Phosphorylation of actin-binding protein (ABP-280; filamin) by tyrosine kinase p56[lck] modulates actin filament cross-linking

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Abstract

Actin-binding protein (ABP-280; filamin) is a phosphoprotein present in the periphery of the cytoplasm where it can cross-link actin filaments, associate with lipid membranes, and bind to membrane surface receptors. Given its function and localization in the cell, we decided to investigate the possibility of whether it serves as substrate for p56[lck], a lymphocyte-specific member of the *src* family of protein tyrosine kinases associated with cell surface glycoproteins. The interaction of p56[lck] with membrane glycoproteins is important for cell development and functional activation. Here, we show that purified p56[lck] interacts and catalyzes in vitro kinase reactions. Tyrosine phosphorylation by p56[lck] is restricted to a single peptide of labeled ABP-280 shown by protease digest. The addition of phorbol ester to cells results in the inhibition of phosphorylation of ABP-280 by p56[lck]. These results show a decrease in phosphorylation suggesting conformationally induced regulation. Dynamic light scattering confirmed increased actin filament cross-linking due to phosphorylation of ABP-280 by p56[lck].
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1. Introduction

Signaling events from the cell surface to the nucleus have remained so far unsolved despite a large body of evidence that suggests that phosphorylation of several cellular proteins is involved in the signal transduction pathway (Hall, 1998; Ktistakis, 1998; Vadlamundi et al., 2002). Several cell surface receptors have been described to have either intrinsic tyrosine kinase activity i.e., VEGF receptors, or to be associated with those that are wholly cytoplasmic or membrane-bound (Klagsbrun and D'Amore, 1996). Several of these kinases belong to the *src* family because of their structural homology with the oncogenic product, pp60[*v-src*] encoded by the Rous sarcoma virus. These kinases are normally plasma membrane-associated enzymes consisting of nine well-characterized members: pp60[src], p56[lyn], p56[lck], p59[fyn], p59[yes], p62[yes], p59[hck], p55[fgr], and p55[blk] (Ziegler et al., 1989; Schieven et al., 1992; Venkitaraman and Cowling, 1992; Sleckman et al., 1992; Torigoe et al., 1992; Pleiman et al., 1993; Levin et al., 1993; Wechsler and Monroe, 1995; Henning and Cantrell, 1998). Among the *src* kinases, p56[lck] is expressed in high amounts in T-lymphocytes and plays a vital role in T-cell activation. p56[lck] has a unique N-terminal sequence which is involved in the physical association with specific cell receptors like CD4 and CD8. Further, it has been shown that p56[lck] interacts with IL-2 receptor and is indirectly associated with GPI-anchored surface receptors (August and Dupont, 1996; Tosello et al., 1998).

Despite progress in the elucidation of surface receptors interacting with p56[lck], little is known about substrate specificity of this tyrosine kinase either in vitro or in vivo. Thus, one substrate has been identified as the

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Nomenclature

(ABP-280; filamin)	actin-binding protein
CEM	T-cell line
CHAPS	(3-[(3-cholamidopropyl)dime-thylammonio]-1propanesulfo-nate)
GAP	GTPase-activating protein
HLA	hybridoma (monoclonal anti-body)
MAPK	mitogen activated protein kinase
NRS	normal rabbit serum
NS1	non-secreting (clone) 1
PBL	peripheral blood lymphocytes
PMA	phorbol myristate acetate
RPMI 1640	Roswell Park Memorial Insti-tute (medium)
TCR	T-cell receptor

ζ-subunit of the TCR complex, which when phosphor-ylated, is increased three- to fourfold after cross-linking with CD4 (Brenner et al., 1996). Other substrates identified by in vitro p56[lck] kinase reaction i.e., p21*ras*, GTPase-activating protein (GAP), and mitogen activated protein kinase (MAPK) were found to be phosphorylated specifically at tyrosine residues within one tryptic peptide (Loo et al., 1998). Purified p56[lck] both tyrosine phosphorylated and stimulated the seryl-threonyl phosphorylase activity of purified 44 kDa MAPK.

Notwithstanding these identified substrates, the downstream signal via cytoskeletal proteins from the surface to the nucleus has never been addressed. We, therefore focused on whether actin-binding protein (ABP-280; filamin) is a probable substrate for p56[lck]. Our assumption was based on the reported function and localization of ABP-280 in lymphocytes (Sharma et al., 1995). Here, this protein was described to be localized in the periphery of the cell under the plasma membrane and attached p56[lck] to the cytoplasmic face of the plasma membrane through the myristoylated N-termi-nal glycine. The investigation into utilizing immuno-purified ABP-280 and purified p56[lck] by in vitro kinase reaction showed that ABP-280 is a major substrate of p56[lck] that binds specifically to immunopurified ABP-280. We discussed possible regulation of the association of p56[lck] with ABP-280 and its phosphorylation in terms of actin filament cross-linking.

2. Materials and methods

2.1. Cells

Peripheral blood lymphocytes (PBL) were isolated by Iso-Hypaque centrifugation and either used as resting cells or stimulated with PMA at 100 ng/ml for different time periods. CEM cells were grown in RPMI 1640 with 10% fetal calf serum containing 1000 IU of penicillin and 10 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% H_2O.

2.2. Antibodies and purified proteins

The monoclonal antibodies, M5 against ABP-280 were a generous gift from Dr. J.H. Hartwig, Brigham and Women's Hospital, Boston, and TS1/18a were raised against CD18 provided by Dr. S. Burakoff, Dana Farber Cancer Institute, Boston. Polyclonal rabbit anti-p56[lck] serum was purchased from Oncogene Science, Uniondale, NY. Recombinant human p56[lck] was ex-pressed in baculovirus expression system and kindly provided by Dr. J. Watts, Biomedical Research Center, Vancouver, Canada. NS1 and HLA were gifts from Dr. J.-L. Alonso, Children's Hospital, Boston. For dynamic light scattering experiments ABP-280 was essentially pre-pared as described in http://iprotocol.mit.edu/protocol/302.htm and actin was purified according to the pro-cedure in http://iprotocol.mit.edu/protocol/299.htm.

2.3. Immunoprecipitation and kinase assay

PBLs ($3-5 \times 10^7$) were solublized in lysis buffer containing Triton X-100 or 1% CHAPS as described previously by Tauber et al. (1989). After preclearing the lysate with Sepharose coupled protein G, immunopre-cipitation was performed using 5 ml ascite and 50 ml protein G coupled with Sepharose for 4 h at an ambient temperature or overnight at 4 °C. After immunoprecip-itation, the immune complexes were washed twice in lysis buffer and once in kinase buffer containing 20 mM Hepes, pH 7.2; 5 mM $MnCl_2$, 5 mM $MgCl_2$, 0.5 mM sodium orthovanadate, 0.1% Triton X-100, and in-cubated in 50 ml kinase buffer containing 10 μM ATP, 10 μCi [^{32}P]-ATP ($\sim$5000 Ci/mmol; *New England Nu-*clear, (NEN) Boston). After 15 min incubation at 25 °C, immunoprecipitates were washed twice in lysis buffer, eluted in Laemmli buffer, and then subjected to SDS-PAGE and autoradiography prior to transferring the proteins onto a Immobilon-P membrane. In a typical kinase reaction performed in the presence of p56[lck], 5–10 ng of purified kinase and 50 μM ATP were included in the kinase reaction mixture. Thereafter, immuno-precipitates were washed and processed as described above.

2.4. Western blot and tryptic peptide map and phosphoamino acid analyses

The proteins were transferred onto a Immobilon-P membrane, which were then briefly stained with Coomassie blue. The portion of the membrane corre-sponding to ABP-280 and p56[lck] was cut out and probed

with antibodies against phosphotyrosine and p56[lck], respectively, using the enhanced chemiluminescent method recommended by the supplier (ECL, Amersham). The tryptic peptide map of the labeled protein was essentially performed as described previously by Tauber et al. (1989). For phosphoamino acid analysis, the radiolabeled ABP-280 was cut out from the membrane and hydrolyzed in 6 N HCl for 2 h at 110 °C. The hydrolysates were analyzed by two-dimensional thin layer chromatography.

2.5. Dynamic light scattering

The basic set-up of the light scattering apparatus was as described by Goetter et al. (1995) with the following modifications: the 488 nm spectral line of an Innova 70-4 (Coherent) laser with an output of ∼1.2 W was used as the light source. Signal detection was achieved by an autocorrelator ALV 3000 supplied by ALV, Langen, Germany. This system allowed the recording of photon autocorrelation functions in real time on a linear time scale of 1024 channels covering a time span from 4 μs to 20 ms within a single measurement. A purpose-written computer program based on the commercially available IGOR Pro software was used for analysis on an Apple Macintosh.

3. Results

3.1. Phosphorylation of ABP-280 by p56[lck]

To investigate whether ABP-280 is a potential substrate of p56[lck], in vitro kinase reactions were performed utilizing immunopurified ABP-280 and purified p56[lck]. Fig. 1a illustrates a major band on a Coomassie blue stained gel at 280 kDa when the monoclonal antibody, M5 against ABP-280 was applied. When kinase reactions of the immunoprecipitates in Fig. 1a were performed in the presence of purified p56[lck], ABP-280 was predominately phosphorylated (Fig. 1b). Analyzing the amino acids that were phosphorylated by p56[lck] in this assay showed that tyrosine residues were essentially affected (Fig. 1c). Further, tryptic and V8 map analysis of ABP-280 revealed that a single peptide was phosphorylated at tyrosine residue(s) despite a total of 33 tyrosine residues in the entire molecule. This is indicative of higher specificity for p56[lck] (data not shown). To rule out the possibility that phosphorylation of ABP-280 by p56[lck] is due to endogenous kinase activity of ABP-280, it was immunopurified, and divided into a 3 to 1 ratio, and then incubated in kinase buffer containing labeled ATP in the presence and absence of human recombinant p56[lck]. Results from these experiments are shown in Fig. 2a–d. The intensity of phosphorylated ABP-280 was many times higher when kinasing was performed in the presence of p56[lck] despite the fact that the amount of ABP-280 was three times less than that in the absence of p56[lck]. The net difference of phosphorylation in presence and absence of p56[lck] was larger than a factor of 1000.

3.2. Co-immunoprecipitation and interaction of ABP-280 and p56[lck]

To demonstrate in vivo interactions of ABP-280 and p56[lck], CHAPS lysate of lymphocytes and CEM cells were utilized for co-immunoprecipitation. As shown in
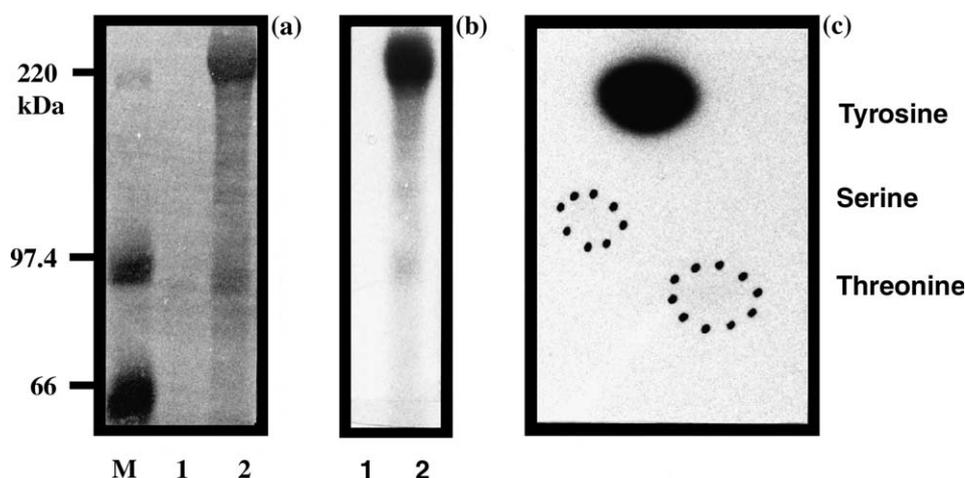
Fig. 1. In vitro phosphorylation of ABP-280 by purified p56[lck]. β2 Integrins and ABP-280 were immunopurified using 5 μl of monoclonal antibodies TS1/18a and M5, respectively. The immunobeads were suspended in kinase buffer and incubated with 5 ng of purified p56[lck] in a final volume of 50 μl containing 10 μCi [^{32}P]-ATP. The incubation was carried out for 10 min at 25 °C under constant agitation. The immunoprecipitates were washed and analyzed by SDS-PAGE, stained with Coomassie blue, and exposed on X-ray films. (a) Coomassie blue stained gel, (b) corresponding autoradiogram, and (c) phospho amino acid analysis of [^{32}P] labeled ABP-280. Lane M = marker; lane 1 = TS1/18a; and lane 2 = M5. Note: the position of ninhydrin stained phospho amino acids is depicted by dotted lines.
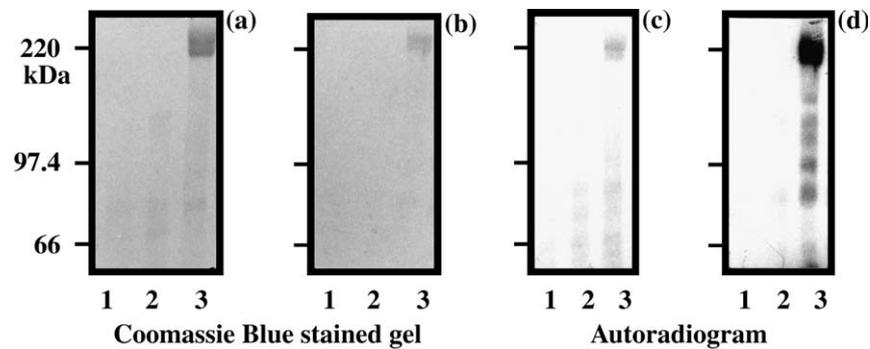
Fig. 2. Comparison of in vitro phosphorylation of ABP-280 in the presence and absence of p56[lck]. A kinase reaction was performed in the absence (a, c) and presence (b, d) of 5 ng purified p56[lck]. In order to demonstrate the specificity of phosphorylation, three times the amount of ABP-280 was included in the reaction in the absence of p56[lck]. The reactions were separated by SDS-PAGE and stained with Coomassie blue (a, b) and processed for autoradiography (c, d). Lane 1 = NS1; lane 2 = HLA; and lane 3 = M5.

Fig. 3, ABP-280 is associated with p56[lck] when the lysate immunoprecipitates with anti-p56[lck] antiserum, thus when the monoclonal antibody M5 is used, p56[lck] does not co-immunoprecipitate. No association of these species was observed using non-ionic detergents like digitonin, Brij-96, Triton X-100, and NP-40.

Since p56[lck] phosphorylates ABP-280, we were anxious to find out how these proteins associate. For this purpose, we immunopurified ABP-280 from cell lysates and incubated it with p56[lck] either at 4 °C for 2 h or at room temperature for 15 min. Immunobeads containing ABP-280 were then washed three times in lysis buffer and analyzed by SDS-PAGE and Western blotting. Fig. 4 shows that p56[lck] interacted directly with the immunobeads containing ABP-280 but did not bind when an antibody was used.

3.3. Regulation of the interaction of p56[lck] and ABP-280

As an *src* kinase, p56[lck] has a SH-2 and SH-3 domain in the N-terminal half of the molecule. It has

been established that the SH-2 domain interacts with different tyrosine phosphorylated proteins and that SH-3 associates with the cytoskeleton (Brenner et al., 1996). To investigate whether phosphorylation of tyrosine residue(s) on ABP-280 catalyzed by p56[lck] increases the affinity for the SH-2 domain, we performed a binding assay. After incubating ABP-280 with p56[lck], we immunopurified ABP-280 from cells treated with and without PMA, washed with 0.5 M NaCl, and then incubated it with 10 ng of purified p56[lck] in the presence and absence of 50 μM ATP. Results obtained from these experiments are shown in Fig. 5. Equal amounts of ABP-280 were immunoprecipitated from cells treated with and without 100 ng/ml PMA, as judged by Coomassie blue stained gel (Fig. 5A). Western blot demonstrated that the interaction of p56[lck] with ABP-280 was not influenced by the phosphorylation catalyzed by p56[lck] (Fig. 5B). The

Fig. 3. Co-immunoprecipitation of p56[lck] and ABP-280. 3 × 10^{7} PBLs were lyzed in CHAPS lysis buffer, the lysate was immunoprecipitated with NRS (control) or antiserum against p56[lck]. The immunoprecipitate was separated on SDS-PAGE and transferred to the membrane. Portions of the membrane were probed using antiserum against ABP-280.
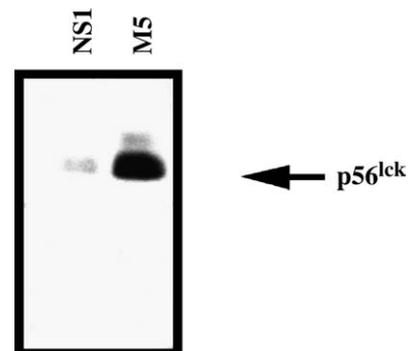
Fig. 4. In vitro interaction of purified p56[lck] with ABP-280. The in vitro interaction was studied by direct binding. To the purified ABP-280 in lysis buffer, 10 ng of purified p56[lck] was added to a final volume of 200 μl and incubated at 25 °C for 2 h, washed three times in 0.5 ml lysis buffer, eluted in Laemmli buffer, and then analyzed by SDS-PAGE and Western blotting. The portion of the membrane presenting molecular weights between 45 kDa and 65 kDa was cut out and probed against anti-rabbit p56[lck] antiserum using antibodies NS1 and M5.
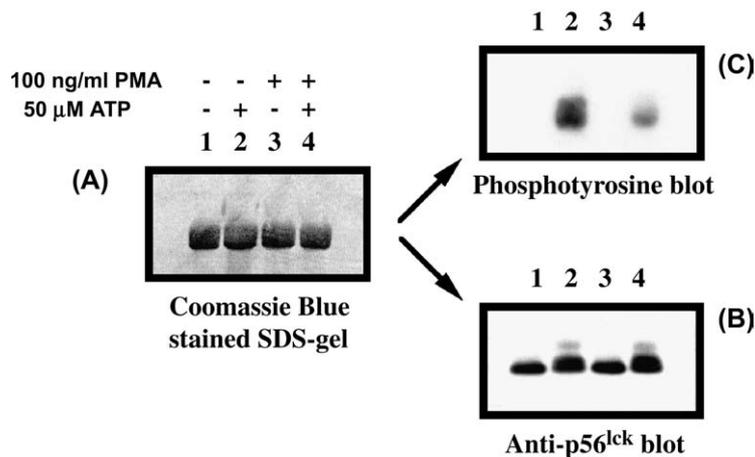
Fig. 5. Regulation of phosphorylation of ABP-280 by p56[lck]. 5×10^7 PBLs were incubated with (lane 3 + 4) and without (lane 1 + 2) 100 ng/ml PMA for 10 min at 37 °C and ABP-280 was isolated from the lysate. The ABP-280 containing immunobeads and 10 ng of purified p56[lck] was incubated in 50 µl kinase buffer in the presence (lane 2 + 4) and absence (lane 1 + 3) of 50 µM ATP (A). After incubating for 30 min at 25 °C under constant agitation, the immunobeads were washed three times in lysis buffer, separated on SDS-PAGE, and transferred onto Immobilon-P membrane. The membrane was briefly stained with Coomassie blue, and portions of the membrane representing p56[lck] and ABP-280 were blotted with antiserum against p56[lck] (B) and monoclonal antibodies against phosphotyrosine (C), respectively.

only visible difference in the association of p56[lck] with ABP-280 under these conditions was the appearance of a phosphorylated form of p56[lck], which was also found to be associated with ABP-280 but at higher mobility. These results suggest that the SH-2 domain in p56[lck] does not have a preference for tyrosine phosphorylation of ABP-280, or that the interaction of p56[lck] with ABP-280 does not require the SH-2 domain. Although equal amounts of p56[lck] and ABP-280 were utilized as shown by Western blots, tyrosine phosphorylation of ABP-280 by p56[lck] decreased fourfold when the ABP-280 was isolated from cells treated with PMA (Fig. 5C).

3.4. Modulation of actin-ABP-280 binding by p56[lck] phosphorylation

To measure actin filament dynamics in the presence of ABP-280 and after phosphorylation of ABP-280 by p56[lck], we applied dynamic light scattering. Using this approach, we observed the following: when plotting the normalized dynamic structure factor $g(q,t)$ against decay time at a scatter angle of 90° for 0.3 mg/ml pure actin ($\diamond$), actin in the presence of pure ABP-280 ($\square$), and actin in the presence of phosphorylated ABP-280 ($\bigcirc$) at a molar ratio, $r_{A:ABP} = 20$, the decay time for actin in the presence of pure ABP-280 had only a small influence on the internal dynamic behaviour of actin filaments compared with pure actin filaments. However, when ABP-280 was phosphorylated by p56[lck], the bending stiffness of actin filaments showed a 50% increase compared to pure actin (Fig. 6).

4. Discussion

Based on the localization of both p56[lck] and ABP-280, we were interested to determine whether ABP-280 can serve as a substrate for p56[lck]. We addressed this possibility by utilizing immunopurified ABP-280 and recombinant p56[lck] in vitro kinase assays. In the present investigation, we demonstrated that ABP-280 specifically phosphorylates tyrosine residue(s) and that the phosphorylation is restricted to a single tryptic or V8 peptide observed by Cleveland peptide mapping. The phosphorylation of ABP-280 was not due to the
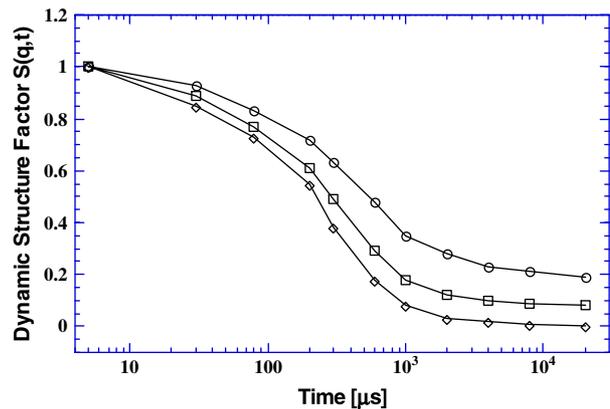
Fig. 6. Dynamic light scatter measurements. The normalized dynamic structure factor $g(q,t)$ at various decay times for pure actin solutions ($\diamond$), for actin solutions containing pure ABP-280 ($\square$), and actin solutions in the presence of phosphorylated ABP-280 ($\bigcirc$) at a molar ratio, $r_{A:ABP} = 20$. Conditions: F-buffer (150 m KCl, 2 mM MgCl$_2$; 20 mM Tris−HCl, pH 7.4, 20 °C); actin concentration 0.3 mg/ml; scatter angle of 90°.

endogenous kinases associated with the ABP-280, as phosphorylation increases by one order of magnitude when kinasing is performed in the presence of p56[lck].

Using antiserum against p56[lck], we successfully co-immunoprecipitated ABP-280 from PBL and CEM cell lysates, however, co-immunoprecipitation of p56[lck] was not observed when antibodies against ABP-280 were used to immunoprecipitate p56[lck] from supernatant of different cells and in the presence of different detergents. The inability to co-immunoprecipitate p56[lck] with ABP-280 is not yet understood, despite the direct interaction of purified p56[lck] with immunopurified ABP-280.

One possible function of the phosphorylation of ABP-280 by p56[lck] is the protection of ABP-280 from proteolysis, as has been described for the phosphorylated ABP-280 from blood platelets (cf. Yada et al., 1990). That the phosphorylation of ABP-280 by p56[lck] did not alter the susceptibility toward calpain suggests that phosphorylation of ABP-280 in T-lymphocytes has a different role to play. At this point, it is tempting to speculate that the increased avidity of lymphocytes after cross-linking with CD-3 and PMA could be due to the activation of p56[lck], which then phosphorylates the ABP-280 present in the periphery of cells. Events of phosphorylation and dephosphorylation of ABP-280 have been postulated to play a dynamic role in the organization of the cytoskeleton (Kovacsovics and Hartwig, 1996). In the present investigation, this observation is substantiated by the finding that phosphorylation of ABP-280 by p56[lck] is less effective when ABP-280 isolated from cells is treated with PMA. These results suggest that phosphorylation of ABP-280 by purified p56[lck] and that which is induced by PMA probably target different residues on ABP-280. It is, therefore, intriguing to speculate that PMA may activate or inactivate other kinases or influence the confirmation of the ABP-280 that impairs the activity of p56[lck]. Recent reports have shown that PMA activates protein kinase C which does not phosphorylate ABP-280; thus, PMA might affect other kinases which may have an effect on the conformation of ABP-280 (Wang et al., 1997).

Another possible role of the phosphorylation of ABP-280 by p56[lck] is that it regulates its association with surface receptors i.e., β2-integrins and modulates cross-linking of actin filaments into network or both. First results from dynamic light scattering indicated that phosphorylated ABP-280 seems to bind and cross-link actin filaments more readily. In a previous study, we and others demonstrated that ABP-280 induces an increase in filament stiffness when mixed with actin (Goldmann et al., 1997). Hence, it was of interest to investigate the internal dynamics of actin filaments in relation to ABP-280 phosphorylation. The dynamic light scattering data analyzed here by a stretched exponential fit (Goldmann et al., 1998) showed that the influence of ABP-280 on the flexibility of actin filaments/networks is phosphorylation-dependent. Further rheological examination of this effect on the viscoelasticity of filamentous actin networks is now needed to support this observation.

Acknowledgments

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