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Caldesmon: A Common Actin-Linked Regulatory Protein in the Smooth Muscle and Nonmuscle Contractile System

Kenji Sobue, Keiko Kanda, Toshihiko Tanaka, and Noboru Ueki

Department of Neurochemistry and Neuropharmacology, Institute of Higher Nervous Activity, Osaka University Medical School, Osaka 530, Japan

Caldesmon was originally purified from gizzard smooth muscle as a major calmodulin-binding protein which also interacts with actin filaments. It has an alternative binding ability to either calmodulin or actin filaments depending upon the concentration of Ca^{2+} ("flip-flop binding"). Two forms of caldesmon (Mr's in the range of 120–150 kDa and 70–80 kDa) have been demonstrated in a wide variety of smooth muscles and nonmuscle cells. Immunohistochemical studies suggest that caldesmon is colocalized with actin filaments in vivo. Considering its abundance, the Ca^{2+}-dependent flip-flop binding ability to either calmodulin or actin filaments, and its intracellular localization, caldesmon is expected to be involved in contractile events. Recent results from our laboratory have led to the conclusion that caldesmon regulates the smooth muscle and nonmuscle actin-myosin interaction and the smooth muscle actin-high Mr actin-binding protein (ABP or filamin) interactin in a flip-flop manner. It might function in cell motility by regulating the contractile system.

Key words: actin-linked regulation, myosin-linked regulation, Ca^{2+} regulation of actomyosin, calmodulin, high Mr actin-binding protein (ABP or filamin)

Cell motility, including contraction, exocytosis, endocytosis, shape change, and movement, is considered to involve cytoskeleton. Actually, cytoskeletal elements are distributed in a wide range of tissues and cells [1]. Actomyosin, which converts the chemical energy of ATP into mechanical force, plays an important role in cell motility. In striated muscles such as skeletal and cardiac muscles, the actomyosin system is highly specialized and its regulatory mechanism by Ca^{2+} has been well documented [2]. However, in smooth muscle and nonmuscle cells the involvement of Ca^{2+}-dependent regulatory components such as troponin is unclear. The regulatory mechanism of actomyosin in these tissues and cells seems to be distinctively different from that in striated muscles.

There had been a dispute on the Ca^{2+}-dependent regulation of the smooth muscle actin-myosin interaction. The most popular theory is the myosin-linked mechanism based on phosphorylation of myosin by Ca^{2+}- and calmodulin-dependent myosin light chain kinase (MLC kinase) and dephosphorylation by myosin phosphatase [3–5]. In contrast

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to this theory, the involvement of actin-linked mechanism was proposed, but no specific actin-linked protein other than leiotonin was identified [6]. Recent studies led to the conclusion that the actomyosin system in smooth muscles and nonmuscle cells was dually controlled by Ca^{2+} via the myosin- and actin-linked mechanisms [7–9]. Nevertheless, until the identification of caldesmon, little was known concerning a specific actin-linked regulatory protein. In this brief article, we will demonstrate the molecular and functional aspects of caldesmon.

TWO FORMS OF CALDESMON OF DIFFERING Mr

In 1981, caldesmon, which is composed of a doublet with subunits of 150 and 147 kDa in equal molar ratio, was purified from gizzard smooth muscle as a calmodulin-binding and actin-interacting protein [10]. We named this protein caldesmon, a combination of the words calmodulin and *desmos* (a Greek word which means binding). Caldesmon is a major target of calmodulin in gizzard smooth muscle. The concentration of caldesmon in gizzard is calculated to be 9.7 μM [11]. Since 2 mol of calmodulin bind to 1 mol of caldesmon heterodimer, the concentration of caldesmon in gizzard smooth muscle is equivalent to 70% of the total calmodulin in this tissue. It has an alternative binding ability to either calmodulin or actin filaments depending upon the concentration of Ca^{2+}. At less than 1 μM of free Ca^{2+} concentration, caldesmon binds to actin filaments, whereas at a higher concentration of Ca^{2+} (>1 μM), calmodulin activated by Ca^{2+} forms a complex with caldesmon and this complex is freed from actin filaments ("flip-flop binding") [10,12]. Subsequently, caldesmon with a similar Mr (120–150 kDa) has been identified in, or isolated from, smooth muscles [11,13–17]. The great differences in the reported Mr might be due to the use of different gel systems. However, gizzard and aorta proteins do not exactly comigrate using the same gel. According to our data, two proteins are a 150/147-kDa heterodimer for gizzard caldesmon and a 148-kDa homodimer for aorta caldesmon [11]. In agreement with this, amino acid analysis and peptide mapping have revealed some differences between the two proteins [17]. Recent results indicate the presence of low Mr caldesmon (Mr in the range of 70–80 kDa) in nonmuscle tissues and cells [18–22]. The low-Mr caldesmon has been purified from bovine adrenal medulla [19] and porcine platelets [23] and compared it with gizzard protein. The low-Mr protein possesses a number of features in common with gizzard protein, including the flip-flop binding ability to either calmodulin or actin filaments depending upon the concentration of Ca^{2+} and immunological cross-reactivity with antigizzard caldesmon antibody [19]. We termed the high- and low-Mr proteins *h*- and *l*-caldesmons, respectively.

Table I shows the distribution of caldesmons in tissues and cells. The concentrations of caldesmons were determined by densitometric scanning of immunoblotting data [24]. In the smooth muscles, *h*-caldesmon was detected in large quantities. Contrarily, *l*-caldesmon was not detected in these tissues. In nonmuscle tissues, the content of *l*-caldesmon was much higher than that of *h*-caldesmon; *l*-caldesmon was primarily found in lymphocytes, platelets, and cultured cells. These results indicate that the expression of *h*-caldesmon might be more specific to smooth muscles. The relationship between *h*- and *l*-caldesmons is still unclear. There are three possibilities as follows: (1) *l*-caldesmon is a proteolytic fragment of *h*-caldesmon; (2) both caldesmons are derived from the different genes; and (3) *h*-caldesmon results from a posttranslational modification of *l*-caldesmon. We have recently observed with interest that the interconversion of

TABLE I. Distribution of Caldesmons in Various Tissues*

Tissues	μM	
	h-caldesmon	*l*-caldesmon
Chicken		
Gizzard	9.2	< 0.06
Bovine		
Aorta (media)	3.0	< 0.06
Adrenal medulla	0.04	0.82
Adrenal cortex	0.05	0.68
Anterior pituitary	0.03	0.44
Posterior pituitary	0.03	0.64
Rat		
Spleen	0.05	0.43
Brain	< 0.01	0.25
Liver	0.03	0.78
Kidney	0.03	0.66
Cultured cells		
balb/c 3T3	0.03	0.62
NRK	< 0.01	0.35
BHK	< 0.01	0.53
MDCK	< 0.01	0.40
CHO-K1	< 0.01	0.48

*The contents of caldesmons were determined by immunoblotting with the caldesmon antibody by using ^{125}I-labeled protein A [24].

h- and *l*-caldesmons shows a good relationship between growth and cytodifferentiation of smooth muscle cells [24]. As the first example, we have found that in developing gizzards the expression of caldesmon switches from the *l*- to *h*-forms. Chamley et al. demonstrated that vascular smooth muscle cells undergo marked changes in phenotype [25]. When grown in cell culture, smooth muscle cells convert from the contractile to the synthetic state in association with cell growth. In accordance with this change, the expression of *h*-caldesmon decreased and that of *l*-caldesmon increased in primary cultures of rat aortic smooth muscle cells. Additionally, the levels of those mRNAs which direct the synthesis of both caldesmons were apparently in proportion to the quantities of proteins as determined by use of an in vitro translation system. These results suggest that the expression of caldesmons during phenotypic modulation of smooth muscle cells is regulated at the level of mRNA. We then investigated the expression of both caldesmons by using a smooth-muscle-like cell line (BC_3H1 cells) during differentiation as a counterpart of primary culture (Fig. 1). When serum was deprived, BC_3H1 cells ceased to grow and began to differentiate. This change was confirmed by the morphology and by a dramatic increase in the numbers of acetylcholine receptors. In the growing state, *l*-caldesmon was predominantly detected. Accompanying the serum-deprived cytodifferentiation, the expression of *h*-caldesmon was enhanced. These results suggest that the expression of *h*-caldesmon is closely associated with cytodifferentiation of smooth muscle cells.

Immunohistochemically, caldesmon is localized along cellular stress fibers and in leading edges of cultured fibroblast cell lines [18,20]. This indicates that caldesmon colocalizes with actin filaments in cells. Bretscher and Lynch have performed a detailed morphological analysis, pointing out that the distribution of caldesmon along stress fibers is periodic, coinciding with that of tropomyosin but complementary to α-actinin [20].

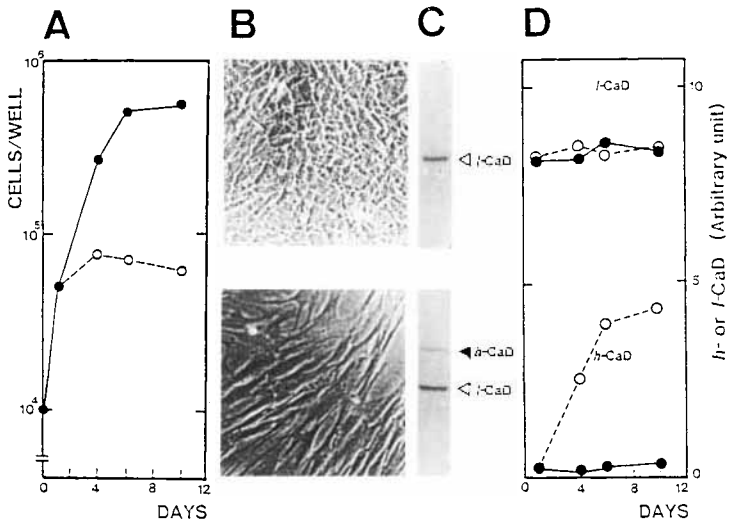

Fig. 1. Expression of *h*- and *l*-caldesmons during cytodifferentiation of BC_3H1 cells. Cells were cultured in 20% (●) or (○) 1% fetal calf serum. **A**: Effect of fetal calf serum on cell growth. **B**: Morphological change following differentiation. Upper and lower panels indicate the growing and differentiated states of BC_3H1 cells. **C**: Immunoreplica using anticaldesmon IgG. Upper and lower immunoreplicas indicate the detection of caldesmons in the growing and differentiated states of BC_3H1 cells. **D**: Contents of *h*- and *l*-caldesmons in two states of BC_3H1 cells.

The topographical distribution of caldesmon and actin was investigated in various organs such as the thyroid gland, intestine, submandibular gland, adrenal gland, pancreas, ovary, kidney, esophagus, oviduct, aorta, skeletal muscle, and heart [26–28]. The coexistence of caldesmon and actin was demonstrated in smooth muscles and numerous nonmuscle cells and tissues. Caldesmon was not detected in the skeletal muscle or heart. In contrast Ngai and Walsh have reported the detection of caldesmon in the skeletal and heart muscle and in any other organs examined with the immunoblotting technique using antigizzard caldesmon antibody [29]. Based on our observations, the results by Ngai and Walsh might have arisen from contaminating smooth muscle cells and endothelial cells in skeletal muscle and heart. Therefore, we conclude that caldesmon is a ubiquitous protein in smooth muscle and nonmuscle cells and that it colocalizes with actin filaments in cells.

CALDESMON IS AN ACTIN-LINKED REGULATORY PROTEIN

As mentioned in the beginning of this paper, there was only little data concerning the actin-linked regulatory mechanism by Ca^{2+} in the smooth muscle actomyosin system. In 1982, we tried to determine whether caldesmon is a prime candidate for an actin-linked regulatory protein. Using desensitized gizzard actomyosin, we found that *h*-caldesmon inhibits the actin-myosin interaction and that this inhibitory effect of caldesmon is overcome by calmodulin [30]. Caldesmon, therefore, seems to regulate the actin-myosin interaction in a Ca^{2+}- and calmodulin-dependent flip-flop manner. In this assay system, phosphorylation of myosin was essential for the actin-myosin interaction. We also suggested that tropomyosin is possibly required for the action of caldesmon. Subsequently, using a complete reconstitution system in which myosin was preferentially phosphorylated, we clearly demonstrated the regulatory mechanism of *h*-caldesmon in

the actin-myosin interaction [31]; *h*-caldesmon inhibited the tropomyosin-enhanced actomyosin ATPase activity with or without Ca^{2+}. This inhibition was overcome by addition of calmodulin only in the presence of Ca^{2+} but not in the absence of Ca^{2+}. From these observations, it is apparent that the regulatory mode of *h*-caldesmon is the control of tropomyosin function through the actin site and that calmodulin confers Ca^{2+} sensitivity upon the effect of *h*-caldesmon. We have hypothesized that, like troponin C and troponin I in striated muscles, calmodulin and caldesmon regulate the actin-myosin interaction in smooth and nonmuscle cells (Fig. 2). Actually, recent observations support this idea. Szpacenko and Dabrowska demonstrated that only the 40-kDa fragment of caldesmon preserves functional properties of the parent molecule [32]. Furthermore, we have found that the functional domain (35-kDa fragment) of caldesmon, which retains alternative binding ability to either calmodulin or actin filament depending upon the concentration of Ca^{2+}, shows the same regulatory effect on the actin-myosin interaction in a flip-flop manner [33]. Additionally, the amino acid composition of the 35-kDa fragment is very similar to that of troponin I. In future studies, sequencing analysis of caldesmon will be required to eludicate the molecular similarity.

Ngai and Walsh reported that the inhibitory effect of caldesmon on smooth muscle actomyosin ATPase activity could not be overcome by Ca^{2+} and calmodulin but that phosphorylation of caldesmon by Ca^{2+}- and calmodulin-dependent caldesmon kinase could reverse this inhibition [34]. Although caldesmon kinase has been identified, no investigator has demonstrated the reversal effect of caldesmon mediated through phosphorylation by this kinase [35,36]. Marston and Lehman purified Ca^{2+}-sensitive thin filaments from vascular smooth muscle and identified caldesmon as a regulatory component of thin filaments [15]. They have proposed a model for smooth muscle thin filaments in which Ca^{2+} regulates contractile activity by converting the inhibited actin-tropomyosin-caldesmon complex to the active complexes [37]. According to their model, it is necessary to guess the presence of the actin-tropomyosin-caldesmon-calmodulin-Ca^{2+} complex. Further studies are required to define the protein-protein interactions. Most recently, Lash et al. have demonstrated that caldesmon can enhance cross-bridge activity between actin and myosin which inhibits actomyosin ATPase activity [36]. They have speculated that this effect of caldesmon may involve the intriguing "latch" state [38].

The presence of a myosin-linked mechanism has been demonstrated in nonmuscle cells [4,5]. We found that *l*-caldesmon also might be involved in the nonmuscle actin-myosin interaction as an actin-linked regulatory protein. In bovine adrenal medulla, medullary *l*-caldesmon suppresses the actin-myosin interaction determined by superpre-

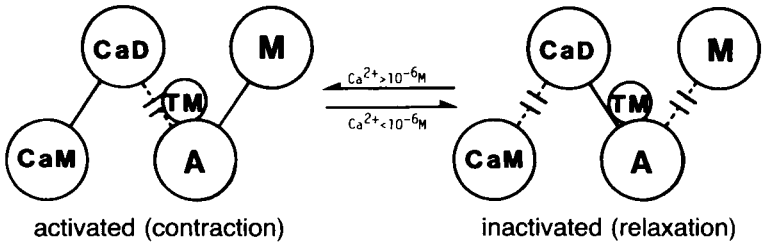

Fig. 2. Ca^{2+}-dependent regulation of smooth and nonmuscle actin-myosin interaction by calmodulin- and caldesmon-linked flip-flop mechanism. **CaM**, calmodulin; **CaD**, caldesmon; **A**, F-actin; **TM**, tropomyosin; **M**, phosphorylated myosin.

cipitation and the actin-activated myosin ATPase activity [19,39]. Dingus et al. have purified *l*-caldesmon from human platelets and have showed the flip-flop binding of *l*-caldesmon to either calmodulin or actin filaments [22]. Onji et al. have purified *l*-caldesmon from porcine platelets and investigated the effect of *l*-caldesmon on reconstituted platelet actomyosin [23]. Platelet *l*-caldesmon shows the specific inhibition of the tropomyosin-enhanced actomyosin ATPase activity. In this assay system, it is also proven that the effect of *l*-caldesmon is the control of tropomyosin function through the actin site. These studies support the idea that caldesmon is a common actin-linked regulatory protein in the smooth muscle and nonmuscle actomyosin systems.

During contraction in vivo, an actin filament attachment site is required for the generation of force. In striated muscles, α-actinin is a well-known thin filament anchorage protein in the Z-band [40]. However, in smooth muscles and nonmuscle cells, a fulcrum-like protein between actin filaments is still unclear. High-Mr actin-binding protein (ABP or filamin) and α-actinin are postulated to be potent candidates for this protein. We found that caldesmon also regulates the ABP-actin interaction in a flip-flop manner [41]. We have furthermore demonstrated that the regulatory effect of caldesmon on the ABP-actin interaction becomes stronger in the presence of tropomyosin [42]. We have established a three-dimensional contractile model system including actin, tropomyosin, ABP, myosin, MLC kinase, and calmodulin and investigated the involvement of caldesmon in this system [43]. Caldesmon inhibits both the actin-myosin and actin-ABP interactions, resulting in the inhibition of three-dimensional contraction. Further, this inhibitory effect of caldesmon can be overcome by calmodulin in a Ca^{2+}-dependent manner. Taken together with these results, the ABP-induced gelation and the actin-myosin interaction are carried out in the contraction state. In the relaxation state, caldesmon inhibits the gelation-contraction coupling processes. And calmodulin confers Ca^{2+} sensitivity on the interconversion of three-dimensional contraction and relaxation (Fig. 3). Considering the

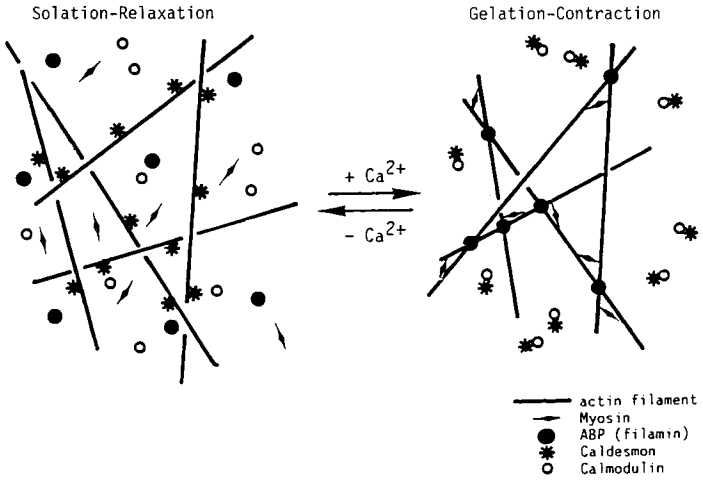

Fig. 3. Possible hypothesis of Ca^{2+}-dependent three-dimensional contraction. At a lower concentration of Ca^{2+} (>1 μM), caldesmon binds to actin filaments, resulting in the inhibition of ABP-induced actin filament cross-linking and of the actin-myosin interaction (solation-relaxation). At more than 1 μM of Ca^{2+}, caldesmon is freed from actin filaments. Consequently, the ABP-induced gelation of actin filaments and the actin-myosin interaction take place (gelation-contraction).

wide distribution of the above contractile elements in smooth muscle and nonmuscle cells, a three-dimensional contractile model may be applicable to other nonmuscle contractile events.

INVOLVEMENT OF CALDESMON IN BIOLOGICAL PHENOMENA

In spite of much biochemical evidence, little was known concerning the involvement of caldesmon in biological phenomena. However, recent investigations have shown the possible biological significance of caldesmon.

As mentioned above, *l*-caldesmon is localized along the stress fibers and the leading edges of cultured cells. In contrast, the intracellular distribution of *l*-caldesmon dramatically changed to a diffuse and blurred appearance in *onc* gene virus-transformed cells, and the cellular content of *l*-caldesmon in transformed cells decreased to about one-third of that in normal counterparts [18]. These changes in *l*-caldesmon may correlate to the loss of Ca^{2+} regulation in the transformed cells.

Stimulation of adrenal chromaffin cells causes a transient increase in the intracellular free Ca^{2+}, resulting in catecholamine secretion. Burgoyne et al. have identified a chromaffin granule-binding protein with an Mr of 70 kDa as *l*-caldesmon [21]. They also have demonstrated that *l*-caldesmon is localized at the periphery of chromaffin cells. From these observations, they have speculated that *l*-caldesmon may control a reorganization of F-actin at the cell periphery following the transient increase in intracellular free Ca^{2+} during stimulation.

In lymphocytes, multivalent ligands, such as lectins and antibodies against cell surface receptors, form aggregates (cap formation) in energy- and temperature-dependent processes [44]. Regarding such cell surface receptor mobility, it has been postulated that there might be transmembrane linkages between surface receptors and contractile elements just beneathe plasma membranes, and that receptors might be actively collected into caps by the force generated by actin-myosin interaction analogous o the muscle contraction. It has been demonstrated that myosin-linked components such as myosin, MLC kinase, and calmodulin are accumulated into subcaps in accompanying with cell surface receptor aggregation [45]. Then, we have examined the involvement of actin-linked components (caldesmon, tropomyosin, and actin filaments) in concanavalin A (Con A) receptor capping of T-lymphocytes [46]. During Con A-receptor capping, caldesmon, tropomyosin, and actin are found to redistribute into subcaps (Fig. 4). In addition, these redistributions of actin-linked components and Con A capping are inhibited by calmodulin antagonists. These results suggest that the actin-linked regulatory components such as caldesmon, tropomyosin, and calmodulin might also be involved in the ligand-induced cell surface receptor mobilization.

CONCLUDING REMARKS

The molecular and functional aspects of caldesmon discussed above are based on the results of its abundance and localization in cells and the molecular mechanism of the in vitro reconstitution system. Caldesmon plays a major role in the regulation of smooth muscle and nonmuscle contractile events, resulting in the involvement of a wide variety of biological phenomena including contraction, cell movement, shape change, exocytosis, and endocytosis. Further studies are required to determine the actual involvement of caldesmon in the corresponding biological processes.

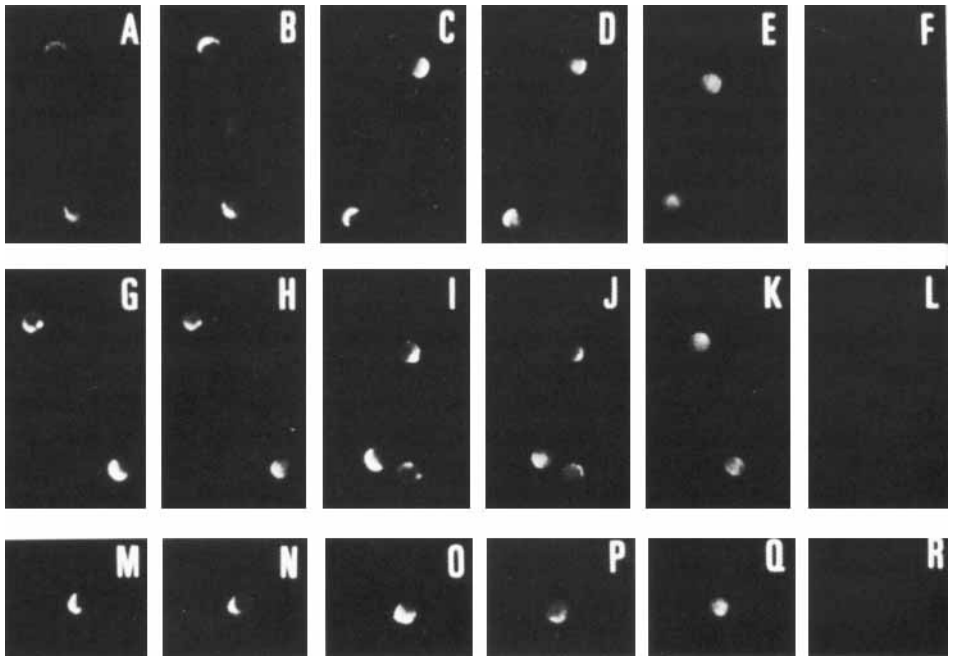

Fig. 4. Redistribution of actin-linked regulatory components (caldesmon and tropomyosin) during concanavalin A (Con A) receptor capping. Double-fluorescence micrographs of splenic T-lymphocytes stained with rhodamine-Con A for Con A-receptor (**A**,**C**,**G**,**I**,**M**, and **O**) and with fluorescein-anti-IgG for the anticaldesmon (**B**,**D**, and **E**), antitropomyosin (**H**,**J**, and **K**), and antiactin (**N**,**P**, and **Q**) IgGs. E,K, and Q indicate the distribution of caldesmon, tropomyosin, and actin in untreated T-lymphocytes, respectively. As a control, the anticaldesmon, tropomyosin, or actin IgG preabsorbed with respective protein showed background levels of staining (**F**,**L**, and **R**). ×500.

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