dependent antibody verified roughly equal immunoprecipitation of Fyn from the two WT lysates and no immunoprecipitation from $Fyn^{-/-}$ cells.

A simple mechanism to explain the difference in the requirement for Src and Fyn in caveolin phosphorylation would be

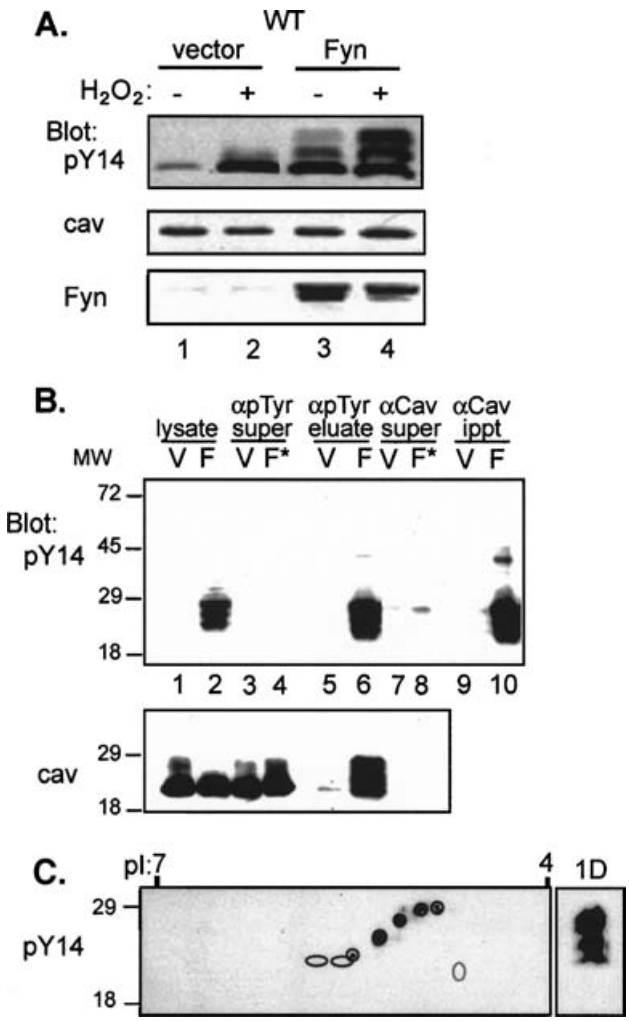


Figure 4 Overexpression of Fyn is sufficient to induce caveolin phosphorylation and hyperphosphorylation in response to stress

(**A**) Cell lysates from WT mouse embryonic fibroblasts infected with empty vector or Fyn-expressing adenovirus. Cells were incubated in medium alone (−) or medium with H_2O_2 (+). (**B**) Sequential immunoprecipitations from primary HF cells infected with empty vector (V) or adenovirus expressing Fyn (F). To enrich for phosphorylated proteins, anti-phosphotyrosine immunoprecipitations were performed, and bound proteins were eluted with phenyl phosphate. Anti-caveolin immunoprecipitations were then performed on the eluates. Shown are the original lysates (lanes 1 and 2), the supernatants after anti-phosphotyrosine immunoprecipitation (αpTyr super, lanes 3 and 4), the phenyl phosphate eluates (lanes 5 and 6), and the supernatants (αCav super, lanes 7 and 8) and pellets [αCav immunoprecipitate (ippt), lanes 9 and 10] after anti-caveolin immunoprecipitation. (**C**) Two-dimensional gel electrophoresis phenyl phosphate eluates from the anti-phosphotyrosine immunoprecipitates. Samples were analysed by Western blotting with antibodies against phosphocaveolin (PY14), caveolin (cav) and Fyn. A sample lane of the original eluate was added during the second-dimension SDS/PAGE separation step as a control (1D).

selective activation of Fyn in response to stress. To determine if Src as well as Fyn is activated in response to oxidative stress, we repeated the same experiments, immunoprecipitating Src. We found that phosphorylation on the active site of Src also increased in response to oxidative stress, indicating that, in these cells, Src is activated in response to cellular stress (Figure 5B, pY416). These results are in agreement with a previous report that showed Src is required for oxidative-stress-mediated activation of BMK1 [4], and show that differential activation does not account for their differential requirements in caveolin phosphorylation.

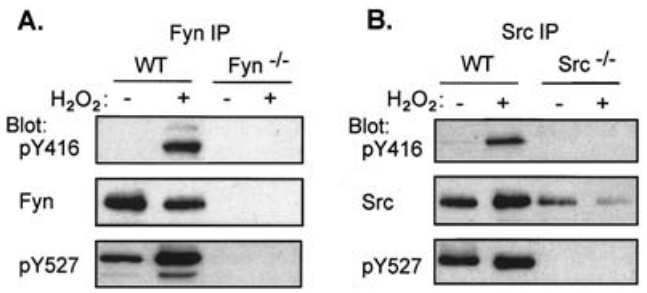


Figure 5 Fyn is auto-activated then rapidly inactivated by Csk in response to oxidative stress

Fyn (**A**) or Src (**B**) immunoprecipitates from WT, Fyn$^{-/-}$ or Src$^{-/-}$ cells. Immunoprecipitates were analysed by Western blotting with phospho-specific antibodies against Src phosphorylated on its autophosphorylated activation site, Tyr^{416} (pY416), or at its inhibitory Csk site, Tyr^{527} (pY527). Immunoprecipitates were also analysed for total levels of Fyn or Src.

Inactivation of Src family kinases is achieved through phosphorylation on an inhibitory tyrosine residue. Phosphorylation on the inhibitory residue (Tyr^{527} in Src) triggers an intramolecular interaction between the C-terminal tail and the SH2 domain of the kinase. We have recently described an inhibitory feedback loop in which activation of Src-family kinases leads to caveolin phosphorylation. This in turn leads to recruitment and activation of Csk in caveolae via direct binding to phosphocaveolin [17]. Recruitment of Csk leads to selective phosphorylation of resident Src-family kinases, attenuating Src-kinase activity in these structures. Consistent with this model, we found that oxidative stress also increased phosphorylation of the inhibitory site of Fyn indicating that activation of Fyn is rapidly followed by inactivation (Figure 5A, pY527). Importantly, we found that, in contrast to Fyn, phosphorylation on the inhibitory site of Src was not increased by stress, although Src was constitutively phosphorylated on this residue (Figure 5B, pY527). Since caveolin phosphorylation recruits Csk to caveolae membranes and Csk phosphorylates Src as well as Fyn, these data suggest that Src is not co-localized with the pool of Fyn that is activated and the pool of caveolin that is phosphorylated in response to oxidative stress. This would account for our finding that Fyn, and not Src, is required for caveolin phosphorylation under conditions of cellular stress. Our data indicate that Src and Fyn can be differentially regulated in cells.

Overexpression of Fyn leads to phosphorylation of caveolin and recruitment of Csk to caveolae-enriched complexes

Overexpression of Fyn in HF cells is sufficient to induce high levels of caveolin phosphorylation in these cells (Figure 4B). Our model predicts that increased caveolin phosphorylation should cause the translocation of a pool of Csk to caveolin-enriched fractions. Caveolae-enriched cell fractions were isolated by flotation on sucrose density gradients, based on their low-density and resistance to detergent solubilization. After centrifugation, 12 fractions of 1 ml were collected (starting from the top of the gradient) and fractions analysed by Western blotting. The caveolae-enriched cell fractions were identified as fractions 4, 5 and 6 by the marker protein caveolin and the appearance of a band of flocculent material at the 5%/15% sucrose interface. The caveolae-enriched fractions contain very low levels of total protein, with nearly all of the cellular protein remaining at the bottom of the gradient in the detergent-soluble protein fractions 9–12 (results not shown). Overexpression of Fyn did not

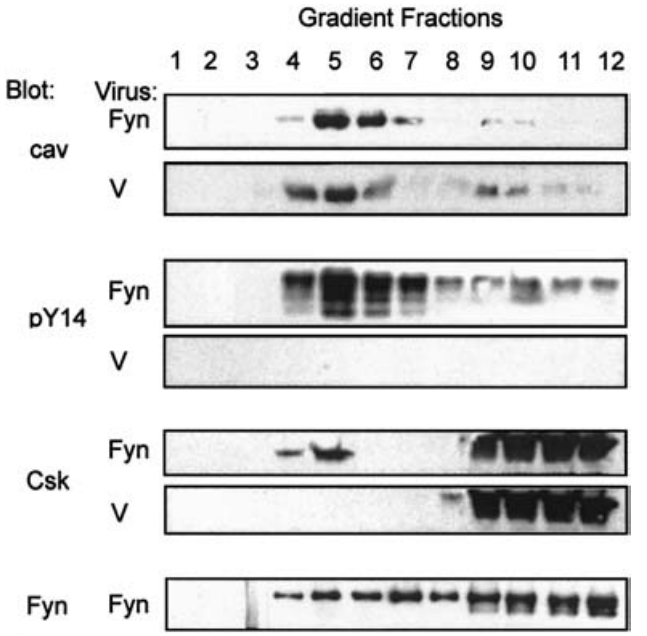


Figure 6 Overexpression of Fyn induces hyperphosphorylation of caveolin-1 and translocation of Csk to caveolin-enriched low-density complexes

Primary HF cells were infected with empty-vector adenovirus (V) or Fyn virus. Cell lysates were fractionated by sucrose density centrifugation to isolate low-density, detergent-resistant complexes. Gradients were fractionated from top to bottom into 12 fractions of 1 ml. Equal volumes of each fraction were analysed by Western blotting with antibodies against caveolin (cav), phosphocaveolin (pY14), C-terminal Src kinase (Csk) and Fyn (Fyn). Fractions 4, 5 and 6 contain low-density, detergent-resistant complexes and very little protein. Fractions 9, 10, 11 and 12 contain the Triton-X-100-solublized proteins and the majority of the protein in the cell lysate.

change caveolin expression or distribution compared with vector-infected cells (Figure 6, cav). Overexpression of Fyn led to phosphorylation of caveolin on Tyr^{14} (pY14). The phosphocaveolin distributed primarily to the low-density fractions. No phosphocaveolin was detected in vector-infected cells. The overexpressed Fyn was also localized to the low-density fractions, but unlike caveolin, it was also found in other fractions (Figure 6, Fyn). Most importantly, overexpression of Fyn led to the recruitment of a pool of Csk from the soluble-protein fractions into the phosphocaveolin-enriched fractions of the gradient, whereas no Csk was detected in these fractions from vector-infected cells (Csk). The amount of Csk detected in the caveolin-enriched fractions of the Fyn gradient represents an enrichment over the soluble fractions when corrected for total protein. The distribution of Csk in the gradients was much more restricted than Fyn. This indicates that it is a substrate of Fyn and not Fyn itself that is directing the translocation of Csk. Consistent with this, it has been shown that Csk must interact with a membrane-associated phosphoprotein to be targeted to its substrates, and that Csk does not bind to the Src-family kinases themselves [25,26]. Phosphocaveolin and Fyn itself were the two major tyrosine-phosphorylated proteins detected in the caveolae fractions from the Fyn-overexpressing cells (results not shown), and we have shown previously that Csk binds to phosphocaveolin [17]. These data strongly suggest that it is the Fyn-catalysed phosphorylation of caveolin that induces translocation of Csk to caveolin-enriched membranes. We have also observed translocation of Csk when cells are exposed to oxidative stress (H. Cao, A. R. Sanguinetti and C. C. Mastick, unpublished work).

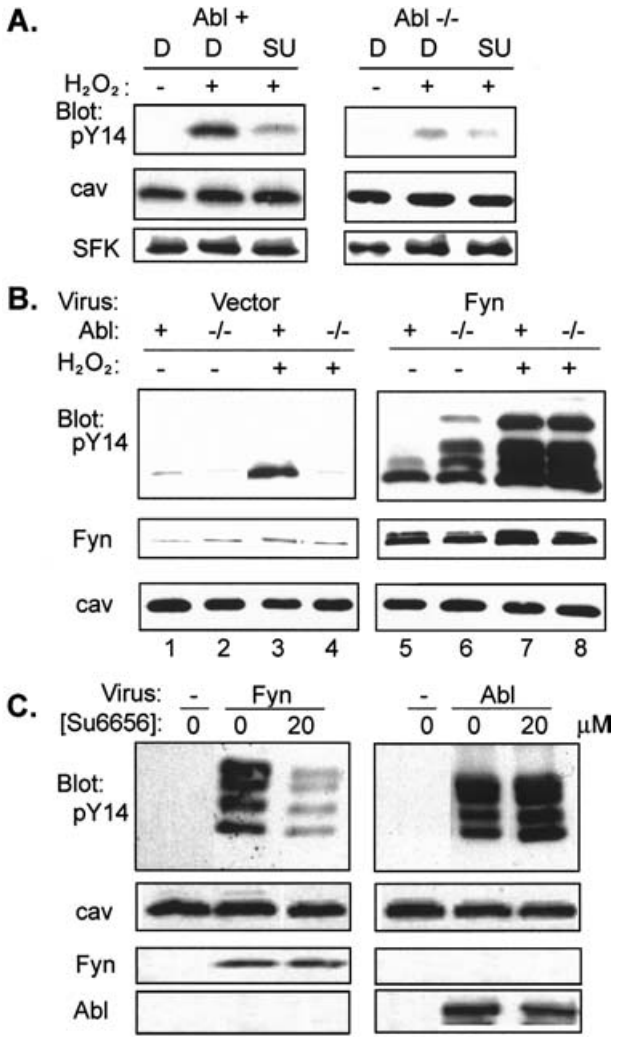


Figure 7 Oxidative-stress-induced caveolin phosphorylation requires both Fyn and Abl

Cells derived from Abl$^{-/-}$ mice or the same cells infected with a retrovirus expressing Abl (Abl^{+}) were (**A**) pre-treated with 10 μM Sugen 6656 (SU) or DMSO (D) for 30 min, or (**B**) infected with empty vector or Fyn-expressing adenovirus for 2 days, and then were incubated with (+) or without (−) 2 mM H_2O_2 for 5 min. (**C**) HF cells were infected with empty vector (−) or adenovirus expressing Fyn or Abl for 2 days, then were incubated with Sugen 6656 for 2 h. After incubation, cells were washed and lysed in solubilization buffer and were analysed by Western blotting with antibodies against phosphocaveolin (PY14), caveolin (cav), Src-family kinases (SFK), Fyn or Abl, as indicated.

Oxidative stress-induced caveolin phosphorylation requires both Src-kinase and Abl activity

We had previously shown that oxidative stress failed to induce caveolin phosphorylation in cells derived from Abl-knockout mice [34]. Reconstitution of Abl expression in these cells via stable retroviral-mediated transfection restored oxidative-stress-induced caveolin phosphorylation. In the present paper, we show, using inhibitors and cells derived from knockout animals, that Fyn expression is also required for oxidative-stress-induced caveolin phosphorylation. To determine whether both Fyn and Abl activities are required in a single cell type, we analysed the effect of Src-kinase inhibitors on caveolin phosphorylation in Abl-reconstituted cells (Figure 7A). Pre-treatment of Abl^{+} cells with SU6656 abolished oxidative-stress-induced caveolin phosphorylation, indicating that Abl and Fyn co-operate in some way in this pathway. One mechanism to explain the requirement for both Abl and Fyn would be that they act sequentially in a single pathway. For example, Fyn might be required for activation of Abl, and Abl might actually be the stress-induced caveolin-kinase (stress → Fyn → Abl → phosphocaveolin). This model predicts that Fyn would require Abl expression to induce caveolin phosphorylation. To test this, we overexpressed Fyn in the Abl$^{-/-}$ cells and these same cells reconstituted with Abl (Figure 7B). Oxidative stress induced caveolin phosphorylation in the Abl reconstituted cells (Figure 7B, lanes 1 and 3). In contrast, vector-infected Abl$^{-/-}$ cells showed no increase in caveolin phosphorylation in response to oxidative stress (Figure 7B, lanes 2 and 4). As was previously observed in WT mouse embryonic fibroblasts, overexpression of Fyn in the Abl^{+} cells was sufficient to increase basal phosphocaveolin levels to the same level as the oxidative-stress-induced levels in the vector infected cells (Figure 7B, lane 5). However, inconsistent with the proposed linear pathway, Fyn-overexpression-induced caveolin phosphorylation did not require expression of Abl. In fact, it was significantly higher in the Abl$^{-/-}$ cells (Figure 7B, lane 6) than in the Abl^{+} cells, indicating that Abl plays a negative regulatory role for Fyn.

An alternative explanation for the requirement for both kinases is that Abl activation may be required for activation of Fyn (stress → Abl → Fyn → phosphocaveolin). However, inconsistent with this model, oxidative stress induced very high levels of hyperphosphorylation of caveolin in both the Abl$^{-/-}$ and the Abl^{+} cells when Fyn was overexpressed in these cells (Figure 7B, lanes 7 and 8), indicating that Abl is not required for stress-induced activation of Fyn. To test whether Src-kinase activity was required downstream of Abl, the effect of the Src inhibitors on Abl-induced caveolin phosphorylation was examined (Figure 7C). HF cells were infected with control virus or adenovirus expressing Abl or Fyn. After 2 days, the cells were treated with SU6656 for 2 h, then lysates were analysed by Western blotting. While SU6656 blocked Fyn-induced caveolin phosphorylation, Abl-induced phosphorylation was unaffected. These results indicate that Abl, like Fyn, acts directly on caveolin and does not require activation of a downstream kinase.

DISCUSSION

In this report, we show by several means that activity of Src-family kinases is required for caveolin phosphorylation after exposure to either oxidative stress or hyperosmotic stress. The Src-family-kinase inhibitor Sugen 6656 abolished caveolin phosphorylation on Tyr^{14} in response to oxidative stress and hyperosmotic stress (Figure 1). In addition, cells lacking expression of three of the Src-family kinases, Src, Yes and Fyn, failed to catalyse caveolin phosphorylation in response to oxidative stress. We explored the requirement for each of these kinases individually using cell lines derived from Src-, Yes- or Fyn-knockout mice. We found that of the three single knockout cell lines, only the cells lacking Fyn failed to catalyse caveolin phosphorylation in response to oxidative stress or osmotic shock (Figure 2). The Fyn$^{-/-}$ cells were still able to respond to stress, as indicated by an increase in the phosphorylation of ERK. These data indicated that Fyn is a stress-activated caveolin kinase. A previous report had implicated Src as the hyperosmotic-stress-induced caveolin kinase [23]. However, this was based on experiments utilizing dominant-negative, kinase-inactive Src to block phosphorylation. It has been shown that dominant-negative Src will block the activation of Src, Yes and Fyn [41], therefore the results from these two studies are consistent. Using the same lines of knockout cells, Kapus et al.

[5,6] showed that Fyn, but not Src, is required for hyperosmotic-stress-induced phosphorylation of cortactin, a protein that is important for the rearrangement of the cortical actin cytoskeleton. Phosphorylation of cortactin in response to hyperosmotic stress was blocked in cells lacking Fyn, but not Src. Fyn, but not Src, was also shown to be required for stress-induced activation of JAK2, Ras, p90 ribosomal S6 kinase, and the osmotic-response-element-binding protein [7,10,11]. Together, these data indicate an important role for Fyn in stress-induced signalling pathways.

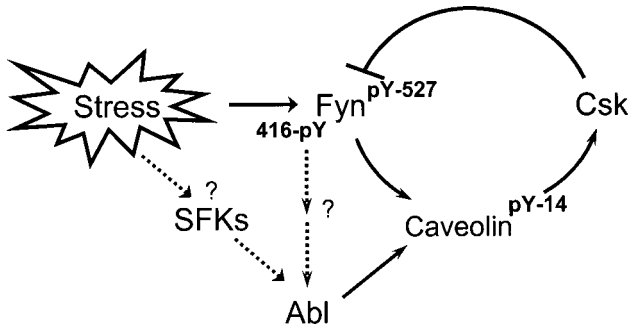
To verify that $Fyn^{-/-}$ cells and $SYF^{-/-}$ cells fail to phosphorylate caveolin in response to stress specifically due to their lack of Fyn expression, we reconstituted expression of Fyn in each of these cell lines. Expression of Fyn restored stress-induced caveolin phosphorylation in both Fyn-deficient cell lines (Figure 3). Therefore Fyn is sufficient for caveolin phosphorylation in response to cellular stress in the absence of Src and Yes. Overexpression of Fyn in mouse embryonic fibroblasts derived from either WT (Figure 4A) or Abl-deficient mice (Figure 7B) was sufficient to induce caveolin phosphorylation and lead to hyperphosphorylation of caveolin after exposure to oxidative stress. We also observed very high levels of hyperphosphorylation of caveolin in primary HF cells overexpressing Fyn (Figures 4B and 7C). However, a previous study using COS cells [31] reported that overexpression of Src, but not Fyn, was sufficient to increase basal caveolin phosphorylation. Based on these results, the authors concluded that Src, but not Fyn, is a caveolin kinase. There are two possible reasons for the discrepancy in the data from the two laboratories. One could be that the levels of overexpression were different in the two studies. Alternatively, it could represent a cell-type difference. We previously reported that stable overexpression of Fyn in 3T3-L1 adipocytes increased basal phosphorylation of caveolin, and led to hyperphosphorylation of caveolin in response to insulin [20]. In the same study, we observed that caveolin phosphorylation was not elevated in the transfected fibroblast cells before differentiation, and there was no insulin-stimulated caveolin phosphorylation in either the control or transfected fibroblasts. These results indicate that different cell types have different intrinsic abilities to regulate Src-kinase activity. We also observed cell-type differences in the ability to regulate Abl-kinase activity. Overexpression of Abl in primary HF cells leads to very high levels of phosphorylation of caveolin (Figure 7C; [34]). However, overexpression of Abl in a number of different mouse embryonic fibroblast cell lines, or in NIH-3T3 cells, did not lead to elevated caveolin phosphorylation (results not shown). Our data indicate that, in the context of cellular stress, Fyn, and not Src, is the caveolin kinase. However, they do not rule out the possibility that Src acts as a caveolin kinase under other types of stimuli, for example during cell attachment and spreading on fibronectin.

In cell lines that overexpress Fyn, we saw hyperphosphorylation of caveolin after induction of oxidative stress, indicating activation of this kinase (Figure 4A). We verified activation of Fyn in response to oxidative stress as indicated by an increase in phosphorylation on its activation site (Figure 5A). Consistent with these findings, a number of other studies have shown activation of Fyn or a requirement for Fyn in response to osmotic stress [5,7] and oxidative stress [10,11]. However, Src is also required for stress-induced responses in cells, notably for the activation of BMK1 [4,9]. Consistent with this finding, we found that Src is also activated in response to oxidative stress in mouse embryonic fibroblasts (Figure 5B), but activation of Src in the absence of Fyn is insufficient to induce caveolin phosphorylation in these cells (Figure 2). Therefore differential activation of Src and Fyn in response to stress is not the reason that there is a difference in the requirement for these two kinases in stress-induced caveolin phosphorylation.

Our previous report indicated that caveolin phosphorylation would recruit Csk to caveolae, which would phosphorylate and inactivate resident Src-family kinases [17]. Consistent with this, activation of Fyn and subsequent phosphorylation of caveolin is followed rapidly by phosphorylation of Fyn on its inhibitory Csk site (Figure 5A). These data support the idea that Fyn and caveolin are co-localized in cells, and that oxidative stress recruits Csk to these complexes (Csk is the only kinase known to phosphorylate Src-family kinases at the C-terminal site). Csk phosphorylates both Src and Fyn equally well. However, the phosphorylation of Src by Csk did not increase after oxidative stress (Figure 5B), indicating that Src may not be co-localized with the pool of Fyn that is activated and the pool of caveolin that is phosphorylated in response to oxidative stress. This differential localization may explain the differences in requirement for these two kinases in response to stress. We are currently investigating this possibility further.

Fyn is targeted to caveolae/lipid rafts due to post-translational modification by myristate and subsequently by palmitate. Addition of palmitate is essential for co-fractionation of both Fyn and other proteins with caveolin. Conversely, kinases such as Src that are only singly acylated with a myristate moiety have less affinity for membranes and do not co-purify with caveolae/lipid rafts, as determined by a number of different biochemical methods to study these domains [27,28]. Although suggestive, biochemical fractionation techniques do not prove or disprove co-localization. Co-localization of dually acylated proteins such as Lyn (or Fyn) with caveolin has recently been confirmed using FRET (fluorescence resonance energy transfer) [42]. Importantly, FRET occurs only between fluorophores that are 1–10 nM apart. Although it was shown that geranyl/geranylated proteins are not co-localized with caveolin, the behaviour of singly acylated proteins, such as Src, was not determined. A number of reports have shown direct binding of Src to caveolin, which would be an alternative method for localization to caveolae. This association inhibits the activity of Src [29]. Our data do not rule out co-localization of Src and caveolin in cells. Instead, they suggest that there are distinct signalling complexes containing caveolin and Src or caveolin and Fyn that are differentially activated in response to stress.

We have identified a requirement for two different non-receptor tyrosine kinases, Abl and Fyn, in oxidative-stress-induced caveolin phosphorylation. Cell lines lacking expression of either kinase fail to phosphorylate caveolin in response to oxidative stress (Figures 2 and 7; [34]), overexpression of either kinase can induce caveolin phosphorylation (Figure 7C), and Src-kinase inhibitors block stress-induced caveolin in cells where this process has previously shown to be Abl-dependent (Figure 7A). The simplest explanation for the requirement of both kinases would be a linear pathway in which activation of one kinase was required for activation of the next kinase, which is required for the phosphorylation of caveolin. However, our data indicate that this is not the case. Activation of Fyn by overexpression leads to increased caveolin phosphorylation (Figures 4 and 7). This does not require expression of Abl (Figure 7B), indicating that Abl is not an obligate downstream kinase for Fyn. Conversely, overexpression of Abl in primary HF cells also leads to increased caveolin phosphorylation, and this does not require Src-kinase activity (Figure 7C), indicating that Fyn is not an obligate downstream kinase for Abl. Also inconsistent with these models, activation of Fyn by oxidative stress does not require expression of Abl (Figure 7B). We are currently investigating whether or not activation of Abl in response to oxidative stress requires Src-kinase activity. However, it has been shown that Src-kinase activation is required for activation of



Scheme 1 Model for the requirement of both Fyn and Abl in stress-induced caveolin phosphorylation

Exposure of cells to oxidative stress leads to activation of Fyn and autophosphorylation at Tyr^{416} (416-pY). The activated Fyn is resident in caveolae, and phosphorylates the caveolar-coat protein caveolin-1 on Tyr^{14} (pY-14). This phosphorylation leads to the recruitment and activation of Csk, a negative regulator of Src-family kinases (SFKs) including Fyn. This leads to the phosphorylation of Fyn on its inhibitory site, Tyr^{527} (pY-527), and attenuation of both the kinase activity and caveolin phosphorylation. At the same time, oxidative stress leads to the activation of Abl and recruitment into caveolae, through a process that may require activation of Fyn or another SFK. This leads to the stable phosphorylation of caveolin and the stable inhibition of Fyn through Csk.

Abl in response to PDGF (platelet-derived growth factor) [43]. Additionally, expression of activated Src or Fyn leads to activation of Abl, and Src and Fyn kinases can directly phosphorylate Abl *in vitro*. Furthermore, Abl lies downstream of Src kinase activation in growth-factor-induced c-myc expression, DNA synthesis and membrane ruffling [43,44].

Our current model is that Fyn and Abl act synergistically in the phosphorylation of caveolin (Scheme 1). Activation of Fyn in caveolae by oxidative stress leads directly to caveolin phosphorylation. This leads to the recruitment and activation of Csk, which attenuates Src-kinase activity, and limits the extent of phosphorylation of caveolin. At the same time, oxidative stress induces the activation of Abl and/or the translocation of Abl to caveolae where Abl also phosphorylates caveolin-1. Abl is not regulated by Csk, and would remain active in these complexes, maintaining a high level of caveolin phosphorylation. This represents a novel mechanism through which Abl might regulate Src-family-kinase activity: phosphorylation of a common scaffolding protein and recruitment of Csk. Paxillin, a substrate of both Abl and Src-family kinases, organizes a similar regulatory complex containing Abl, Src and Csk [45]. Csk is recruited to this complex after phosphorylation of paxillin on Tyr^{118}.

Why are both Abl and Fyn required for caveolin phosphorylation? Our model would predict that, at least in the case of the Fyn-deficient cells, activation of Abl by stress would lead to caveolin phosphorylation. It is possible that Fyn is required for stress-induced Abl activation, explaining the requirement for both kinases in caveolin phosphorylation. Alternatively, Fyn-catalysed phosphorylation of caveolin may be required for recruitment of Abl as well as Csk to caveolae. Tyr^{14} in caveolin-1 is in the context of Tyr-Xaa-Xaa-Pro, a consensus binding site for the Abl SH2 domain. Additionally, phosphocaveolin is co-immunoprecipitated with epitope-tagged Abl when this kinase is overexpressed in HF cells (results not shown). A third possibility is that the phosphorylation of caveolin by either kinase alone may simply be much less than the phosphorylation when both kinases are activated, and our methods of detection may not be sensitive enough to detect low-level phosphorylation induced by a single kinase. Interestingly, Fyn and Abl are required for opposing stress-induced pathways in cells. Activation of Fyn is required for the induction of survival pathways [10,11], whereas activation of Abl induces cell death [14,15]. Activation of Fyn and Abl are also temporally different. Fyn is activated rapidly, while Abl activation takes longer (maximal activation after 30 min). Low-level exposure to oxidative stress (low concentration or short duration) stimulates survival pathways, whereas higher doses or longer exposures induce apoptotic pathways [1,2]. It is possible that low-level oxidative stress activates only Src-family kinases, hence survival pathways, whereas higher exposures are required to activate Abl and apoptotic pathways. Abl-induced recruitment of Csk to complexes containing Src-family kinases (i.e. to phosphocaveolin or paxillin) would be a mechanism to ensure that survival pathways are turned off, allowing apoptosis to proceed. A similar mechanism may explain the observation that Abl and Src-family kinases have opposing effects on regulation of the actin cytoskeleton and cell migration [46]. Counter-regulatory effects of Abl and Src-family kinases have been observed in a number of additional signalling pathways and negative-regulatory pathways involving Abl, Fyn and their substrates have been identified genetically in second-site repressor screens in *Drosophila* [44,47–50]. Our work indicates that this regulatory circuit may work in part through stable activation and recruitment of Csk.

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