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Analysis of the Role of the Leucine Zipper Motif in Regulating the Ability of AFAP-110 to Alter Actin Filament Integrity

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Abstract AFAP-110 has an intrinsic ability to alter actin filament integrity as an actin filament crosslinking protein. This capability is regulated by a carboxy terminal leucine zipper (Lzip) motif. The Lzip motif facilitates self-association stabilizing the AFAP-110 multimers. Deletion of the Lzip motif (AFAP-110$^{\Delta lzip}$) reduces the stability of the AFAP-110 multimer and concomitantly increases its ability to crosslink actin filaments, in vitro, and to activate cSrc and alter actin filament integrity, in vivo. We sought to determine how the Lzip motif regulates AFAP-110 function. Substitution of the c-Fos Lzip motif in place of the AFAP-110 Lzip motif (AFAP-110^{fos}) was predicted to preserve the α-helical structure while changing the sequence. To alter the structure of the α-helix, a leucine to proline mutation was generated in the AFAP-110 α-helical Lzip motif (AFAP-110^{581P}), which largely preserved the sequence. The helix mutants, AFAP-110$^{\Delta lzip}$, AFAP-110^{fos}, and AFAP-110^{581P}, demonstrated reduced multimer stability with an increased capacity to crosslink actin filaments, in vitro, relative to AFAP-110. An analysis of opposing binding sites indicated that the carboxy terminus/Lzip motif can contact sequences within the amino terminal pleckstrin homology (PH1) domain indicating an auto-inhibitory mechanism for regulating multimer stability and actin filament crosslinking. In vivo, only AFAP-110$^{\Delta lzip}$ and AFAP-110^{581P} were to activate cSrc and to alter cellular actin filament integrity. These data indicate that the intrinsic ability of AFAP-110 to crosslink actin filaments is dependent upon both the sequence and structure of the Lzip motif, while the ability of the Lzip motif to regulate AFAP-110-directed activation of cSrc and changes in actin filament integrity in vivo is dependent upon the structure or presence of the Lzip motif. We hypothesize that the intrinsic ability of AFAP-110 to crosslink actin filaments or activate cSrc are distinct functions. J. Cell. Biochem. 91: 602–620, 2004.

Key words: adaptor protein; AFAP-110; leucine zipper motif; actin filaments; lamellipodia; Src

The actin filament associated protein AFAP-110 was originally identified as a binding partner for activated forms of the Src oncoprotein [Reynolds et al., 1987; Flynn et al., 1993]. The domain structure of AFAP-110 indicates that it can function as a scaffolding or adaptor protein. AFAP-110 binds to actin filaments via a carboxy terminal actin-binding domain and can interact with protein-binding partners via protein-binding modules that are amino terminal to the actin-binding domain [Baisden et al., 2001b]. These protein-binding modules include two SH2-binding motifs, an SH3-binding motif, two pleckstrin homology (PH) domains, and a leucine zipper motif. An analysis of potential protein-binding partners demonstrated that activated forms of the Src nonreceptor tyrosine kinase (Src^{527F}) will phosphorylate AFAP-110 on tyrosine and bind to the AFAP-110 SH3- and SH2-binding motifs. AFAP-110 is also a

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substrate and binding partner for PKCα, in vitro and in vivo. The amino terminal pleckstrin homology (PH1) domain of AFAP-110 will facilitate protein–protein interactions with PKCα [Qian et al., 2002]. Deletions between amino acids 180–226 in the PH1 domain prevent activated PKCα (myrPKCα) from binding to AFAP-110. Collectively, these data indicate that AFAP-110 can link cSrc or PKCα kinases to actin filaments.

It has also been established that interactions with PKCα will affect AFAP-110 structure and function, in vitro [Qian et al., 2002]. Gel filtration analysis of recombinant AFAP-110 (rAFAP-110) demonstrated that it exists predominantly as a larger multimer, predicted to be a nonamer, within the context of aqueous actin filament-binding buffer. Phosphorylation of rAFAP-110 by PKCα, in vitro, destabilizes the multimer as evidenced by a subpopulation of smaller multimers. It has also been shown that actin-binding proteins that are able to multimerize will crosslink actin filaments. Smaller multimers will crosslink actin filaments more efficiently than larger multimers due to the shorter spacing accommodated between crosslinked actin filaments by a smaller actin filament crosslinking protein [Meyer and Aebi, 1990; Matsudaira, 1991; Wachsstock et al., 1993]. Because AFAP-110 can bind actin filaments directly and can multimerize, it was hypothesized that AFAP-110 was an actin filament crosslinking protein. Further, phosphorylation by PKCα may increase the actin filament crosslinking potential of AFAP-110 by reducing the stability and size of the multimer. In agreement, rAFAP-110 was shown to be capable of crosslinking actin filaments and PKCα-phosphorylated rAFAP-110 demonstrated increased actin filament crosslinking ability, in vitro [Qian et al., 2002]. In cells, AFAP-110 is associated with both stress filaments and the cell membrane and is strongly represented in lamellipodia and filopodia at the leading edge of a motile cell [Qian et al., 1998, 2000, 2002; Baisden et al., 2001a]. As activation of PKCα induces the formation of motility structures such as lamellipodia and filopodia at the leading edge of the cell [Dwyer-Nield et al., 1996; Coghlan et al., 2000] and these structures are rich in newly forming actin filaments and a site for actin filament crosslinking [Condeelis, 1993; Cramer, 1997; Roberts and Stewart, 2000], AFAP-110 would be positioned to affect changes in actin filament crosslinking in response to PKCα. In theory, increased actin filament crosslinking may be important for providing the protrusive force that is associated with lamellipodia formation and extension, as has been demonstrated for actin filament-binding proteins such as MSP and ABP-120 [Condeelis, 1993; Cox et al., 1995, 1996]. Unique to AFAP-110, this function appears to be regulated.

The leucine zipper (Lzip) motif of AFAP-110 is placed in the carboxy terminus, immediately upstream of the actin-binding domain, and regulates self-association and actin filament crosslinking capability [Qian et al., 1998, 2000, 2002; Baisden et al., 2001a]. When the carboxy terminal 177 amino acids, which include the Lzip motif, are expressed as a GST-encoded fusion protein (GST-Cterm), GST-Cterm can affinity absorb AFAP-110 from cell lysates, while deletion of the Lzip motif from GST-Cterm abrogates binding to AFAP-110, indicating a role for the Lzip motif in facilitating self-association of AFAP-110 [Qian et al., 1998]. Gel filtration analysis confirms that AFAP-110 self-associates and that the Lzip motif plays a role in multimer formation [Qian et al., 1998, 2002]. Deletion of the Lzip motif will reduce multimer stability in a manner similar to PKCα, whereby recombinant AFAP-110$^{\Delta lzip}$ (rAFAP-110$^{\Delta lzip}$) segregates in populations of larger and smaller multimers. Furthermore, rAFAP-110$^{\Delta lzip}$ is able to crosslink actin filaments, in vitro, more efficiently than rAFAP-110. These data indicate that the carboxy terminus, including the Lzip motif, plays an important role in facilitating self-association of rAFAP-110, the stability of the AFAP-110 multimer, and its ability to crosslink actin filaments. Loss of this interaction is predicted to destabilize the multimer, increasing the ability of AFAP-110 to crosslink actin filaments. Interestingly, deletion of the Lzip motif enables AFAP-110$^{\Delta lzip}$ to activate cSrc and alter actin filament integrity, in vivo, in a RhoA-dependent manner [Baisden et al., 2001a]. Wild type AFAP-110 does not affect actin filament integrity, in vivo. These data indicate that AFAP-110 may have an intrinsic ability to activate cSrc and alter actin filament integrity that is regulated through intramolecular or intermolecular interactions involving the Lzip motif.

In this report, we sought to determine how the Lzip motif is able to regulate the ability of AFAP-110 to crosslink actin filaments, in vitro,

and alter actin filament integrity, in vivo. Leucine zipper motifs are defined as α-helical sequences that are highly charged and defined by a heptad repeat of leucine residues [Hodges, 1996]. These motifs are found in a variety of proteins, including transcription factors and cytoskeletal-associated proteins. Leucine zipper motifs form homodimeric interactions with other leucine zipper motifs, such as those defined by c-Jun homodimers, or they forge heterodimeric interactions, such as those defined by c-Fos/c-Jun binding [Hurst, 1994]. Leucine zipper motifs can also bind to opposing sequences that are not themselves leucine zipper motifs. For example, the heat shock protein HSP90 self-associates via interactions between its leucine zipper motif and amino terminal sequences that do not define a leucine zipper motif [Nemoto et al., 1995]. We hypothesize that the Lzip motif of AFAP-110 may be contacting opposing sequences within the AFAP-110 protein and that loss of this interaction may serve to destabilize the AFAP-110 multimer, which in turn would increase its ability to crosslink actin filaments. Here, we sought to determine whether the sequence or the structure of the AFAP-110 α-helical Lzip motif was important for regulating its ability to crosslink actin filaments and to affect actin filament integrity, in vivo. To test this theory, we predicted that substituting the Lzip motif with the analogous sequence from c-Fos would preserve the α-helical structure with a different sequence. Further, mutating the 3rd leucine in the heptad repeat to a proline would be predicted to alter the structure of the α-helix, but have only a minimal effect on sequence. By identifying how the Lzip motif could facilitate self-association, we were able to formulate a hypothesis as to how this motif may regulate the ability of AFAP-110 effect actin filament integrity.

EXPERIMENTAL PROCEDURES

Recombinant Proteins and Constructs

GST-Cterm was generated by amplifying the sequences that encode the AFAP-110 leucine zipper motif by PCR, as previously described [Qian et al., 1998]. GST-Cterm^{fos} was created by amplifying by PCR the sequence encoding the Lzip motif of avian c-Fos and subcloning it in place of the Lzip motif of AFAP-110 within GST-Cterm. The fusion proteins were analyzed by 15% SDS–PAGE and Coomasie staining for purity and size. Coomasie staining consistently demonstrated purified proteins with a M_r consistent with predictions. The only other bands detected were smaller proteins that are predicted to be proteolytic breakdown products generated in the bacteria (data not shown). Concentrations of the fusion proteins were estimated based on an analysis of BSA standards, performing scanning densitometry and comparing with the intensity of the GST-Cterm^{fos} and GST-Cterm fusion proteins. GST-PH1, GST-PH1$^{\Delta 180-226}$, GST-PH1$^{\Delta 226-240}$, and GST-PH1^{W240A} were generated as previously described [Baisden et al., 2001a]. Recombinant AFAP-110 (rAFAP-110) and rAFAP-110$^{\Delta lzip}$ were generated using the *pGex-6P-1* system, as previously described [Qian et al., 2002]. The rAFAP-110^{fos} chimera was created by amplifying the sequence that encodes the avian c-Fos leucine zipper motif by PCR and subcloning this sequence into AFAP-110$^{\Delta lzip}$, in frame, substituting in place where the Lzip motif is normally encoded. Thus, AFAP-110^{fos} conserves the α-helical structure of a Lzip motif, but does not conserve the sequence of the AFAP-110 Lzip motif. To alter the Lzip structure, site-directed mutagenesis was used to change the 3rd leucine in the heptad repeat (Leu^{581}) to a proline residue (AFAP-110^{581P}), as previously described [Qian et al., 1998]. The rAFAP-110 and rAFAP-110 helix mutant chimeric constructs were proteolytically removed from GST sequence using Precision cut enzyme (Pharmacia) and were generated as previously described [Qian et al., 2002]. The rAFAP-110 and rAFAP-110 helix mutant proteins were purified away from immobilized GST protein (which was bound to glutathione-coated sepharose beads) and dialyzed into actin filament-binding buffer (5 mM $MgCl_2$, 1 mM EGTA, 2 mM ATP, and 50 mM KCl, pH 7.5), as previously described [Qian et al., 2002]. Recombinant AFAP-110 proteins were analyzed for purity by separation on 8% SDS–PAGE and Coomasie staining, which also revealed protein concentration relative to BSA controls.

The AFAP-110, AFAP-110$^{\Delta lzip}$, and AFAP-110$^{\Delta 553-730}$ (AFAP$^{\Delta Cterm}$) were expressed in Cos-7 cells using the pCMV-1 vector, as previously described [Guappone et al., 1996; Guappone and Flynn, 1997; Qian et al., 2000; Baisden et al., 2001a]. GFP-AFAP-110$^{\Delta 1-552}$ (AFAP^{Cterm}) expresses only the carboxy terminus of AFAP-110 as a GFP fusion protein and

was generated as previously described [Qian et al., 2000].

Analysis of Multimer Formation and Actin Filament Crosslinking

Multimer formation was analyzed by gel filtration analysis using FPLC and Superose 6 chromatography, as previously described [Qian et al., 2002; Xie et al., 2002]. Fractions were collected, analyzed by Western blot analysis and fractions correlated to predicted molecular weight based on an analysis of molecular weight standards, as previously described [Qian et al., 2002; Xie et al., 2002]. Actin filament crosslinking assays were performed as previously described [Qian et al., 2002]. These include actin filament pelleting assays (biochemical analysis) and light microscopy analysis of rhodamine-labeled actin filaments (microscopy) as well as electron microscopy (EM), negative stain analysis.

Calculation of a Stokes Radius

Using the data obtained from the gel filtration analysis which determined retention of rAFAP-110 in a Superose 6 chromatography column (Pharmacia), a Stokes radius was calculated. Details of the procedure can be found at a website (http://itsa.ucsf.edu/~hdeacon/Stokesradius.html) and the calculations were generated as previously described [Xie et al., 2002]. The data were plotted by running known standards through the Superose 6 column and using a 500 μl injection port on the FPLC. The known standards used were Thyroglobulin (669 kDa, Stokes radius = 8.5 nm), Ferritin (440 kDa, Stokes radius = 6.1 nm), Catalase (232 kDa, Stokes radius = 5.22 nm), Aldolase (158 kDa, Stokes radius = 4.81 nm), and Albumin (BSA; 67 kDa; Stokes radius = 3.55 nm). The standards were run through the column by FPLC and the fraction in which they eluted (elution volume or Ve) identified by UV spectra. Retention was calculated using the formula [−log Kav] where Kav = [Ve−Vo]/[Vt−Vo]. Ve = elution volume of the standard or rAFAP-110 samples, which was revealed by analysis of fractions eluted from the column by either UV spectra or Western blot analysis. Vo = the void volume (7.32 ml); Vt = the total volume (24.7 ml). The gel bed volume (Vg = 4.9 ml) is not used for calculating the Kav by the Laurent and Killander solution for a Stokes radius. Retention in terms of [−log Kav] was graphed against known Stokes radii and the Stokes radius for rAFAP-110 estimated from this graph based on Ve = 8.6 ml (verified by Western blot analysis of fractions collected and UV spectra from the FPLC).

Cell Culture, Immunofluorescence, and Western Blot Analysis

Cos-7 cells were used for expression of AFAP-110 constructs for affinity absorption experiments, as previously described [Qian et al., 1998]. Immunofluorescence analysis was performed as previously described [Qian et al., 2002]. Western blot analysis was performed, as previously described [Qian et al., 1998]. The polyclonal antibody, F1, was used to detect AFAP-110 constructs by Western blot analysis and was prepared as previously described [Flynn et al., 1993; Qian et al., 1999]. Previous publications demonstrate the ability of Ab F1 to uniquely detect AFAP-110 overexpressed in Cos cells [Flynn et al., 1995; Qian et al., 1998]. Actin filament integrity can be revealed with AFAP-110 and the helix mutants, which always colocalize with actin filaments [Qian et al., 2000 and data not shown]. cSrc was detected with mAb EC10 followed by anti-mouse Ig conjugated to Cy5 and false-colored red. Cellular phosphotyrosine or cSrc activation were revealed separately using rabbit anti-phosphotyrosine or rabbit anti-phosphoY416, respectively, then detected with anti-rabbit Ig antibodies conjugated with rhodamine and false-colored blue.

RESULTS

Changing the Sequence or Structure of the AFAP-110 Lzip Motif

The leucine zipper motif of AFAP-110 is defined by amino acids 553–595 and contains a heptad repeat of five leucine residues. Deletion of the Lzip motif was accomplished as previously described, whereby a *Bgl*II site defined the carboxy terminal end of the Lzip motif and a second *Bgl*II site was engineered on the amino terminal side, which resulted in a silent change that did not alter the coding sequence [Qian et al., 1998]. Digestion with *Bgl*II followed by religation removed the Lzip motif and preserved the downstream amino acid sequence, in frame. To change the sequence while preserving the structure of the α-helical leucine zipper

motif, the avian c-Fos leucine zipper motif was amplified such that the sequence which encode the analogous 43 amino acids of the c-Fos Lzip motif were subcloned into the *Bgl*II site of AFAP-110$^{\Delta lzip}$ (Fig. 1). The avian c-Fos leucine zipper sequence was chosen as an analogous sequence to substitute in place of the AFAP-110 Lzip motif because it is not predicted to facilitate a homodimeric leucine zipper interaction (i.e., self-association) [Hurst, 1994]. In order to alter the structure of the AFAP-110 Lzip motif with minimal affects on sequence, the 3rd leucine residue in the heptad repeat was mutated to a proline, which was predicted to place a kink in the α-helical structure (Fig. 1A).

To ensure that the avian c-Fos leucine zipper motif did not have the potential to facilitate interactions with AFAP-110, GST-Cterm was generated, immobilized on glutathione-coated sepharose beads and used to affinity absorb AFAP-110 from Cos-7 cell lysates, as previously described [Qian et al., 1998]. GST-Cterm^{fos} was also generated, which contains the c-Fos Lzip motif substituted for the AFAP-110 Lzip motif. GST-Cterm, but not GST-Cterm^{fos}, was able to affinity absorb AFAP-110 (Fig. 1B), demonstrating that the avian c-Fos leucine zipper motif does not facilitate self-association with AFAP-110. By contrast, immobilized GST-Cterm^{581P}, which has the 3rd leucine residue in the heptad repeat mutated to a proline, will affinity absorb cellular AFAP-110 [Qian et al., 1998]. Thus, disruption of affinity absorption requires (a) large-scale changes in the Lzip domain or (b) changes in the sequence along the length of the Lzip domain. An analysis of

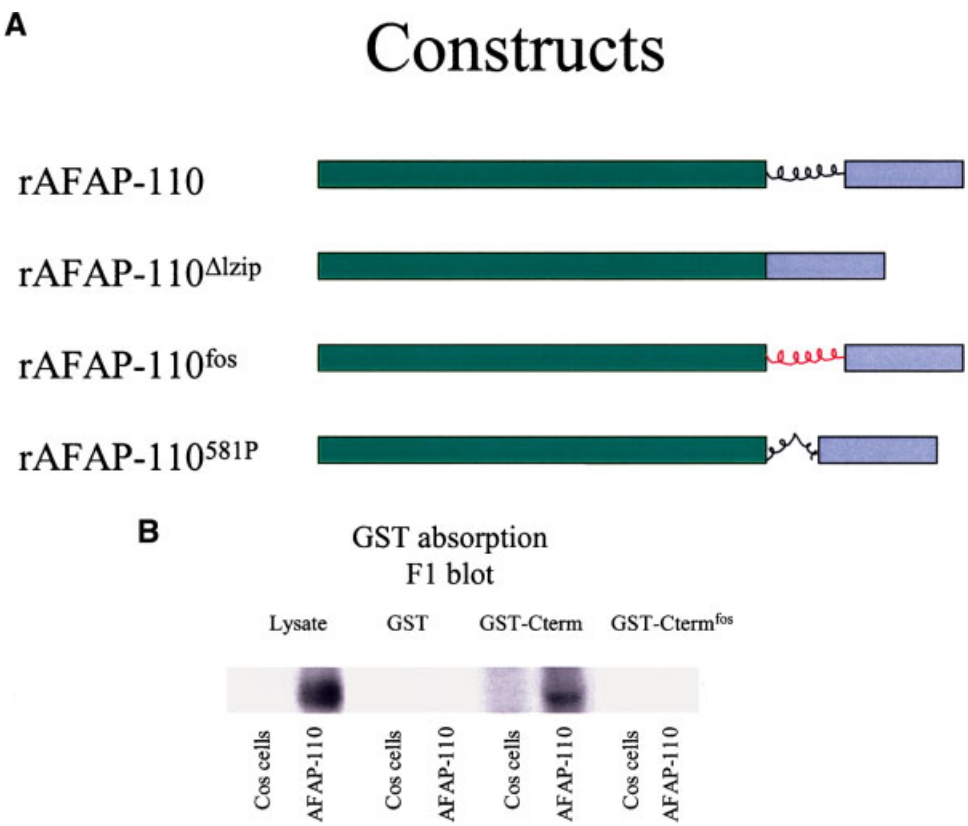

Fig. 1. **A**: Recombinant AFAP-110 constructs. AFAP-110 contains a carboxy terminal leucine zipper motif, shown as an α-helical structure near the carboxy terminus (''squiggly'' line with five loops). The leucine zipper motif was deleted (AFAP-110$^{\Delta lzip}$) or substituted with the c-Fos leucine zipper motif to preserve structure and change the sequence (AFAP-110^{fos}). The sequence was largely preserved and the structure changed by mutating the 3rd leucine residue to a proline within the heptad repeat that defines the leucine zipper motif (AFAP-110^{581P}). These constructs were expressed as recombinant fusion proteins in *E. coli*, as described in Materials and Methods. **B**: Affinity absorption of AFAP-110. GST-Cterm^{fos} was affinity purified from lysed bacteria and equal amounts of fusion protein (2 μg) absorbed to glutathione-coated sepharose beads. AFAP-110 was expressed in Cos-7 cells, the cells lysed in NP-40 buffer and 500 μg of cell lysates incubated with GST, GST-Cterm, or GST-Cterm^{fos}. Absorbed proteins were resolved by 8% SDS–PAGE and Western blot analysis performed using Ab F1 to detect bound AFAP-110. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

[^{35}S]-methionine labeled proteins indicates that GST-Cterm has the potential to affinity absorb other cellular proteins, in addition to AFAP-110 (data not shown, see Discussion).

In order to determine whether substitution of the c-Fos Lzip motif or mutagenesis of Leu → Pro in the 3rd heptad repeat of the Lzip motif could affect multimer stability, recombinant AFAP-110 proteins were analyzed by gel filtration analysis. rAFAP-110, rAFAP-110^{fos}, and rAFAP-110^{581P} were generated, enzymatically removed from GST, purified, dialyzed into actin filament-binding buffer, and resolved by gel filtration analysis. Figure 2A demonstrates that rAFAP-110 migrated as a narrow peak (fractions #23–#35), whereas AFAP-110^{fos} and AFAP-110^{581P} migrated as a main peak (fractions #23–#35) followed by a succession of smaller peaks (fractions #36–#47) similar to results reported for AFAP-110$^{\Delta lzip}$ [Qian et al., 2002]. These latter data are consistent with the separation profile of AFAP-110$^{\Delta lzip}$ [Qian et al., 2002]. The relatively narrow range of separation for rAFAP-110 cannot be attributed to differences in loading, as identical concentrations and volumes of the recombinant fusion proteins were loaded onto the FPLC Superose 6 column prior to gel filtration. The first peak represents a population of multimeric rAFAP-110 that we predict correlate with nonamers; thus, the later eluting protein peaks represent smaller multimers and/or monomers. Although the helix mutants, AFAP-110$^{\Delta lzip}$, rAFAP-110^{fos}, or rAFAP-110^{581P}, apparently form similar sized multimers (predicted to be nonamers) they are much less stable than the multimers formed by native rAFAP-110.

Both rAFAP-110^{fos} and rAFAP-110^{581P} Are Efficient Actin Filament Crosslinking Proteins

Based on our previous results demonstrating that the helix deletion mutant, rAFAP-110$^{\Delta lzip}$, is a destabilized multimer that crosslinks actin filaments more efficiently than rAFAP-110 [Qian et al., 2002], we predicted that the helix altered mutants rAFAP-110^{fos} and rAFAP-110^{581P} would increase actin filament crosslinking as well. We tested this hypothesis using two different techniques, a biochemical analysis that analyzes the ability of actin filament-binding proteins to pellet crosslinked actin filaments at low speed (20,800*g*) and fluorescence microscopy analysis of crosslinked actin filaments. At low speed, actin filaments will not efficiently pellet unless bound to actin filament crosslinking proteins [Cooper and Pollard, 1982; Pollard and Cooper, 1982; Qian et al., 2002]. Using pure proteins, a low-speed centrifugation assay showed that approximately 25% of the rAFAP-110 and 50% of the actin filaments sedimented. By contrast, nearly 100% of the helix mutants, rAFAP-110^{fos}, rAFAP-110^{581P}, and AFAP-110$^{\Delta lzip}$, cosedimented with greater than 80% of the actin filaments pelleted (Fig. 3A). The results for AFAP-110 and AFAP-110$^{\Delta lzip}$ were consistent with earlier studies of this mutant [Qian et al., 2002]. Like rAFAP-110, rAFAP-110^{fos} and rAFAP-110^{581P} do not efficiently pellet in the absence of actin filaments ([Qian et al., 2002] and data not shown). These data demonstrate that relative to rAFAP-110, the rAFAP-110$^{\Delta lzip}$, rAFAP-110^{fos}, and rAFAP-110^{581P} helix mutant proteins are more efficient actin filament crosslinking proteins, indicating that both the sequence and structure of the Lzip motif may be important for actin filament crosslinking.

To verify these results, we analyzed crosslinking by fluorescence microscopy (Fig. 3B). Here, randomly oriented rhodamine-labeled actin filaments in solution can be seen by confocal microscopy to coalesce into distinctive patterns in the presence of proteins that crosslink [Bachmann et al., 1999; Qian et al., 2002]. Incubation with rAFAP-110 resulted in some coalescence of actin filaments indicating crosslinking, based on a qualitative analysis. Here, the settings on the immunofluorescence microscope were kept constant, to permit a visual analysis of changes in actin filament coalescence. Incubation with rAFAP-110$^{\Delta lzip}$ significantly upregulated actin filament crosslinking, in agreement with our previous report [Qian et al., 2002]. A different staining pattern was observed with rAFAP-110$^{\Delta lzip}$, rAFAP-110^{fos}, and rAFAP-110^{581P}, which showed a greater coalescence of actin filaments than achieved by rAFAP-110. The strong coalescence of actin filaments is consistent with AFAP-110$^{\Delta lzip}$, rAFAP-110^{fos}, and rAFAP-110^{581P} being more efficient actin filament crosslinking proteins than rAFAP-110, perhaps owing to mutation induced destabilization of rAFAP-110 multimers.

Smaller multimers of actin crosslinking proteins are predicted to crosslink actin filaments more efficiently compared to larger multimers by facilitating closer associations between adjacent actin filaments [Meyer and Aebi, 1990;

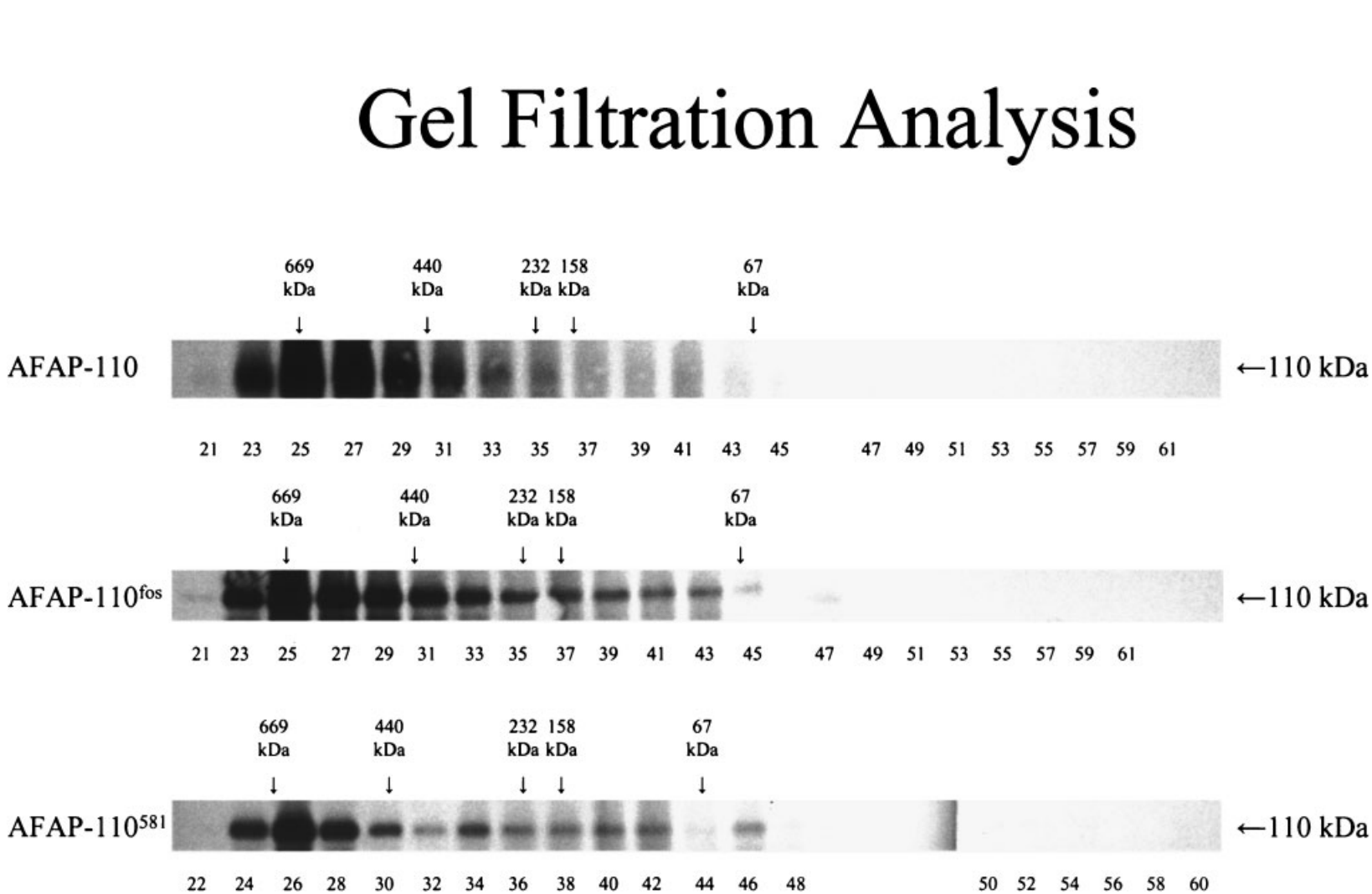

Fig. 2. Gel filtration analysis of rAFAP-110. **A**: rAFAP-110, rAFAP-110^{fos}, and rAFAP-110^{581P} were separated by gel filtration using FPLC and Superose 6 chromatography. Resolved fractions were collected in 250 µl aliquots and Western blot analysis performed on each fraction. The elution fraction of the molecular weight markers is shown.

Matsudaira, 1991; Wachsstock et al., 1993]. EM analysis of negative-stained preparations demonstrated that rAFAP-110 decorates the sides of actin filaments as aggregates that ranged in size from 10×10 nm to 10×30 nm (Fig. 4A), in agreement with previous findings [Qian et al., 2002]. For reference, actin filaments have a known diameter of 7 nm [Milligan et al., 1990; Holmes et al., 1993; Schmid et al., 1994]. Like rAFAP-110$^{\Delta lzip}$, rAFAP-110^{fos} and rAFAP-110^{581P} also appeared to induce crosslinking of actin filaments (Fig. 4A) and the size of the discrete aggregates were significantly smaller relative to rAFAP-110 and could not be accurately resolved for size determination, likely owing to a reduction in size of the multimers.

A Stokes radius was determined for rAFAP-110 in solution in order to determine whether a calculated size of the rAFAP-110 multimer may approximate the size of the rAFAP-110 aggregates detected on the sides of actin filaments. rAFAP-110 eluted from a Superose 6 column with 8.6 ml of actin filament-binding buffer, which was used to calculate $[-\log Kav] = 1.14$. On a standard curve ($[-\log Kav]$) versus known Stokes radii (Fig. 4B), the 1.14 value of rAFAP-110 corresponds to an estimated Stokes radius of 8.6 nm. This estimation is in agreement with the radius range of rAFAP-110 particles (5–15 nm) discerned by EM analysis (see Discussion).

Carboxy Terminus/Lzip Motif Contacts Sequences in the PH1 Domain

It is possible that the Lzip motif forms homodimeric interactions by binding to an opposing AFAP-110 leucine zipper motif, to other motifs defined within the AFAP-110 protein, or possibly a combination. To analyze this further, we incubated GST-Cterm with Cos-7 cell lysates expressing AFAP-110 or AFAP-110$^{\Delta lzip}$ (Fig. 5A). GST was unable to bind AFAP-110 and other studies have demonstrated that GST is unable to bind AFAP-110$^{\Delta lzip}$ or any other Cos cellular protein (Flynn, data not shown). The data demonstrate that the GST-Cterm can affinity absorb both AFAP-110 and AFAP-110$^{\Delta lzip}$. Thus, GST-Cterm may bind to sequences in AFAP-110 that are not a leucine zipper motif. Given that the Lzip domain of GST-Cterm binds the Lzip deletion mutant, AFAP-110$^{\Delta lzip}$, we surveyed a series of protein-binding modules from AFAP-110 to determine if they could affinity absorb cellular AFAP-110 from cell lysates (data not shown). In our survey, only the amino terminal pleckstrin homology (PH1) domain was able to bind to AFAP-110 from cell lysates (Fig. 5B). An analysis of GST-PH1 demonstrated that it could affinity absorb full-length AFAP-110, but was unable to affinity absorb a mutant of AFAP-110 that lacked the carboxy terminal 177 amino acids, AFAP-110$^{\Delta 553-730}$. This deletion mutant does not encode the Lzip motif or adjacent carboxy terminal sequences [Qian et al., 2000]. The 82-kDa protein detected in the AFAP-110 lysate lane is hypothesized to be a proteolytic breakdown product, as previously described [Flynn et al., 1995; Qian et al., 1999]. These data indicate that

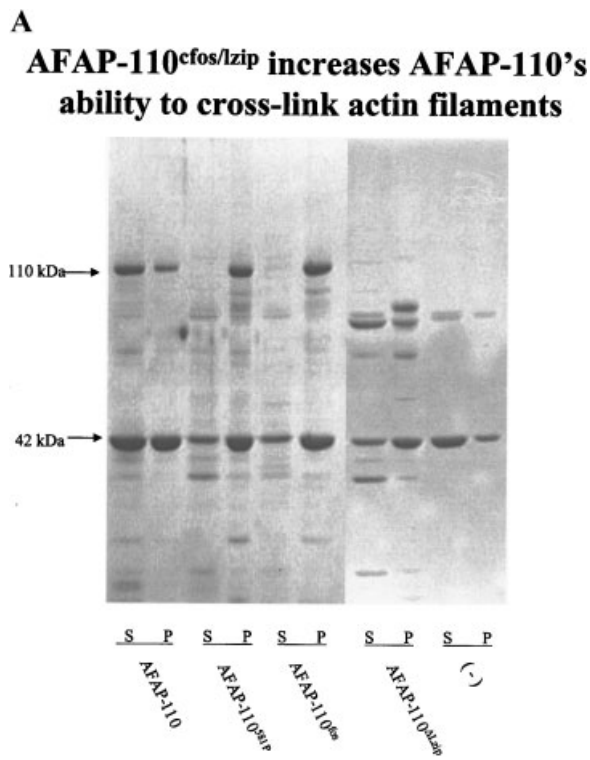

Fig. 3. Actin filament crosslinking analysis. **A**: Biochemical analysis. The recombinant AFAP-110 proteins were purified and incubated with polymerized G-actin (F-actin) in the presence of actin filament crosslinking buffer. The mixture was pelleted at 20,800*g* and pelleted fractions resolved by 8% SDS–PAGE followed by Coomasie staining to identify the 110 kDa rAFAP-110 proteins and the 42 kDa actin protein. S = supernatant (not crosslinked F-actin) and P = pellet (crosslinked actin filaments fraction). **B**: Fluorescence microscopy analysis. G-actin was polymerized into actin filaments in the presence of a 1:10 ratio of rhodamine labeled G-actin to unlabeled G-actin. Recombinant AFAP-110 proteins (2 μg) were incubated with actin filaments and placed on coverslips for analysis using immunofluorescence confocal microscopy. Coalescence of ''red'' indicated cross-linked actin filaments. Bar indicates 10 μm.

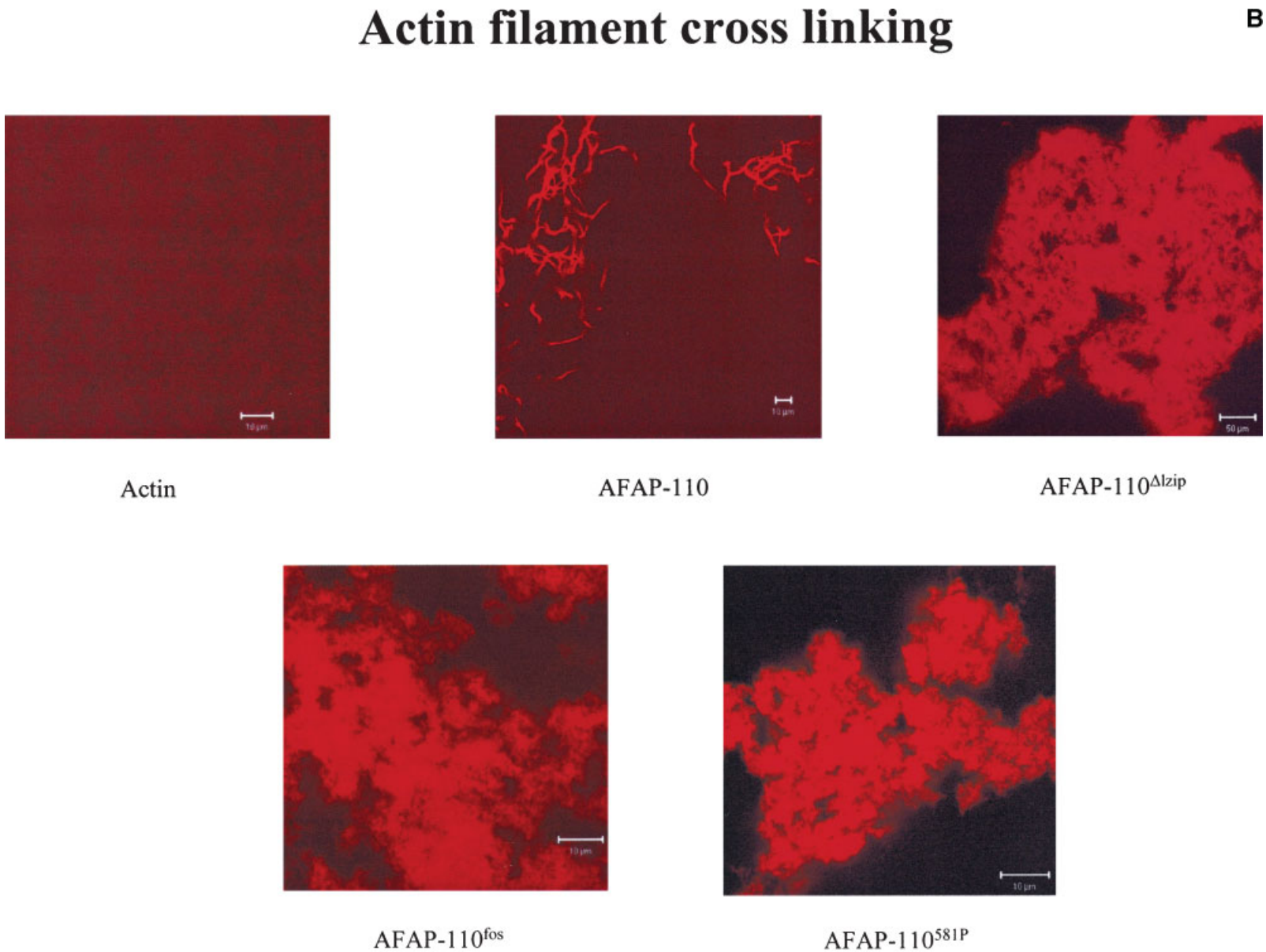

Fig. 3. (*Continued*)

A

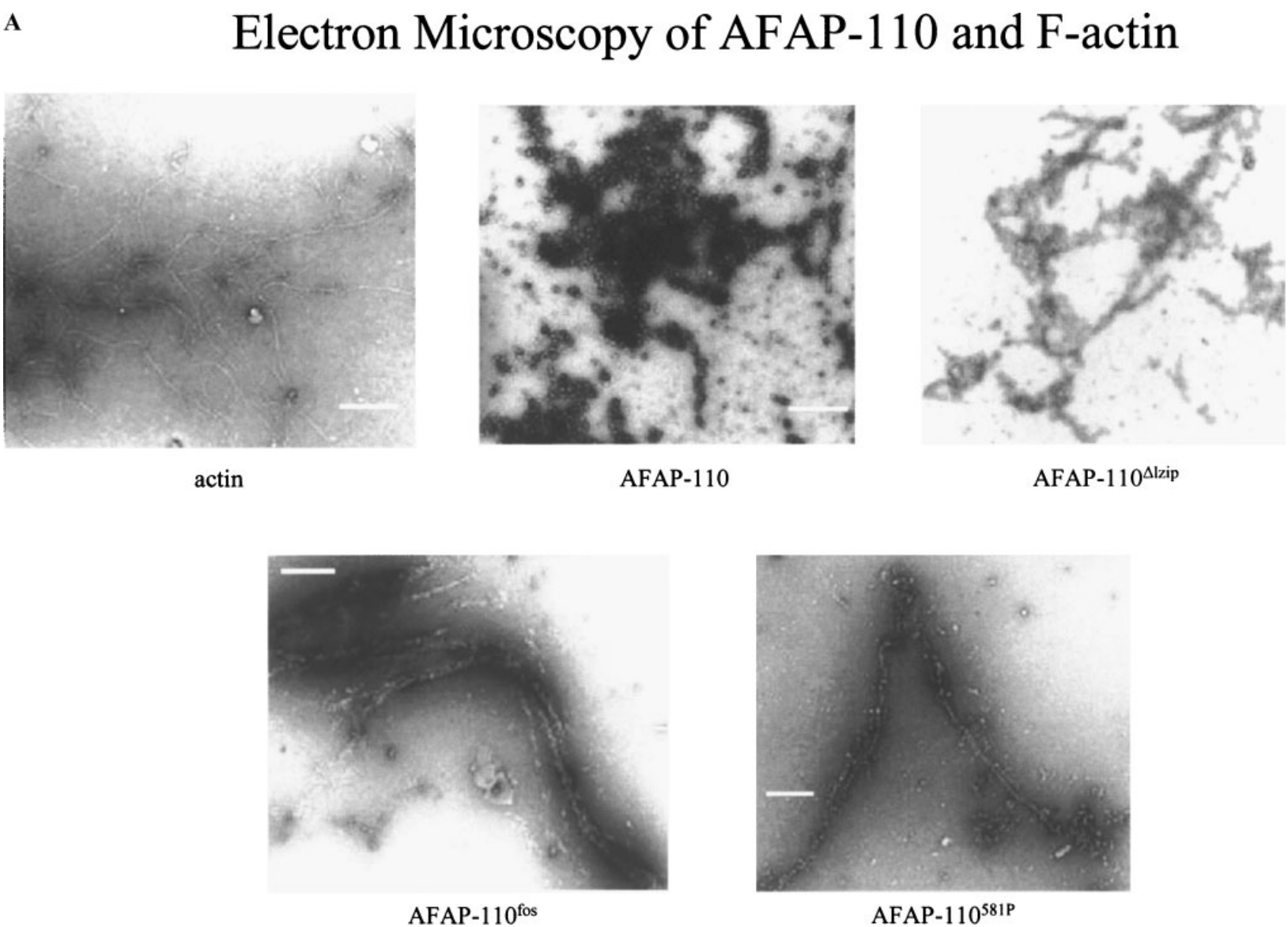
Electron Microscopy of AFAP-110 and F-actin
actin
AFAP-110
AFAP-110Δlzip
AFAP-110fos
AFAP-110581P

Fig. 4.

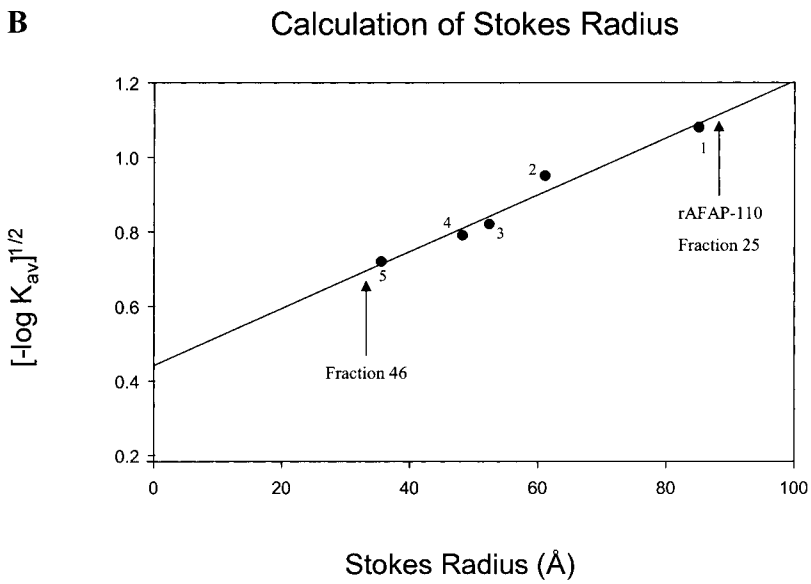

Fig. 4. (*Continued*)

the PH1 domain can affinity absorbs AFAP-110 and this interaction is dependent upon the carboxy terminal 177 amino acids that include the Lzip motif.

Deletional analysis of the PH1 domain demonstrate that removal of amino acids 180–226 abrogated binding to cellular AFAP-110, while deletion of amino acids 226–240 or point mutation of amino acid 240 to Ala had no effect (Fig. 5C). The significance of these residues is that amino acids 180–226 define the PKCα binding site [Qian et al., 2002], while amino acids 226–240 define sequences downstream of the PKCα binding site. Further, Trp^{240} represents a conserved Trp residue found in all pleckstrin homology domains [Shaw, 1996]. Mutation of this conserved Trp to Ala affects the way the conserved, carboxy terminal α-helix folds across the main body of the PH domain. Thus, these data indicate that the PKCα binding site overlaps with sequences that facilitate interactions with the carboxy terminus.

To determine whether the PH1 domain could uniquely contact sequences in the carboxy terminus of AFAP-110, a GFP fusion protein was created that only expressed the carboxy terminal 177 amino acids of AFAP-110, GFP-AFAP-$110^{\Delta 1-552}$ [Qian et al., 2000]. Both GST-Cterm and GST-PH1 were able to affinity absorb GFP-AFAP-$110^{\Delta 1-552}$, indicating that both the Lzip motif and the PH1 domain have the potential to contact sequences in the carboxy terminus of AFAP-110. These data indicate that the Lzip may stabilize the AFAP-110 multimer by contacting amino acids 180–226, which are defined within the PH1 domain.

Effect of Helix Mutants Upon Activation of Cellular Tyrosine Kinases

Deletion of the Lzip motif enables AFAP-$110^{\Delta lzip}$ to alter actin filament integrity, in vivo, by activating cSrc [Baisden et al., 2001a]. To determine whether AFAP-110^{fos} or AFAP-110^{581P} could affect actin filament integrity and activate cellular tyrosine phosphorylation and cSrc, MEF cells were transiently transfected with AFAP-110 or the helix mutant constructs as GFP-fusion proteins. Changes in actin filament integrity were associated with intracellular localization of AFAP-110 and the helix mutant constructs, which each retains a strong association with stress filaments and the cell membrane. An associated increase in cellular phosphotyrosine levels (Fig. 6A) was

Fig. 4. **A**: Electron microscopy analysis of crosslinked actin filaments. G-actin was polymerized into actin filaments and incubated with recombinant AFAP-110 proteins and pelleted at 20,800*g*. The crosslinked actin filaments were analyzed by negative stain analysis and electron microscopy. Bar = 100 nm. **B**: Stokes radius. A Stokes radius was calculated based on the gel filtration analysis of Figure 2. rAFAP-110 has a [−log Kav] = 1.14, indicating a predicted Stokes radius of 8.6 nm. Fraction 46 represents a 2nd peak detected in the rAFAP-110^{581P} sample seen in Figure 2 and correlates with a Stokes radius of 3.6 nm.

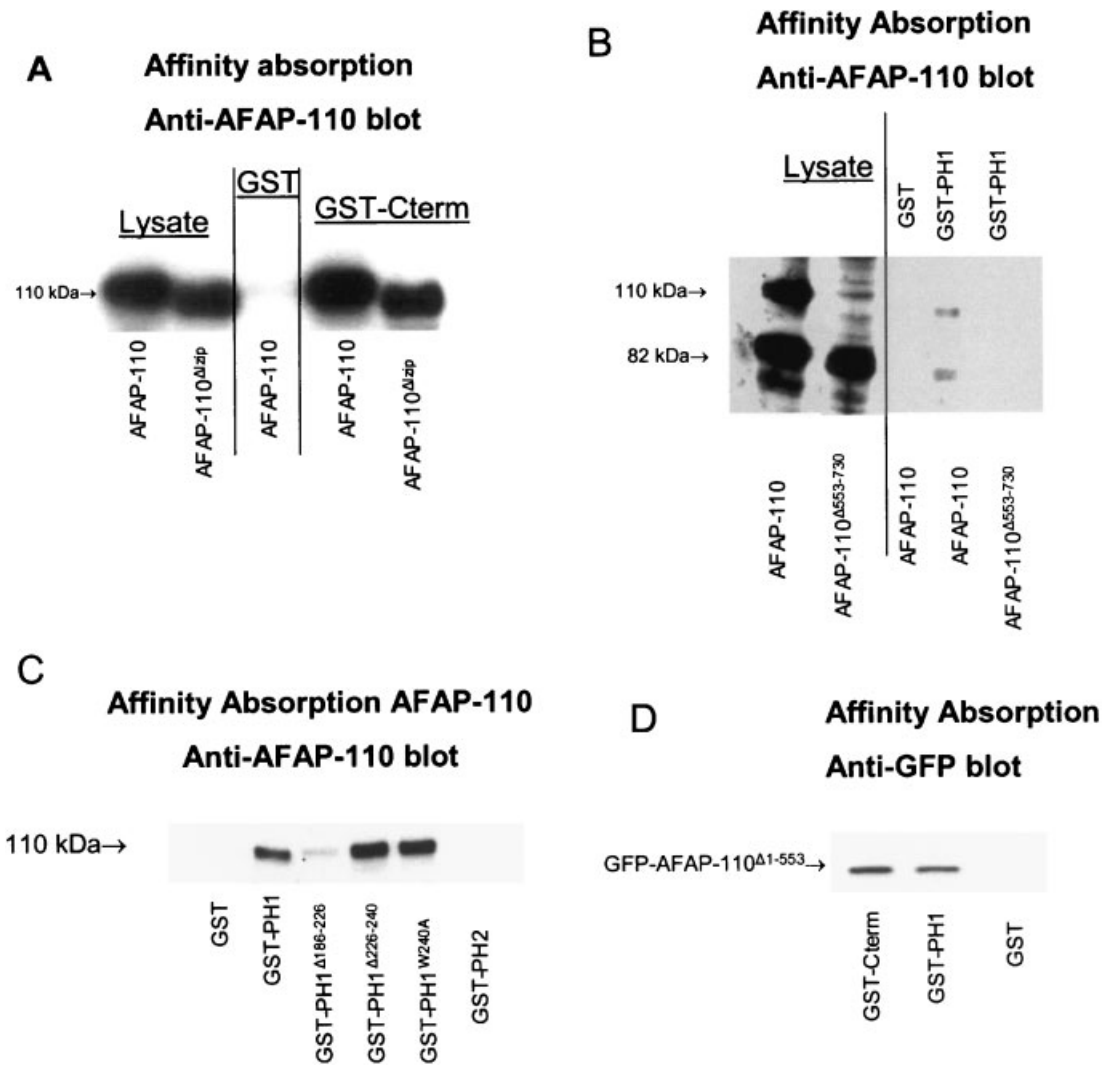

Fig. 5. The PH1 domain can act as an opposing binding site for the carboxy terminus/Lzip motif. **A**: AFAP-110 or AFAP-110$^{\Delta lzip}$ were expressed in Cos-7 cells. Five hundred micrograms of cell lysates was incubated with immobilized GST or GST-Cterm. Affinity-absorbed proteins were resolved by 8% SDS–PAGE and Western blot analysis performed with Ab F1. **B**: The PH1 domain was expressed as a GST fusion protein and incubated with Cos-7 cell lysates expressing AFAP-110 or AFAP-110$^{\Delta 553-730}$. Bound proteins were resolved by 8% SDS–PAGE and Western blot analysis performed with Ab F1. Twenty-five micrograms of lysates were also analyzed. **C**: The fusion proteins were incubated with 500 μg of Cos-7 cell lysates expressed AFAP-110. Bound proteins were resolved by 8% SDS–PAGE and Western blot analysis performed with Ab F1. **D**: GFP-AFAP-110$^{\Delta 1-553}$ expresses only the carboxy terminal 177 amino acids of AFAP-110 as a GFP-encoded fusion protein in Cos-7 cells. Five hundred micrograms of cell lysates were incubated with GST-Cterm (encoding the AFAP-110 leucine zipper motif), GST-PH1, or GST. Bound proteins were resolved by 8% SDS–PAGE and Western blot analysis performed with Ab F1.

correlated with expression of AFAP-110 and the helix mutants in MEF cells. Activation of cSrc was also qualitatively measured using anti-phospho-Y416 antibodies (Fig. 6B). AFAP-110 has no affect upon cellular phosphotyrosine levels or cSrc activation, similar to normal, untransfected MEFs, as previously described [Baisden et al., 2001a] (Fig. 6A,B). Conversely, AFAP-110$^{\Delta lzip}$ was able to direct upregulation of cellular phosphotyrosine levels and cSrc activation. AFAP-110^{fos} was unable to affect actin filament integrity or direct activation of cellular

Fig. 6. Analysis of the affects of sequence or structural changes of the Lzip motif upon changes in actin filament integrity, in vivo. GFP-tagged AFAP-110, AFAP-110$^{\Delta lzip}$, AFAP-110^{fos}, and AFAP-110^{581P} (green) were transiently transfected with cSrc into MEF cells. Changes in (**A**) cellular tyrosine phosphorylation or (**B**) cSrc activation, based on immunoreactivity with anti-phosphotyrosine or anti-phosphoY416, respectively. cSrc is shown in red, while phosphotyrosine or cSrc activation are shown as blue.

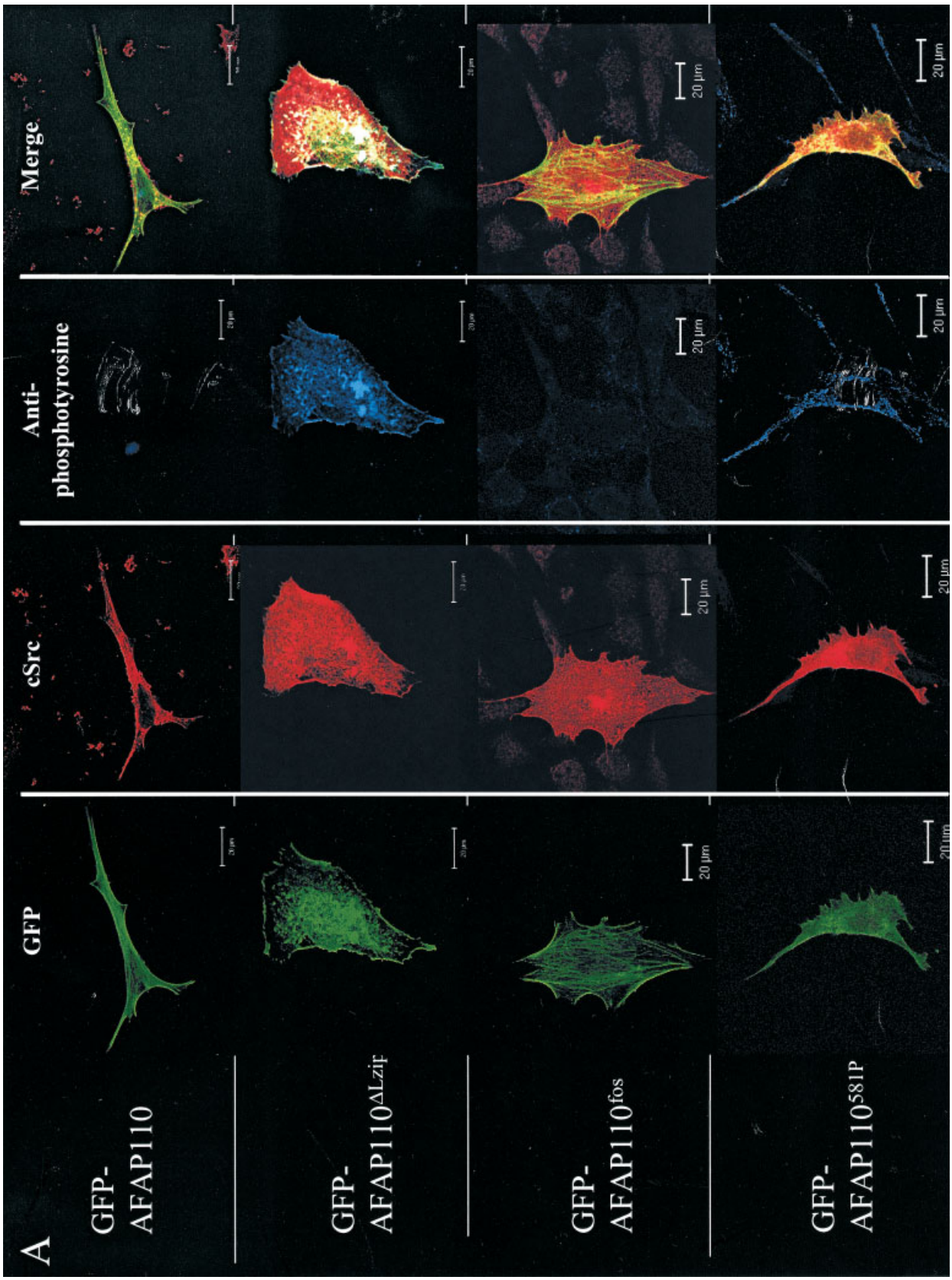
A
GFP
cSrc
Anti-phosphotyrosine
Merge
GFP-AFAP110
GFP-AFAP110ΔLzif
GFP-AFAP110fos
GFP-AFAP110581P
20 μm

Fig. 6.

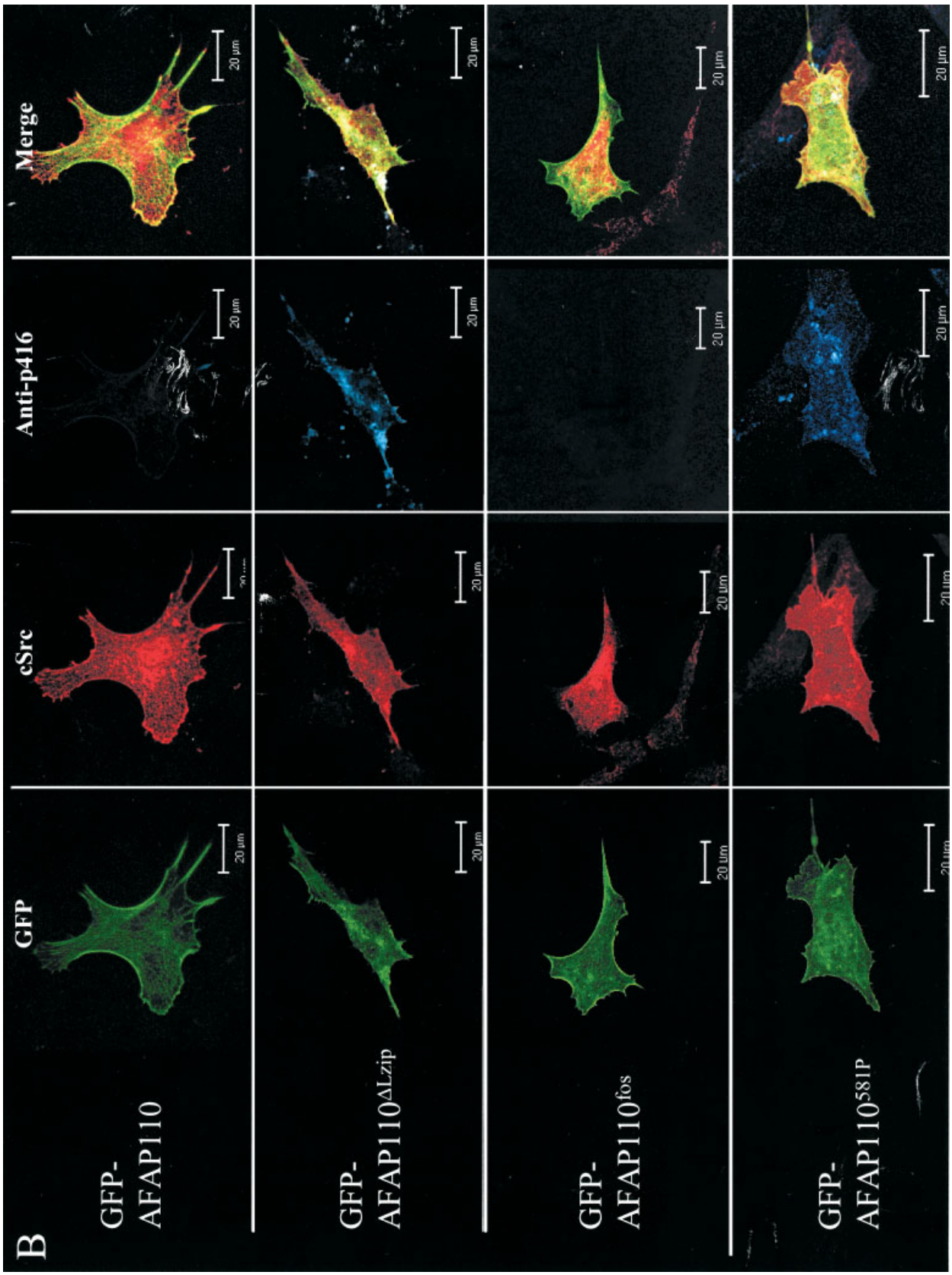

Fig. 6. (*Continued*)

tyrosine phosphorylation or cSrc; however, AFAP-110^{581P} was able to direct increased cellular tyrosine phosphorylation and cSrc activation. In addition, prior work has demonstrated that AFAP-110$^{\Delta lzip}$ directs formation of actin filament rosettes in every transfected cell [Qian et al., 1998, 2000, 2002]. Further, these same reports demonstrate that AFAP-110 and AFAP-110$^{\Delta lzip}$ each decorate stress filaments and the cortical actin matrix. In addition, our data indicate that AFAP-110^{fos} and AFAP-110^{581P} similarly decorate actin filaments and the cortical actin matrix (Gatesman and Flynn, data not shown). These data indicate that the ability of the Lzip motif to regulate AFAP-110 activation of cSrc in vivo is dependent upon the presence or structure of the Lzip motif.

DISCUSSION

Sequence and Structure of the Lzip Stabilize the AFAP-110 Multimer

In this report, we addressed how the Lzip motif of AFAP-110 regulates its ability to alter actin filament integrity. Previous work demonstrated that AFAP-110 binds to actin filaments directly is able to multimerize, and that deletion of the Lzip motif decreased the stability of the multimer and increased actin filament crosslinking capability [Qian et al., 1998, 2000, 2002]. Because the Lzip motif is required for self-association and multimer stability, we determined whether the sequence of the Lzip motif, or its α-helical structure, were important for regulating the ability of AFAP-110 to crosslink actin filaments. The stabilities of multimers were measured using gel filtration analysis and the results indicated that rAFAP-110 (within the context of actin filament-binding buffer) exists predominantly as a multimer, which we predict could be a nonamer based on gel filtration analysis. Deletion of the Lzip motif destabilizes the rAFAP-110$^{\Delta lzip}$ multimer, resulting in populations of both larger and smaller multimers [Qian et al., 2002]. The data presented here confirm that AFAP-110 exists as a large multimer. Interestingly, gel filtration analyses of rAFAP-110^{fos} and rAFAP-110^{581P} revealed destabilized multimers, as exhibited by a broader range of molecular weights, in contrast to rAFAP-110 which was more concentrated in a narrow range of fractions. From this, we hypothesize that changing the sequence of the Lzip motif by substitution with an analogous motif or changing the conformation of the Lzip motif by mutagenesis destabilizes the AFAP-110 multimer.

Destabilized Multimers Are Efficient Actin Filament Crosslinking Proteins

The rAFAP-110$^{\Delta lzip}$ protein is a more efficient actin filament crosslinking protein than rAFAP-110 [Qian et al., 2002], concomitant with being a more destabilized multimer relative to rAFAP-110. Thus, we predicted that changes in the Lzip sequence or structure may promote actin filament crosslinking if these changes are able to destabilize the multimer. In agreement, rAFAP-110^{fos} and rAFAP-110^{581P} each crosslinked actin filaments as efficiently as AFAP-110$^{\Delta lzip}$ and more efficiently than rAFAP-110 based on biochemical and light microscopy analysis. Compared with rAFAP-110, the helix mutants appear more compact in structure based on EM analysis, consistent with our hypothesis that changes in the sequence and/or structure of the Lzip motif can destabilize the AFAP-110 multimer. The size of the rAFAP-110 aggregates ranged from 10×10 nm to 10×30 nm in diameter. A Stokes radius was calculated using the gel filtration data and predicted a radius of 8.6 nm, which is consistent with the size of the aggregates of rAFAP-110 visualized on actin filaments. Small discrepancies in size for rAFAP-110 may be attributed to the fact that the measurement of the Stokes radius was accomplished in solution (in actin filament-binding buffer), while upon actin filaments, rAFAP-110 may either undergo a shape change upon binding to actin filaments that alters its diameter or when bound to actin filaments, it may recruit in additional rAFAP-110 resulting in apparently larger aggregates. The former possibility might be supported based on how the rAFAP-110 multimer contact actin filaments. Actin filaments are represented as two strands of polymerized G-actin that come together as an α-helical structure with a pitch of 36 nm containing six to seven actin monomers/strand/pitch or 13 actin monomers per double strand/pitch. As rAFAP-110 spans 10–30 nm, and it exists as a multimer (predicted to be a nonamer) in which each subunit has one actin filament-binding domain, it could contact one or more sites on the actin filament which could affect some distortion upon the rAFAP-110 and how it interacts with the actin filament. Conversely, it is also possible that when rAFAP-110

binds to actin filaments, it may facilitate recruitment of additional AFAP-110 molecules which would increase the apparent size of rAFAP-110 when bound to actin filaments. This hypothesis is consistent with a calculated Hill coefficient of 4.2 for AFAP-110, which predicts that AFAP-110 can facilitate recruitment of additional AFAP-110 molecules to actin filaments [Qian et al., 2002]. Collectively, these data confirm that AFAP-110 has an intrinsic capability to crosslink actin filaments and we hypothesize that the Lzip motif plays an important role in regulating this function by affecting the stability of the AFAP-110 multimer through both its sequence and structure by contacting sequences defined within the PH1 domain. Here, the Lzip motif may stabilize the rAFAP-110 aggregate as a large multimer with a Stokes radius of 8.6 nm. Loss of the Lzip motif may lead to a destabilized multimer that, when bound to actin filaments, assumes a smaller size, as evidenced by EM. The smaller sized rAFAP-110$^{\Delta lzip}$, rAFAP-110^{fos}, or rAFAP-110^{581P} may facilitate more efficient crosslinking of actin filaments by permitting closer asscociations between packed actin filaments, while a larger rAFAP-110 aggregate may affect different spacing between crosslinked actin filaments. A reduction in multimerization size has been hypothesized to increase the ability of an actin-binding protein to crosslink actin filaments by reducing the spacing between packed actin filaments [Meyer and Aebi, 1990; Matsudaira, 1991; Wachsstock et al., 1993].

Lzip Motif Contacts Sequences in the PH1 Domain

These experiments led us to ask whether the Lzip motif might be contacting internal sequences in AFAP-110 as a mechanism to stabilize the multimer. AFAP-110 multimer formation is clearly complex and we do not yet know the exact sequences that modulate higher order multimer formation. We hypothesize that although the Lzip motif may stabilize the AFAP-110 multimer, it may not play a direct role in the formation of the larger multimer. This hypothesis is based on the results of the gel filtration analysis, which reveal a significant subpopulation of rAFAP-110$^{\Delta lzip}$ multimers that can form larger multimers with similar elution fractions to rAFAP-110 [Qian et al., 2002]. The same is true for rAFAP-110^{fos} and rAFAP-110^{581P}. Because the Lzip motif, when expressed as a GST-encoded fusion protein, can affinity absorb full-length AFAP-110, and both the sequence and structure of the Lzip motif are important for multimer stability, we hypothesized that the Lzip motif may be stabilizing the multimer by contacting an internal sequence, either through intramolecular or intermolecular interactions, through sequence-specific interactions. This hypothesis would be supported by the residual-binding capacity of GST-Cterm^{581P} for AFAP-110, as previously described [Qian et al., 1998]. It was also possible that deletion of the α-helical Lzip motif may have a global effect on AFAP-110 structure; however, it is important to note that the Lzip motif is encoded within a single exon [Clump et al., 2003]. Thus, it could be hypothesized that the Lzip motif may have evolved as a self-folding modular domain and deletion of the Lzip may not necessarily affect the structure of other functional domains. Nevertheless, our analysis indicates that the Lzip motif can contact sequences in either the carboxy terminus or the PH1 domain. Conversely, the PH1 domain has the capacity to contact sequences in the carboxy terminus, which could stabilize the AFAP-110 multimer. Thus, we hypothesize that one way the Lzip motif may regulate AFAP-110 actin filament crosslinking ability is by stabilizing the AFAP-110 multimer and contacting sequences in the PH1 domain. It is possible that the Lzip motif may also homodimerize or contact adjacent sequences in the carboxy terminus, which may also contribute to stabilizing the AFAP-110 multimer. However, it is important to point out that GST-Cterm can affinity absorb other cellular proteins which may also promote or link interactions between the Lzip motif and AFAP-110. We consider this less likely, as deletion of the Lzip motif from GST-Cterm abrogates binding to AFAP-110 [Qian et al., 1998] and the data of Figure 5 would further support a direct link between the Lzip motif and the PH1 domain.

Lzip Motif May Provide an Auto-Inhibitory Mechanism to Regulate Actin Filament Crosslinking by AFAP-110

From these studies, we hypothesize that carboxy terminal sequences, which include the Lzip motif, regulate the actin filament crosslinking ability of AFAP-110 by mediating contact between the carboxy terminus and/or the

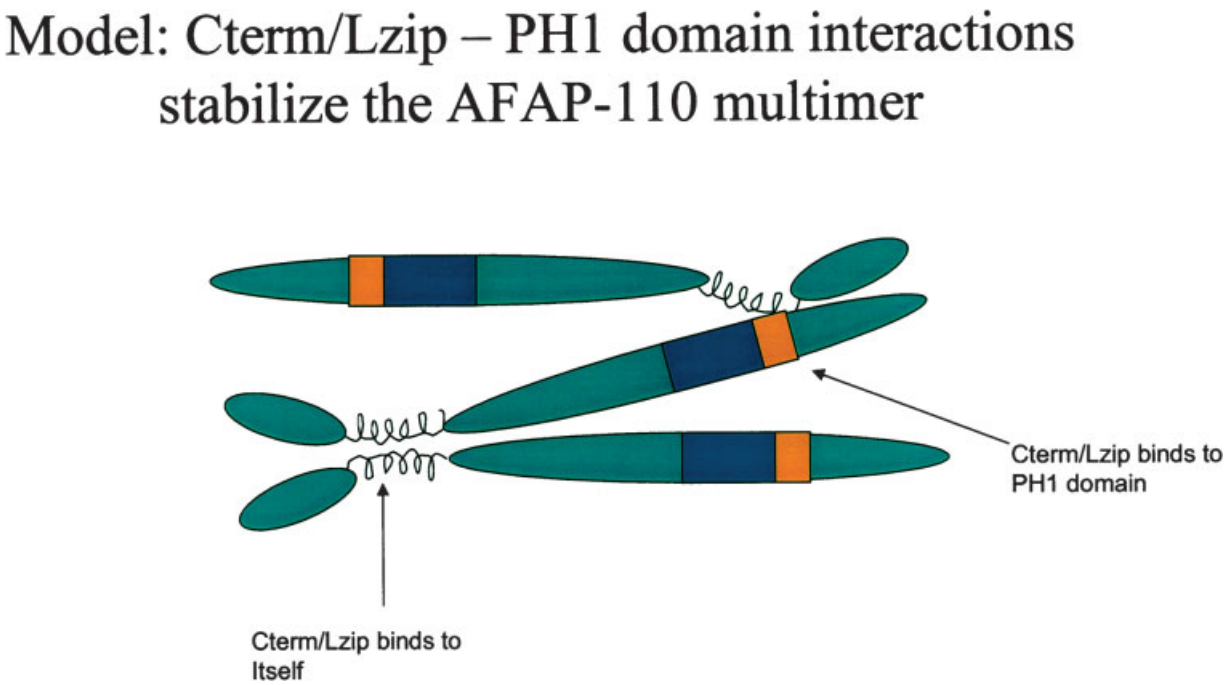

Fig. 7. Model of proposed mechanism of Lzip function. We propose that the carboxy terminus/leucine zipper motif has the capability to contact opposing sequences in the carboxy terminus of AFAP-110, or the PH1 domain. Loss of this interaction could destabilize the AFAP-110 multimer. Upon contact with actin filaments, the destabilized AFAP-110 protein assumes a smaller multimeric size, which enables it to efficiently crosslink actin filaments. The Lzip motif is shown as a heptad helix, the PH1 domain is shown in orange and the STK region is shown in blue. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com]

PH1 domain, through either intra- or intermolecular interactions (Fig. 7). This is a novel finding for a pleckstrin homology domain. It is not clear if sequences specific to the Lzip motif or adjacent carboxy terminal sequences are responsible for contacting the PH1 domain. Future studies will focus on discerning the multimeric status of AFAP-110 derived from cells and uncovering the exact sequences that modulate multimer stability and multimer formation. In this regard, AFAP-110 appears to have some unique properties, relative to other actin filament crosslinking proteins. For example, many actin filament crosslinking proteins that contain a single actin-binding domain, like AFAP-110, will dimerize (e.g., α-actinin [Wachsstock et al., 1993]) or form a tetramer (e.g., VASP [Bachmann et al., 1999]). We are not aware of other actin filament crosslinking proteins which form large multimers that can be regulated in response to cellular signals, analogous to AFAP-110. Thus, these studies presented here offer some important biological implications. Increased actin filament crosslinking is known to occur in the leading edge of a motile cell and specifically within lamellipodia and filopodia. Crosslinking of actin filaments may help in providing the protrusive force necessary to extend a lamellipodia and filopodia. Many signaling proteins have been implicated in promoting the formation of motility structures and facilitating cell motility. One of these proteins is PKCα. From our data, we predict that binding partners for the PH1 domain or for the carboxy terminus/Lzip motif could displace interactions designed to stabilize the AFAP-110 multimer, resulting in a destabilized multimer with increased actin filament crosslinking capability. To this end, it cis important to note that AFAP-110 is strongly represented in lamellipodia and filopodia, AFAP-110 is a substrate and binding partner for PKCα and that PKCα will bind to exactly the same sequences in the PH1 domain as the carboxy terminus/Lzip motif [Qian et al., 2000, 2002; Baisden et al., 2001a]. It is also possible that post-translational modifications, for example, phosphorylation, could affect the ability of the carboxy terminus/Lzip motif to contact sequences like the PH1 domain, resulting in a destabilized multimer that promotes actin filament crosslinking. Thus, PKCα, or possibly other potential PH1 domain binding partners for AFAP-110, such as PKCβ, PKCγ, PKCλ, or

phosphoinositides, could play a role in affecting AFAP-110 multimer stability and promote actin filament crosslinking ability by displacing Lzip/PH1 interactions [Baisden et al., 2001a,b; Qian et al., 2002]. As each of these proteins/lipids is associated with signals that promote cell motility, it is possible that AFAP-110 might play an important role in regulating actin filament crosslinking at the leading edge of a motile cell, in response to cellular signals that destabilize the AFAP-110 multimer and promote actin filament crosslinking.

Ability of the Lzip Motif to Regulate Actin Filament Crosslinking and cSrc Activation Are Distinct Functions

Interestingly, the ability of the Lzip motif to regulate actin filament crosslinking, in vitro, and cSrc activation and actin filament integrity, in vivo, appear to be distinct functions. AFAP-110^{fos} was able to efficiently crosslink actin filacments but was unable to direct activation of cSrc, unlike AFAP-$110^{\Delta lzip}$ or AFAP-110^{581P}. Based on our analysis of AFAP-110, we predict that the Lzip motif, and/or adjacent carboxy terminal sequences, can contact sequences in the PH1 domain and this could serve to stabilize the AFAP-110 multimer. Deletion of the Lzip motif or changing the sequence or structure may displace this intra- or intermolecular interaction and destabilizes the multimer, promoting actin filament crosslinking. However, this in itself may not be sufficient to activate cSrc and direct changes in actin filament integrity. AFAP-110^{581P} was able to affect actin filament rosette formation and cSrc activation, unlike AFAP-110^{fos}. Previous data demonstrated that AFAP-$110^{\Delta lzip}$ was able to activate cSrc via SH3-binding and point mutations that prevent AFAP-110 from being an SH3-binding partner for cSrc also block the ability of AFAP-$110^{\Delta lzip}$ from activating cSrc and affecting changes in actin filament integrity [Baisden et al., 2001a]. We hypothesize that changes in the α-helical structure associated with the Lzip motif may be sufficient to direct AFAP-110 to activate cSrc, either through deletion of the Lzip motif or through point mutations that are predicted to alter the structure. It may be possible that changes in the structure of the α-helix may promote activation of a signal that enables AFAP-110 to activate cSrc, or conversely, that a conformational change associated with the Lzip structure may promote the ability of AFAP-110 to activate cSrc. This hypothesis is currently under investigation.

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