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Studying protein isoforms of the adaptor SETA/CIN85/Ruk with monoclonal antibodies☆

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Abstract

SETA/CIN85/Ruk is a multifunctional adaptor protein involved in signal transduction and attenuation downstream of receptor tyrosine kinases. It has a modular structure, and various isoforms that combine different protein–protein interaction domains have been proposed based on cDNA analysis. As a first step towards understanding SETA/CIN85/Ruk isoforms at the protein level, we have characterized 5 monoclonal antibodies against this protein. Three of these were used to study lysates fractionated on a pH gradient, leading to the identification of various SETA/CIN85/Ruk proteins on the basis of p*I* and apparent molecular weight. While good correspondence with proteins predicted from cDNA analysis was found for two isoforms, in most cases it was not possible to make an unequivocal assignment. We conclude that additional splice variants remain to be described, and that a deeper understanding of SETA/CIN85/Ruk post-translational processing and modification is necessary to gain further understanding of this complex gene product.

Keywords: SETA; CIN85; Ruk; SH3KBP1; CD2BP3; Monoclonal antibody; Adaptor; Domain structure; Protein isoforms

SETA/CIN85/Ruk/SH3KBP1 is a multifunctional adaptor protein that interacts with many molecules engaged in a variety of cellular processes. It was identified as a gene whose expression was associated with the ability of in vitro transformed $p53^{-/-}$ astrocytes to form gliomas in vivo [1]; as a c-Cbl interacting protein of 85 kDa (CIN85), present in Cbl protein complexes on the activated epidermal growth factor receptor (EGFR) [2,3]; as Ruk, via interaction with the phosphatidylinositol 3 kinase (PI3K) subunit p85α, and shown to be a negative regulator of PI3K activity [4]; and as a CD2 interacting protein CD2BP3 [5]. Yeast two-hybrid screens have demonstrated SETA interactions with a growing list of proteins via its various protein–protein interaction domains (Fig. 1). SETA acts by bringing together elements of various signal transduction pathways that may then act upon one another, including receptor tyrosine kinases and the machinery that mediates their internalization (for review see [6]). The primary structure of the SETA protein (Fig. 1) reveals its modular nature: three N-terminal src homology 3 (SH3) domains, a proline rich region with several P-X-X-P motifs known to bind to SH3 domains, a serine-rich region, and a C-terminal coiled coil thought to be involved in multimer formation

☆ *Abbreviations:* Alix, apoptosis linked gene X; CC, coiled coil; CD2BP3, CD2 binding protein 3; CIN85, Cbl interacting protein of 85 kDa; EGFR, epidermal growth factor receptor; GST, glutathione-*S*-transferase; IP, intraperitoneally; MAb, monoclonal antibody; PBS, phosphate-buffered saline; PI3K, phosphatidylinositol 3 kinase; Ruk, regulator of universal kinase; SB1, SETA binding protein 1; SETA, SH3 domain encoding, expressed in tumorigenic astrocytes; SH3, src homology three; WB, Western blot.

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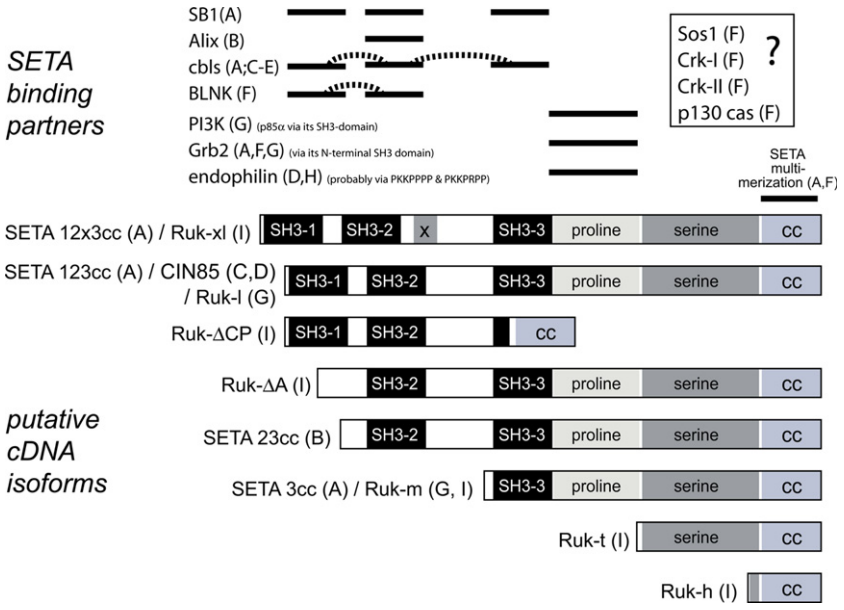

Fig. 1. Schematic showing interactions and cDNA isoforms of SETA/CIN85/Ruk. The location of the smallest known regions of interaction of SETA binding proteins are shown above schematic SETA proteins. Those with no known site of interaction are shown in the box with a question mark. Dotted lines show potential co-operativity between binding interactions. The SH3 domains, proline and serine rich regions, and the coiled-coil at C-terminus (cc) are indicated on the SETA isoforms based on evidence from cDNA analysis. References indicated in parentheses are: A, [7]; B, [9]; C, [2]; D, [3]; E, [18]; F, [8]; G, [4]; H, [19]; and I, [10]. For protein sequence alignment see Fig. S1. See also Table 2 for biochemical characteristics of these predicted isoforms.

[7,8]. Evidence for the existence of different isoforms of SETA as a result of alternative exon usage has come from the sequencing of cDNAs isolated from several libraries and RT-PCR together with analysis of the structure of the genomic locus [4,7,9,10]. These isoforms differ in the domains that they encode (see Fig. 1 for cartoon; see Fig. S1 for alignment of predicted protein sequences). As each domain has distinct binding preferences, different forms of SETA would be predicted to have significantly divergent functions. For example, SETA 3cc/Ruk-m, which lacks the two N-terminal SH3 domains [4,7,10], would not to be able to bind to BLNK or Alix, which bind the missing domains [8,9], and perhaps retains only weak affinity for Cbl proteins, which require co-operative binding to more than one SH3 domain for high affinity [2,3,9]. However, this isoform might still bind PI3K, Grb2 or endophilins which bind the proline rich region C-terminal to the SH3 domains [4,7,8]. Therefore, understanding the nature of the SETA isoforms present in a given cell at the protein level is important to understanding what the role of this adaptor is in the biology of specific processes. Here we report the generation of 5 monoclonal antibodies raised against the rat SETA 123cc protein that show domain specificity, present their characterization, and use them to investigate SETA/CIN85/Ruk proteins displayed on a pH gradient. The majority of the protein isoforms identified by this approach do not map straightforwardly onto molecules predicted on the basis of cDNA sequences, and we conclude that additional splice variants remain to be discovered. In addition, a greater understanding of the post-translational processing and modification of SETA/CIN85/Ruk will be necessary for a complete understanding of how this complex and versatile adaptor protein functions.

Results and discussion

A cDNA encoding full-length rat SETA (SETA 123cc/Ruk-l), with an N-terminal His tag, was expressed in bacteria, and the protein was purified and used to immunize mice and generate pools of hybridoma cell lines (for details see Materials and methods). These were screened by ELISA using bacterially expressed SETA 123cc protein generated from the same cDNA cloned as a GST-fusion protein, as well as by Western blot with bacterially expressed SETA domains, immunoprecipitation from cell lysates and cell staining. Five pools were selected on the basis of these data, and clonal lines were isolated by two rounds of dilution cloning using ELISA as a primary screening tool. Following a second round of dilution cloning, greater than 99% of the wells with cells showed a strong positive ELISA signal in each case, suggesting that clonality had been achieved. The 5 clones are summarized in Table 1.

To establish the domain specificity of the antibodies Western blot analysis against purified bacterial GST-fusion proteins encoding full length or regions of the SETA protein was performed (Fig. 2A). The 5 monoclo-

Table 1
Characteristics of the SETA MAbs

MAb	Isotype	WB vs bacterial SETA proteins				Comment	
		123cc	SH3-1	SH3-2	SH3-3		
50.1.E2	IgG_3 (κ)	+	+	−	−	SH3-1 specific	
61.6.D8	$IgG2_a$ (κ)	+	−	−	−	Recognizes KKVKG	
84.2.A7	IgG_1 (κ)	+	−	−	−	Recognizes KKVKG	Upstate, Cat# 05-733
155.6.F4	IgG_3 (κ)	+/−	+	−	+	SH3-1 and -3	
179.1.E1	$IgG2_b$(κ)	+	+	+	+	SH3-1, -2, and -3	Upstate, Cat# 05-731

The full clone designation, isotype, and summary of reactivity with bacterial SETA-GST proteins (see Fig. 2A for details) as well as deduced specificity in Western blot (WB) are summarized. MAbs 84 and 179 are commercially available through Upstate.

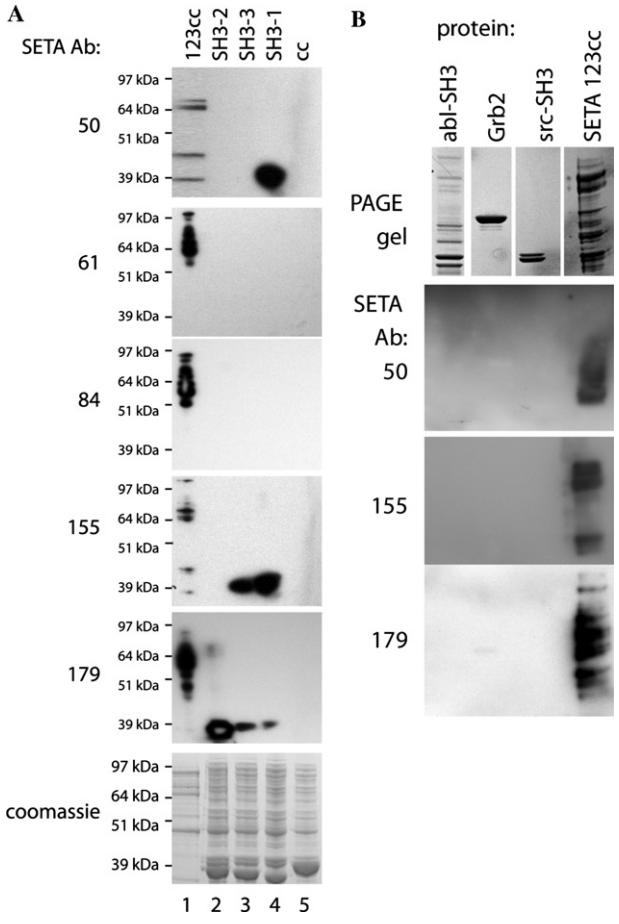

Fig. 2. Region specificity of SETA MAbs. (A) Western blots of bacterially expressed SETA-GST fusion proteins encoding either isolated SH3 domains, the SETA 123cc/CIN85/Ruk-l isoform or the isolated coiled coil (cc) probed with SETA MAbs. MAbs 50, 155, and 179 recognize the SH3 domains, in different combinations; MAbs 61 and 84 recognize only SETA 123cc. (B) Bacterially expressed SH3-domain containing GST-fusion proteins representing the isolated SH3 domains of abl and src, or the entire Grb2 protein or SETA 123cc were separated by PAGE and stained with Coomassie (upper panel) or subjected to Western blot with SETA MAbs as indicated. The three MAbs, 50, 155, and 179, which recognize SETA SH3 domains do not cross-react with these other SH3-domain proteins.

nal antibodies showed different patterns: MAbs 50, 155, and 179 all recognized SH3 domains, with 50 showing a strong preference for SH3-1. MAb 155 also recognized SH3-3 and 179 recognized all three equally. MAbs 61 and 84 did not recognize the SH3 domains, but did react strongly with the full-length SETA protein (Fig. 2A).

SH3 domains are highly conserved, raising the possibility that antibodies will cross-react with other SH3 domain-containing proteins. Western blot analysis of GST fusion proteins of the abl and src SH3 domains, as well as full-length Grb2, which contains two SH3 domains, failed to reveal such cross-reactivity (Fig. 2B), suggesting that MAbs 50, 155, and 179 are SETA-specific.

MAbs 61 and 84 did not recognize SH3 domains, but did recognize the full-length SETA 123cc protein. To narrow down the region that they recognized Western blots were performed with HEK293 cells expressing two shorter SETA constructs, SETA SH3-23 and 23cc. Both constructs start close to the N-terminal border of SH3-2, and SH3-23 terminates at the C-terminal end of the SH3-3, while 23cc extends to and includes the C-terminal coiled coil. Both proteins were recognized by MAb 179 (Fig. 3A) presumably because of its reaction with the two SH3-domains present in both constructs. Interestingly, they were also both recognized by MAbs 61 and 84, suggesting that these antibodies recognize the region between the 2nd and 3rd SH3 domain. To further narrow down the region recognized by MAbs 61 and 84, epitope mapping was performed. A cDNA encoding SETA 123cc was DNase digested into fragments of between 50 and 150 base pairs and cloned into a bacterial expression vector. Bacterial colonies were screened individually with the two antibodies and the inserts of positive clones were sequenced. The smallest sequence retrieved spanned amino acids 208–241 of SETA 123cc (KKV KG...FLPVE; data not shown) and reacted with both antibodies. To refine this region we constructed GST fusion proteins with 10 amino acid segments from this region (Fig. 3B). These consisted either of two GST moieties flanking the 10mer peptide (61.1, 61.2, 61.3, and 61.6) or of the 10mer at the N-terminus of a single GST fusion protein (61.5). Western blot analysis of these bacterially produced proteins demonstrated that both MAbs 61 and 84 specifically recognized the GST-61.1 encoding KKVKGVGFGD (Fig. 3B), but not the adjacent GST-61.2, suggesting that the epitope is centered on KKVKG. To confirm this the 61.1 peptide was used to

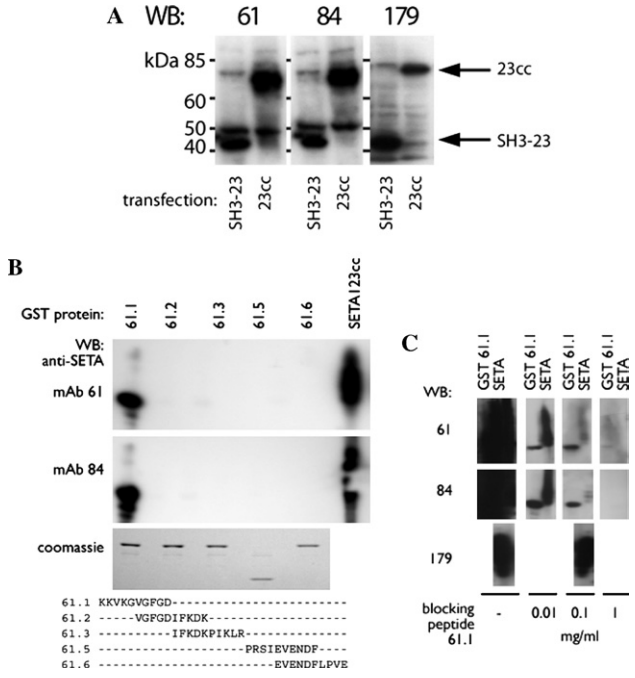

Fig. 3. Determining the epitope for MAbs 61 and 84. (A) Western blot of lysates from SETA 23cc or SETA SH3-23 transfected HEK 293 cells with three MAbs shows that both MAbs 61 and 84 recognize sequences between the second and third SH3 domains of SETA. (B) Western of GST-peptide fusion proteins expressed in bacteria and encoding portions of epitope 61 identified initially in an epitope mapping experiment with MAb61. Fusions of the sequences shown at the bottom of the panel either between two GST moieties (61.1, 61.2, 61.3, and 61.6) or at the N-terminus of one GST protein (61.5) were run on a PAGE and stained with Coomassie or subjected to Western blot analysis alongside a control of bacterially expressed SETA 123cc. Only GST-61.1 was recognized by MAbs 61 and 84. (C) Peptide 61.1 blocked the recognition of bacterial GST-61.1 or SETA 123cc by MAbs 61 and 84 but not 179, in a dose-dependent manner in Western blot. The peptide was pre-incubated with the antibodies for 1 h prior to application to the membrane, at the concentration indicated. All panels are from the same exposure, which was relatively long to allow stringent examination of the highest peptide concentration, and so resulted in overexposure of the negative control panels.

block recognition in western blot (Fig. 3C). Peptide 61.1 was able to block MAbs 61 and 84 from recognizing both bacterial GST-61.1 and SETA 123cc in transfected cell lysates in a concentration dependent manner. However, peptide 61.1 was not able to block the recognition by MAb 179, which recognizes SH3 domains outside of the 61.1 peptide (and does not recognize GST-61.1). MAbs 61 and 84 are different isotypes and so do not represent a duplicate isolation of the same clone.

Antibodies were then tested for their ability to recognize endogenous SETA proteins from glioma cells (Fig. 4). Analysis of lysates from tumorigenic $p53^{-/-}$ astrocytes that had been cultured for over 30 passages in serum-containing medium [11,12], and were known to express SETA mRNA [1] consistently showed the strongest signal in Western blot (Fig. 4A). In these cells the polyclonal anti-SETA antibody recognized a predominant band at 85 kDa, with additional bands near 50 and 40 kDa, as well as at approximately 160 kDa which most likely represents a stable multimer [8,13]. This antibody recognized a similar pattern in human glioma cell lines U87, LNZ308, and LN443, although in some instances additional bands around 85 kDa were observed, and the relative intensities of the 50 and 40 kDa bands differed. MAbs 50, 155, and 179 yielded similar patterns, with predominant signals at 85 kDa, and additional bands near 50 and 40 kDa. Mabs 50 and 155 appeared to show a strong preference for mouse protein in Western blots.

Immunoprecipitation with the MAbs of protein from human and mouse glioma cell lines showed a predominant band at 85 kDa in a SETA polyclonal Western blot (Fig. 4B). Interestingly, MAbs 50, 155, and 179 also efficiently immunoprecipitated a SETA isoform migrating at approximately 55 kDa, which was only faintly detected in the other immunoprecipitates (Fig. 4B). These three antibodies recognize SETA's SH3 domains (Fig. 2), suggesting that this shorter form contains some or all of these domains. It appears likely that the most N-terminal SH3-1 domain is present, as the intensity of the 55 kDa band in the immunoprecipitates with MAb 50 is consistently similar to the 85 kDa band. Overall, the complexity of the pattern of SETA/CIN85/Ruk isoforms observed after immunoprecipitation with all the antibodies was much lower than that observed in Western blot (compare Figs. 4A and B), suggesting that approaches such as mass spectrometric analysis of proteins recovered by antibody interaction would not yield much information in this instance. Therefore, we decided to proceed by fractionating proteins and analyzing them by Western blot.

Fractionation on a pH gradient of cellular lysates from $p53^{-/-}$ astrocytes, which express endogenous SETA, allowed further dissection of SETA protein isoforms. These experiments were done with MAbs 61, 84, and 179, as 50 showed low efficiency in Western blot and 155 showed a pattern essentially identical to 179 (Fig. 4A). First, lysates were separated according to pH on the first dimension of the Beckman–Coulter PF2D system, which generated 30 fractions (for details see Materials and methods). These were 9 fractions before and 4 fractions after a 17-fraction pH gradient from 8.4 to 3.9 (Fig. 5). Aliquots of each fraction were subjected to Western blot analysis with SETA polyclonal and monoclonal antibodies, using a 96-well gel format, allowing the entire gradient to be represented on a single membrane (Fig. 5). This analysis showed that the patterns for SETA protein expression were relatively similar, with reactivity found both in the region above the start of the gradient at pH 8.4, and at pH 5.5 and below. However, there were also noticeable differences between the patterns obtained with some of the antibodies. In particular, MAb 84 recognized proteins in fractions 7,

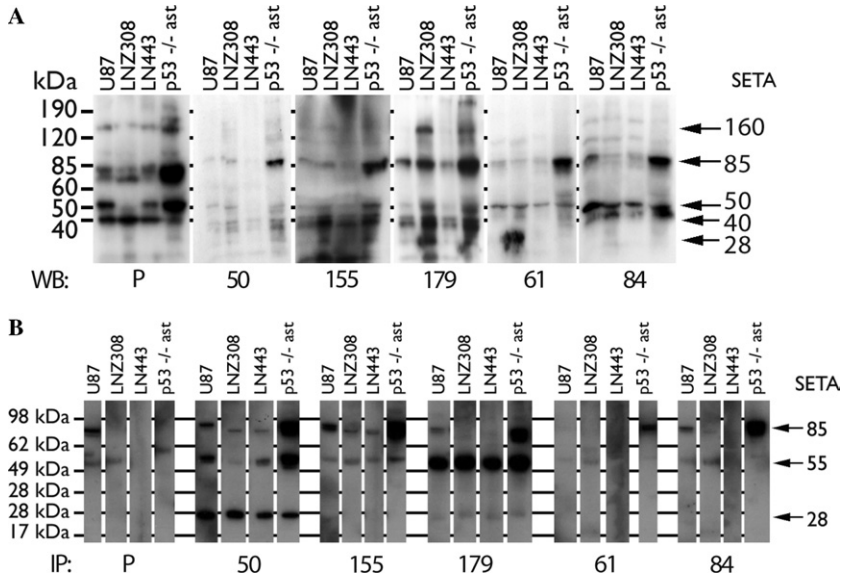

Fig. 4. Western blot and immunoprecipitation of cell lysates with SETA antibodies. (A) Whole cell lysates from human glioma cell lines U87, LNZ308, LN443 or transformed mouse $p53^{-/-}$ astrocytes were probed with polyclonal (P) or monoclonal anti-SETA antibodies as indicated. (B) Lysates from the same cells were subjected to immunoprecipitiation with the antibodies indicated, and the pellets were subjected to Western blot analysis with the polyclonal anti-SETA antibody.

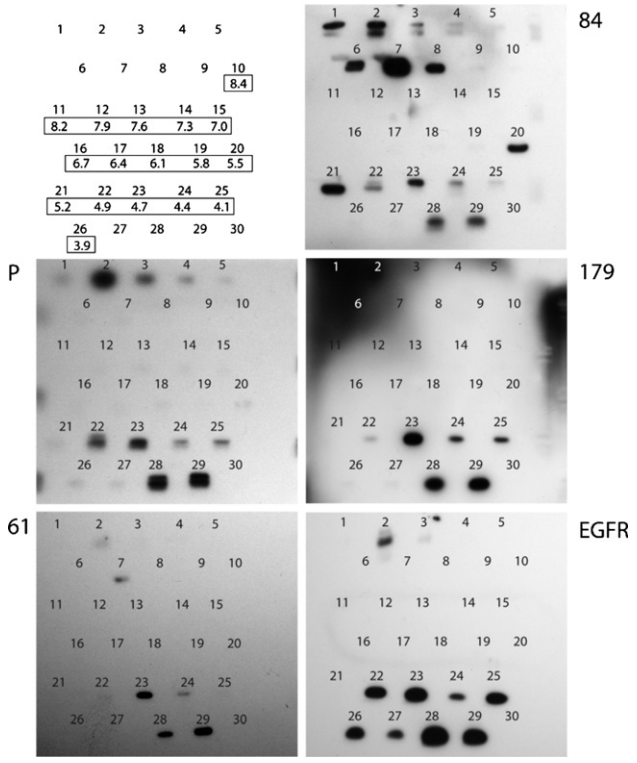

Fig. 5. Chromatofocusing gradients analyzed by Western with SETA MAbs. Fractions collected from the separation of proteins according to isoelectric point on a chromatofocusing column were subjected to Western blot analysis with the antibodies shown, including anti-SETA polyclonal (P) and MAbs 61, 84, and 179, as well as an antibody against the EGFR (Oncogene Science). The panel at the top left identifies the position of fractions in the wells of the gel, as well as the pH of the fractions in the gradient region of the separation. Please note that mobility separation is minimal in this gel format.

20, and 21 that the other antibodies, including MAb 61, did not. This demonstrates that although MAbs 61 and 84 recognize a common sequence (Fig. 3), they do not recognize the exact same epitope, with MAb 84 having a wider spectrum of isoforms that it recognizes. Comparison of the different profiles, overall, as well as more detailed analysis (Fig. 6) suggests that MAb 84 is SETA-specific, as almost all the bands it recognizes are also recognized by at least one other anti-SETA antibody. As a control we also performed Western blot analysis for EGFR on this fractionated lysate, and detected it in fraction 2, as well as at pH 4.9 and below.

To investigate the specific isoforms of SETA further, the pH fractions that were found to contain SETA proteins (Fig. 5) were subjected to conventional Western blot (Fig. 6). Again the overall patterns of SETA proteins identified by the 4 antibodies in the analysis were similar, with the major band at 85 kDa common to all. This band, which was seen prominently in fraction 23, and in fractions 28 and 29 with all antibodies and at similar relative intensities (labeled 'A' in the composite image at the bottom of Fig. 6), most likely represents the SETA 123cc/CIN85/Ruk-l isoform (see Table 2). This conclusion is also based on many experiments where the transfection of cDNA constructs encoding SETA 123cc/CIN85/Ruk-l results in a band that migrates at 85 kDa, despite the fact that it encodes a protein of predicted molecular weight of around 73 kDa (e.g. [2–4,9]). The predicted isoelectric point of SETA 123cc/CIN85/Ruk-l, which is around pH 6, also does not agree very closely with what was observed; in our fractionation the 85 kDa appears at around pH 4.7 and has another

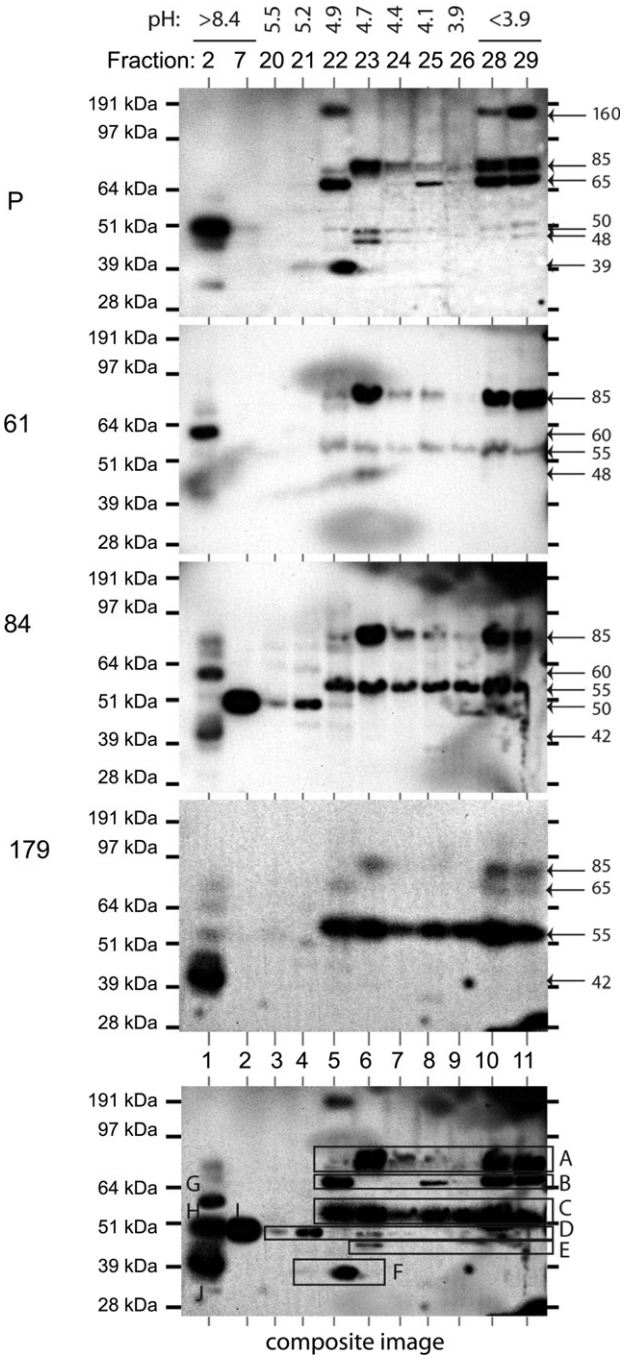

Fig. 6. Western blot analysis of selected fractions from the chromatofocusing gradient with SETA MAbs. Fractions from the gradients shown in Fig. 5 that showed the presence of a SETA band were selected and subjected to Western blot analysis with anti-SETA polyclonal (P) and MAbs 61, 84, and 179 as indicated. Fraction number and approximate pH of the fractions are indicated at the top. The molecular weight of the proteins identified is shown. A composite of the images of the 4 Western blots was created using the Photoshop darken blend mode and is presented as an annotated composite image for ease of identification of isoforms (for details see text).

major peak below pH 4. However, a similar shift towards low pH was observed for the EGFR (Fig. 5; EGFR has a predicted isoelectric point of pH 6.3). The presence of SETA 123cc/CIN85/Ruk-l through a wide spectrum of fractions, spanning over one pH unit, further suggests that post-translational modifications may affect this characteristic. To date, no evidence that SETA is phosphorylated has been reported, and direct investigation of this by our group has failed to detect it [9,14]. Therefore, other kinds of modifications may underlie this variation.

A less clear correspondence exists between the prominent band at 65 kDa that is observed in the Western blot probed with the polyclonal anti-SETA antibodies, and fainter with MAb 179. This band, labeled 'B' in Fig. 6, appears in fractions 22 and 25 in the gradient, as well as in 28 and 29, and so has a similar distribution to the 85 kDa isoform, although the first band appears one fraction more basic. Although these characteristics resemble both Ruk-ΔA and SETA 23cc, which are more basic and smaller than the 85 kDa form (Table 2), the fact that this band is only very weakly recognized by MAbs 61 and 84 (which recognize SETA 23cc; Fig. 3A) suggests that it is neither of these isoforms. Similarly, the smaller and more acidic Ruk-ΔCP also encodes the epitope for MAbs 61 and 84, and so is not a good candidate. On the other hand, the 65 kDa protein is too large and too acidic to be a good match for the SETA 3cc/Ruk-m proteins. We postulate that the 65 kDa SETA contains SH3 domains, as it is recognized by MAb 179, but lacks the 9th exon described by Buchman et al. (Fig. 1 in [10]), which encodes the dominant epitope of MAbs 84 and 61. It is not clear why a cDNA encoding such a protein was not identified by the RT-PCR experiments reported by Buchman and colleagues, but it is possible that it is not present in the tissues they studied. Alternately, this 65 kDa form could represent a heavily modified form of SETA 3cc/Ruk-m proteins, with the modifications making the protein's apparent size significantly larger and its isoelectric point more acidic. Another interesting observation is that the presence of the 65 kDa form correlates, with the exception of fraction 25, with the presence of a higher molecular weight band at 160 kDa, which probably represents the stable multimer which has been observed before in Western blot analysis, and whose existence is dependent on the presence of the coiled coil domain (see Fig. 3 in [13]). Interestingly, this is not observed in fraction 23, where the 85 kDa SETA 123cc is found, but can be seen in fraction 22 where there is little of this isoform, but predominantly the 65 kDa protein. This allows that the 65 kDa form, and not the commonly encountered 85 kDa form, is primarily involved in multimerization [8].

The band at around 55 kDa ('C' in Fig. 6) is interesting in that it is not recognized by the polyclonal anti-SETA antibody, but is the dominant band in the

Table 2
Characteristics of SETA/CIN85/Ruk proteins predicted from cDNAs

	Amino acids	Predicted MW (kDa)	Observed MW (kDa)	Predicted isoelectric point	KKVKG	Bands (Fig. 6)
SETA 12x3cc/Ruk xl	709	78.0		6.64	Y	
SETA 123cc/CIN85 / Ruk-l	665	73.1–73.2	85	5.95–6.25	Y	A
Ruk-ΔA	628	68.8		7.19	Y	
SETA 23cc	592	64.5		6.47	Y	
SETA 3cc/Ruk-m3	427	46.8		8.89–9.01	N	
Ruk-m1	404	44. 4	42	9.03	N	J
Ruk-ΔCP	354	39.6		5.45	Y	
Ruk-t	241	26.0	39	7.05	N	F (?)

Summary of length, predicted and in some cases observed molecular weight, predicted p*I*, and whether the KKVKG epitope recognized by MAbs 61 and 84 is present is given for SETA/CIN85/Ruk isoforms based on cDNA sequencing. In addition, the bands in Fig. 6 to which some of the isoforms correspond are given. For details see Results. For references to different isotypes, see legend to Fig. 1. In some instances p*I* is given as a range as the predicted protein sequences varied slightly between reports, either due to species differences or polymorphisms. For details of protein sequences see Fig. S1 and text.

Western blot with MAb 179. This is consistent with the presence of multiple SH3 domains, which were not recognized by the polyclonal antibody (data not shown). It is also possible that this variant does not express the coiled coil, which was recognized by the polyclonal, but none of the MAbs (Fig. 2; no cDNA without the coiled coil has been identified by RT-PCR, however [10]). This band is also strongly recognized by MAb 84 but only weakly by MAb 61, so unlike the 85 kDa isoform which is recognized equally strongly by these two antibodies, it probably does not contain the KKVKG sequence. As described above, MAb84 appears to be able to recognize a broader set of epitopes around this motif, and such an epitope is likely to mediate recognition here. If these deductions are correct, it is again difficult to map the 55 kDa form onto one of the known splice variants, as all the predicted isoforms that are acidic and encode SH3 domains also contain the KKVKG sequence. A similar issue arises for proteins at 50, 48, and 39 kDa ('D', 'E, and 'F' in Fig. 6, respectively), which are not efficiently recognized by MAb 61, yet are acidic. However, as MAb 179 also does not recognize these proteins, it is likely that they represent isoforms that do not encode any SH3 domains. One such isoform, Ruk-t, has been proposed—it also does not encode the KKVKG motif, and is predicted to have a neutral p*I*. The 50, 48, and 39 kDa isoforms do preferentially occur near the higher end of the pH gradient, and are found about 2 pH units away from their predicted pI (around pH 5 rather than 7), which is similar to the 85 kDa form (around 4 instead of 6). Therefore, while it is possible that Ruk-t is the protein labeled 'F' in Fig. 6, to which it is closest in size, other splice variants similar to Ruk-t are likely to encode the other small acidic non-SH3 domain encoding SETA isoforms. It should be noted that we also have never observed any smaller SETA proteins that could be encoded by the Ruk-h cDNAs in agreement with Buchman et al. [10].

On the basic side of the gradient, 4 proteins are identified, with apparent molecular weights of 60, 50, 50, and 42 kDa, labeled 'G', 'H', 'I,' and 'J,' respectively (Fig. 6). The 60 kDa form is recognized strongly by MAbs 61 and 84, and so is likely to contain the KKVKG sequence, making it a poor match for the basic SETA 3cc or Ruk-m proteins. In addition, the 60 kDa and the two 50 kDa proteins are not recognized by MAb 179, which binds to all three SH3 domains of SETA, again making a relatively poor fit for the SETA 3cc/Ruk-m isforms. The best match for Ruk-m1 is the 42 kDa protein, labeled 'J', which is of an appropriate size and p*I*, and is recognized by MAb 179 but not by MAb 61 as predicted (Table 2). In general, it is also possible that the bands observed are not stable isoforms encoded by a specific spliced mRNA, but could also be derived from processing or degradation of SETA proteins, in which case a clear correspondence to cDNA sequences would not be expected.

Lastly, MAbs were tested for their ability to recognize SETA/CIN85/Ruk in cells by immunohistochemistry. Both MAbs 61 and 84 showed staining of endogenous SETA in confocal analysis (Fig. 7), confirming the cytoskeletal staining pattern observed previously with the polyclonal antibody and described in detail [13].

In summary, our analysis of SETA/CIN85/Ruk protein isoforms with new monoclonal antibodies has revealed a complex pattern of variants, only some of which map readily on the isoforms identified at the cDNA level. Our data therefore suggest that there is ample room for additional, yet to be discovered, cDNA sequences. In particular, we predict that additional differential splicing of exon 9, which encodes the dominant epitope for MAb 61, is likely to be encountered. In addition, a deeper understanding of protein processing and modifications of SETA/CIN85/Ruk is likely to be necessary to complete this complex picture. Additional proteomic approaches will also be useful in this question, and the new reagents we present here may well aid in these studies.

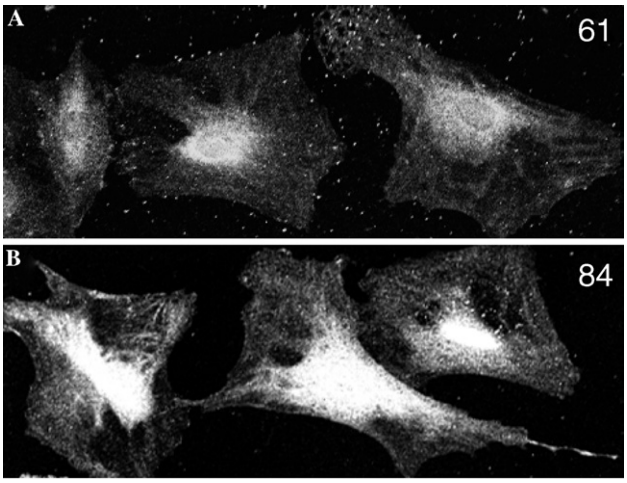

Fig. 7. Confocal image of astrocytes stained with MAbs 61 and 84. Primary rat cortical astrocytes were grown on coverslips and stained with antibodies as indicated, followed by fluorescently conjugated secondary antibodies. They were then imaged by confocal microscopy using a Bio-Rad MRC microscope. (A) Cells stained with MAb 61 and imaged at the level of the nucleus. (B) Cells stained with MAb 84 and imaged near the surface of the coverslip. The different planes of imaging result in images that reveal different regions of the cytoskeleton.

Materials and methods

Generation of purified bacterial proteins. A rat SETA 123cc cDNA in pET30a (Novagen, Madison, WI) was expressed in *Escherichia coli* BL21 DE3. The His-tagged protein was purified with a BugBuster His-Bind kit (Novagen). The same cDNA as well as shorter constructs were also cloned in-frame with glutathione-*S*-transferase (GST) in pGEX-KG, expressed in *E. coli* BL21 DE3, and purified using the BugBuster GST-bind kit (Novagen). All purified protein was dialyzed against PBS prior to use.

Generation of mouse hybridomas. Purified antigen was shipped to Dr. Anthony Siadak at the Hope Heart Institute, Seattle, WA, where immunizations, cell fusions, and master ELISA were performed. Five female BALB/c mice were injected intraperitoneally (IP) on day 1, 21, and 42 with 50 μg His-SETA 123cc in R-700 adjuvant (Ribi ImmunoChem, Hamilton, MT). On day 56 the two mice with the strongest anti-SETA antibody titer were administered an IP dose of 50 μg of His-SETA 123cc in PBS as a pre-fusion booster immunization. Four days following pre-fusion booster immunization, splenocytes from these two mice (#4966 and #4968) were harvested and fused at a 2:1 ratio with the P3-X63-Ag.653 mouse myeloma cell line [15] according to published procedures [16] and plated at 1.2×10^5 cells/well in rich hybridoma culture medium [Iscove's modified Dulbecco's medium; IMDM, Invitrogen, Carlsbad, CA; with 10% (v/v) fetal clone I serum (HyClone Laboratories, Loga, UT), 10% (v/v) hybridoma cloning supplement (BM Condimed H1, Roche Diagnostics, Indianapolis, IN, cat. #1088947), 2 mM L-glutamine, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (all Invitrogen)]. Fusion culture medium was also supplemented with HAT (0.1 mM hypoxanthine, 0.4 mM aminopterin, and 0.016 mM thymidine; Invitrogen) as a selective agent against unfused myeloma cells. The master ELISA was performed using GST-SETA 123cc as target antigen to avoid detection of those antibodies that were reactive with the histidine tag. The second step antibodies for this assay were specific for mouse IgG and as such only anti-rat SETA mAbs of the IgG class were detected in 191 master wells whose culture supernatants produced an OD $\geqslant$ 0.4 (versus a background OD of 0.050–0.100). Cells from each of these 191 master wells were successfully expanded and retested. Frozen cells and supernatants were shipped to this laboratory for further analysis. Five wells were selected for cloning by two rounds of dilution cloning, based on Western blot and immunohistochemistry results. In every case more than 95% of the wells with cells that were obtained after the second round showed a positive titer by ELISA. These 5 lines were then weaned stepwise from 10% to 1% (v/v) hybridoma cloning supplement (BM Condimed H1, Roche Diagnostics). Concentrated supernatant was generated with CL350 system (Integra Biosciences) for the analyses presented here.

ELISA. The plate-bound rat GST-SETA 123cc ELISA for detection of anti-rat SETA MAbs was performed as follows. The wells of Immulon II plates (Dynex Technologies, VWR) were coated overnight at 4 °C with 1 μg/ml of purified recombinant GST-SETA 123cc protein in PBS, the wells were washed twice with PBS containing 0.05% (v/v) Tween 20 (Sigma, St. Louis, MO; PBST), and blocked with 200 μl/well of 1% (w/v) bovine serum albumin (Sigma, cat. A-9647) in PBST for one hour at room temperature. The wells were washed four times with PBST and 55 μl/well hybridoma culture supernatant was applied in duplicate. After incubation at room temperature for one hour the wells were again washed four times with PBST and 55 μl/well of an HRP-conjugated goat anti-mouse IgG second step antibody (Southern Biotechnology Associates, Birmingham, AL) diluted 1:4000 in IMDM supplemented with 2% (v/v) fetal calf serum was added. The secondary antibody was removed after one hour, and the plates were washed five times with PBST, 55 μl/well of a 1:100 dilution of TMB substrate (EIA Chromagen, Genetic Systems, Redmond, WA, or ImmunoPure TMB substrate kit, Pierce, Rockford, IL) diluted in substrate buffer according to the manufacturer's instructions (Genetic Systems or Pierce) was added and incubated for 10–15 min at room temperature in the dark. Color development was stopped by adding 55 μl/well of 1 N H_2SO_4 and the optical density was read on a plate reader at 450 and 650 nm. The final OD measurement was calculated by subtracting the OD_{650} from the OD_{450}.

Epitope mapping and isotyping. Epitope mapping was performed using the NovaTope Protein Domain Mapping System kit from Novagen with the SETA 123cc cDNA. Positive clones were identified essentially as described below in Western blot analysis. Isotyping of the antibodies was performed using the IsoStrip mouse monoclonal antibody isotyping kit (Roche Biochemicals) according to the manufacturer's instructions.

Immunoprecipitation and Western blot analysis. Lysates were prepared in RIPA buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% NP-40, 0.1% SDS, 1% deoxycholate, 0.025% NaN_3, 1 mM DTT, protease, and phosphatase inhibitor cocktails; Sigma) from untransfected cells, or from cells transfected by the calcium phosphate method for 48 h, with various SETA cDNAs (described previously [9]). Briefly, lysates were sheared 10 times through 18G1 and IM1 needles, and cleared by centrifugation for 10 min at 12,000*g* at 4 °C. The supernatants were incubated with antibody (100 μl of hybridoma supernatant for SETA MAbs/1 ml lysate) for 4 h at 4 °C with continuous rotation. Complexes were collected with 50 μl protein A agarose (Roche Biochemicals) at 4 °C overnight with continuous rotation. Lysates or immunoprecipitates were separated by polyacrylamide gel electrophoresis and Western blotted to PVDF membrane, exposed to primary antibodies as described, and detected by chemiluminescence (Bio-Rad, Hercules, CA), following standard protocols [17]. In some instances proteins were electrophoresed using the E-Gel 96 Protein Electrophoresis System (Invitrogen) and Western blotted to PVDF membrane according to the manufacturer's protocol.

pH fractionation of proteins. The separation of protein lysates according to p*I* was done using the Beckman–Coulter PF2D first dimension. Cell lysates (7.5 M urea, 2.5 M thiourea, 12.5% glycerol, 50 mM Tris, 2.5% *n*-octylglucoside, 6.25 mM TCEP Tris-(carboxyethyl)phosphine hydrochloride, and 1.2 mM protease inhibitor) were precleared by centrifugation at 20,000*g* for 60 min at 4 °C. The lysis buffer was exchanged with start buffer (pH 8.5 ± 0.1; Beckman–

Coulter, Fullerton CA) using a PD-10 column (Amersham–Pharmacia Biotech, Piscataway NJ), and the first 3.5 ml, collected, injected on the chromatofocusing column in the first dimension compartment of the PF2D instrument, and the separation method was initiated. This method, which operates at a constant flow rate of 0.2 ml/min, starts by washing the column with 100% start buffer pH 8.5 ± 0.1 for 45 min. Fractions are collected at 5 min intervals and are approximately 1 ml. Then a gradient to 100% eluent buffer pH 4 ± 0.1 (Beckman–Coulter), which takes approximately 60 min to complete, and fractions are collected every 0.3 pH units, resulting in approximately 17 fractions ranging in size from 0.6 to 1 ml. After the pH of the eluent is at pH 4.0, column is washed with 10 column volumes of high ionic strength wash to remove residual proteins, and fractions are collected by time as before, resulting in the collection of another 3–5 fractions of approximately 1 ml.

Immunohistochemistry of cells and sections. Cells were grown on glass coverslips overnight, washed with PBS, and fixed in 4% paraformaldehyde in PBS. Primary antibodies were detected with fluorescently conjugated secondary antibodies (Southern Biotechnology Associates) and viewed using a Zeiss Axiophot microscope.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2004.10.007.

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