Targeting of α -kinase-anchoring protein (α KAP) to sarcoplasmic reticulum and nuclei of skeletal muscle

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The sarcoplasmic reticulum (SR) plays a key role in excitation/contraction coupling of skeletal muscle. The SR is composed of two continuous yet heterogeneous membrane compartments, the free or longitudinal SR and cisternal SR. Cisternal SR is made up of free SR membrane, enriched in Ca^{2+} pumps, and junctional SR (jSR) membrane, enriched in ryanodine-sensitive Ca^{2+} -release channels, and contains calsequestrin within its lumen. Protein phosphorylation mediated by the Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase II) has significant, distinct regulatory roles in both Ca^{2+} uptake and Ca^{2+} release. Kinase-anchoring proteins (KAPs) constitute a novel mechanism for achieving cell compartmentalization of effectors in phosphorylation pathways. Here, targeting of α KAP, a CaM kinase II-anchoring protein encoded within the α -CaM kinase II gene, was studied in transgenic skeletal muscle fibres of the adult rat soleus. The transgenes were epitope-tagged versions of α KAP and of a

deletion mutant, allowing their specific immunodetection against the wild-type background. Our results show that α KAP is largely localized at the free SR and thus near the Ca^{2+} pump, a protein that can be modulated by CaM kinase II phosphorylation. Only minor co-localization was observed with the jSR ryanodine-sensitive Ca^{2+} -release channel, which is a potential CaM kinase II target. In non-muscle cells, recombinant α KAP is targeted to endoplasmic reticulum (ER). Both ER and SR targeting requires the N-terminal hydrophobic region of α KAP. An unexpected additional specific localization that does not require the N-terminus was found in the nucleus, providing a first clue of how CaM kinase II can fulfil its nuclear functions in skeletal muscle.

Key words: anchoring, Ca^{2+} /calmodulin-dependent protein kinase (CaM kinase), nucleus, sarcoplasmic reticulum, skeletal muscle, targeting.

INTRODUCTION

The sarcoplasmic reticulum (SR), a network of tubules and cisternae molecularly specialized for the control of rapid, transient changes of cytoplasmic Ca^{2+} , is a sub-compartment of the endoplasmic reticulum (ER) and plays a pivotal part in excitation/contraction coupling of skeletal muscle [1]. The SR is composed of two continuous compartments, the longitudinal SR, enriched in Ca^{2+} pumps, and the cisternal SR, enriched in calsequestrin (CS) within its lumen. Cisternal SR membrane is further subdivided into non-junctional (free SR) membrane and junctional SR (jSR) membrane, the latter being juxtaposed to transverse tubule and enriched in ryanodine receptor (RyR) type 1 Ca^{2+} -release channel (for a review see [2] and references therein). SR proteins are devoted to Ca^{2+} uptake from the cytoplasm (Ca^{2+} -pump protein), intralumen Ca^{2+} storage (CS), Ca^{2+} release to the cytoplasm (RyR1 Ca^{2+} -release channel), signal transduction between transverse tubules and SR (triadin, junctin, RyR1). The protein profile of the different SR compartments is complex and strikingly heterogeneous with respect to both intrinsic and extrinsic proteins ([1–3] and references therein); it includes minor components, such as regulatory/effector/scaffolding proteins, e.g. FK506-binding proteins [4], that cause functional association or 'coupled gating' between RyR1 channels, such as kinases and kinase-anchoring proteins (KAPs) [5–7]. Protein–protein interactions between extrinsic and intrinsic SR proteins are still poorly understood [8–12].

Various proteins (Ca^{2+} pump, RyR1, triadin) of isolated SR membrane fractions are reported to be phosphorylated and thereby regulated by Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase II) [9,13–18], phosphorylase kinase [19] and cAMP-dependent protein kinase [20]. Thus, kinase-dependent protein phosphorylations have significant, distinct regulatory roles in both Ca^{2+} uptake and Ca^{2+} release, although there are still conflicting data as to the substrate(s) being phosphorylated [21].

KAPs constitute a novel mechanism for achieving cell compartmentalization of effectors in phosphorylation pathways [22–25]. Bayer et al. [6] recently demonstrated that α KAP, a non-kinase protein encoded by a gene within the gene of α -CaM kinase II [5], can target CaM kinase II holoenzymes to skeletal muscle SR. It is not known, however, whether α KAP is localized in both free SR and jSR, or is selectively restricted to either membrane domain, which are the specific targeting domains of α KAP *in vivo*, and whether α KAP interacts physically and/or functionally with other known SR proteins. Differential localization among the sub-compartments would impact the potential mechanisms for Ca^{2+} homeostasis in skeletal muscle.

In this paper, we used epitope-tagged α KAP-HA1 and the deletion mutant α KAP-HA1 Δ h (without 21 hydrophobic amino acids at the N-terminus), to investigate topography and intrinsic targeting domains of α KAP. Transient transfection and immunocytochemical analysis were carried out not only on cell lines, e.g. HeLa cells, and primary cultures of rat myotubes, but also on

Abbreviations used: CaM kinase II, Ca^{2+} /calmodulin-dependent protein kinase II; CR, calreticulin; CS, calsequestrin; ER, endoplasmic reticulum; HA, haemagglutinin A; jSR, junctional SR; KAP, kinase-anchoring protein; NLS, nuclear localization signal; RyR, ryanodine receptor; SERCA, sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase; SR, sarcoplasmic reticulum.

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soleus muscle fibres of the adult rat. The latter experimental model allows *in vivo* studies on transgenic skeletal muscle fibres and yields clear-cut data as to SR topography and targeting [26,27]. The present results show that α KAP is localized largely to free SR, whereas only a minor fraction is found at the jSR membrane; in non-muscle cells, overexpressed α KAP targets to the ER; targeting to ER/SR requires the N-terminal hydrophobic region of α KAP. Surprisingly, an additional specific localization of α KAP was found in the nucleus.

EXPERIMENTAL

α KAP expression vectors

cDNAs coding for α KAP-HA1, with the nine amino acid epitope tag of haemagglutinin A (HA1) epitope tag inserted at the C-terminus, and α KAP-HA1 Δ h, without the 21 hydrophobic amino acids at the N-terminus, were developed as described in [6].

Cell cultures and generation of transient transfectants

HeLa or HEK-293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM glutamine and 10 % fetal calf serum. Primary myoblasts were isolated from hind-limb skeletal muscles of 0–3 day-old rats [26] and cultured in DMEM with 20 % fetal calf serum and 20 mM glucose. Differentiation to myotubes was obtained by changing the medium to DMEM with 10–20 % horse serum and subsequently to DMEM with 2 % horse serum. For immunofluorescence studies, cells were transfected by the calcium phosphate precipitation method and processed as described in [26]. For fractionation studies, HeLa or HEK-293T cells were transfected by Lipofectin and PLUS reagents or LipofectAMINE PLUS reagent (Invitrogen), and collected 3 days after transfection.

Immunofluorescence

Cells were fixed in 4 % paraformaldehyde/240 mM phosphate buffer (pH 7.4), for 15 min, and permeabilized with 0.3 % Triton X-100, 20 mM phosphate buffer (pH 7.4), 0.45 M NaCl and 15 % goat serum (buffer A) for 30 min. Both transversal 9 μ m and longitudinal 6 μ m sections were obtained from soleus muscles [26]. Incubation with polyclonal anti-CS [1] and monoclonal anti-CS antibodies (Affinity Bioreagents), monoclonal and polyclonal anti-HA1 antibodies (BabCo and Santa Cruz Biotechnology, respectively), monoclonal anti-RyR1 antibodies (Affinity Bioreagents), monoclonal anti-sarcoplasmic/endoplasmic-reticulum Ca^{2+} -ATPase (SERCA) 2 antibodies (Affinity Bioreagents) or monoclonal anti-calreticulin (CR) antibodies (Affinity Bioreagents) was performed at room temperature for 90 min in buffer A. Images were obtained with either a Leica DMRB fluorescence microscope or a BioRad Radiance 2100 confocal microscope.

Immunoblot analysis

Rat skeletal muscle homogenates were prepared as described in [6]. HeLa and HEK-293T cells were washed in PBS and lysed in 150 mM NaCl, 15 mM MgCl_2 , 1 mM EGTA, 1 mM PMSF, 50 mM Hepes (pH 7.5), 10 % glycerol, 1 % Triton X-100, 2 % SDS and 20 % β -mercaptoethanol. Protein concentration was determined according to Lowry et al. [28] or by densitometric scanning using BSA as standard and NIH image software after SDS/PAGE. SDS/PAGE (either a linear gradient of 5–15 % or 15 %) and immunoblotting with anti-HA1 antibodies, anti-CaM kinase II (M176; Santa Cruz Biotechnology) antibody or anti-

nuclear localization signal (NLS) antibody [6] were carried out as described in [1,6].

Fractionation studies

Subcellular fractions of transfected HeLa or HEK-293T cells were prepared at 0–4 °C using a procedure modified from Kingston [29]. In brief, cells were harvested by centrifugation and resuspended in hypotonic buffer [10 mM Hepes (pH 7.9), 1.5 mM MgCl_2 , 10 mM KCl, 0.2 mM PMSF, 0.5 mM dithiothreitol and 1 $\times$ Complete protease inhibitor cocktail (Roche)]. After 10 min incubation, cells were homogenized and centrifuged for 15 min at 3300 *g*. Low-speed pellets were resuspended in 7.2 ml of sucrose buffer I [0.32 M sucrose, 3 mM CaCl_2 , 2 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris/HCl (pH 8.0), 0.5 % Nonidet P-40 and 1 mM dithiothreitol] and layered on to 4.4 ml of sucrose buffer II [2 M sucrose, 5 mM magnesium acetate, 0.1 mM EDTA, 10 mM Tris/HCl (pH 8.0) and 1 mM dithiothreitol]. After 45 min of centrifugation at 30000 *g*, the pellet (pure nuclei) and sucrose interface (membranes) were collected. The membranes were centrifuged for 1 h at 100000 *g*, washed with low-salt buffer [20 mM Hepes (pH 7.9), 25 % glycerol, 1.5 mM MgCl_2 , 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM dithiothreitol] and pooled with the second membrane fraction (see below). Pure nuclei were resuspended in high-salt buffer (low-salt buffer with 0.6 M KCl). After 30 min of mixing, the nuclear fraction was centrifuged for 1 h at 100000 *g*. Supernatants were collected as the nuclear extract. The nuclear pellet was washed with high-salt buffer and re-centrifuged for 10 min. The initial low-speed supernatant was adjusted to a final KCl concentration of 0.6 M and centrifuged for 1 h at 100000 *g*. Supernatants were collected as cytosol. Pellets were washed with 30 mM Hepes (pH 7.9), 0.6 M KCl and 3 mM MgCl_2 , re-centrifuged for 10 min and pooled to yield the total membrane fraction.

In vivo transfection of skeletal muscle fibres

Male adult Wistar rats were anaesthetized with ketamine and soleus muscles injected with 50 μ g of plasmid cDNA [26]. Transgenic skeletal muscle fibres were obtained by electroporation [30]. Pulse protocols, i.e. six pulses of 220 V/cm, a pulse duration of 20 ms and an interpulse delay of 200 ms, were delivered using an ECM 830 Electro Square Porator (BTX, Genetronics). Soleus muscles were collected after 3 days.

RESULTS

Overexpression of α KAP-HA1 and KAP-HA1 Δ h in HeLa cells

In our analysis of α KAP targeting, we made use of an HA-tagged α KAP (α KAP-HA1; see Figure 1), as the available antibodies against α KAP are not suitable for immunocytochemistry. Furthermore, localization of overexpressed, tagged α KAP mutants can be distinguished, after transfection, from endogenously expressed wild-type α KAP.

In a first set of experiments, α KAP-HA1 and its mutant α KAP-HA1 Δ h were transiently expressed in HeLa cells to define their subcellular localization (Figure 2). α KAP-HA1 was mainly distributed in the ER and in perinuclear membrane domains in continuity with the ER, as judged by ample overlap with CR, a well-known ER marker ([31] and Figure 2A). Some α KAP-HA1 was also detected within the nucleus, where no co-localization with CR was observed. Finally, no plasma membrane localization was detected.

Figure 1 Schematic representation of α KAP: domain structure (top) and amino acid sequence of the N-terminus (bottom)

The C-terminal position of the HA-epitope tag introduced into α KAP-HA1 is indicated. Black box (in both panels), hydrophobic membrane anchor (amino acids 1–22); grey box (in both panels), NLS (amino acids 39–42); open box (in both panels), association domain (amino acids 68–200). Dashed lines (in both panels) indicate α KAP sequences not present in α -CaM kinase II (amino acids 1–25 and 40–50). The arrow indicates a potential alternative start of translation, consistent with a shorter α KAP product seen in cell extracts.

α KAP-HA1 Δ h is a deletion mutant lacking the N-terminal hydrophobic domain (see Figure 1) and, in contrast with wild-type α KAP, is absent from perinuclear membranes and ER (Figure 2B). Nuclear localization of this deletion mutant, however, is maintained; in fact, α KAP-HA1 Δ h was found almost exclusively in the nucleus. These results indicate that the hydrophobic N-terminus lacking in α KAP-HA1 Δ is crucial for targeting of α KAP to the ER in HeLa cells.

Nuclear localization of full-length α KAP

Immunoblotting was used to analyse protein extracts from either HeLa or HEK-293T cells overexpressing α KAP-HA1. Using anti-HA antibody, full-length α KAP-HA1 and an additional

Figure 3 The full-length α KAP is found in nuclear and membrane fractions, but not in cytosol

(A) Immunoblot of cell lysates from HeLa or HEK-293T cells transfected with α KAP-HA1 (wt), α KAP-HA1 Δ h (Δ h) or α KAP-HA1 Δ 33 (Δ 33) using anti-HA or anti-NLS antibodies as indicated. Ab, antibody. (B) Subcellular fractions from HEK-293T cells transfected with α KAP-HA1 (upper panel), α KAP (middle panel) and α KAP Δ h (lower panel) by immunoblot analyses using anti-HA (upper panel) or anti- α KAP (middle and lower panels) antibodies. Molecular-mass markers and the positions of specific recombinant proteins are indicated on the left and right, respectively. Abbreviations: wt, full-length α KAP (α KAP-HA1 in upper panel); Δ h, α KAP Δ h (α KAP-HA1 Δ h in upper panel); Δ 33, α KAP Δ 33 (α KAP-HA1 Δ 33 in upper panel); H, homogenate; NE, nuclear extract; NP, nuclear pellet; C, cytosol; M, membrane fraction.

Figure 2 α KAP is localized at the ER and in the nucleus of transfected HeLa cells

HeLa cells were transiently transfected with cDNA of either α KAP-HA1 (A) or α KAP-HA1 Δ h (B). 2 days after transfection, cells were fixed and decorated with anti-HA1 antibody to detect the transgenes (left-hand panels) and with anti-CR antibody to stain the ER (middle panels). Right-hand panels show the merged images. Overexpression of α KAP-HA1 induced ER rearrangement based on comparison between transfected and untransfected HeLa cells, whereas the α KAP mutant did not. Scale bar, 6 μ m.

Figure 4 α KAP in skeletal muscle fibres after *in vivo* transfection

(A) Soleus skeletal muscle fibres transfected with α KAP-HA1 cDNA were identified by immunofluorescence on transverse sections using anti-HA antibody (left-hand panel), and showed normal morphological appearance under phase contrast (right-hand panel). Scale bar, 16 μ m. (B) Overexpressed α KAP-HA1 (left-hand panel) and endogenous α KAP (right-hand panel) in skeletal muscle compared by immunoblot analysis. The full-length proteins and 33-amino-acid truncations overexpressed in HeLa cells were loaded as molecular-mass indicators. Abbreviations: HA, α KAP-HA1; HA Δ 33, α KAP-HA1 Δ 33; wt, α KAP; Δ 33, α KAP Δ 33; Ab, antibody.

Figure 5 α KAP in nuclei of transgenic muscle

Confocal immunofluorescence of double-labelled soleus muscle fibres after transfection with either α KAP-HA1 (A) or α KAP-HA1 Δ h (B, C) cDNAs. Longitudinal sections were decorated sequentially with either anti-HA1 antibodies (left-hand panels) or anti-CR antibodies (middle panels). Right-hand panels show the merged images. Arrows in all panels point to nuclei. Five soleus muscle preparations were examined. Scale bar, 8 μ m.

shorter polypeptide were detected (Figure 3A). The shorter product is indeed α KAP-specific, because it is also detected by an antibody for the association domain of α KAP and is not present in cells that do not express full-length α KAP (Figure 3B). This shorter α KAP-HA1-related product is N-terminally truncated, since the HA1 epitope is located at the C-terminus. Based on its electrophoretic mobility, the entire hydrophobic region is missing, and the shorter product might be generated by alternative initiation of translation at M50 (see Figure 1). Consistent with this assumption, the truncated product also lacks the NLS, as it does not react with an antibody against this region, in contrast with the full-length polypeptide (Figure 3A). However, it was still possible that only the shorter product would be localized in the

nucleus, whereas the full-length α KAP could be completely anchored in the ER membrane, raising questions about the interpretation of the immunocytochemical data. Therefore, we fractionated transfected HEK-293T cells into four fractions: soluble nuclear extract, nuclear pellet (nuclear envelope membrane and insoluble matter such as chromatin), cytosol and membrane fraction (plasma membrane and intracellular membranes except the nuclear envelope). Figure 3(B) shows immunoblot analysis of such fractions, using an antibody against the association domain shared by α -CaM kinase II and α KAP. The full-length α KAP is indeed enriched in the nucleus compared with cytosol. α KAP-HA and wild-type α KAP show essentially identical distribution among the fractions; the corresponding

full-length proteins are found in each fraction, except in the cytosol. By contrast, the shorter product is present in all fractions. As expected, the α KAP- Δ h mutant was found exclusively in the nuclear fractions. Both nuclear α KAP and nuclear α KAP- Δ h were only partially solubilized by high salt, indicating protein interactions that are specific to the nucleus and independent of the hydrophobic N-terminus. By contrast, α KAP not associated with the nucleus was not solubilized by high salt at all, consistent with membrane insertion of its N-terminus. Taken together, the biochemical data show that full-length α KAP is partially localized in the nucleus.

α KAP-HA1 Δ h is exclusively nuclear in transfected skeletal muscle myotubes

Rat skeletal muscle myotubes in primary culture represent a model system in which developing SR expresses typical SR markers such as CS [26,27]. We argued that α KAP-HA1 Δ h, which is nuclear in HeLa and HEK-293T cells (Figures 2 and 3), might be partially localized at the SR in myotubes if α KAP contains binding sites for interaction with specific SR proteins that are not present or less abundant at the non-specialized ER. Nevertheless, α KAP-HA1 Δ h localization was found to be exclusively nuclear also in transfected myotubes without any staining of developing SR that contained, instead, CS (results not shown, but see below for targeting in skeletal muscle). These experiments indicate that the hydrophobic domain of α KAP is absolutely required for SR targeting, and that any protein–protein interactions of the remaining portion cannot compensate.

α KAP-HA1-positive transgenic skeletal muscle fibres show normal morphology

The *in vivo* transfection approach of rat skeletal muscle allows determination of α KAP-HA1 targeting *in vivo*, including distribution between SR subcompartments, i.e. longitudinal SR and cisternal SR, and between SR membrane domains, i.e. free SR membrane and jSR membrane. A number of fibres indeed expressed the recombinant α KAP-HA1, as judged by indirect immunofluorescence of labelled transverse sections with anti-HA1 antibodies (Figure 4A, left-hand panel). The overall morphological appearance of the muscle was well preserved 3 days after transfection and electroporation, as judged by phase-contrast microscopy (Figure 4A, right-hand panel).

In immunoblot analysis of transfected muscles, a shorter band is detected in addition to the full-length α KAP-HA1 (Figure 4B, left-hand panel), as seen above for transfected HeLa cells. For the endogenous α KAP in skeletal muscle, three shorter bands were also observed, but were barely detectable (Figure 4B, right-hand panel).

Nuclear targeting of α KAP-HA1 and α KAP-HA1 Δ h in transgenic skeletal muscle fibres

Localization of α KAP-HA1 and α KAP-HA1 Δ h, expressed in double-labelled longitudinal sections of transgenic skeletal muscle fibres, was extensively analysed by confocal immunofluorescence, using both the anti-HA1 antibody and an antibody specific for CR, an intraluminal protein distributed throughout the entire SR (Figure 5 and [1]).

Figure 5(A) shows the labelling pattern of transgenic skeletal muscle fibres expressing α KAP-HA1 with anti-HA1 and anti-CR antibodies. It is interesting to note that α KAP-HA1 was detected not only in SR but also in nuclei and perinuclear membranes. Thus, the partially nuclear localization of α KAP is indeed

relevant in the tissue where α KAP is expressed endogenously, and is not restricted to heterologous systems such as HeLa cells.

α KAP-HA1 Δ h-positive fibres displayed a normal SR architecture, based on distribution of CR (Figure 5B). α KAP-HA1 Δ h was completely absent from SR and restricted to nuclei of skeletal muscle fibres, aligned underneath the plasmalemma. At higher magnification, it becomes apparent that α KAP-HA1 Δ h is within the nucleus and that no staining of nuclear envelope or perinuclear membranes is observed (Figure 5C). This indicates that the membrane association of α KAP is solely mediated by the hydrophobic N-terminus, and that there are no additional dominant interactions with intrinsic SR proteins.

α KAP-HA1 is targeted to specific sub-compartments of the SR in transgenic skeletal muscle fibres

Localization of α KAP-HA1 at the SR was thoroughly investigated by confocal immunofluorescence of double-labelled longitudinal sections of soleus transgenic muscle fibres with anti-HA1 antibodies and antibodies for CS (Figure 6A), RyR (Figure 6B) or Ca^{2+} pump (Figure 6C), markers of the cisternal SR lumen, jSR membrane and free SR membrane, respectively.

CS labelling in Figure 6(A) shows the typical pattern, i.e. parallel rows of bright spots, each resulting from clusters of terminal cisternae filled up with CS [27,32]. α KAP-HA1, detected with anti-HA1 antibodies, displayed a regular banding pattern of more diffuse fluorescence at the A–I interface (the interface between the anisotropic band and the isotropic band of the sarcomere) with extension into the A band (Figure 6A). Merged images indicate that recombinant α KAP-HA1 only partially overlaps with endogenous CS and is selectively localized in the central area of the I band. However, since CS is located in the lumen of cisternal SR lined with both free and jSR membranes [1–3], and CS and α KAP-HA1 appeared to overlap on the very edges (Figure 6A), the issue of whether α KAP-HA1 is associated with free SR and/or jSR cannot be assessed with certainty.

With this aim, confocal immunofluorescence analysis was carried out on longitudinal sections of transgenic skeletal muscle fibres sequentially decorated with anti-HA1 antibodies and antibodies specific for RyR1, a well-known jSR membrane marker (Figure 6B). Comparison of the immunostains suggests that the two signals are largely segregated, as RyR1 is absent from the I band. At the edges, however, some overlap was detected.

These data indirectly indicate that recombinant α KAP-HA1 is associated with free SR. Direct evidence was sought with confocal analysis of transgenic skeletal muscle fibres sequentially decorated with anti-HA1 antibodies and antibodies specific for the Ca^{2+} pump. As shown in Figure 6(C), the two signals overlap in the I band. Within the limits of the experimental approach, we draw the conclusion that α KAP-HA1 is largely in the free SR, but may extend into contiguous areas of jSR.

DISCUSSION

In skeletal muscle, CaM kinase II functions both in transcriptional regulation within the nucleus [33,34] and in feedback regulation of excitation/contraction coupling by modulating Ca^{2+} channels and Ca^{2+} pumps of the SR membrane [17,18,21,35,36]. We show here that α KAP, a CaM kinase II-anchoring protein, is targeted to both subcellular locations, and provide the first mechanistic explanation for the dual CaM kinase II function in skeletal muscle. α KAP is largely localized at the longitudinal SR, and this differential distribution among the SR subcompartments sheds light on recent findings of RyR

Figure 6 Confocal immunofluorescence of double-labelled soleus muscle fibres after transfection with α KAP-HA1 cDNA

Longitudinal sections were decorated sequentially with polyclonal anti-HA1 antibodies and monoclonal anti-CS (**A**), monoclonal anti-RyR1 (**B**) or monoclonal anti-SERCA2 (**C**) antibodies. Lower panels represent the merged images. Free SR in slow-twitch muscles is less developed at the A band as compared with fast-twitch muscles [45], and this accounts for the apparent lack of labelling with both anti-SERCA2 and anti-HA1 antibodies. Five soleus muscle preparations were examined. Scale bar, 8 μ m.

channel populations that differ in their regulation by endogenous CaM kinase II [36].

The serum response factor, a nuclear transcription factor, is phosphorylated by CaM kinase II [34], mediates Ca^{2+} -dependent gene expression [33,37], and is also required in myogenic differentiation [38]. Although some splice variants of CaM kinase II (α_B and δ_B) contain a functional NLS [39,40], these variants were not detected in skeletal muscle [6], raising the question of how CaM kinase II interacts with its nuclear substrates in this tissue. The nuclear localization of α KAP shown here provides a mechanism for nuclear targeting of CaM kinase II variants without NLS: α KAP and CaM kinase II can co-assemble in skeletal muscle [6], and CaM kinase II variants without NLS have been shown to be targeted to the nucleus when co-assembled with NLS-containing variants [39]. α KAP and α_B -CaM kinase II actually contain both an identical association domain and an NLS (see Figure 1 and [5]). It has been previously proposed that the hydrophobic N-terminus of α KAP is dominant over the NLS [6]. Indeed, we found most of the α KAP targeted to the SR membrane, whereas deletion of the hydrophobic N-terminus resulted in almost exclusive nuclear targeting. This suggested the possibility that the nuclear α KAP could be a truncated version lacking the hydrophobic region rather than the full-length protein. Such a regulation of protein localization has been described previously for the unfolded protein response. There, proteolytic cleavage of kinases like Ire1, which are integral ER membrane proteins,

leads to release of the kinase domains and to their nuclear translocation, where they can function in transcriptional silencing (for a review see [41]). For α KAP, cell-fractionation experiments (Figure 3) revealed that the full-length protein accounts for the nuclear staining observed in immunofluorescence. In addition, we did detect a truncated form. However, this truncated α KAP lacks not only the hydrophobic N-terminus but also the NLS, and it is not enriched in the nucleus. Its length is consistent with usage of an alternative start codon at M50; no consensus sequences for proteolytic cleavage sites were found in the relevant region of α KAP. The functional significance of the truncated form of α KAP is presently unclear. It appears, thus, that nuclear targeting of CaM kinase II in skeletal muscle could be mediated only by the full-length α KAP.

α KAP is found not only associated with nuclear envelope but also soluble within the nuclear extract. By contrast, outside of the nucleus α KAP is essentially absent from the cytosol and is found only in membrane-bound form. Membrane targeting appears specific only for ER and related membranes, such as SR and nuclear envelope; no α KAP was detected on the plasma membrane or on mitochondria. This indicates that α KAP may either contain some kind of ER retention signal or be rapidly targeted for degradation at other compartments. Retention could be mediated by binding to ER proteins. Such putative interactions are not likely to be very strong, since the NLS becomes dominant after removal of the hydrophobic membrane anchor. Moreover,

the truncated α KAP product, lacking both the NLS and the hydrophobic region, is found at least partially in the cytosol. However, this does not rule out the possibility that lower-affinity interactions with intrinsic ER proteins could become relevant once α KAP is anchored to the ER membrane.

In cardiac muscle, CaM kinase II has been reported to regulate the L-type voltage-gated Ca^{2+} channel [42]; additionally, co-localization of CaM kinase II and L-type Ca^{2+} channel has been reported [43]. According to our study, such CaM kinase II targeting could not be mediated by α KAP, at least in skeletal muscle, and probably requires a different mechanism.

By far, most of the α KAP in skeletal muscle is localized in the membrane of non-junctional, free SR in the I band (Figure 6). The SR Ca^{2+} -pump protein can be activated by CaM kinase II, both by direct phosphorylation [18] and by phosphorylation of the regulator protein phospholamban [35]. Thus CaM kinase II activated by Ca^{2+} /calmodulin can enhance Ca^{2+} re-uptake into the SR, regulating cytosolic Ca^{2+} concentration in a negative feedback loop; α KAP can mediate the proper subcellular targeting of CaM kinase II and may thereby function to ensure speed and specificity of this process. CaM kinase II can also regulate the RyR, the SR Ca^{2+} -release channel. Different types of regulation by CaM kinase II have been described for RyR, apparently dependent on the targeting of the kinase: CaM kinase II associated with jSR preparations inhibits the RyR, thus mediating a negative-feedback loop of Ca^{2+} regulation, similar to the effect on the Ca^{2+} pump. On the other hand, soluble CaM kinase II activity (added to the preparation) actually enhanced RyR activity. It is conceivable that targeting could determine a differential CaM kinase II effect by restricting phosphorylation to a subset of the potential sites on the receptor. The RyR is concentrated in jSR membrane, the SR membrane domain with the lowest amount of α KAP. Significant co-localization was observed only at the edges of jSR, indicating that only a fraction of the RyRs could be regulated by CaM kinase II targeted to the SR by α KAP. Indeed, it has been demonstrated that about 25% of the total RyR is regulated differently by endogenous CaM kinase II [36]. Thus our results provide a mechanistic basis to the contention that RyR1 Ca^{2+} -release channels are heterogeneous with respect to activation and regulation, and fit the quantitative model of the control of Ca^{2+} release, based on dual control of RyR1, primarily by voltage, secondarily by Ca^{2+} [44].

We thank Dr L. Galassi and Dr F. Visentini for carrying out preliminary experiments. K.-U.B. was supported by Deutsche Forschungsgemeinschaft research fellowship BA1647/1-1. This work was supported by funds from the Ministero dell'Università e della Ricerca Scientifica e Tecnologica (2001–2003 Programma di ricerca di rilevante interesse nazionale on Biopathology of the skeletal muscle fiber) to P.V.

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Received 18 October 2002/22 November 2002; accepted 6 December 2002

Published as BJ Immediate Publication 6 December 2002, DOI 10.1042/BJ20021624