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Fibre-type specific concentration of focal adhesion kinase at the sarcolemma: influence of fibre innervation and regeneration

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Summary

In skeletal muscles, focal adhesion complexes (FACs) form part of the costamere, a sarcolemmal protein complex that enables lateral transfer of forces and ensures the stability of the sarcolemma. The present investigation tested whether localisation of a major assembly factor of FACs, focal adhesion kinase (FAK), to the sarcolemma parallels the known modulation of FACs by fibre type (innervation pattern) and fibre regeneration. Immunohistochemical experiments indicated that FAK is preferentially associated with the sarcolemma in a high proportion (>74 %) of the (slow-twitch) type I and (fast-twitch) type IIA fibres in normal rat soleus (N-SOL) muscle and of the type IIA fibres in extensor digitorum longus (N-EDL) muscle. In contrast, a low proportion (<15 %) of fast-twitch type IIB and type I fibres in N-EDL showed sarcolemmal FAK immunoreactivity. Cross-reinnervation of slow-twitch rat SOL muscle with the fast EDL nerve induced slow-to-fast fibre transformation and led to a significant reduction in sarcolemmal FAK immunoreactivity in type I and type IIA fibres. Transplantation of the fast EDL into the slow SOL bed with regeneration and reinnervation of the muscle by the SOL nerve (T-EDL) caused a significant increase in sarcolemmal FAK immunoreactivity in new type I and hybrid I/II fibres and a corresponding reduction in sarcolemmal FAK immunoreactivity in 'normal' IIA and IIB fibres. Conversely, sarcolemmal FAK immunoreactivity in small IIB fibres of T-EDL muscle was increased. Correspondingly, the transplanted and regenerated SOL (reinnervated by the fast EDL nerve) maintained the percentage of FAK-positive sarcolemma in the (regenerated) type I and IIA fibres. Thus, the expression and association of FAK with the sarcolemma are regulated (i) by factors that determine the fibre type and (ii) during fibre regeneration. Our data suggest that the integrity of sarcolemmal FACs is dependent on the fibre type and that FAC turnover is increased during regeneration of muscle fibres.

Key words: focal adhesion complex, costamere, skeletal muscle, focal adhesion kinase, nerve, regeneration, myosin.

Introduction

The structural/functional integrity of skeletal muscle is crucially dependent on the functional adhesion of the fibre's periphery to the surrounding connective tissue and other muscle fibres (Gullberg et al., 1998). Key structures for this adhesion are the costameres. These subsarcolemmal structures contain two different laminin receptors, a dystrophin/glycoprotein complex and an integrin-associated complex, that link *via* the cytoskeleton to the Z-disk and *via* the extracellular matrix (ECM) to adjacent muscle fibres (Fig. 1) (Patel and Lieber, 1997; Huijing, 1999; Morris and Fulton, 1994; Rybakova et al., 2000). Costameres link the lengthening and shortening of skeletal muscle fibres with the sarcolemma (Pardo et al., 1983; Patel and Lieber, 1997). Finally, costamere components and the associated molecules are essential for muscle formation (Gullberg et al., 1998; Huijing, 1999).

There is sparse evidence indicating the fibre-type specificity of costamere components in, for example, the chicken (Bozyczko et al., 1989; Shear and Bloch, 1985). However, the extent to which sarcolemmal components contribute to fibre-type-specific properties is poorly understood (Shear and Bloch, 1985).

Recently, the integrin-associated tyrosine kinase focal adhesion kinase (FAK) was found in an irregular pattern in association with the sarcolemma of chicken slow tonic muscle fibres (Flück et al., 1999); it was concentrated in slow-twitch soleus (SOL) rather than fast-twitch rat muscles (plantaris and gastrocnemius) (Gordon et al., 2001). Intriguingly, FAK was

also demonstrated to be associated with the dystrophin/glycoprotein complex (Cavaldesi et al., 1999) and to localize to the sarcomeric Z-line of cardiac myocytes (Kovacic-Milivojevic et al., 2001). In cultured mesodermal cells, FAK is known to play a central role in the formation and turnover of focal adhesion complexes (FACs) (Cary and Guan, 1999; Ilic et al., 1995; Schlaepfer and Hunter, 1998), which link, in a manner analogous to costameres, the outside of a cell to the extracellular matrix and interact with cytoskeletal elements in its interior (Fig. 1) (Burridge and Chrzanowska-Wodnicka, 1996). Interaction between integrins and the ECM induces rapid phosphorylation of the integrin-associated FAK on distinct tyrosine residues (Cary and Guan, 1999). Such modifications create docking sites for the subsequent recruitment of cytoskeletal and signalling molecules to FACs (Fig. 1) (Giancotti, 1997; Miyamoto et al., 1995). It has also been suggested that FAK controls the cell cycle of myoblasts in culture and is involved in the attachment of muscle fibres to laminin (Disatnik and Rando, 1999; Oktay et al., 1999; Sastry et al., 1999). Furthermore, FAK protein and FAK kinase activity are induced during the formation of muscle fibres in culture and during muscle hypertrophy, as well as in myopathy (Flück et al., 1999; Saher and Hildt, 1999). Thus, FAK plays a critical role in the formation of muscle fibres and could conceivably contribute to the mechanisms that control the fibre-type-specific distribution of sarcolemmal FACs.

Changes in the muscle fibre recruitment pattern, as a consequence of changed innervation, cause transformations of fibre types (Buller et al., 1960; Jolesz and Sreter, 1981; Pette and Staron, 1997; for further references, see Lu et al., 1999). In addition to fibre-type transformation, fibres in transplanted muscle undergo, after an initial degeneration, a regeneration process on the scaffold of the basal lamina (Marshall et al., 1977) that is sometimes accompanied by the deposition of a new basement membrane (Gulati et al., 1983). We have explored the hypothesis that the expression of FAK protein and its association with the sarcolemma in rat skeletal muscle corresponds with fibre types and is affected by the remodelling of the basement membrane during fibre regeneration.

Materials and methods

Immuno-reagents

Rabbit polyclonal antiserum A-17 raised against N-terminal amino acid residues 2–18 of human FAK was obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Monoclonal antibody 2A7, which recognises an epitope within C-terminal amino acid residues 852–1052 of FAK was a gift from Dr Thomas Parsons (Kanner et al., 1990). Rabbit polyclonal antiserum (termed αC-FAK) raised against C-terminal amino acid residues 749–1042 of rat FAK and normal rabbit serum were prepared and affinity-purified as described previously (Flück et al., 1999). Horseradish-peroxidase-coupled recombinant anti-phosphotyrosine antibody RC20:HRPO was obtained from Transduction Laboratories (Lexington, KY, USA). Peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse whole IgG were obtained from ICN Biomedicals GMBH (Germany), and mouse-anti-peroxidase complex was obtained from Jackson Laboratories (West Grove, PA, USA). Monoclonal type I (slow) and type II (fast) myosin-isoform-specific antibodies were obtained from Chemikon (Juro, Lucerne, Switzerland) and Sigma Chemicals (Buchs, Switzerland), respectively.

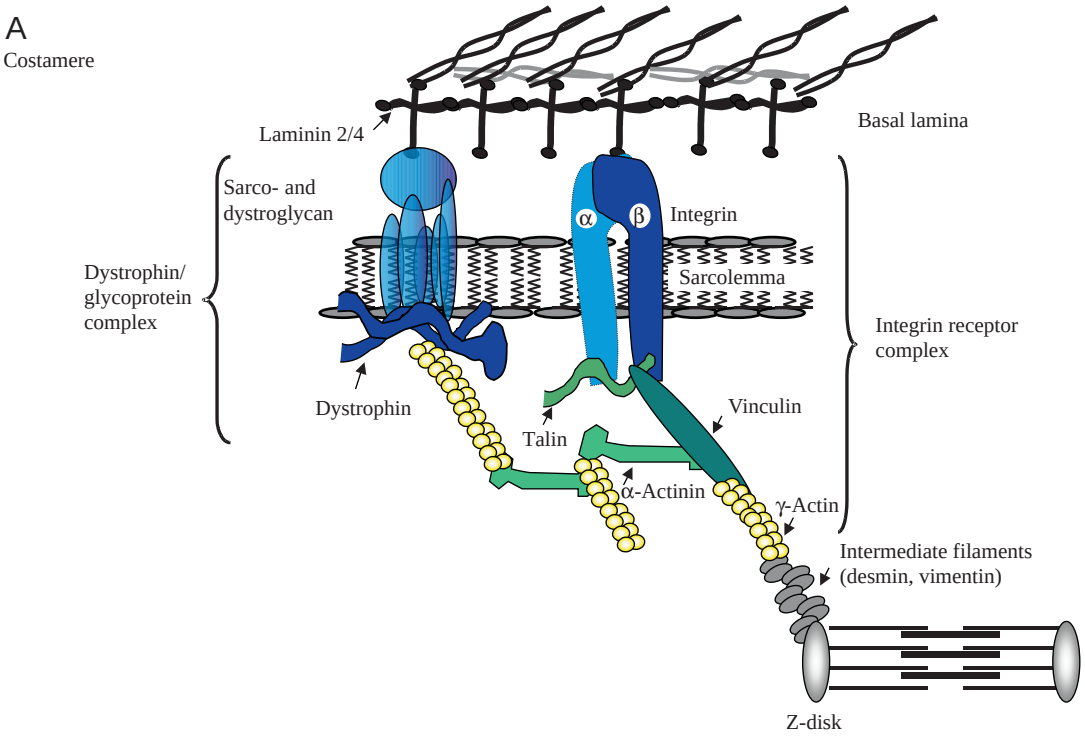
Animals and muscles

The muscle samples originated from a previous experiment, described in detail by Lu et al. (1999), and had been stored for approximately 5 years at –70 °C. All surgical procedures were performed on male rats (strain Zur:SIV; Institute of Laboratory Animal Science, University of Zürich, Switzerland) at the Institute of Anatomy, University of Zürich-Irchel, with the permission of the State Animal Protection Commission. Animals were anaesthetised by injection with 0.25 ml kg^{-1} body mass Innovar-Vet (Pitman-Moore GMBH, Germany) intramuscularly combined with 2.5 mg of Valium (Roche, Reinach, Switzerland) and 7.5 mg of Nembutal (Abbott, Baar, Switzerland) intraperitoneally (for details, see Lu et al., 1999). The following muscles were analysed in the present investigation: (i) soleus (SOL) muscles, which were cross-reinnervated by the extensor digitorum longus (EDL) nerve, termed X-SOL, (ii) SOL muscle grafted into the site of the EDL muscles with foreign reinnervation by the EDL nerve, termed T-SOL, (iii) EDL muscles transplanted into the site of the SOL muscles with foreign reinnervation by the SOL nerve, termed T-EDL, and (iv) normal SOL and EDL muscles, termed N-SOL and N-EDL, respectively. For each treatment, muscles from at least five animals were analysed. X-SOL, T-SOL and T-EDL muscles were analysed only after the muscle fibre transformation process was considered to be complete, i.e. at least 6 months after innervation by the foreign nerve.

Protein extraction and immunoprecipitation

The preparation and denaturing of deoxycholate extracts in SDS–PAGE loading buffer was as described in Flück et al. (2000).

For immunoprecipitation, proteins were extracted from cryosections of the belly portion of the muscle with 500 µl of cold immunoprecipitation buffer (10 mmol l^{-1} Tris-HCl, pH 7.4, 1 % Triton X-100, 150 mmol l^{-1} NaCl, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} EGTA, 1.5 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} Na_3VO_4, 0.2 mmol l^{-1} phenylmethylsulphonyl fluoride, 2.5 µg ml^{-1} aprotinin, 2.5 µg ml^{-1} leupeptin), and the soluble fraction resulting after centrifugation (3 min, 10 000 ***g*** at 8 °C; termed the Triton X-100 extract) was divided into three parts. Each part was incubated with a different rabbit serum (2 h, 8 °C), followed by reaction for 1 h at 4 °C with 100 µl of a 10 % slurry of Protein A crosslinked to agarose (Sigma Chemical, Buchs, Switzerland). The isolation, washing and denaturing of the precipitated antigen/antibody complexes in SDS–PAGE loading buffer were carried out as described previously (Flück et al., 1999).



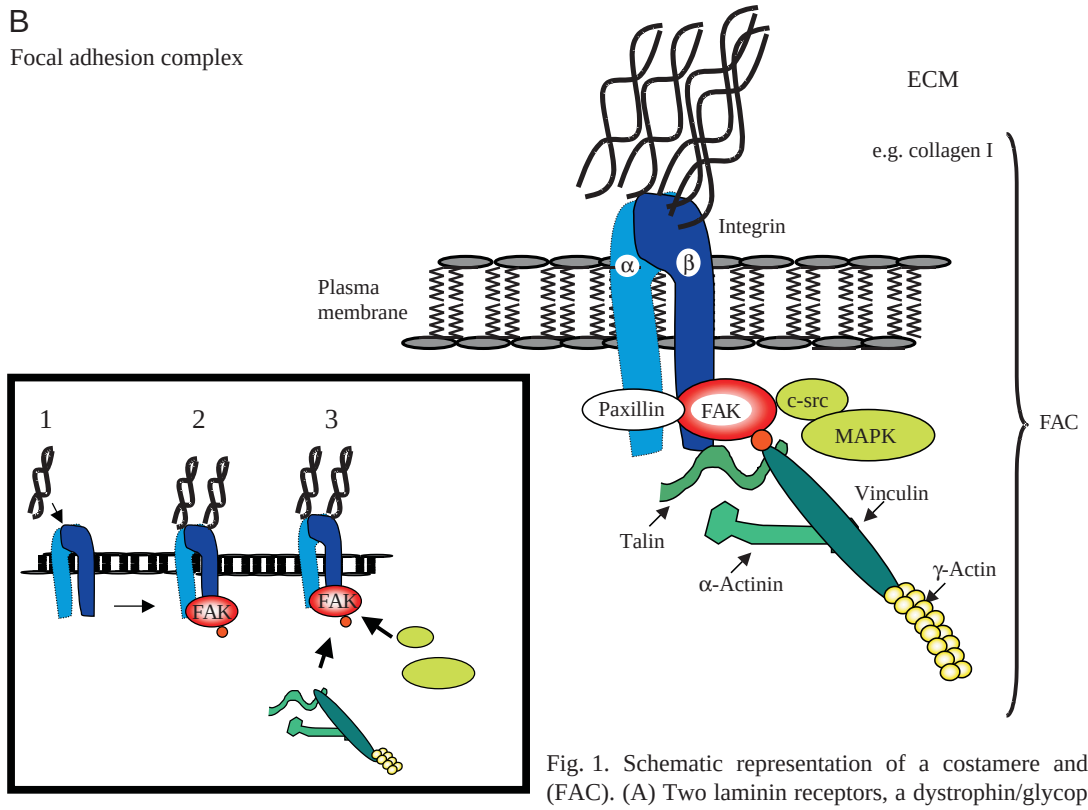


Fig. 1. Schematic representation of a costamere and the focal adhesion complex (FAC). (A) Two laminin receptors, a dystrophin/glycoprotein complex and an integrin receptor complex are among the sarcolemmal structures (Pardo et al., 1983) that link the contractile apparatus of muscle fibres with the surrounding basal lamina. Components of both receptors, i.e. both dystrophin and the integrin-associated cytoskeletal proteins (talin, vinculin, α-actinin), co-localise in subsarcolemmal complexes (Pardo et al., 1983) which connect through γ-actin and the intermediate-filament proteins desmin and vimentin to the Z-disk of skeletal muscle fibres (adapted from Patel and Lieber, 1997; Rybakova et al., 2000). (B) Integrin-based FACs of cultured mesodermal cells bridge cortical γ-actin to the extracellular matrix (ECM). The inset indicates schematically the proposed involvement of FAK (in red) in the formation of FACs. Occupancy of integrins with ECM ligand (1) causes phosphorylation (orange circle) of integrin-associated FAK (2) which, in turn, promotes recruitment of both cytoskeletal (paxillin, vinculin, talin, α-actinin and γ-actin; coloured in dark green) and signalling molecules (3) (e.g. MAPK and c-src kinase, coloured in light green) to integrins (Miyamoto et al., 1995).

SDS–PAGE and immunoblotting

Denatured SDS–PAGE loading buffer samples of deoxycholate extracts (20 μg) or immunoprecipitates were separated on 7.5 % and 5 % SDS–PAGE gels, respectively, and processed for immunoblotting essentially as described previously (Flück et al., 2000). Blots were stained with Ponceau S (Serva Electrophoresis GMBH, Heidelberg, Germany) to verify equal loading and transfer and were subsequently incubated with primary antibodies and secondary peroxidase-conjugated goat anti-rabbit IgG at a dilution of 1:1000 and 1:5000, respectively, in TTBS (20 mmol l^{-1} Tris base, pH 7.5, 150 mmol l^{-1} NaCl, 0.05 % Tween-20) containing 2.5 % non-fat dry milk and 1 % bovine serum albumin (BSA). After washing, bound secondary antibody was detected by enhanced chemoluminescence (ECL; SuperSignal®West Pico from Pierce, Socochim SA, Switzerland) and recorded on X-ray film. The detection of tyrosine-phosphorylated proteins with antibody RC20:HRPO was carried out in a similar manner, but the addition of milk powder to the solution was omitted and the ECL reaction was carried out directly.

Immunocytochemistry

Cryosections (12 μm thick) were prepared from the belly portion of the muscle, mounted on glass slides (SuperFrost®Plus; Menzel-Gläser, Germany), air-dried and stored (for 0.5–5 days) at –20 °C. Detection of FAK with different antibodies was carried out using different protocols.

Visualization of FAK immunoreactivity with antiserum A-17 was carried out using a two-step detection protocol as described by Flück et al. (2000) but with the following modifications. Thawed sections were fixed in cold acetone and wetted in phosphate-buffered saline (PBS); tissue peroxidase activity was then quenched (10 min, 3 % H_2O_2 in methanol), and sections were washed in PBS and blocked with 3 % BSA in PBS for 0.5 h. Subsequently the sections were incubated for 1 h at room temperature (20 °C) with antibody A-17 (diluted 1:100 in 0.3 % BSA/PBS) and, following brief washing in PBS, reacted for 30 min with peroxidase-conjugated goat anti-rabbit IgG (diluted 1:2000 in 0.3 % BSA/PBS) and again washed with PBS. Immunoreactivity was detected with 3-amino-9-ethylcarbazole substrate (Sigma Chemicals, Buchs, Switzerland); the nuclei were counterstained with haematoxylin, and the sections were embedded in Aquamount (BDH Laboratory Supplies Poole, UK). The stain was visualised on film (Ektachrome 64T, Kodak) using a microscope/photograph system (Vanox-S, Olympus). The slides were scanned using a Nikon SF-200 slide scanner operated by a Power Macintosh G3 using the Nikon Scan 2.0 interface and imported in JPEG format into Adobe Photoshop version 5.0.

Detection of FAK with monoclonal antibody 2A7 and slow and fast myosin isoforms with specific antibodies were performed as follows. Sections were quenched and blocked as described above, incubated with a 1:100 dilution of the respective specific antibodies followed by goat anti-mouse whole IgG (dilution 1:500) and then with mouse anti-peroxidase complex (dilution 1:5000) with intermittent washing steps in PBS. Immunoreactivity and nuclei were visualised as described above. For each of the individual protocols, a control reaction was carried out with normal rabbit serum.

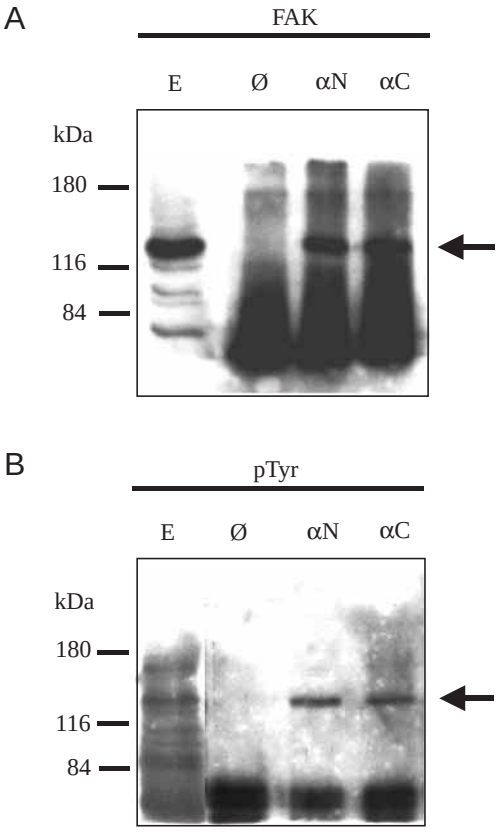


Fig. 2. Characterisation of antibodies. Triton X-100 extracts of rat normal soleus muscle were subjected to immunoprecipitation with normal (Ø), polyclonal rabbit antisera A-17 against the N terminus (αN) or the serum (αC) of focal adhesion kinase (FAK) against the C-terminal portion (αC) of FAK protein. Equal amounts of the precipitates, together with a sample of cell extract (E), were resolved by SDS–PAGE on 7.5 % gels and immunoblotted with either anti-FAK antiserum αC-FAK (A) or anti-phosphotyrosine antibody (pTyr) (B). The sizes of molecular mass markers are indicated.

Fibre analysis

Histochemical analysis of myofibrillar ATPase activity was carried out on consecutive cryosections to those analysed immunocytochemically for expression of type I or type II myosin isoforms and for FAK immunoreactivity (Lu et al., 1999). Micrographs were taken from corresponding fields of the stained sections, and the fibre types were classified. Type I and II fibres were differentiated on the basis of the presence of immunoreactivity for either type I or type II myosin isoforms. Intermediate (hybrid) type I/II (type IIC) fibres were identified by the simultaneous presence of type I and II myosin. Type II fibres were further differentiated into type IIA and IIB fibres by the more robust alkali-stable myofibrillar ATPase

activity of type IIA fibres. The presence of alkali-stable ATPase was demonstrated by pre-incubation of sections at pH 10.4 and 10.5 combined with incubation at pH 9.5 or 9.6 (Baker et al., 1994), respectively. Using these criteria, type IIX fibres could not be differentiated and were, if present, ranked with IIB fibres.

For each fibre type, the total number of fibres and the number of sarcolemmal FAK-positive fibres per field were counted. Fibres were arbitrarily assigned as FAK-positive when at least two boundaries of the sarcolemma showed distinct positive (orange-red) FAK immunoreactivity. An average of 150 fibres was counted per section. Data from different fields were pooled, and the mean percentage and standard error of sarcolemmal FAK-positive fibres per fibre type were calculated. Differences in percentage of sarcolemmal FAK-positive fibres among fibre types and muscles were verified using a bilateral two-by-two χ^2-test for statistical significance (Microsoft Excel 97).

Results

FAK protein expression in rat slow- and fast-twitch muscles

Rabbit polyclonal antisera directed either against the N terminus (A-17) or the C terminus (αC-FAK) of FAK protein were tested for their reactivity towards detergent-extracted FAK from normal rat SOL muscle (N-SOL). Both antisera immunoprecipitated a protein of the correct size, approximately 125 kDa (Fig. 2A), and the protein recognised by both sera was phosphorylated on tyrosine (Fig. 2B). Western blot analysis of detergent extracts of slow-twitch N-SOL and fast-twitch N-EDL muscles revealed that the normal slow-twitch SOL muscles contained higher levels of FAK protein (Fig. 3).

FAK localises to the sarcolemma of slow-twitch fibres

Having established that the antisera recognise the FAK isoform of rat skeletal muscle, we performed experiments to localise the FAK protein in rat skeletal muscle. Immunohistochemical experiments using antiserum A-17 (N terminus) indicated that FAK immunoreactivity in slow-twitch N-SOL muscle was associated with the sarcolemma of most fibres (Fig. 4). Normal SOL muscle contains 97% of slow-twitch (type I) and 3% of fast-twitch type IIA fibres. Using histochemical criteria, i.e. the expression of the slow myosin isoform and ATPase activity, type I fibres of N-SOL muscle were identified and compared with the distribution of FAK-immunoreactive staining. Of type I fibres, 89% were FAK-positive at the sarcolemma, as were 74% of type IIA fibres (see Fig. 9A).

FAK localisation to the sarcolemma in fast-twitch fibres

We characterised the expression of FAK in normal rat EDL muscle (N-EDL), which contains up to 98% of fast-twitch fibres (Lu et al., 1999). Using antiserum A-17 (N terminus), FAK immunoreactivity was detected at the sarcolemma in only approximately half the muscle fibres (Fig. 5A).

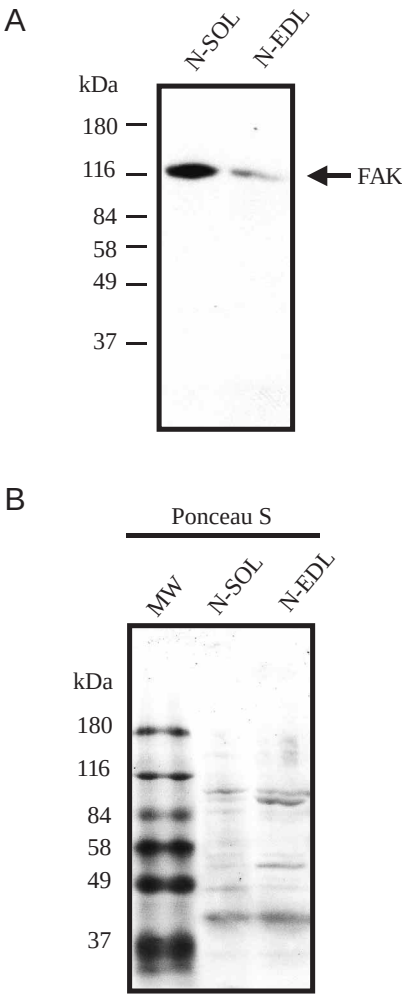


Fig. 3. Focal adhesion kinase (FAK) expression in normal slow- and fast-twitch muscle. (A) Soluble deoxycholate protein extract (10 µg) of slow-twitch normal soleus (N-SOL) and fast-twitch normal extensor digitorum longus (N-EDL) muscle was subjected to immunoblotting analysis using the αC-FAK serum. The position of the FAK protein is indicated by an arrow. (B) A Ponceau-S-stained transfer membrane prior to detection showing that approximately equal amounts of protein were loaded. The sizes of molecular mass (MW) markers are indicated.

To verify the absence of sarcolemmal FAK immunoreactivity in certain fibres of fast-twitch N-EDL muscle, we used a C-terminal FAK-reactive monoclonal antibody, 2A7. This antibody has been used previously to detect FAK in skeletal muscle of non-mammalian species (Baker et al., 1994; Flück et al., 1999; Kanner et al., 1990); it detects FAK protein in extracts of rat skeletal muscle (Fig. 2). Staining revealed expression of FAK along the sarcolemma of the same fibres that reacted with antibody A-17 (Fig. 5B). In addition, punctate staining outside the muscle fibres, which presumably reflects capillaries, was detected with antibody 2A7 (Polte et al., 1994).

The fibres that exhibited sarcolemmal FAK immunoreactivity were fast-twitch type IIA fibres, as detected

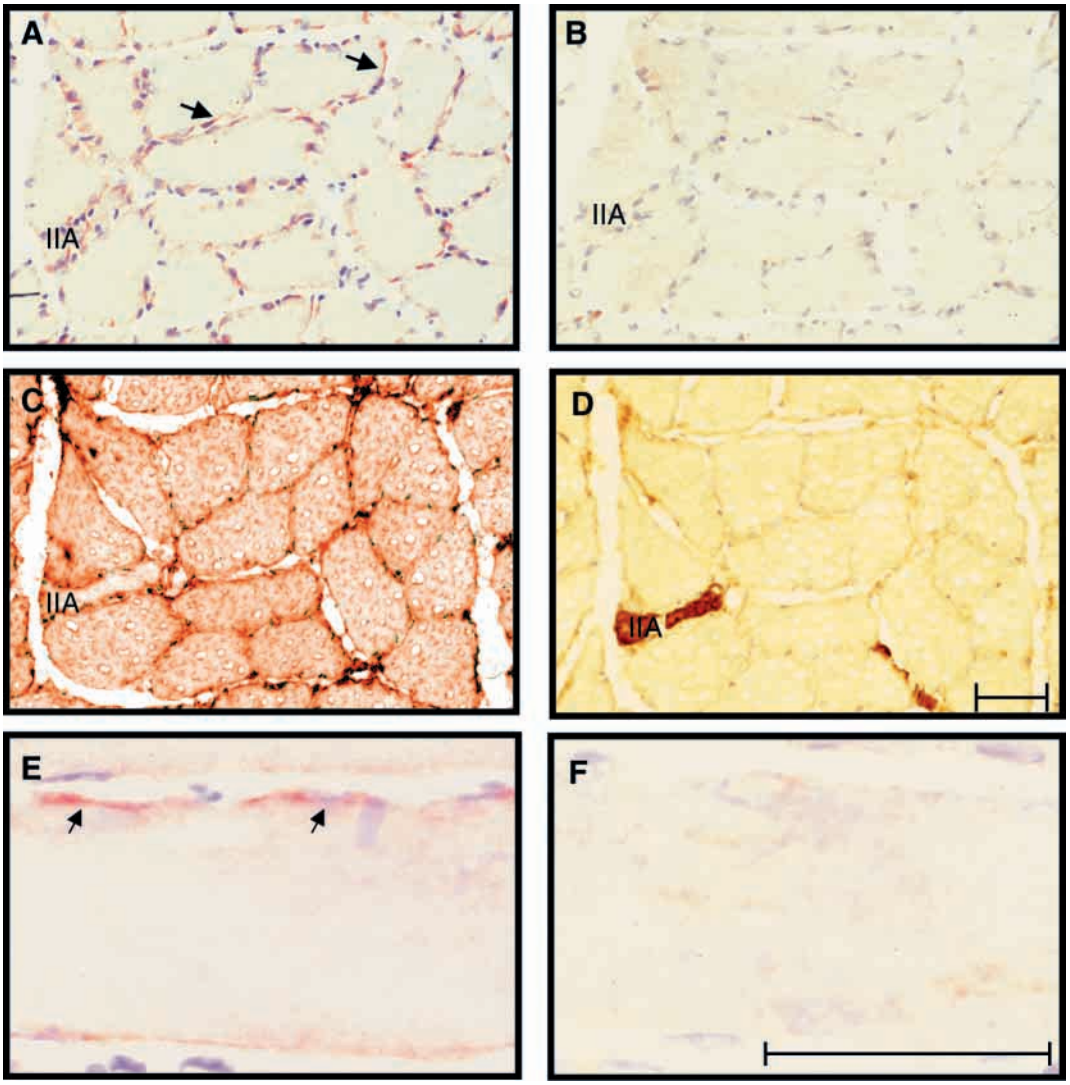


Fig. 4. Focal adhesion kinase (FAK) localisation in normal slow-twitch muscle. Immunocytochemical analysis of cross (A–D) and longitudinal (E,F) sections from slow-twitch normal soleus (N-SOL) muscle with the FAK N-terminal antiserum A-17 (A,E). Positive staining appears orange and nuclei appear blue. Control reactions of consecutive cryosections with normal rabbit serum are also shown (B,F). Arrows point to FAK-immunoreactivity at the sarcolemma. Consecutive sections were also stained for slow (C) or fast myosin (D) isoforms to determine the fibre types. All fibres, with the exception of two denoted type IIA, are of type I. Scale bars, 50 μm.

by myosin isoform staining and myofibrillar ATPase staining (Fig. 6). These fibres comprise up to 43 % of all N-EDL fibres. The rest of the fibres of N-EDL muscle are made up mostly (55 %) of fast-twitch type IIB with approximately 2 % of type I fibres (Lu et al., 1999). Quantitative assessment revealed that approximately 90 % of type IIA and 10 % of type IIB fibres showed sarcolemmal FAK-immunoreactive staining (see Fig. 9B).

Sarcolemmal localisation of FAK in transformed muscle fibres

In cross-reinnervated soleus (X-SOL), the proportion of type I fibres was reduced to 15–25 % (N-SOL, 93–97 %), whereas the proportion of type IIA fibres was increased to 72–85 % (N-SOL 3–6 %) (Lu et al., 1999). After cross-reinnervation, FAK expression at the sarcolemma was reduced to 61 % in the remaining type I fibres and was 28 % in newly transformed type IIA fibres (Fig. 7; see Fig. 9A).

Sarcolemmal localisation of FAK in regenerated and transformed muscle fibres

The slow-to-fast fibre-type transformation in SOL muscle that had been transplanted into the EDL bed (T-SOL) was more pronounced than in X-SOL muscle cross-reinnervated with the fast EDL nerve (Lu et al., 1999). After several months of recovery, the proportion of type I fibres decreased from more than 90 % to approximately 11 %, while the proportion of type IIA fibres increased from less than 10 % to approximately 89 %. Sarcolemmal FAK expression was observed in 90 % of remaining type I fibres and in 84 % of (new) type IIA fibres

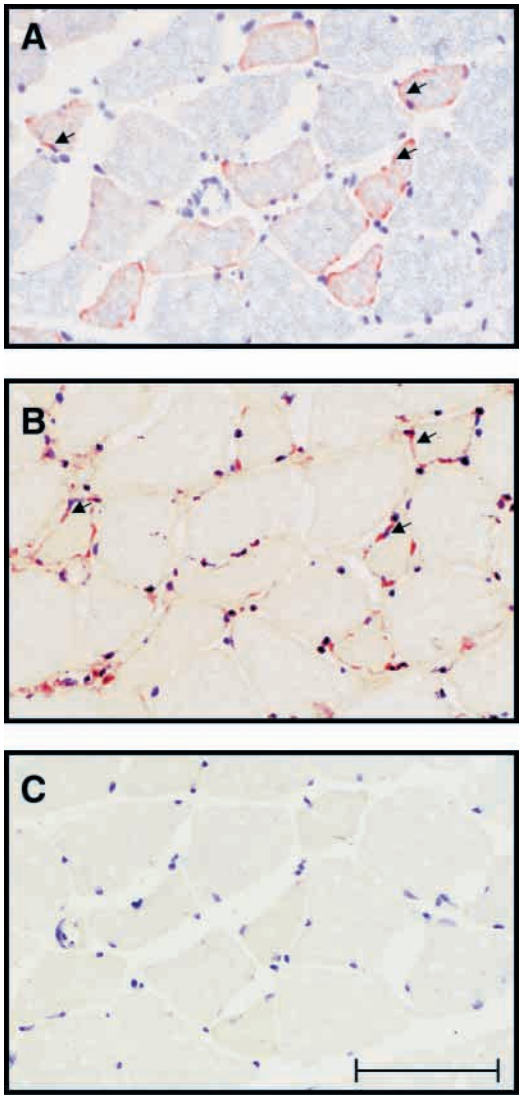


Fig. 5. Staining specificity of different focal adhesion kinase (FAK) antibodies. Immmunocytochemical analysis of FAK in parallel cryosections of fast-twitch normal extensor digitorum longus (N-EDL) muscle with polyclonal A-17 (A) and monoclonal FAK antiserum 2A7 (B). Positive staining is orange and nuclei appear blue. A control reaction with normal rabbit serum is also shown (C). Arrows indicate FAK immunoreactivity at the sarcolemma. Scale bar, 100 μm.

(see Fig. 9). Thus, compared with X-SOL muscle, the levels of sarcolemmal FAK immunoreactivity in type I and IIA fibres were significantly increased in fibres of the transplanted and regenerated T-SOL muscle.

In T-EDL that had regenerated after transplantation into the SOL bed, the proportion of type I and type I/II fibres increased to 93–97 % (N-EDL 2.0–3.5 %), while the proportion of type IIA fibres decreased to 3–7 % (N-EDL 34–50 %); less than 1 % of type IIB fibres persisted (N-EDL 47–55 %). In transplanted T-EDL muscles, FAK expression at the sarcolemma appeared in newly formed intermediate (hybrid) type I/II and type I fibres and was strongly associated with the expression of slow-type myosin (Fig. 8). Of the intermediate (hybrid) type I/II fibres, 88 % showed FAK immunoreactivity; 83 % of type I fibres showed FAK immunoreactivity. A lower percentage (9 %) of FAK immunoreactivity was seen in the remaining type IIA fibres (Fig. 9). The percentage of sarcolemmal FAK immunoreactivity in persisting, normally sized type IIB fibres was low (approximately 11 %), but was increased in small type IIB fibres (to more than 90 %).

Discussion

Differential expression of the enzymes involved in energy metabolism (e.g. aldolase), contraction (e.g. myosin light and heavy chains) and ion homeostasis (e.g. Ca^{2+}-ATPase) are classical features of slow and fast skeletal muscle fibres (Berchtold et al., 2000; Pette and Staron, 1990, 1997). In contrast to the information available about these adaptations in the sarcoplasmic compartment, little is understood about the extent to which differential expression of proteins situated at the sarcolemma parallels the fibre type characteristics. The present study implies that differential localisation of the protein tyrosine kinase FAK at the sarcolemma is associated with different muscle fibre phenotypes.

Immunocytochemical analysis with antibodies against the N and C termini of FAK demonstrated significant FAK immunoreactivity in muscle fibres exclusively in the vicinity of the sarcolemma, suggesting that, in muscle fibres, FAK protein is primarily concentrated at the fibre's periphery.

The muscle fibre types of N-SOL and N-EDL muscles could be divided into two populations according to sarcolemmal FAK immunoreactivity. A high percentage of type I and IIA muscle fibres in N-SOL muscle and type IIA fibres in N-EDL muscle ($\geqslant$74 %) showed sarcolemmal localisation of FAK. In contrast, only a small proportion (<15 %) of the type IIB and type I muscle fibres of N-EDL muscle revealed sarcolemmal FAK expression. The punctate sarcolemmal FAK staining in longitudinal sections of type I fibres of N-SOL (Fig. 4) indicates that a sampling error may explain the lack of FAK immunoreactivity in a proportion of type I and IIA muscle fibres of N-SOL muscle and type IIA fibres of N-EDL.

The levels of FAK protein at the sarcolemma in individual muscle fibre types of rat N-SOL and N-EDL muscle are in good agreement with the firing pattern of the innervating motoneurones during normal motor behaviour of adult rats (Hennig and Lomo, 1985). Recruitment of type I motor units in SOL muscle during free movement and during quiet standing (Armstrong and Laughlin, 1985) is frequent, and these units are active 22–35 % of the total time (Hennig and Lomo, 1985). During free movement, the recruitment of type IIA motor units in EDL muscle was also recorded for a significant fraction of the total time (1.6–5 %), while type IIB units were recruited much less frequently, i.e. 0.04–0.22 % of the total time. To our knowledge, no data have been published on the recruitment pattern of the low-abundance type I fibres of EDL muscle during normal cage activity of laboratory rats. Since, during daily life in cages, control laboratory rats do not

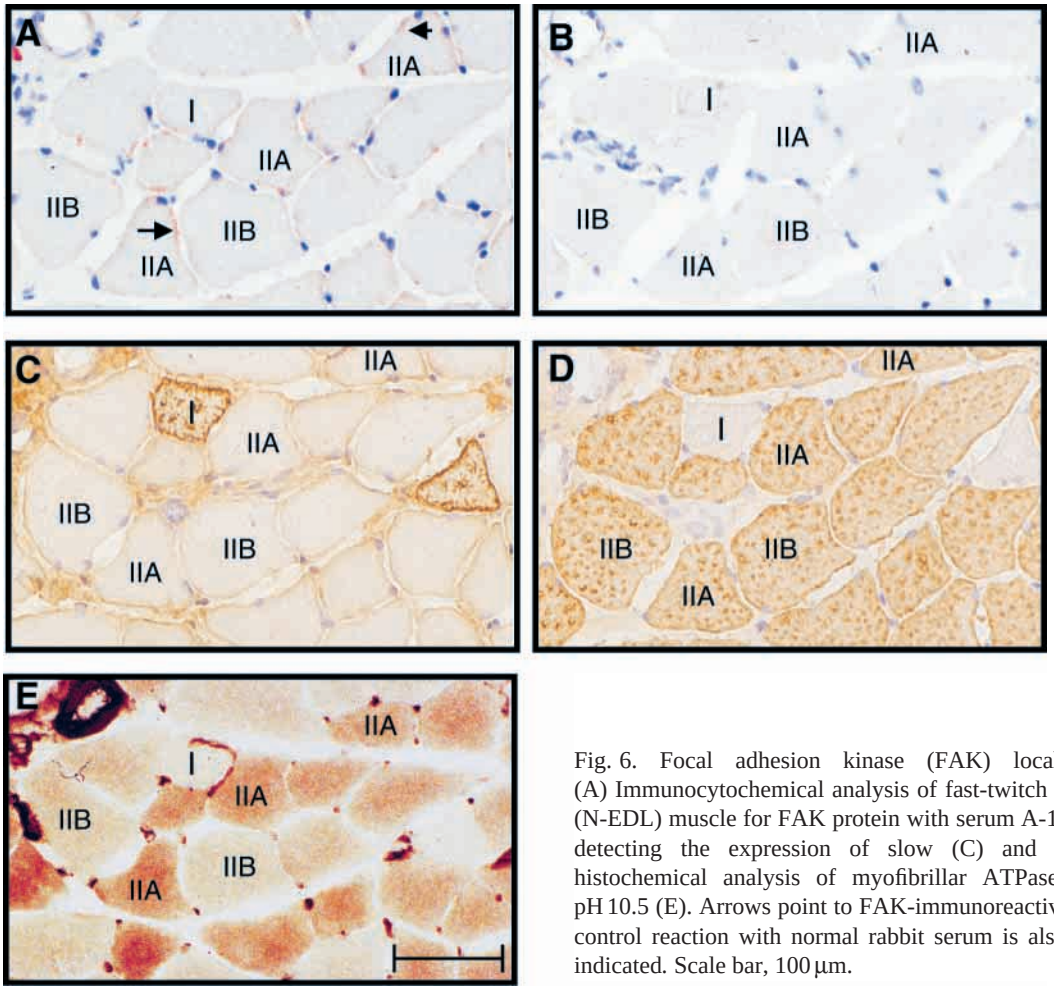


Fig. 6. Focal adhesion kinase (FAK) localisation in fast-twitch muscle. (A) Immunocytochemical analysis of fast-twitch normal extensor digitorum longus (N-EDL) muscle for FAK protein with serum A-17. Fibre typing was carried out by detecting the expression of slow (C) and fast (D) myosin isoforms and histochemical analysis of myofibrillar ATPase activity after preincubation at pH 10.5 (E). Arrows point to FAK-immunoreactivity at the sarcolemma. A negative control reaction with normal rabbit serum is also shown (B). The fibre types are indicated. Scale bar, 100 μm.

perform any type of endurance exercise activity, we speculate that the type I fibres of the EDL, in contrast to their complete recruitment during the 'unusual' locomotory activity of swimming (Yoshimura et al., 1992), are only rarely recruited. It is possible that the recruitment pattern and firing rate of the motoneurons are the main determinants of the localisation of FAK protein at the sarcolemma.

To test this hypothesis, soleus muscles were cross-reinnervated with the nerve of the fast-twitch EDL muscle. The sarcolemmal concentration of FAK in such X-SOL muscles was significantly reduced in type I and IIA fibres (Fig. 9A). These reciprocal changes in sarcolemmal FAK expression after foreign reinnervation of slow-twitch SOL with a nerve supply of higher firing frequency are in good agreement with the presumed decreased recruitment of muscle fibres in X-SOL (Hennig and Lomo, 1985). Similarly, the percentage of sarcolemmal FAK immunoreactivity decreased in IIA fibres and remained low in normal-sized IIB fibres of transplanted T-EDL muscle that had undergone a fast-to-slow fibre transformation as a result of foreign reinnervation with the slow SOL nerve. The remaining type IIB and the IIA fibre types in T-EDL are expected to show a reduced recruitment to compensate for the preferential activation of the newly formed type I fibres. Correspondingly, sarcolemmal FAK immunoreactivity occurred in a high proportion of newly formed intermediate (hybrid) type I/II (88 %) and type I (83 %) fibres in T-EDL, supporting our hypothesis that the firing rate of the innervating nerve, by determining the recruitment pattern, controls FAK localisation at the sarcolemma.

Conversely, FAK immunoreactivity increased in small type IIB fibres of T-EDL, which represent newly regenerated myotubes (Gulati et al., 1983). Similarly, transplanted T-SOL muscle undergoing a slow-to-fast fibre transformation maintained its percentage of fibres with sarcolemmal FAK immunoreactivity in type I and type IIA fibres (Fig. 9). This increase in the percentage of sarcolemmal FAK-positive fibres may be related to changes in the basement membrane zone. Transplantation of rat EDL muscle results initially in fibre degeneration and the disappearance of basement membrane components, i.e. type IV and V collagen and laminin. Over time, a new basement membrane appears and persists in the regenerated fibres (Bassaglia and Gautron, 1995; Gulati et al., 1983). Several laminin chains are re-expressed during regeneration of muscle fibres and probably contribute to induced reconstruction of the basal lamina (Patton et al., 1999).

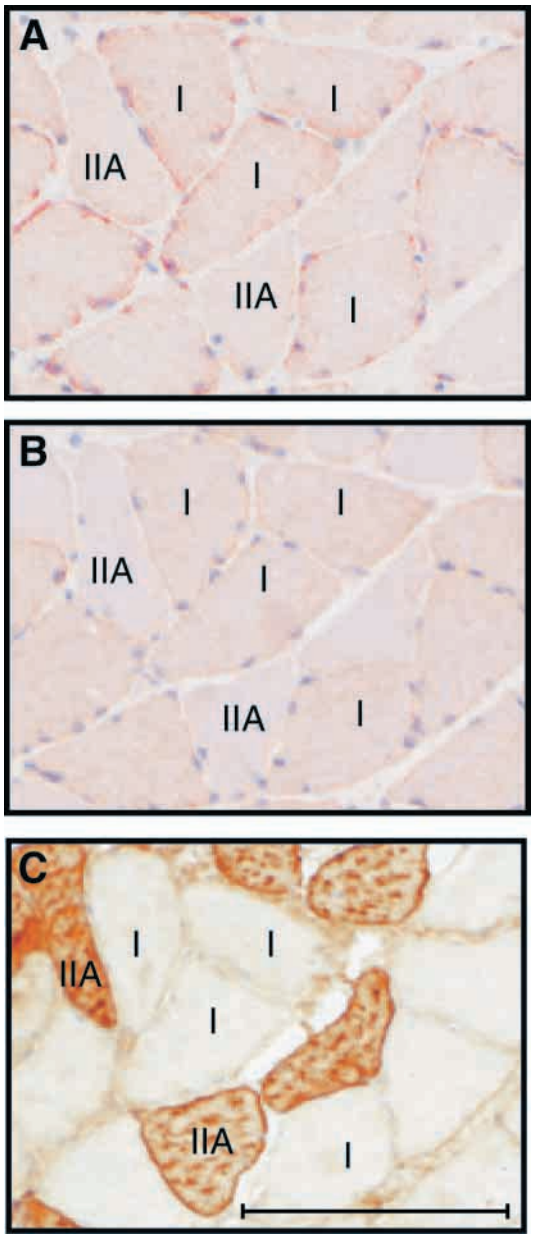


Fig. 7. Sarcolemmal focal adhesion kinase (FAK) immunoreactivity in muscle fibres during the slow- to fast-twitch transformation. Sarcolemmal FAK immunoreactivity (A, FAK antiserum A-17; B, control reaction) and fibre type (C, fast myosin) were determined on consecutive cryosections of soleus muscle 10 months after cross-reinnervation (X-SOL) with the nerve supply of the fast-twitch extensor digitorum longus muscle. The fibre types are indicated. Scale bar, 100 μm.

The presence of laminin isoforms, through binding to their integrin receptors, has been shown to trigger the formation of focal adhesion structures in cultured cells (Sondermann et al., 1999). Such basal lamina remodelling may cause occupancy of integrins with new ECM ligands and induce the formation of FACs involving upregulation of sarcolemmal FAK expression. In the first 2 weeks after injury to rat gastrocnemius and soleus muscle, the FAC component vinculin, together with the extracellular matrix proteins type IV collagen, fibronectin and laminin, accumulate in regenerating (small) fibres in regions corresponding to the costamere (Kaariainen et al., 2000; Kami et al., 1993). This suggests that some fibres showing a high(er) percentage of sarcolemmal FAK immunoreactivity in transplanted compared with normal muscles, i.e. small type IIB fibres in T-EDL and type I and IIA fibres in T-SOL, reflect regenerating fibres that show an increased turnover or density of FACs as a result of changes in basal lamina composition. Alternatively, the reduced sarcolemmal FAK immunoreactivity in type IIA fibres of T-EDL could reflect fibres with reduced costamere density, similar to the situation observed in injured rat muscle after regeneration (Kaariainen et al., 2000).

The association of FAK with integrin-based focal adhesions of cultured cells is through paxillin-mediated interaction between the C-terminal (focal adhesion targeting) domain of FAK. It is negatively regulated by the level of specific C-terminal products of the FAK gene, which are derived from alternative transcriptional initiation or postranslational processing (Cary and Guan, 1999). Immunoblotting experiments did not indicate enhanced expression of proteins related to the FAK C terminus in N-EDL *versus* N-SOL muscle (Fig. 3). In contrast, immunoblotting analyses indicated that the level of full-length 125 kDa FAK protein was elevated in slow-twitch N-SOL relative to fast-twitch N-EDL muscle (Fig. 3). These latter differences are in agreement with the different percentages of sarcolemmal FAK-positive fibres in normal muscles. For example, only 45 % of fibres in N-EDL muscle (the product of the percentage of sarcolemmal FAK-positive fibres in a fibre type and the abundance of fibre type relative to total fibres) *versus* 89 % of total fibres in N-SOL muscle were sarcolemmal FAK-positive. These data suggest that an increased level of FAK protein, rather than modification of FAK localisation by FAK C-terminal products, causes the high level of sarcolemmal expression of FAK in rat fibre types.

A major biological function of FAK is the control of FAC turnover, and localisation of FAK is in many cell types restricted to FACs (Cary and Guan, 1999; Ilic et al., 1995; Schlaepfer and Hunter, 1998). Thus, the sarcolemmal concentration of FAK in rat muscles probably reflects the concentration of FAK to sarcolemmal focal adhesions (Pardo et al., 1983). It has been proposed that the costameres transmit physical forces laterally to the connective tissue and to adjacent muscle fibres (Huijing, 1999; Monti et al., 1999; Patel and Lieber, 1997). Fast- and slow-twitch skeletal muscle fibres contract at different velocities (Burke et al., 1971; Close, 1965). It is therefore conceivable that focal adhesions of the sarcolemma of fast- and slow-twitch fibres are subjected to different profiles of mechanical forces. Intriguingly, mechanical forces induce FAK phosphorylation in many cells, and physical load has recently been shown to induce phosphorylation and expression of FAK in skeletal muscle (Flück et al., 1999; Gordon et al., 2001; Li et al., 1997; Tang et al., 1999). We speculate that the higher sarcolemmal FAK

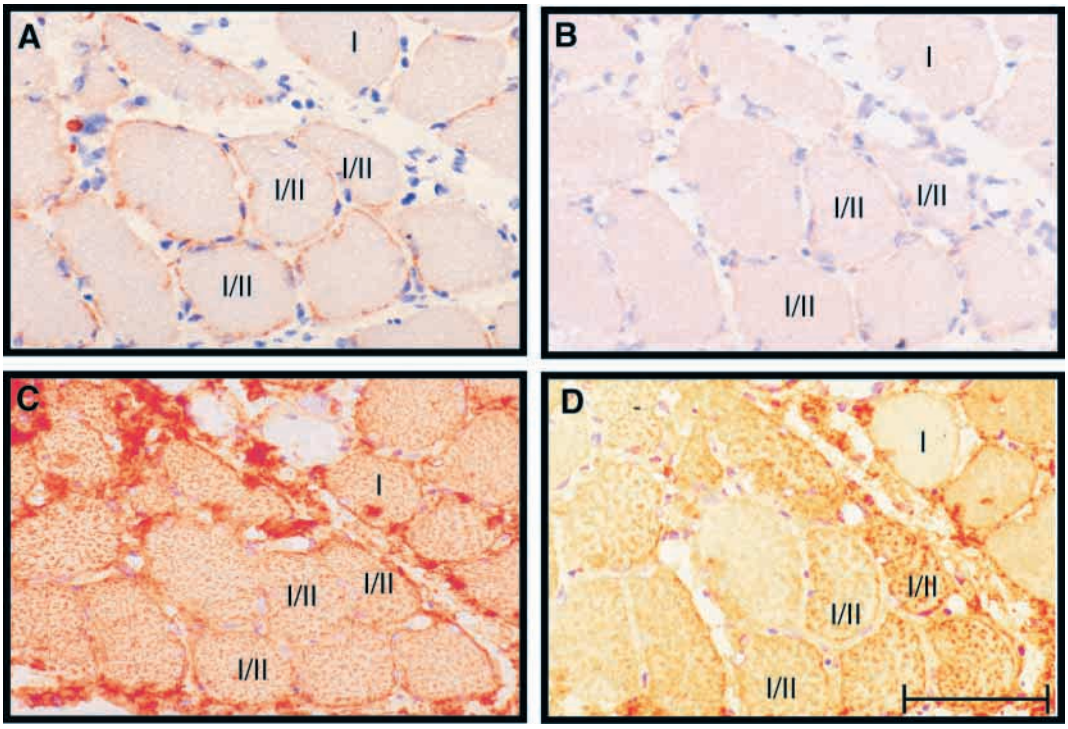
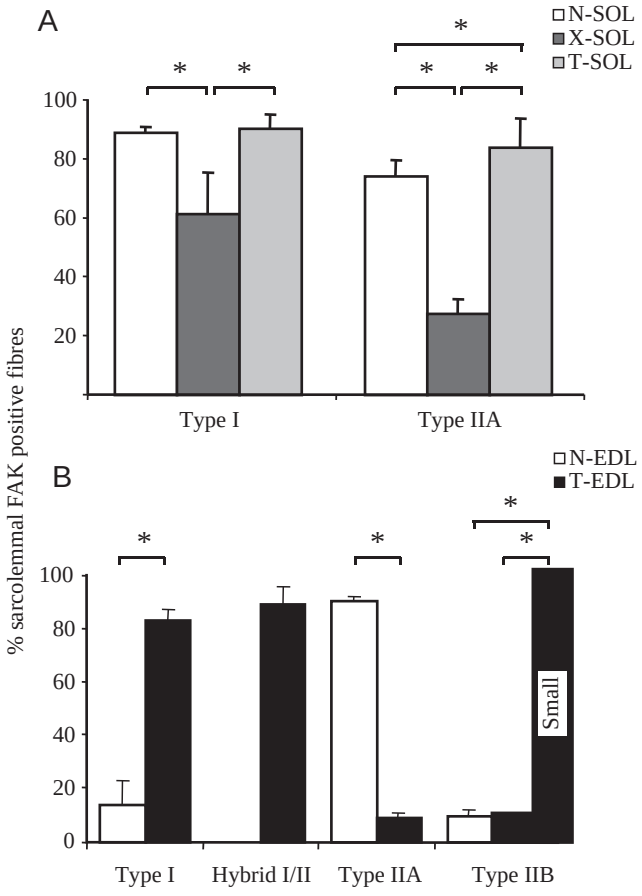


Fig. 8. Sarcolemmal focal adhesion kinase (FAK) immunoreactivity in muscle fibres during the fast- to slow-twitch transformation. Sarcolemmal FAK immunoreactivity (A, serum A-17; B, control reaction) and fibre type (C, slow myosin; D, fast myosin) were determined on consecutive cryosections from an extensor digitorum longus muscle 8 months after autografting and reinnervation (T-EDL) with the nerve of the slow-twitch soleus muscle. The fibre types are indicated. Scale bar, 100 μm.



immunoreactivity in frequently recruited fibres of normal rat muscle reflects an adaptive increase in costamer density or turnover needed to stabilise the fibres against the higher overall mechanical load. However, increased sarcolemmal FAK immunoreactivity in transplanted rat muscles may reflect increased turnover and the formation of new costameres during remodelling of the basement membrane in regenerating fibres (Fig. 10). This is consistent with the hypothesis that FAK is

Fig. 9. Quantification of sarcolemmal focal adhesion kinase (FAK) expression in different fibre types of normal and foreign-reinnervated rat skeletal muscles: Fibres were classified into different types and subdivided on the criteria of FAK immunoreactivity into a sarcolemmal FAK-positive and sarcolemmal FAK-negative fibre population. The percentage of sarcolemmal FAK-positive fibres for each fibre type was then counted as described in Materials and methods. The histograms display the mean + S.E.M. (N=4–6) of the calculated percentage of sarcolemmal FAK-immunoreactive fibres in (A) normal soleus muscle (N-SOL, white columns), cross-reinnervated (X-SOL) soleus muscle (black columns) and transplanted and foreign-reinnervated (T-SOL) soleus muscle (grey columns), and in (B) normal extensor digitorum longus (N-EDL, white columns) and transplanted and reinnervated (T-EDL) extensor digitorum longus muscle (black columns). Individual values were compared using a bilateral χ^2-test for statistical significance. An asterisk denotes a significant difference (P<0.001) in the percentage of sarcolemmal FAK-immunoreactive fibres in a muscle fibre type between the experimental muscle types marked with a bracket.

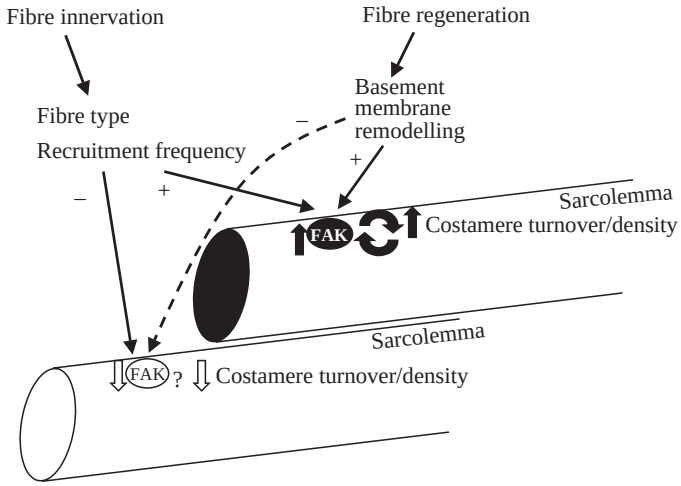


Fig. 10. Summary of the findings on the regulation of sarcolemmal focal adhesion kinase (FAK) immunoreactivity by fibre type (innervation pattern) and regeneration. A model is proposed whereby an increased association between FAK and the sarcolemma is explained by frequent fibre recruitment and basement membrane remodelling and is correlated with increased turnover and density of costameres.

involved in the fibre-type-specific assembly of sarcolemmal integrin receptor complexes and in their eventual modulation through fibre regeneration and fibre recruitment pattern.

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