Metalloprotease–disintegrin ADAM 12 binds to the SH3 domain of Src and activates Src tyrosine kinase in C2C12 cells

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ADAM 12, a member of the ADAM (protein containing **a** **d**isintegrin **a**nd **m**etalloprotease) family of metalloprotease–disintegrins, has been implicated in the differentiation and fusion of skeletal myoblasts, and its expression is dramatically up-regulated in many cancer cells. While the extracellular portion of ADAM 12 contains an active metalloprotease and a cell-adhesion domain, the function of the cytoplasmic portion is much less clear. In this paper, we show that the cytoplasmic tail of ADAM 12 mediates interactions with the non-receptor protein tyrosine kinase Src. The interaction is direct, specific, and involves the N-terminal proline-rich region in the cytoplasmic tail of ADAM 12 and the Src homology 3 (SH3) domain of Src. ADAM 12 and Src co-immunoprecipitate from transfected C2C12 cells, suggesting that the two proteins form a complex *in vivo*. Co-expression of Src and ADAM 12, but not ADAM 9, in C2C12 cells results in activation of the recombinant Src. Moreover, endogenous ADAM 12 associates with and activates endogenous Src in differentiating C2C12 cells. These results indicate that ADAM 12 may mediate adhesion-induced signalling during myoblast differentiation.

Key words: co-immunoprecipitation, myogenesis, protein–protein interaction, signal transduction.

INTRODUCTION

The ADAMs (proteins containing **a** **d**isintegrin **a**nd **m**etalloprotease) are cell surface receptors that have been implicated in cell adhesion, communication and fusion [1–3]. To date, almost 30 members of the ADAM family have been identified in a variety of animal species. The characteristic feature of ADAM proteins is their unique domain organization, consisting of a pro-domain, metalloprotease, disintegrin-like and cysteine-rich domains and, in most cases, epidermal growth factor-like, transmembrane and cytoplasmic domains. ADAM 12 is expressed primarily in skeletal muscle, where it has been postulated to promote the differentiation and fusion of myoblasts [4–6]. Indeed, the spatio–temporal pattern of expression of ADAM 12 in mouse embryos suggests its involvement in myogenesis [5]. In the myoblastic cell line C2C12, ADAM 12 is up-regulated at the onset of cell differentiation [4,7]. Overexpression of an ADAM 12 fragment extending from the disintegrin domain to the cytoplasmic domain leads to accelerated fusion of C2C12 myoblasts, whereas transfection of the cells with the same construct in an antisense orientation results in inhibition of fusion [4]. ADAM 12 is also found, at lower levels, in placenta [6], bone [8] and macrophage-derived giant cells and osteoclasts [9]. Moreover, ADAM 12 expression is dramatically up-regulated in many tumour cell lines [10].

Recent studies have shed some light on the function of the extracellular portions of several ADAMs, including ADAM 12. The metalloprotease domains of ADAMs 9, 10 and 17 and a member of the ADAMTS (ADAM with thrombospondin motifs) subfamily are involved in cell surface proteolysis and shedding of the ectodomains of several membrane proteins ([1,11], and references therein). ADAM 12 appears to be an active metalloprotease as well [12], but its physiological substrates have not been identified. In addition, the disintegrin-like and/or cysteine-rich domains of ADAMs 1 [13], 2 [14–17], 9 [18], 12 [10,19,20], 15 [21,22] and 23 [23] have been shown to support cell–cell adhesion and communication.

The function of the cytoplasmic portions of ADAM proteins is much less clear. The cytoplasmic tails of ADAM 9 and ADAM 15 bind endophilin I and a novel Src homology 3 (SH3) domain- and Phox homology (PX) domain-containing protein, SH3PX1 [24]. ADAM 17 (tumour necrosis factor α convertase; TACE) interacts with mitotic-arrest-deficient 2 protein (MAD2), and ADAM 9 binds to a novel MAD2-related protein [25]. Recently the cytoplasmic domain of ADAM 12 has been shown to bind α-actinin-2 [7] and α-actinin-1 (Y. Cao and A. Zolkiewska, unpublished work), suggesting a link between ADAM 12 and the actin cytoskeleton.

The cytoplasmic domain of ADAM 12 contains eight copies of the sequence PXXP (where X denotes any amino acid residue). The PXXP motif constitutes a core consensus sequence that is required for binding to SH3 domains [26,27]. Amino acids flanking the PXXP region confer the binding specificity for individual SH3 domains [28–30]. SH3 ligands can bind in either an N $\rightarrow$ C (class I) or a C $\rightarrow$ N (class II) orientation [31,32]. The minimal consensus binding motifs for the SH3 domain of Src have been determined as RXXPXXP (class I ligands) or PXXPXR (class II ligands [31]). Both of these motifs are present in the cytoplasmic tail of ADAM 12 (see Figure 1, below).

Src (also called c-Src), the product of the cellular *src* gene, is the prototype member of a family of non-receptor protein tyrosine kinases that regulate cellular responses to extracellular stimuli [33–35]. Src contains a unique N-terminal region, an SH3 domain, an Src homology 2 (SH2) domain, a catalytic (tyrosine kinase) domain and a short C-terminal tail. The SH3 domain is important for inter- as well as intra-molecular interactions which regulate Src catalytic activity, cellular localization and recruitment of protein substrates [33–36].

Abbreviations used: ADAM, protein containing **a** **d**isintegrin **a**nd **m**etalloprotease; SH3, Src homology 3; SH2, Src homology 2; GST, glutathione S-transferase; CBP, calmodulin-binding peptide; DMEM, Dulbecco's modified Eagle's medium.

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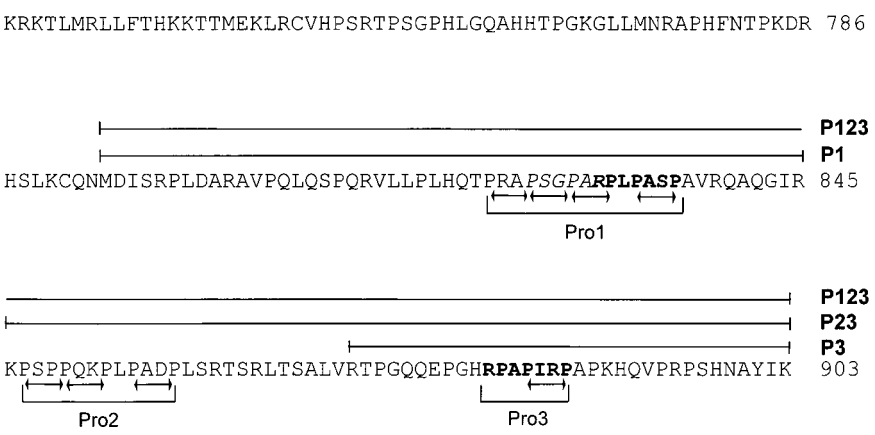

Figure 1 Amino acid sequence of the cytoplasmic tail of mouse ADAM 12

The eight minimal consensus PXXP motifs that are recognized by SH3 domains are indicated by the symbol ↔. Pro1, Pro2 and Pro3 indicate the three regions in which PXXP motifs are grouped. Two sites within the Pro1 and Pro3 regions that conform to the consensus sequence of class I ligands for Src SH3 (RXXPXXP) are shown in **bold**. A site within the Pro1 region that matches the consensus sequence of class II Src SH3 ligands (PXXPXR) is shown in *italics*. P123, P23, P1 and P3 are different fragments of the ADAM 12 cytoplasmic domain that have been generated and used in this study. P123 (residues 794–903) spans Pro1, Pro2 and Pro3; P23 (residues 846–903) includes Pro2 and Pro3; P1 (residues 794–845) and P3 (residues 871–903) contain the Pro1 and Pro3 regions respectively.

Despite the presence of SH3 binding motifs in the cytoplasmic tail of ADAM 12, no SH3-containing proteins have been reported to interact with ADAM 12. In the present study, we show that the cytoplasmic tail of ADAM 12 binds to the SH3 domain both *in vitro* and *in vivo*. This binding involves the N-terminal proline-rich region in the cytoplasmic tail of ADAM 12. Interaction with ADAM 12 results in stimulation of Src tyrosine kinase activity, as co-expression of ADAM 12 and Src leads to activation of the recombinant Src. Moreover, after induction of differentiation, the endogenous ADAM 12 in C2C12 cells interacts with and activates endogenous Src. These results provide insight into the function of the cytoplasmic domain of ADAM 12, and indicate that ADAM 12 may play a role in transmembrane signalling during myoblast differentiation.

EXPERIMENTAL

Antibodies

Rabbit anti-(ADAM 12) antibodies were generated against a peptide from the cytoplasmic domain of mouse ADAM 12 (amino acids 774–791) and affinity purified (Bethyl Laboratories, Montgomery, TX, U.S.A.). Rabbit anti-(ADAM 9) antibodies were raised against the whole cytoplasmic domain of mouse ADAM 9, which was expressed in *Escherichia coli* as a 6 × His-tagged protein, and affinity purified. Mouse monoclonal anti-6 × His antibody, recognizing five consecutive histidine residues (Penta-His), was obtained from Qiagen (Valencia, CA, U.S.A.). Goat polyclonal antibodies against glutathione S-transferase (GST) were from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.); mouse monoclonal anti-Src antibody (clone GD11) was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.); rabbit polyclonal anti-Src antibody (sc-18) was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.); rabbit polyclonal anti-(Src pY423) phosphospecific antibody was from Biosource International (Camarillo, CA, U.S.A.); and mouse anti-phosphotyrosine antisera PY20 and 4G10 were from ICN Biomedicals (Costa Mesa, CA, U.S.A.) and Upstate Biotechnology respectively.

Bacterial expression constructs

cDNAs encoding fragments P123 (residues 794–903), P1 (residues 794–845), P23 (residues 846–903) and P3 (residues 871–903) of mouse ADAM 12 (see Figure 1) were amplified by PCR using mouse skeletal muscle cDNA (Clontech, Palo Alto, CA, U.S.A.) as template, *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA, U.S.A.), and appropriate sets of primers. Similarly, cDNA fragments encoding the SH3 domain of mouse Src (residues 74–154), the N-terminal SH3 domain of mouse Crk (residues 121–192), and the cytoplasmic domain of mouse integrin β1A (residues 752–792) were amplified. PCR products were cloned into the pGEX-2T vector (Amersham Phamacia Biotech) between *Bam*HI and *Eco*RI sites for expression of GST fusion proteins in *E. coli*. In addition, cDNAs encoding the P123 fragment of ADAM 12 and the cytoplasmic tail of integrin β1A were cloned into the pCAL-n vector (Stratagene) between *Bam*HI and *Eco*RI sites for expression of calmodulin-binding peptide (CBP)-tagged proteins.

Mammalian expression constructs

cDNA fragments encoding the full-length mouse ADAM 12 (residues 1–903), a truncated form of mouse ADAM 12 extending from the disintegrin to the cytoplasmic tail and lacking the pro- and metalloprotease domains [residues 425–903; ADAM 12 (Δ1–424)], and a similarly truncated form of mouse ADAM 9 [residues 417–845; ADAM 9 (Δ1–416)] were amplified by PCR using a mouse skeletal muscle cDNA library and *Pfu* Turbo DNA polymerase. PCR products, containing mouse Igκ secretion signal, were cloned into the pIRESpuro vector (Clontech) between *Cla*I and *Not*I sites. A Src expression vector containing mouse Src cDNA (wild type) in a pUSEamp plasmid was purchased from Upstate Biotechnology. To add a 6 × His tag at the C-terminus of Src, Src cDNA was amplified by PCR using pUSEamp-Src vector as a template and a 3′-end primer with a Src-specific sequence followed by a sequence encoding a 6 × His tag. The amplification product was cloned into the pIRESpuro vector between *Cla*I and *Not*I sites.

Protein expression and purification

All GST fusion proteins were expressed in DH5α *E. coli* cells (Life Technologies, Rockville, MD, U.S.A.) as soluble proteins and purified on glutathione–Sepharose columns (Amersham Pharmacia Biotech) according to the manufacturer's instructions. All CBP-tagged proteins were expressed in BL21 *E. coli* cells (Novagen, Madison, WI, U.S.A.) as soluble proteins and purified on a calmodulin affinity resin (Stratagene) according to the manufacturer's instructions.

Cell culture, labelling and transfections

C2C12 and COS-7 cells were incubated in growth medium [Dulbecco's modified Eagle's medium (DMEM) with 10 % (v/v) fetal bovine serum] in the presence of 5 % CO_2 in a humidified atmosphere. Confluent C2C12 cells were transferred to differentiation medium (DMEM with 2 % horse serum). For metabolic labelling, confluent non-differentiating or differentiating C2C12 cells were incubated for 16 h in growth medium or differentiation medium respectively, which contained 10 % of the normal concentration of methionine and cysteine and was supplemented with Easy Tag Express-[^{35}S] Protein Labeling Mix (40 μCi/ml; NEN, Boston, MA, U.S.A.). C2C12 or COS-7 cells (5×10^5 cells/100-mm plate) were transfected with expression vectors using LIPOFECTAMINE™ Plus reagent (Life Technologies) according to the manufacturer's instructions. Expression of the recombinant proteins was analysed 38 h after transfection.

Cell extracts and immunoprecipitation

C2C12 cells were lysed with lysis buffer [50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 % (v/v) Triton X-100, 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride (AEBSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin and 10 μg/ml pepstatin A; 2 ml of lysis buffer/100-mm plate]. In the experiments where tyrosine phosphorylation was examined, the following phosphatase inhibitors were included: 50 mM NaF, 2 mM Na_3VO_4 and 10 mM $Na_4P_2O_7$. Cell extracts were subjected to centrifugation (15000 ***g***, 20 min) and the supernatant (1 ml) was mixed with Protein G–Sepharose (20 μl; Amersham Pharmacia Biotech) and incubated for 1 h at 4 °C (pre-clearing). After removal of Protein G–Sepharose, the cell lysate was incubated with anti-(ADAM 12) antibody (5 μg/ml) or monoclonal anti-Src antibody (3 μg/ml) for 4 h at 4 °C, and then with Protein G–Sepharose (20 μl) for 30 min at 4 °C. The immunoprecipitates were washed four times with buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl and 1 % Triton X-100, and eluted with SDS-gel loading buffer. To deplete ADAM 12-bound Src prior to Src immunoprecipitation (the experiment shown in Figure 10), the C2C12 cell lysate was incubated with anti-(ADAM 12) antibody (5 μg/ml) for 2 h at 4 °C, followed by addition of Protein G–Sepharose (20 μl/ml of lysate; repeated twice) and removal of the anti-(ADAM 12) immunocomplexes.

Protein binding under native conditions

CBP–P123 or CBP–β1 (see the Results section) was immobilized on calmodulin affinity columns (7 μg of protein/100 μl of resin; 0.3 ml column bed volume). Purified GST–SrcSH3 or GST (0.2 mg each) in PBS containing 1 % (w/v) BSA and 1 % Triton X-100 was loaded on the columns, followed by washing of the columns with PBS containing 1 % (v/v) Triton X-100, and elution with SDS-gel loading buffer. To study the interaction of endogenous Src with the cytoplasmic tail of ADAM 12, the lysate from non-differentiated C2C12 cells was subjected to centrifugation (15000 ***g***, 20 min), and the supernatant was incubated with glutathione–Sepharose (50 μl/ml of lysate) for 1 h. Pre-cleared cell lysate (6 ml) was applied on to columns (0.2 ml bed volume) containing GST fusion proteins (0.6 mg), and then the columns were washed with buffer containing 50 mM Tris/HCl (pH 7.4), 150 mM NaCl and 1 % Triton X-100, and eluted with buffer containing 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 20 mM glutathione and 0.1 % Triton X-100.

Blot overlays with GST–SrcSH3

Purified proteins (1–2 μg) were resolved by SDS/PAGE and transferred to a nitrocellulose membrane. The membrane was first incubated in blocking buffer [50 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 2 % (w/v) BSA and 0.1 % (v/v) β-mercaptoethanol] at room temperature for 1 h, and then with GST–SrcSH3 or GST alone in blocking buffer (1 μg of protein/ml) for 16 h at 4 °C. The membrane was washed with buffer containing 50 mM Tris/HCl (pH 8.0), 50 mM NaCl, 1 % Triton X-100 and 1 % (w/v) I-Block (Tropix, Bedford, MA, U.S.A.), followed by incubation with either goat anti-GST antibody or mouse anti-Src antibody, then with anti-goat or anti-mouse IgG horseradish peroxidase-conjugated antibody respectively, and visualization by a chemiluminescence detection method (SuperSignal West Pico; Pierce, Rockford, IL, U.S.A.).

Measurement of tyrosine phosphorylation and Src kinase activity *in vivo*

C2C12 cells transfected with Src and ADAM 12 or ADAM 9 expression vectors were lysed with lysis buffer containing phosphatase inhibitors. The lysate was centrifuged (15000 ***g***, 20 min) and the supernatant was subjected to SDS/PAGE and transferred to a nitrocellulose membrane. Tyrosine phosphorylation of cellular proteins was analysed by Western blotting with anti-phosphotyrosine antibodies PY20 and 4G10. The activation status of Src was analysed by Western blotting with anti-(Src pY423) phosphospecific antibody.

Immunoblotting

Proteins were resolved by SDS/PAGE and transferred to a nitrocellulose membrane. The membrane was incubated first in blocking buffer [PBS containing 1 % (w/v) I-Block and 0.3 % (v/v) Tween-20], then in blocking buffer supplemented with a primary antibody, followed by incubation with a horseradish peroxidase-conjugated secondary antibody, and visualization with a chemiluminescence detection method. The following concentrations or dilutions of primary antibodies were used: anti-(ADAM 12) antibody, 0.3 μg/ml; Penta-His antibody, 0.1 μg/ml; anti-GST antibody, 1:2000 dilution; monoclonal anti-Src antibody, 1:240 dilution; polyclonal anti-Src antibody, 1:200 dilution; polyclonal anti-(Src pY423) phosphospecific antibody, 1:1000 dilution; anti-phosphotyrosine antibody PY20, 1:100 dilution; anti-phosphotyrosine antibody 4G10, 1:1000 dilution.

RESULTS

The cytoplasmic tail of ADAM 12 contains eight minimal consensus PXXP motifs that are required for interaction with SH3 domains. These PXXP motifs are conserved between mouse and human ADAM 12 and are grouped into three proline-rich regions, designated Pro1, Pro2 and Pro 3 (Figure 1). Moreover, two sites, one in the Pro1 and one in the Pro3 region, conform to the consensus sequence for class I ligands for the Src SH3

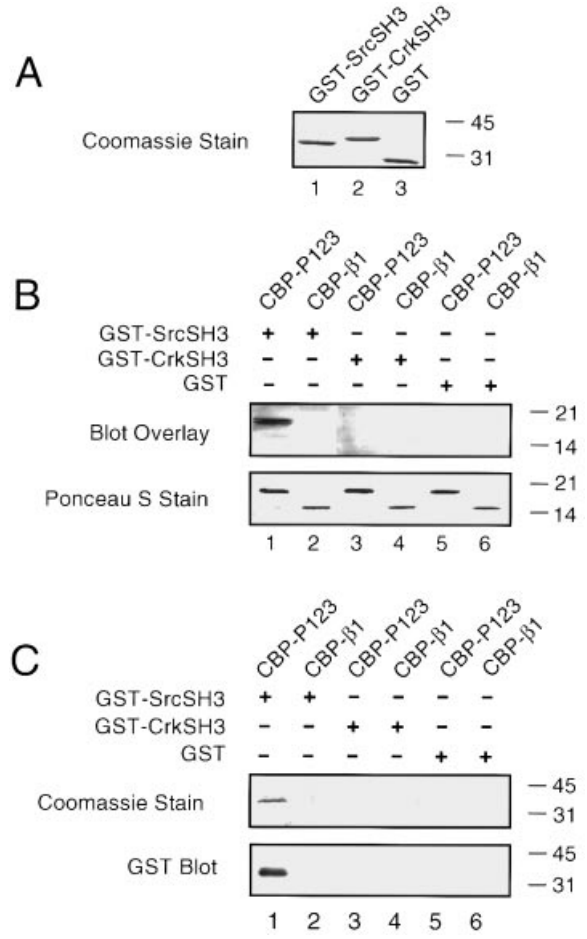

Figure 2 Interaction between the cytoplasmic tail of ADAM 12 and the Src SH3 domain

(**A**) Coomassie Blue-stained gel containing purified GST–SrcSH3 (lane 1), GST–CrkSH3 (lane 2) and GST (lane 3). (**B**) Direct interaction between ADAM 12 and the Src SH3 domain detected by blot overlay. The P123 fragment of the cytoplasmic domain of ADAM 12 (lanes 1, 3 and 5) or the cytoplasmic tail of integrin β1 (lanes 2, 4 and 6), each fused to CBP, was expressed in *E. coli*, purified using a calmodulin affinity resin, subjected to SDS/PAGE and transferred to a nitrocellulose membrane. The membrane was stained with Ponceau S, destained and incubated with purified GST–SrcSH3 (lanes 1 and 2), GST–CrkSH3 (lanes 3 and 4) or GST (lanes 5 and 6), followed by incubation with goat anti-GST polyclonal antibody and anti-goat IgG horseradish peroxiadse-conjugated secondary antibody, and visualization using a chemiluminescence detection method. (**C**) Binding of the Src SH3 domain to ADAM 12 under non-denaturing conditions. CBP–P123 (lanes 1, 3 and 5) or CBP–β1 (lanes 2, 4 and 6) was retained on calmodulin affinity resin. Bacterial lysates containing equivalent amounts of GST–SrcSH3 (lanes 1 and 2), GST–CrkSH3 (lanes 3 and 4) or GST (lanes 5 and 6) were loaded on the columns, followed by washing of the columns and elution with SDS-gel loading buffer. The eluates were analysed by SDS/PAGE and Coomassie Blue staining of the gel (upper panel) or Western blotting using anti-GST antibody (lower panel). Molecular masses (kDa) are indicated on the right of the gels.

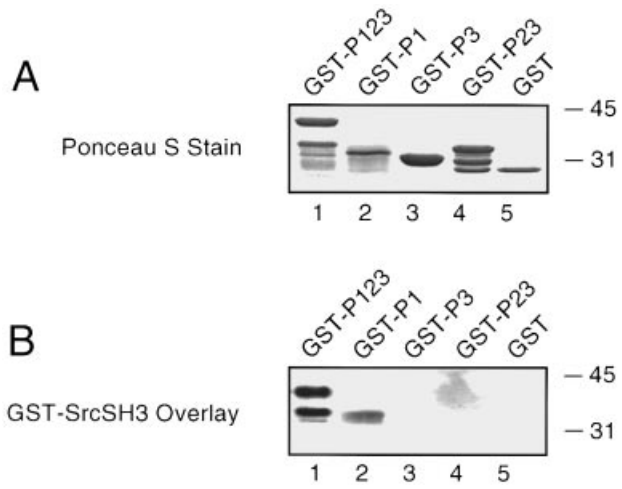

Figure 3 Localization of the Src SH3 binding site in the cytoplasmic tail of ADAM 12

GST fusion proteins containing the Pro1, Pro2 and Pro 3 regions of ADAM 12 (GST–P123; lane 1), Pro1 (GST–P1; lane 2), Pro3 (GST–P3; lane 3) or Pro2 and Pro 3 (GST–P23; lane 4), or GST alone (lane 5), were electrophoresed, transferred to a nitrocellulose membrane, stained with Ponceau S (**A**), destained, and incubated with GST–SrcSH3 protein, followed by incubation with mouse anti-Src antibody, anti-mouse IgG horseradish peroxidase-conjugated secondary antibody, and visualization by a chemiluminescence detection method (**B**). Molecular masses (kDa) are indicated on the right of the gels.

domain (RXXPXXP), and one site within the Pro1 region matches the consensus sequence for class II Src SH3 ligands (PXXPXR).

To examine whether the Src SH3 recognition motifs located in the Pro1 and Pro3 regions constitute functional binding sites for the Src SH3 domain, we performed a series of *in vitro* and *in vivo* binding assays. First, we expressed and purified the P123 fragment of the cytoplasmic tail of mouse ADAM 12 (residues 794–903; see Figure 1), which spans all three proline-rich regions, and was fused to a CBP tag at the N-terminus (CBP–P123 construct). As a control, we expressed and purified the cytoplasmic tail of mouse integrin β1A, which lacks any PXXP motifs and was fused to the same CBP tag (CBP–β1). CBP–P123 and CBP–β1 proteins were electrophoresed, transferred to a nitrocellulose membrane and incubated with the following proteins in a blot overlay assay: GST–(Src SH3 domain) fusion protein (GST–SrcSH3), GST–(Crk N-terminal SH3 domain) fusion protein (GST–CrkSH3) or GST alone (Figure 2A). The consensus sequence recognized by the N-terminal SH3 domain of the adapter protein Crk is PXXPXK [37], which corresponds to amino acids 847–852 of the mouse ADAM 12 sequence (see Figure 1). As shown in Figure 2(B), GST–SrcSH3, but not GST–CrkSH3 or GST, interacted with CBP–P123 protein. No interaction was detected for the control CBP–β1 protein. Consistently, GST–SrcSH3, but not GST–CrkSH3 or GST, bound to CBP–P123 immobilized on the calmodulin affinity column; no binding was observed between GST–SrcSH3 and CBP–β1 proteins (Figure 2C). These results supported a direct and specific interaction between the P123 fragment of ADAM 12 and the SH3 domain of Src.

To localize the site of interaction with the Src SH3 domain within ADAM 12, we compared the binding of GST–SrcSH3 to four different fragments of the cytoplasmic tail of ADAM 12: P123, P1, P3 and P23. While the P123 fragment spanned all three Pro1, Pro2 and Pro3 regions, P1 (residues 794–845) contained Pro1 only, P3 (residues 871–903) contained Pro3 only, and P23 (residues 846–903) included Pro2 and Pro3 (see Figure 1). P123, P1, P3 and P23 were expressed as GST fusion proteins, purified, electrophoresed and subjected to the GST–SrcSH3 blot overlay assay. Binding of GST–SrcSH3 was detected by probing the blot with an anti-Src antibody that recognized the Src SH3 domain. As shown in Figure 3, only the GST–P123 and GST–P1 proteins, but not GST–P3, GST–P23 or GST, inter-

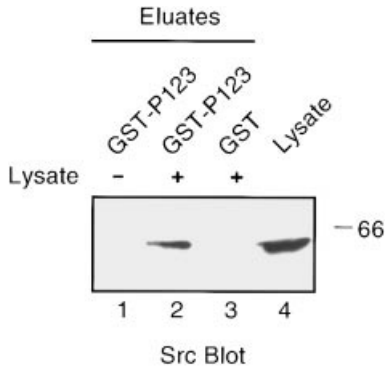

Figure 4 Binding of full-size Src to the cytoplasmic tail of ADAM 12

GST–P123 (lanes 1 and 2) or GST alone (lane 3) was immobilized on glutathione columns. A C2C12 cell lysate (6 ml) was loaded on the columns, followed by washing of the columns and elution with glutathione-containing buffer. The eluates were subjected to SDS/PAGE and Western blotting with mouse anti-Src antibody. Lanes 1–3 contain 50 μl of the eluates (0.5 ml total volume); lane 4 contains 50 μl of the lysate. Molecular mass (kDa) is indicated on the right of the gel.

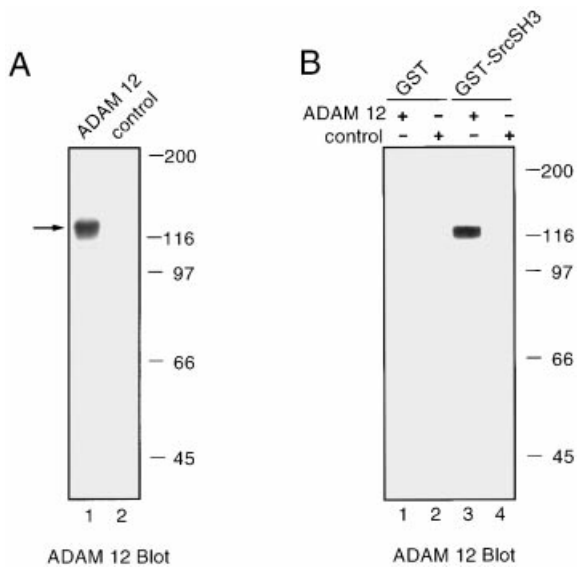

Figure 5 Binding of full-size ADAM 12 to the Src SH3 domain

(**A**) COS-7 cells were transiently transfected with a vector encoding full-size ADAM 12 (lane 1) or a vector with no insert (lane 2). At 36 h after transfection, expression of ADAM 12 was analysed by subjecting the cell lysate to Western blotting with anti-(ADAM 12) antibody. The position of ADAM 12 is indicated by an arrow on the left. (**B**) Lysates from ADAM 12-transfected (lanes 1 and 3) or vector-only-transfected (lanes 2 and 4) COS-7 cells were loaded on glutathione columns with immobilized GST (lanes 1 and 2) or GST–SrcSH3 (lanes 3 and 4). The columns were washed, eluted with glutathione-containing buffer, and analysed by SDS/PAGE and Western blotting using anti-(ADAM 12) antibody. Molecular masses (kDa) are indicated on the right of the gels.

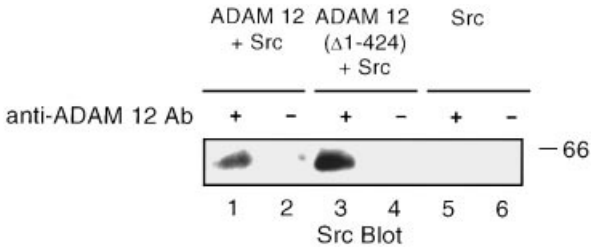

Figure 6 Interaction of ADAM 12 with Src *in vivo*

Lysates from C2C12 cells co-transfected with full-length ADAM 12 plus Src (lanes 1 and 2) or ADAM 12 (Δ1–424) plus Src (lanes 3 and 4), or singly transfected with Src (lanes 5 and 6), were incubated with (lanes 1, 3 and 5) or without (lanes 2, 4 and 6) anti-(ADAM 12) antibody (Ab), followed by incubation with Protein G–Sepharose. The immunoprecipitates were analysed by Western blotting using mouse anti-Src antibody. Molecular mass (kDa) is indicated on the right of the gel.

acted with GST–SrcSH3, suggesting that the site of interaction with the SH3 domain of Src is located within the Pro1 region of ADAM 12. Consistently, a major proteolytic product of GST–P123 that was retained on the glutathione affinity column (Figure 3A, lane 1), and therefore must have contained the Pro1 region proximal to GST, also bound to GST–SrcSH3.

Next, we investigated whether endogenous, full-size Src was capable of interacting with the cytoplasmic domain of ADAM 12. GST–P123 and GST alone were immobilized on glutathione columns. The columns were loaded with a C2C12 cell lysate that contained endogenous Src, and then washed, eluted and examined by Western blotting with anti-Src antibody. As shown in Figure 4, the endogenous, full-size ($\sim$ 60 kDa) Src protein was retained on the GST–P123 column, but not on the GST column, consistent with the interaction of the SH3 domain of Src with the cytoplasmic tail of ADAM 12, as established earlier in the experiment shown in Figure 2.

To examine whether the full-size, transmembrane ADAM 12 binds to the Src SH3 domain, we overexpressed ADAM 12 in COS-7 cells and passed the lysate from transfected and control cells through GST–SrcSH3 and GST affinity columns. Expression of ADAM 12 ($\sim$ 120 kDa) in transfected cells was confirmed by Western blotting with anti-(ADAM 12) antibody (Figure 5A). As shown in Figure 5(B), ADAM 12 was retained on the GST–SrcSH3 column, but not on the GST column, consistent with an interaction between full-size ADAM 12 and the SH3 domain of Src.

Having established the interaction between the cytoplasmic tail of ADAM 12 and the Src SH3 domain *in vitro*, we investigated whether ADAM 12 and Src interact *in vivo*. C2C12 cells were co-transfected with vectors encoding full-size ADAM 12 and Src, with vectors encoding ADAM 12 containing a 424-amino-acid N-terminal deletion [ADAM 12 (Δ1–424)] and Src, or with the Src expression vector alone. The region of ADAM 12 that was deleted in the ADAM 12 (Δ1–424) construct spans the signal peptide, the pro-domain and the metalloprotease domain. To allow proper intracellular processing and localization, an exogenous signal sequence was added to this truncated version of ADAM 12 (see the Experimental section). Altogether, ADAM 12 (Δ1–424) corresponded to a form of ADAM 12 that stimulated the differentiation and fusion of C2C12 myoblasts, as reported previously [4]. As shown in Figure 6, Src was co-immunoprecipitated with ADAM 12 or ADAM 12 (Δ1–424) from doubly transfected cells (lanes 1 and 3), suggesting the presence of complexes between Src and ADAM 12 *in vivo*. The suitability of the anti-(ADAM 12) antibody for immunoprecipitation was confirmed in an independent experiment, where cells were transfected with ADAM 12 containing a C-terminal 6 × His tag and subjected to immunoprecipitation, and the presence of ADAM 12 in the immunoprecipitate was examined by Western blotting with anti-6 × His tag antibody (results not shown).

To determine whether binding of ADAM 12 to the SH3 domain of Src has any effect on the tyrosine kinase activity of

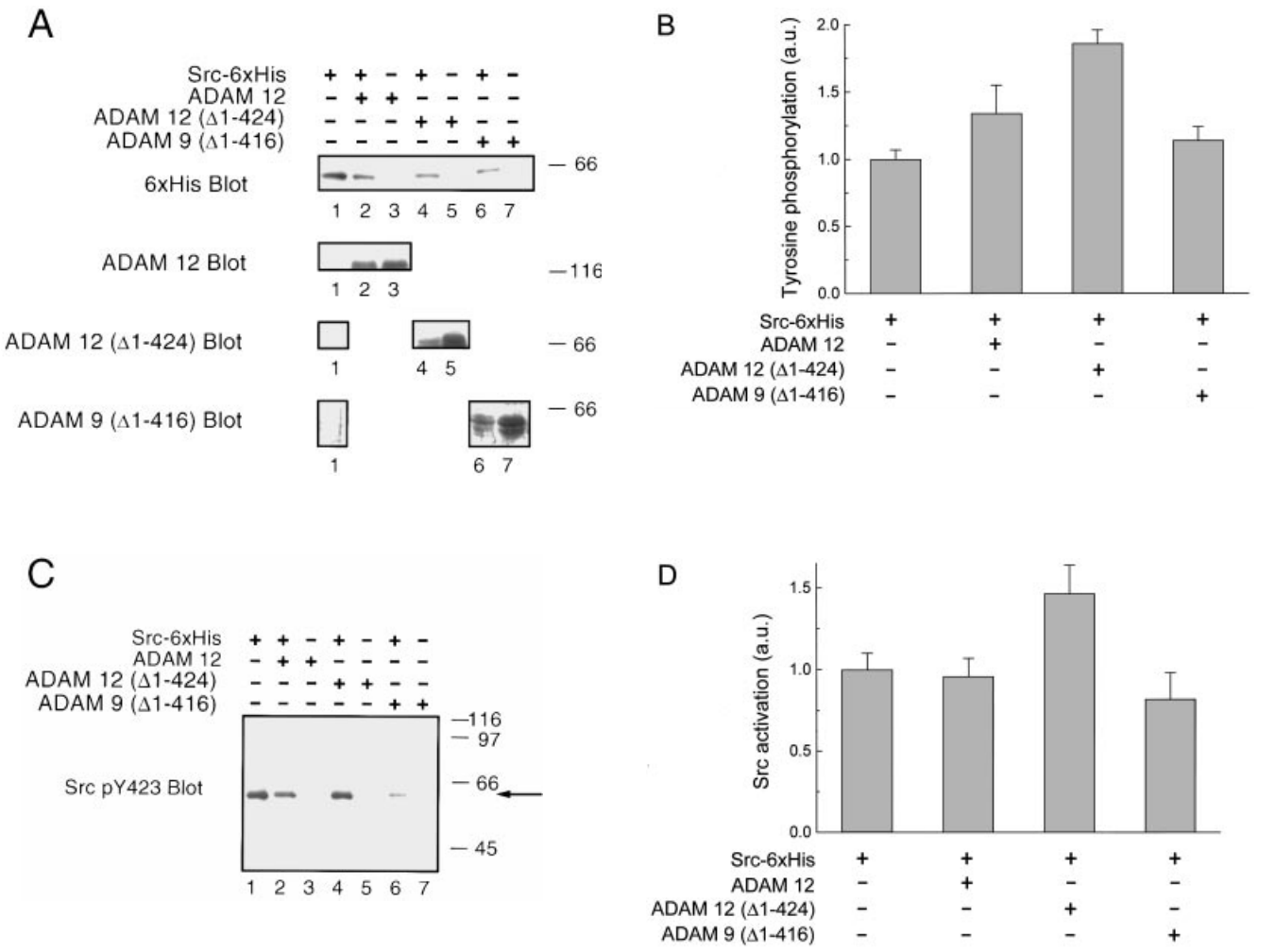

Figure 7 Activation of Src tyrosine kinase *in vivo* by ADAM 12

(**A**) C2C12 cells were transfected with a vector encoding Src with a C-terminal 6 × His tag (Src–6 × His; lane 1), Src–6 × His plus full-length ADAM 12 (lane 2), full-length ADAM 12 only (lane 3), Src–6 × His plus ADAM 12 (Δ1–424) (lane 4), ADAM 12 (Δ1–424) only (lane 5), Src–6 × His plus ADAM 9 (Δ1–416) (lane 6), or ADAM 9 (Δ1–416) only (lane 7). Expression of recombinant Src was detected by subjecting cell lysates to Western blotting with anti-6 × His antibody. Expression of recombinant ADAM 12 and ADAM 12 (Δ1–424) was detected by Western blotting with anti-(ADAM 12) antibody. Expression of ADAM 9 (Δ1–416) was detected with anti-(ADAM 9) antibody. Molecular masses (kDa) are indicated on the right of the gels. (**B**) Stimulation of protein tyrosine phosphorylation by co-expression of Src and ADAM 12. C2C12 cells were transfected with the same constructs as described in (**A**). Tyrosine phosphorylation of cellular proteins was analysed by subjecting total cell lysates to SDS/PAGE and Western blotting with anti-phosphotyrosine antibodies PY20 and 4G10. The extent of total protein phosphorylation was determined by densitometry (a.u., arbitrary units). (**C**) Activation status of Src in transfected C2C12 cells. C2C12 cells were transfected with the same constructs as described in (**A**). The activation status of Src was examined by subjecting total cell lysates to Western blotting with an antibody specific for phosphotyrosine-423 in mouse Src. Molecular masses (kDa) are indicated on the right of the gel. (**D**) Quantification of the effect of co-expression of ADAM 12 or ADAM 9 on Src activation. The amount of active Src shown in (**C**) and the amount of recombinant Src protein shown in (**A**) were quantified by densitometry (a.u., arbitrary units). Src activation for each transfectant was calculated as the ratio of the amount of active Src to the amount of recombinant Src protein. In (**B**) and (**D**), results are shown as the means of three measurements (± S.E.M.).

Src, we investigated protein phosphotyrosine content and the activation status of exogenous Src either expressed alone in C2C12 cells or co-expressed with ADAM 12. The exogenous Src contained a C-terminal 6 × His tag (Src–6 × His) to allow its discrimination from endogenous Src and to allow the evaluation of the expression level after transfection. ADAM constructs used for co-transfection with Src–6 × His included full-length ADAM 12, ADAM 12 (Δ1–424) and ADAM 9 (Δ1–416). The last of these constructs represents mouse ADAM 9 containing an N-terminal deletion equivalent to the deletion in ADAM 12 (Δ1–424). Since ADAM 9 is the only member of the ADAM family that has been reported to interact with Src, at least *in vitro* [38], we decided to test its ability to regulate Src kinase activity *in vivo* and to compare it with that of ADAM 12.

The expression levels of all the exogenous proteins following transfection are shown in Figure 7(A). Cell lysates were subjected to SDS/PAGE and Western blotting with anti-6 × His antibody to detect Src–6 × His (Figure 7A, top panel), with anti-(ADAM 12) antibody to detect ADAM 12 and ADAM 12 (Δ1–424) (Figure 7A, middle panels) or with anti-(ADAM 9) antibody to detect ADAM 9 (Δ1–416) (Figure 7A, bottom panel). As shown in Figure 7(A), Src–6 × His and ADAM 12 migrated as single protein species of ~ 60 kDa and ~ 120 kDa respectively. ADAM 12 (Δ1–424) and ADAM 9 (Δ1–416) gave rise to several protein bands, ranging from 60 to 70 kDa for ADAM 12 (Δ1–424) and from 55 to 65 kDa for ADAM 9 (Δ1–416), which might have been a result of varying protein glycosylation or partial protein degradation.

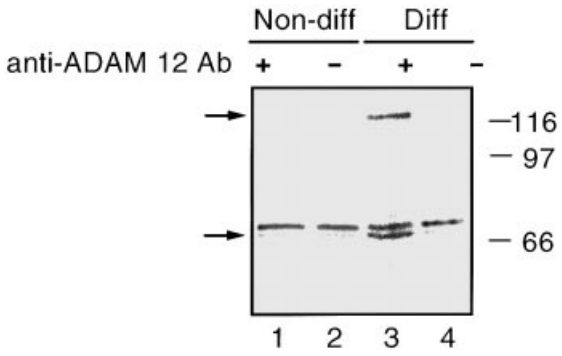

Figure 8 Expression of endogenous ADAM 12 in C2C12 cells

Confluent, non-differentiated C2C12 cells in growth medium (Non-diff; lanes 1 and 2) or cells incubated for 3 days in differentiation medium (Diff; lanes 3 and 4) were metabolically labelled with [^{35}S]methionine plus [^{35}S]cysteine, lysed, and incubated with (lanes 1 and 3) or without (lanes 2 and 4) anti-(ADAM 12) antibody (Ab), followed by incubation with Protein G–Sepharose. The immunoprecipitates were resolved by SDS/PAGE, and gels were dried and subjected to autoradiography. The arrows indicate the positions of the full-size (upper arrow) and processed (lower arrow) forms of endogenous ADAM 12. The radioactive protein band observed in all four samples and present above the processed form of ADAM 12 results from non-specific binding to Protein G–Sepharose. Molecular masses (kDa) are indicated on the right of the gel.

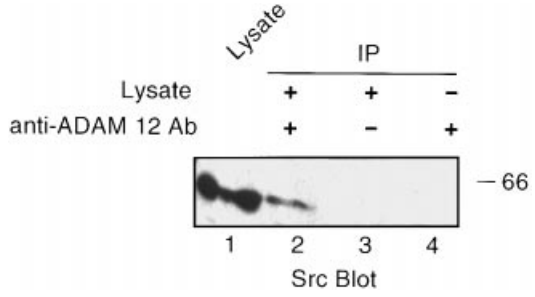

Figure 9 Interaction between endogenous ADAM 12 and endogenous Src *in vivo*

C2C12 cells were incubated for 3 days in differentiation medium and then lysed with lysis buffer. The lysate (1 ml) was incubated with (lane 2) or without (lane 3) anti-(ADAM 12) antibody (Ab), followed by incubation with Protein G–Sepharose. As a control, anti-(ADAM 12) antibody and Protein G–Sepharose were added to lysis buffer (lane 4). Material retained on Protein G was eluted with SDS-gel loading buffer and analysed by SDS/PAGE and Western blotting with mouse anti-Src antibody. Lane 1 contains total cell lysate (50 μl) before immunoprecipitation (IP). Molecular mass (kDa) is indicated on the right of the gel.

To examine the effect of the expression of Src–6 × His on protein tyrosine phosphorylation in C2C12 cells, we subjected the lysates from transfected cells to SDS/PAGE and Western blotting with anti-phosphotyrosine antibodies. The extent of total protein tyrosine phosphorylation in Src–6 × His-transfected cells or cells that were co-transfected with Src–6 × His and ADAM constructs was quantified and normalized to the amount of expressed Src–6 × His protein, as determined in Figure 7(A). As shown in Figure 7(B), protein tyrosine phosphorylation was stimulated almost 2-fold by co-expression of ADAM 12 (Δ1–424), with the full-size ADAM 12 and ADAM 9 (Δ1–416) having smaller effects.

To evaluate directly the effects of ADAM 12 and ADAM 9 on the activation status of Src, we measured the amount of active Src in transfected C2C12 cells using an antibody specific for the phosphorylated form of the tyrosine residue (Tyr-418 in human Src, Tyr-423 in mouse Src and Tyr-416 in chicken Src) in the so-called activation loop of Src. The activation status of Src is correlated directly with phosphorylation of this tyrosine residue; the site is not phosphorylated when Src is inactive, and is phosphorylated when Src is active [33–36]. Figure 7(C) shows the results of Western blot analysis of total cell lysates using anti-(Src pY423) antibody. Due to high levels of Src–6 × His expression and a relatively high threshold level of detection by anti-(Src pY423) antibody, the contribution of the endogenous, active Src (Figure 7C, lanes 3, 5 and 7) to the signals observed for Src–6 × His-transfected cells (Figure 7C, lanes 1, 2, 4 and 6) was negligible. This allowed us to determine the activation status of the recombinant Src (Figure 7D) by quantifying the band intensities in the Western blot obtained with anti-(Src pY423) antibody and normalizing them to the amount of Src–6 × His protein expressed. Specifically, the effect of co-expression of ADAM 12, ADAM 12 (Δ1–424) or ADAM 9 (Δ1–416) on the activation status of recombinant Src was calculated as the ratio of the amount of active Src (see Figure 7C) to the amount of recombinant Src protein (see Figure 7A). As shown in Figure 7(D), co-expression of ADAM 12 (Δ1–424), but not the full-length ADAM 12 or ADAM 9 (Δ1–416), resulted in activation of the recombinant Src.

Next, we asked whether endogenous ADAM 12 can interact with and activate endogenous Src in C2C12 cells. To address this question, it was critical to know at what stage during the differentiation of C2C12 cells ADAM 12 is expressed. The only two published reports on the expression of ADAM 12 in C2C12 cells give an unclear and somewhat contradictory picture. In the first report, ADAM 12 protein was detected in C2C12 cells after differentiation; its presence before differentiation was not tested [4]. According to the other study, ADAM 12 was expressed at low levels in undifferentiated C2C12 myoblasts, was transiently up-regulated at the onset of differentiation, and had decreased to low levels after 2 days of differentiation [7]. To examine the expression of endogenous ADAM 12 in C2C12 cells, non-differentiating confluent cells or cells incubated for 3 days in differentiation medium were metabolically labelled with [^{35}S]methionine and [^{35}S]cysteine and subjected to immunoprecipitation with anti-(ADAM 12) antibody. As shown in Figure 8, radioactive species of ~ 120 kDa and ~ 70 kDa were observed in anti-(ADAM 12) immunoprecipitates from differentiating, but not from non-differentiating, cells. The 120 kDa protein corresponded to full-size ADAM 12, and the 70 kDa species represented an N-terminally truncated form of ADAM 12 with a molecular mass similar to that of ADAM 12 (Δ1–424).

To establish if endogenous ADAM 12 binds to endogenous Src *in vivo*, we immunoprecipitated ADAM 12 from C2C12 cells that were incubated for 3 days in differentiation medium and were expressing endogenous ADAM 12 (as documented in Figure 8). We then analysed the Src content in the anti-(ADAM 12) immunoprecipitates. As shown in Figure 9, Src was co-immunoprecipitated with ADAM 12, consistent with the presence of complexes between ADAM 12 and Src *in vivo*. The percentage of Src associated with ADAM 12 was low (1–2 % of total Src). This amount is similar to the percentage of Src found in complexes with other proteins [39].

In order to determine whether the interaction of endogenous ADAM 12 with endogenous Src in differentiating C2C12 cells is correlated with the activation of Src, we investigated the effects of depletion of ADAM 12 and ADAM 12-associated Src on the amount of activated Src that was subsequently immuno-

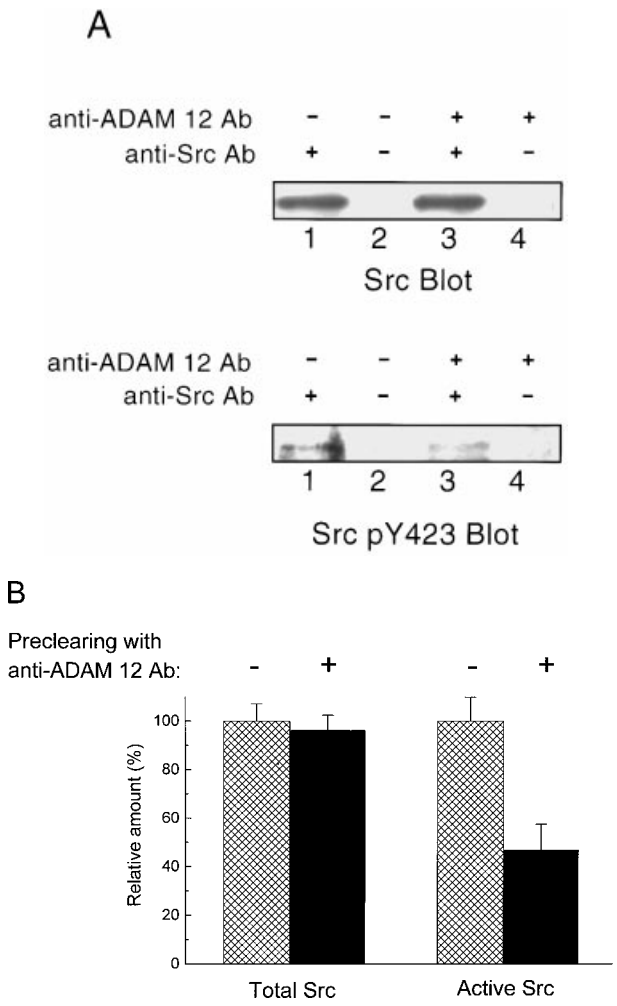

Figure 10 Activation of endogenous Src tyrosine kinase by endogenous ADAM 12 in differentiating C2C12 cells

C2C12 cells were incubated for 3 days in differentiation medium and then lysed with lysis buffer. The lysate (1 ml) was incubated without (lanes 1 and 2) or with (lanes 3 and 4) anti-(ADAM 12) antibody (Ab), followed by incubation with Protein G–Sepharose. Sepharose beads containing ADAM 12 and associated Src protein were removed and the supernatant was further incubated with (lanes 1 and 3) or without (lanes 2 and 4) mouse anti-Src antibody and Protein G–Sepharose. (**A**) The material precipitated with mouse anti-Src antibody was subjected to SDS/PAGE and Western blotting with rabbit anti-Src antibody (upper panel) or an antibody specific for phosphotyrosine-423 in mouse Src (lower panel). (**B**) Relative intensities of the immunoreactive bands shown in (**A**) were quantified by densitometry. Results are shown as the means of three measurements ($\pm$S.E.M.).

precipitated with anti-Src antibody. A C2C12 cell lysate was first incubated with or without anti-(ADAM 12) antibody, followed by removal of anti-(ADAM 12) immunocomplexes, and further immunoprecipitation of Src. While the amounts of total Src present in the anti-Src immunoprecipitates obtained from ADAM 12-pre-cleared and control cell lysates were similar (Figure 10A, upper panel), the amounts of activated, Tyr-423-phosphorylated Src were clearly different (Figure 10A, lower panel). As quantified and shown in Figure 10(B), pre-clearing with anti-(ADAM 12) antibody resulted in the removal of a very small amount of total Src (consistent with only 1–2 % of total Src co-immunoprecipitating with ADAM 12 in Figure 9), but this corresponded with the removal of $\sim$ 50 % of Tyr-423-phosphorylated Src. This suggested that ADAM 12-associated Src represented predominantly the activated form of Src protein.

DISCUSSION

The present study reports the first demonstration of a direct interaction between ADAM 12 and Src protein tyrosine kinase. This interaction involves the N-terminal proline-rich region (Pro1) in the cytoplasmic tail of ADAM 12 and the SH3 domain of Src. *In vivo*, we detected an interaction of exogenously expressed ADAM 12 and Src, as well as an interaction of endogenous ADAM 12 and Src in differentiating C2C12 cells. Binding of ADAM 12 to Src *in vivo* was correlated with the activation of Src protein tyrosine kinase.

Although two different proline-rich regions (i.e. Pro1 and Pro3) in the cytoplasmic tail of ADAM 12 contain consensus binding motifs for the SH3 domain of Src, we determined that the site responsible for the interaction with the Src SH3 domain is located in the Pro1 region. Moreover, the presence of a consensus binding sequence for the SH3 domain of Crk in the cytoplasmic tail of ADAM 12 did not ensure an interaction of ADAM 12 with Crk. This indicates that the specificity of SH3–ADAM 12 interactions is dictated by the overall structure of the cytoplasmic tail of ADAM 12, and is not merely a consequence of the presence of distinct SH3-binding motifs.

The mechanism of activation of Src by ADAM 12 can be envisioned based on our current understanding of Src structure and function. The SH3 domain, together with the SH2 domain, plays a central role in regulating the catalytic activity of Src. The high-resolution crystal structure of human Src provides evidence that the intramolecular interaction of the SH3 domain with the sites in the catalytic domain and in the linker region between the SH2 and catalytic domains stabilizes the inactive conformation of the kinase [40,41]. Interaction of the SH3 domain with other proteins causes displacement of the SH3 domain and disruption of such intramolecular interactions. Src then adopts an 'open', active conformation, leading to autophosphorylation of the tyrosine residue in the activation loop and a further increase in catalytic activity.

We observed that the N-terminal pro- and metalloprotease domains of ADAM 12 were not required for the activation of Src. In fact, the truncated version of ADAM 12, ADAM 12 (Δ1–424), seemed to be more potent in activating Src in C2C12 cells than was full-size ADAM 12 (see Figures 7B and 7D). Since it has been reported previously that expression of a similarly truncated ADAM 12 stimulated C2C12 cell differentiation, whereas expression of the full-size ADAM 12 led to inhibition of differentiation [4], it is tempting to speculate that the 'active' form of ADAM 12 lacks the pro- and metalloprotease domains. It has to be stressed, however, that, in order to compare the intrinsic abilities of the truncated and the full-length ADAM 12 to activate Src or any other signalling pathway in intact cells, it is necessary to secure the same levels of cell surface expression of the two ADAM 12 forms. Work on the intracellular trafficking of full-size ADAM 12 and ADAM 12 (Δ1–424), and the significance of the metalloprotease domain in ADAM 12-mediated transmembrane signalling, is currently in progress.

What is the biological significance of the activation of Src? The answer is related directly to the question of the function of Src in a given tissue or cell type. In skeletal muscle, the tissue in which ADAM 12 is primarily expressed, the distinct and specific role of Src has not been fully established. While expression of the oncogenic version of Src, v-Src, in skeletal myoblasts leads to the inhibition of differentiation into myotubes [42], and while expression of the same protein in myotubes results in the inhibition

of transcription of many muscle-specific genes [42–44] and the disruption of sarcomeres and triads [45,46], it is difficult to correlate the functions of a mutant, constitutively active v-Src with those of its transiently activated cellular homologue, c-Src. More recently, it has been demonstrated that phosphorylation of the acetylcholine receptor in myotubes by Src and the related kinases Fyn and Fyk is important for receptor aggregation and post-synaptic differentiation during synaptogenesis at the neuromuscular junction [39,47]. In addition, in differentiated C2C12 cells, Src co-fractionates with caveolin-3 [48], a muscle-specific form of caveolin that seems to be critical for myoblast fusion [49]. These results indicate that, in addition to their universal roles in cell signalling, Src and other members of the family of related kinases may be programmed to exert more specific functions in skeletal muscle cells, and therefore may be subject to tissue-specific regulation by ADAM 12.

The significance of the interaction between ADAM 12 and Src reported in the present study may not be limited to skeletal muscle. The transmembrane form of ADAM 12 is dramatically up-regulated in many tumour cells, including breast carcinoma cells [10]. Therefore it is of particular interest that a member of the ADAM family has recently been reported to shed the L1 adhesion molecule from breast carcinoma cells, leading to the promotion of cell migration, and that the shedding was regulated by Src [50]. Although the identity of the ADAM protein and the mechanism of activation of the ADAM-mediated release of L1 by Src are currently unknown, one may speculate that ADAM 12 plays a role in this process.

In summary, a long list of receptors that couple to Src family kinases, which includes, among others, cytokine receptors, integrins, receptor protein tyrosine kinases, G-protein-coupled receptors and major histocompatibility receptors [33,34], should be now extended to the ADAM family of metalloprotease–disintegrin receptors.

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