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α-Cardiac Actin (*ACTC*) Binds to the Band 3 (*AE1*) Cardiac Isoform

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Abstract The band 3 protein is the major integral protein present in the erythrocyte membrane. Two tissue-specific isoforms are also expressed in kidney alpha intercalated cells and in cardiomyocytes. It has been suggested that the cardiac isoform predominantly mediates the anion exchange in cardiomyocytes, but the role of the cytoplasmic domain of the band 3 (CDB3) protein in the cardiac tissue is unknown. In order to characterize novel associations of the CDB3 in the cardiac tissue, we performed the two-hybrid assay, using a bait comprising the region from leu 258 to leu 311 of the erythrocyte band 3, which must also be present in the cardiac isoform. The assay revealed two clones containing the C-terminal region of the α-cardiac actin. Immunoprecipitation of whole rat heart using an anti-actin antibody, immunoblotted with anti-human band 3, showed that actin binds to band 3 which was confirmed in the reverse assay. The confocal microscopy showed band 3 in the intercalated discs. Thus, besides the in vivo physical interaction in the *Saccharomyces cerevisiae* cell, we demonstrated using immunopreciptation that there is a physical association of band 3 with α-cardiac actin in cardiomyocyte, and we suggest that the binding occur ''in situ,'' in the intercalated disc, a site of cell–cell contact and attachment of the sarcomere to the plasma membrane. J. Cell. Biochem. 89: 1215–1221, 2003.

Key words: cardiac muscle; intercalated disc; thin filament; yeast two-hybrid screening

Band 3 is the first member of the anion exchanger gene family (*AE1*). It is an integral membrane protein, first characterized in erythrocytes [Fairbanks et al., 1971] and composed structurally and functionally by two domains: the transmembrane domain, highly conserved and responsible for the electroneutral Cl^-/HCO_3^- exchange across the plasma membrane of vertebrate cells [Salhany, 1990], and the cytoplasmic domain (CDB3), which is less conserved and plays specific roles in different cells.

The cytoplasmic domain of the erythrocyte band 3 protein (CDB3e) has been implicated in several functions as glycolysis control through the phosphorylation of tyrosine 8 [Harrison et al., 1991] and cytoskeleton anchoring to the membrane, a structural role played through the ankyrin [Batrukova et al., 2000], protein 4.1 [von Ruckmann et al., 1997], and protein 4.2 associations [Rybicki et al., 1995]. CDB3e binds to many red cell proteins, including aldolase, glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase, deoxyhemoglobin, hemichromes [Low, 1986], protein tyrosine kinase (p72syk) [Harrison et al., 1994], carbonic anhydrase II [Vince and Reithmeier, 2000] and it has recently been implicated in the export of nitric oxide bioreactivity of the red blood cells [Pawloski et al., 2001].

A truncated isoform of the band 3 protein was characterized in the renal alpha intercalated cell where it drives bicarbonate generated in the cell to the blood, through the basolateral membrane [Kudrycki and Schull, 1993].

Recently, it has been suggested that a truncated form of band 3 is the predominant anion exchanger in ventricular myocytes and although this new isoform has not been cloned, it has been demonstrated that the mRNA is similar to the erythroid band 3 mRNA downstream the exon 7 [Richards et al., 1999].

Although the transport activity of the band 3 protein has been studied in a variety of cells,

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the structural properties based on cytoskeleton binding has been extensively examined only in the erythrocyte membrane. Thus, to better understand the band 3 physiology in the cardiac muscle, we performed the two-hybrid assay to screen proteins from a heart library that binds to the CDB3.

MATERIALS AND METHODS

Yeast Two-Hybrid Screening

The yeast two-hybrid screening was performed using the Matchmaker Two-hybrid System 3® from Clontech (Palo Alto, CA). The procedures were based on the Matchmaker users protocol and all controls were performed.

Nucleotides encoding the region comprising the leu 258 to leu 311 based on erythrocyte band 3 cDNA sequence were amplified by PCR with a pair of band 3 specific primers (Forward: 5′CC GAA TTC CTG CCG GTG CCT ATA CGC TT 3′; Reverse: 5′AA GGA TCC TAG GGA GTG CAG CAG CTC CC 3′) from commercial human heart cDNA (QUICK-Clone cDNA-Clontech, Palo Alto). An *Eco*RI site is present in the forward primer and a *Bam*HI site in the reverse primer as shown underlined. The above region constitutes the beta sheet 10 and the alpha helix 7, 8, and 9 of the cytoplasmic domain of erythrocyte band 3–AE1, according to the protein crystallography [Zhang et al., 2000] represented in Figure 1. After digestion with *Eco*RI and *Bam*HI, the PCR product was subcloned into the GAL4 DNA binding domain plasmid, pGBKT7 (Clontech, Palo Alto, CA). The pGBKT7[CDB3] construct was transformed as previously described [Ito et al., 1983 and modified Schiestl and Gietz, 1989; Hill et al., 1991; Gietz et al., 1992] into yeast strain AH109 and mated with the GAL4 activation domain [James et al., 1996] plasmid pACT2[heart library] pre-transformed into yeast strain Y187 (Clontech), a suitable *Saccharomyces cerevisiae* yeast strain for the mating assay. The mated yeast was plated onto synthetic dropout medium lacking adenine, histidine, leucine, and tryptophan to allow high stringency selection. After a 8-day incubation at 30°C, interaction was determined by re-streaking the clones onto X-α-galactosidase indicator plates. In yeast, α-galactosidase is a secreted reporter gene that is expressed from the *MEL1* gene in response to GAL4 activation. X-α Gal can be used with Clontech's GAL4-based Matchmaker products to confirm protein interactions [Aho et al., 1997]. The inserts of positive colonies were identified by PCR and automated sequencing.

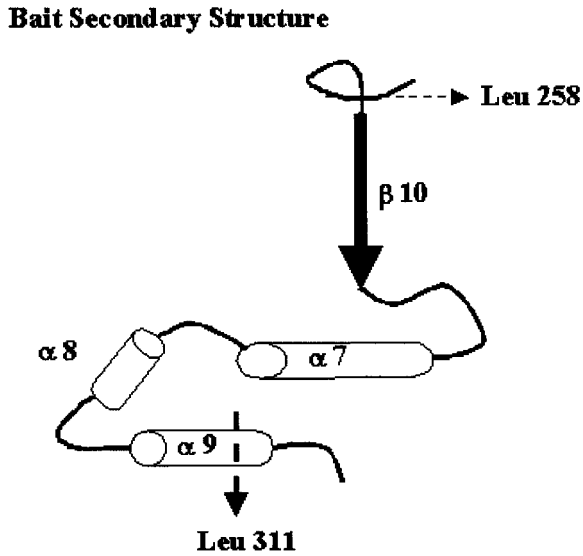

Fig. 1. Secondary structure diagram of the region β $10^{263-271}$, α $7^{278-290}$, α $8^{292-300}$ e α $9^{304-316}$.

Immunoprecipitation

For immunoprecipitation, a whole-heart homogenate was prepared from one anesthetized and PBS-perfused adult Wistar rat. The organ was minced in a plate containing cold PBS, extensively washed, and homogenized by using a polytron at maximum speed for 20 s in an iced extraction buffer (pH 7.5), containing Trizma base 100 mmol/L, pH 7.5; sodium fluoride 100 mM; tetrasodium pyrophosphate 10 mmol/L; sodium orthovanadate 10 mmol/L; phenilmetilsulphonilfluoride (PMSF) 2 mmol/L, aprotinine 0.1 mg/ml, and triton X-100 (1%). The homogenate was incubated on ice for 2 h, centrifuged at 4°C for 20 min at 12,000*g* and the clear supernatant was used as total extract. Two milligrams of total proteins were incubated with 15 μg of the antibody, overnight at 4°C. The immune complexes were precipitated with protein A-Sepharose 6 MB (Amersham, Upsala, Sweden) and washed three times with 50 mM Tris (pH 7.4) containing 2 mmol/L sodium vanadate and 0.1% Triton X-100.

Immunoblotting

After washing, immunoprecipitated proteins were denatured with loading buffer

and resolved by SDS–PAGE (10% or 15% polyacrylamide gels) in a Bio-Rad miniature slab gel apparatus. Electrotransfer was performed in a Bio-Rad miniature transfer apparatus (Mini-Protean). For detecting band 3 or actin, mouse monoclonal anti-human CDB3 (a kind gift from Dr. Philip Low), and goat polyclonal anti-human actin (C11-Santa Cruz Biotechnology, Santa Cruz, CA) were used, and the protein A-I^{125} radioisotope labeling was performed.

Immunohistochemistry

For immunofluorescence study, an adult rat was CO_2 anesthetized, decapitated, and the heart was flash frozen in liquid nitrogen. The whole organ was sliced in 6 μm using a microtome cryostat (Cryo-Star HM 560 V, Microm, Walldorf, Germany). The slices collected in silanized smears were fixed with 4% of formaldehyde in phosphate-buffered saline (PBS) for 30 min. The basal fluorescence was killed, incubating the smears in perhydrol 3%. The material was blocked with PBS containing 3% BSA, during 2 h. The primary polyclonal rabbit anti-band 3 antibody, against the whole region of CDB3, in 1:1,000 dilution, was incubated overnight. The secondary antibody to rabbit IgG, Cy5-linked, (Amersham Pharmacia Biotech, Uppsala, Sweden) in 1:200 dilution, was added and incubated for 2 h. Repeated washes with PBS were done after each step of the immunolabeling procedure. The preparations were mounted in glycerol 80%. The sections of the ventricles were examined by laser scanning confocal microscopy LSM 510 (Carl Zeiss, Jena, Germany) using a 63×1.2 objective lens. The recorded image was taken using dual-channel scanning and consisted of 512×512 pixels.

RESULTS

In this study, we used the yeast two-hybrid system to identify the potential protein partners for the CDB3 in the cardiac tissue. We screened $\sim 9.2 \times 10^5$ clones from a human heart cDNA library (Clontech) and obtained 25 *HIS3*+ and *ADE2*+ clones. Only 13 putative positive AD/library plasmids were further characterized and confirmed to be true positives by X-α-Gal assay (*MEL1*+). These clones were sequenced and the fragments were classified into 11 genes (Table I).

Two of such isolates contained a C-terminus region of the human α-cardiac actin (*ACTC*) gene, introduced into the *Xho*I/*Eco*RI sites of pACT2, with a correct ORF. The clones containing the α-cardiac actin sequences demonstrated a common region from histidine 277 to phenilalanine 354, illustrated in the Figure 2 (697–1062 nt. and 828–1261 nt.). Thus, by the two-hybrid screening, we identified an α-cardiac actin domain (78 aa, *ACTC*) that binds to the cardiac isoform of the band 3 protein (*AE1*) in a site probably surrounding the myocyte membrane, located within the region called "bait" comprising 54 residues from erythrocyte band 3 leu 258 to leu 311.

Confirmation of the Association by Immunoprecipitation

To confirm the physical interaction in vivo between the band 3 protein and actin, we performed immunoprecipitation experiments. The results are presented in the Figure 3. We demonstrated that the cardiac isoform of the band 3 protein efficiently immunoprecipitated with anti-actin antibody (Fig. 3A), and the reverse assay confirmed this physical interaction (Fig. 3B). The band 3 and actin do not

TABLE I. Two Hybrid Clones

Clones	GenBank Match	Genes	Domain/DNA
1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1	*ATP5C1*	D87–Y196
2	Eukaryotic translation elongation factor 1 alpha 1, tu domain 2	*EF1α*	V264–I353
3	Electron-transfer flavoprotein alpha polypeptide (glutaric aciduria II)	*ETFA*	V129–T191
5 and 11	Ferritin, light polypeptide	*FTL*	S28–G130 M1–G75
7	Cytochrome P450	*CYP1A1*	13766–13887 nt.
8	Hypothetical protein of chromosome 14	Unknown	136698–137080 nt.
9	Nuclear receptor subfamily 1, group H, member 3 (LXR)	*NR1H3*	P76–K86
10	Enoyl Coenzyme A hydratase, short chain, 1, mitochondrial (nuclear gene encoding mitochondrial protein)	*ECHS1*	D91–V192
15 and 18	Alpha-cardiac actin	*ACTC*	H277–F354
16	Transcription factor 15 (basic helix-loop-helix) 3′untranslated region	*TCF15*	635–762 nt.
17	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip) subunit	*SDHB*	K152–L258

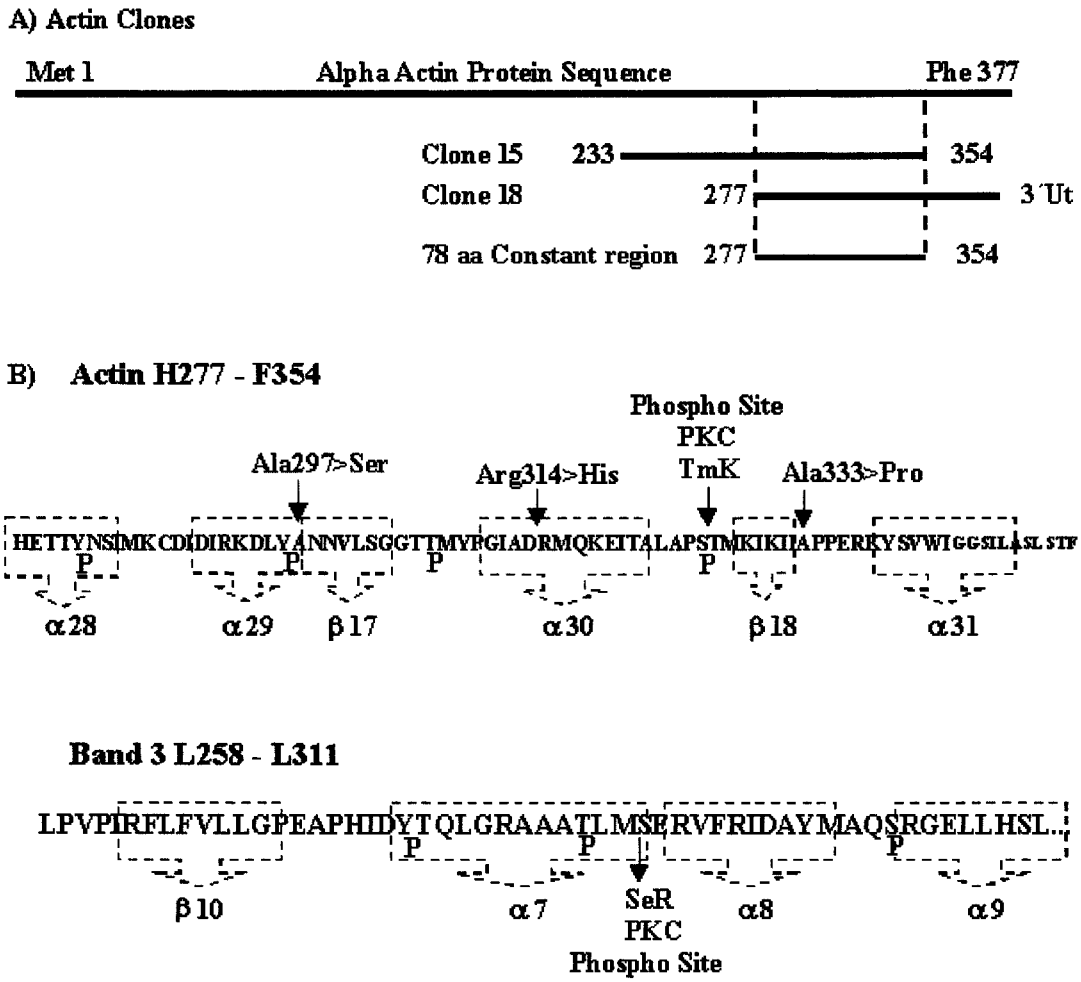

Fig. 2. **A**: Actin clones: The clones containing the cardiac muscle alpha actin sequences demonstrated a common region from histidine 277 to phenilalanine 354. **B**: Functional domains of the band 3 and α-cardiac actin: Primary and secondary structures of the α-cardiac actin [Kabsch et al., 1990] and the band 3 "bait" [Zhang et al., 2000]. P, Predicted phosphorylation sites according to NetPhos 2.0; PKC, protein kinase C phosphorylation site according ScanProsite. Missense mutations affecting the α-cardiac actin (Ala 297 > Ser, Arg 314 > His, and Ala 333 > Pro).

co-immunoprecipitated with a nonspecific primary antibody (anti-c-Abl, Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Double Immunostaining for Actin and Band 3 of the Rat Heart

The confocal microscopy showed the band 3 blue decorated in intercalated discs of the rat heart, and red fluorescent phalloidin-TRITC staining the actin filaments (Fig. 4).

DISCUSSION

In the present study, we found that the α-cardiac actin, a major constituent of the contractile apparatus, interacts with the band 3 protein in the yeast cell. In addition to the in vivo physical interaction into the *Saccharomyces cerevisiae* cells, we demonstrated by immunopreciptation that, in cardiomyocyte, there is a physical association between band 3 and actin.

We propose that the α-cardiac actin sequence H277 to F354 is a functional domain which presents one or more binding sites to the L258–L311 region of the CDB3, and we suggest that the band 3 and actin associations could take place in the cytoplasm, surrounding the sarcolemma, at the intercalated discs. The fact that we found two clones containing the carboxi terminus of cardiac alpha actin and the immunoprecipitation result suggest that band 3 and α-cardiac actin could interact.

The confocal microscopy showed the cardiac band 3 (*AE1*) in the intercalated discs of the rat

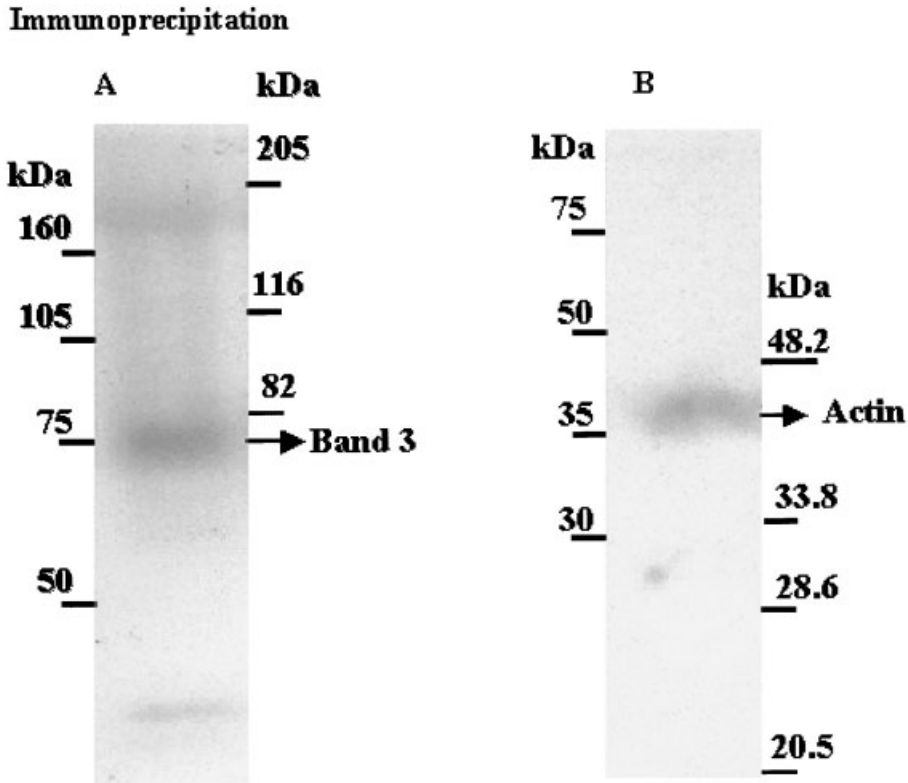

Fig. 3. **A**: Two milligrams of protein of a rat whole-heart homogenate were immunoprecipitated (IP) with goat polyclonal anti-actin antibody mapping the carboxy terminus of human actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and subjected to Western analysis as described in ''Materials and Methods.'' After separation on SDS–PAGE gel (10% Tris-acrylamide), blots were probed with mouse monoclonal anti-band 3 antibody (a gift of Dr. Philip Low) mapping the carboxy terminus of human cytoplasmic domain of band 3 (CDB3). **B**: In parallel, the extracts were immunoprecipitated with anti-band 3 antibody and subjected to Western analysis. After separation on SDS–PAGE gel (15% Tris-acrylamide), blots were probed with anti-actin antibody.

heart. The intercalated discs of cardiomyocytes contain three main junctional complexes: (1) gap junctions, which chemically couple neighboring cardiomyocytes; (2) fascia adherens, which connect the actin cytoskeleton to the membrane; and (3) desmosomes, which attach intermediate filaments to the muscle termini [Clark et al., 2002]. We suggest that the band 3 cardiac isoform could play a role similar to the N-cadherin protein which is the primary receptor at fascia adherens that links the membrane to the contractile apparatus [Volk and Geiger, 1986].

Korichneva et al. [1995], interpreting the presence of band 3-like proteins in the myofibril fraction prepared from cultured neonatal cardiomyocytes, also raised the possibility that besides exchanging anions, the band 3-like proteins could also exert an anchorage function for the contractile apparatus and the cytoskeleton to the membrane.

In cardiomyocytes, cardiac actin is the main component of the thin filament of the sarcomere. One end of the polarized actin filaments forms cross-bridges with myosin, and the other end is immobilized, attached to a Z band or an intercalated disc [Lu et al., 1992; Gregorio, 1997].

Although the band 3-actin interaction has not been evaluated in functional studies, the dilated cardiomyopathy, a heritable form of heart failure, has been associated with cardiac actin (*ACTC*) mutations. The Arg 314 His mutation substitutes an arginine located in the center of the actin domain that we found interacting with band 3. This mutation affects a universally conserved amino acid in the subdomain 3 of actin, which form the immobilized end of the actin filament. The altered actin was suggested to impair force transmission by defective attachment to the anchor polypeptides [Olson et al., 1998].

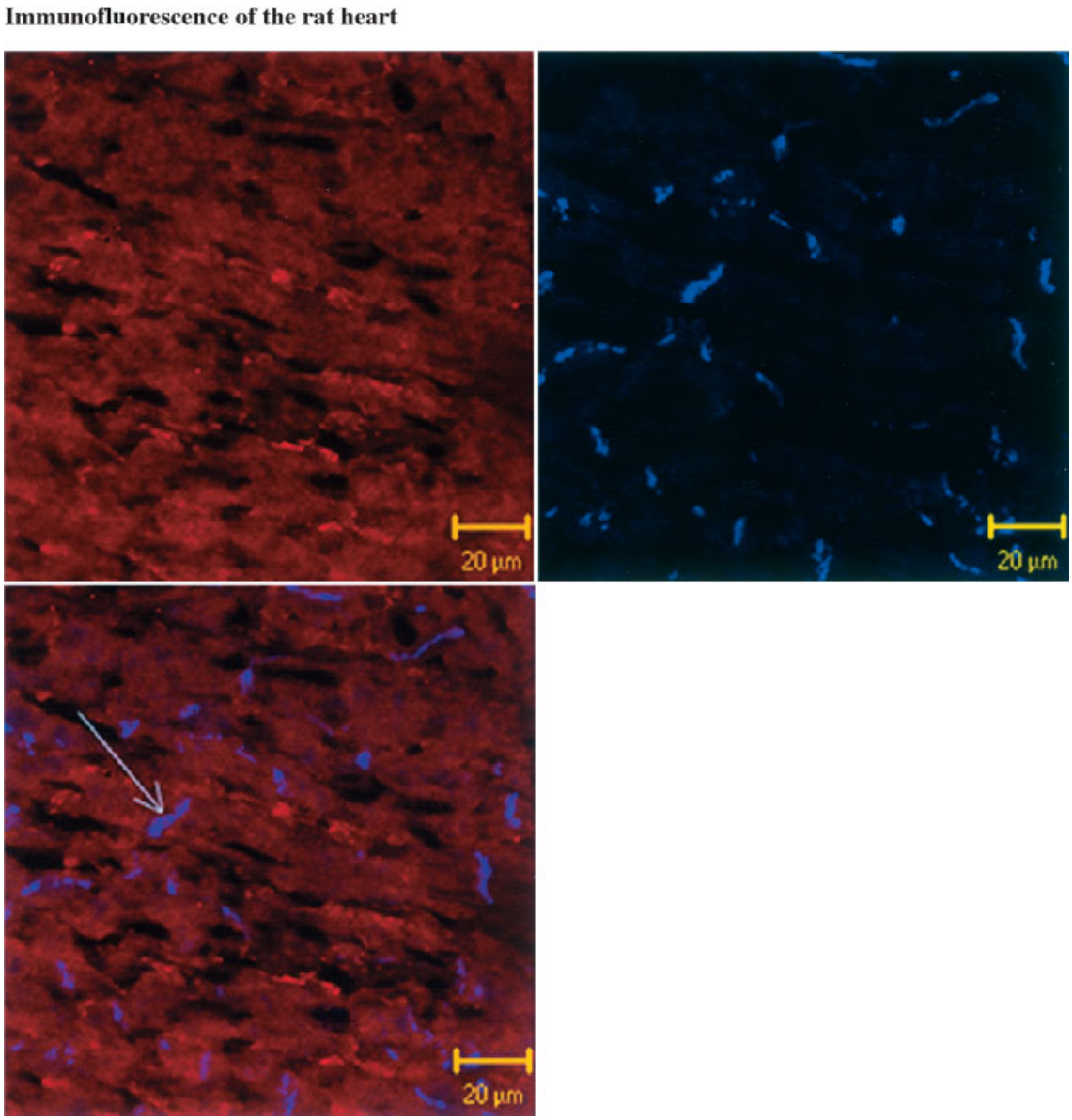

Fig. 4. Immunofluorescence of the rat heart. **Left**: The actin filaments in the myocyte were stained with fluorescent phalloidin-TRITC to visualize all polymeric actin. **Right**: Band 3 was labeled with a rabbit polyclonal anti-band 3 and revealed with an anti-rabbit-Cy5. **Down**: The double channel (TRITC + Cy5) with an arrow showing an intercalated disc, a site of cell–cell contact and attachment of the sarcomere to the plasma membrane.

The dilated cardiomyopathy is a disease of the myocardial force generation or force transmission [Fatkin and Graham, 2002], and mutations in the *ACTC* gene could be a model of the functional consequences in the disruption of the cardiac actin and band 3 protein interaction.

Recently, three mutations in the α-cardiac actin gene (*ACTC*) were also associated with familial hypertrophic cardiomyopathy. The missense mutations Ala295Ser [Mogensen et al., 1999] and Ala331Pro [Olson et al., 2000] are located within the region which we found interacting with the cardiac band 3 protein.

However, unlike the mutations affecting force transmission from the sarcomere, in the idiopathic dilated cardiomyopathy, Mogensen et al. [1999] hypothesized that mutations in *ACTC* lead to familial hypertrophic cardiomyopathy when they involve force generation within the sarcomere.

Finally, besides the structural function of the cytoplasmic domain of the cardiac band 3 in the myofibril attachment to the membrane, it is possible that this domain could be involved in additional myocyte functions as glycolysis and anion transport regulation.

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