CHARACTERIZATION OF p87C3G, A NOVEL, TRUNCATED C3G ISOFORM THAT IS OVEREXPRESSED IN CHRONIC MYELOID LEUKEMIA AND INTERACTS WITH BCR-ABL

Javier Gutiérrez-Berzal,[1] Esther Castellano,[1] Susana Martín-Encabo,[1] Noelia Gutiérrez-Cianca,[1] Jesús M. Hernández,[2,1] Eugenio Santos,[1] and Carmen Guerrero[1]

[1]Centro de Investigación del Cáncer, IBMCC, University of Salamanca-CSIC; and [2]Servicio de Hematología, Hospital Universitario de Salamanca, 37007-Salamanca, Spain.

Correspondence:
Carmen Guerrero
Centro de Investigación del Cáncer, IBMCC
Universidad de Salamanca-CSIC
Campus Miguel de Unamuno
37007 Salamanca, Spain
tel: 34-923-294801
fax: 34-923-294743
cguerrero@usal.es

Abstract

A novel C3G isoform, designated p87C3G, lacking the most amino terminal region of the cognate protein has been found to be overexpressed in two CML cell lines, K562 and Boff 210, both expressing Bcr-Abl p210. p87C3G expression is also highly augmented in patients diagnosed with chronic myeloid leukemia (CML) Ph+, in comparison with healthy individuals, and returns to basal levels after treatment with STI571. p87C3G coimmunoprecipitates with both CrkL and Bcr-Abl in CML cell lines and coimmunoprecipitation between p87C3G and Bcr-Abl was also detected in primary cells from CML patients. These interactions have been confirmed by *in vitro* pull down experiments. The interaction between p87C3G and Bcr-Abl involves the SH3-binding domain of p87C3G and the SH3 domain of Abl and depends mostly on the first polyproline region of p87C3G. Furthermore, we also demonstrated that p87C3G is phosphorylated *in vitro* by a Bcr-Abl-dependent mechanism. These results indicate that p87C3G overexpression is linked to CML phenotype and that p87C3G may exert productive functional interactions with Bcr-Abl signaling components suggesting the implication of this C3G isoform in the pathogenesis of chronic myeloid leukemia.

Keywords: C3G, Bcr-Abl, chronic myeloid leukemia, Hck, protein interaction

Introduction

C3G is a Crk-binding protein that shows transformation suppressor activity towards several oncogenes through a GEF-independent mechanism [1, 2] involving inhibition of ERK1/2 phosphorylation and cyclin A expression and resulting in lost of anchorage independent growth [1, 2].

However, C3G also participates in proliferative pathways through the activation of Rap1 [3, 4], and has been described as an essential gene in embryonic development [5] and integrin-mediated cellular adhesion and migration [5-8].

Chronic myeloid leukemia (CML) is a clonal disorder of hematopoietic stem cells caused by the Philadelphia chromosome (Ph) translocation [9], which results in the generation of the Bcr-Abl fusion oncogene [10]. The N-terminal segment of Abl includes a SH3 domain which regulates the tyrosine kinase function of Abl. The fusion of Abl to Bcr interferes with the SH3 domain regulatory function causing the Bcr-Abl kinase to become constitutively active [11].

C3G gene localizes to 9q34.3 and is telomeric to the c-*abl* locus (9q34.1), whose gene product is also a Crk-binding protein. In cells derived from CML, CrkL is constitutively phosphorylated and is one of the main substrates of Bcr-Abl tyrosine kinase [12]. In most cell types C3G is constitutively associated to CrkL, but in cells expressing Bcr-Abl this association decreases significantly probably due to the fact that both proteins bind CrkL in a mutually exclusive manner [6]. C3G is phosphorylated on Tyr-504 when coexpressed with Crk [13] and also C3G is phosphorylated upon integrin-mediated cell adhesion [6]. Moreover, it has been recently described that the Src family tyrosine kinase Hck, which activity is upregulated in Bcr-Abl expressing cells [14, 15], phosphorylate C3G in hematopoietic cells, where both proteins play a role in apoptosis [16].

Here we describe a novel C3G isoform, p87C3G, highly overexpressed in two hematopoietic cell lines related to CML, K562 and Boff 210, as well as in primary cells from CML patients at the time of diagnosis. p87C3G, lacks the N-terminal region and the first stretch of polyprolines of the SH3-binding (SH3-b) domain and is much more abundant than the full length p140C3G form in these cells. p87C3G, interacts with both CrkL and Bcr-Abl through its SH3-b domain and is phosphorylated in a Bcr-Abl-dependent manner, possibly involving Hck. These observations raise interesting possibilities for the putative participation of p87C3G in the pathogenesis of CML.

Materials and methods

Cell lines and tissue samples

K562, a human cell line derived from a patient with chronic myelogenous leukemia in terminal blast crisis was obtained from the ATCC (CCL 243) and maintained in RPMI 1640 (Life Technologies) containing 10% fetal bovine serum (FBS). Boff 210 is an interleukine 3- (IL3) independent cell line derived from the hematopoietic cell line BaF/3 [17] that expressed Bcr-Abl as a tetracycline-regulated transgene (Bcr-Abl expression is constitutive in absence of tetracycline or doxycycline). Boff 210 was maintained in DMEM (Life Technologies) supplemented with 10% FBS.

Mononuclear cells were obtained from blood samples of healthy individuals and CML patients in chronic phase (i) before treatment (more than 90% Ph+), (ii) treated with STI571 for two weeks (~50% Ph+) or (iii) with complete cytogenetic response after 6 months treatment with STI571 (0% Ph+) by lysing the red cells in a buffer containing 0.13 mM EDTA, 10 mM $HKCO_3$ and 0.15 M $ClNH_4$. All primary samples were provided by Hospital Universitario de Salamanca (Salamanca, Spain).

Agonists

IFN-α was supplied by Calbiochem (Darmstadt, Germany) and used at 10^4 U/ml for 5 min. EGF was purchased from Becton Dickinson (Franklin Lakes, NJ) and used at 200 ng/ml for 2 min.

Kinase inhibitors

STI571 (imatinib mesylate), an inhibitor of the Bcr-Abl kinase activity, was a gift of Novartis Pharma Ag, Basel, Switzerland. STI571 was stored in DMSO as a 10 mM

stock solution at -20ºC. Cells were treated for 24 h with 1 μM STI571 or with solvent only.

5´ RACE

5´RACE (rapid amplification of 5´cDNA ends) was performed with a kit by Roche Molecular Biochemicals, following manufacturer's recommendations, using two sets of three neighbouring primers: (1) mC3G-N1 (GGTCACCCTCTGAGATGTTGTCATAC), mC3G-N2 (ACTGTGAGGGGTGTCGCTCATAGG) and mC3G-N3 (AAA**GGATCC**ATAGGACACCCTGCAGCCAGAG) corresponding to positions 1482-1507, 1460-1483 and 1443-1464 respectively of mouse C3G gene (accession number: AF348669) and (2) mC3G-C1 (GGTCTTCTCTTCGCGGTCTGTTTTCC), mC3G-C2 (CAGAGAGAGTTCCCACAGGGCTTC) and mC3G-C3 (AAA**GGATCC**AGAGTTCCCACAGGGCTTCCTC) corresponding to positions 3233-3258, 3181-3204 and 3178-3199 respectively. mC3G-N3 and mC3G-C3 were designed with extra 9 nucleotides including a restriction site for *Bam*HI (in bold). After cloning into *Cla*I-*Bam*HI sites of plasmid pSP72 (Promega), fragments were sequenced using primers SP6 (ATTTAGGTGACACTATAGAACTC) and T7 (TAATACGACTCACTATAGGGAGA) in an automatic sequencer.

Northern blot

Total RNA from Boff 210 cells (20 μg) was loaded into a formaldehyde agarose gel and blotted to a Hybond-N+ membrane (Amersham Biosciences). The blot was hybridized with a 0.96 Kb cDNA fragment, containing nucleotides 2272 to 3231 of human C3G. The cDNA probe was ^{32}P-labelled by random priming (Stratagene) and used under high stringency hybridization conditions at 42^{o}C in the presence of 50%

(vol/vol) formamide, 5x SSPE (1x SSPE = 150 mM NaCl, 10 mM sodium phosphate pH 7.4, 1 mM EDTA), 100 μg/ml herring testes DNA, 1% SDS, and 5x Denhardt's solution. After 24-36 h of hybridization, the membrane was washed three times in 0.1x SSC, 0.1% SDS at 50°C and autorradiographed at -70°C in the presence of intensifying screens.

Antibodies and immunological studies

We have used the following antibodies: anti-C3G (c-19), anti-C3G (H-300), anti-c-Abl (24-11), anti-Bcr (G6), anti-Rap1 (121), anti-Actin (c-11), anti-tubulin (tu-02), anti-Crk-L (H-62), anti-Hck (N-30) and anti-phospho-tyrosine (PY20) from Santa Cruz Biotechnologies (Santa Cruz, CA) and anti-Hck mAb from BD transduction laboratories (Cat. No: 610277).

Whole cell lysates were prepared by extracting with Cell Lysis Buffer purchased from Cell Signaling Technology, Inc. (#9803).

Immunoprecipitations and In vitro phosphorylation

Immunoprecipitations and *in vitro* Kinase assays were performed as described [16]. For the *in vitro* kinase assays either, 20 μCi [γ^{32}P] ATP or 10 μM cold ATP were used. The samples were resolved by SDS-PAGE and exposed to autoradiography or revealed with anti-phospho-tyrosine (p-Tyr) antibodies.

Constructs in pGEX vectors

A fragment containing part of the catalytic region of human C3G gene was cloned into pGEX-4T-3 (Amersham Biosciences) as a *Sma*I (n2479)-*Bam*HI (n3247) fragment obtaining construct pGST-CAT. Full length p87C3G was cloned as a *Bam*HI-*Eco*RV

fragment into *Bam*HI-*Sma*I of plasmid pGEX-3X (Amersham Biosciences), obtaining construct pGST-p87. pGST-P2 contains the second polyproline stretch of C3G as a *Bam*HI (n840)-*Bsr*B1 (n1437) fragment cloned into *Bam*HI-*Sma*I of pGEX-3X. Entire C3G SH3-binding domain was cloned by PCR using primers AF45 (GCTC**GGATCCA**TGAAGCAGGGACGTC) and AF46R (CTTC**AGATCT**TTACGACTGAGCCGACTC), containing respectively *Bam*HI and *Bgl*II sites (in bold), and introduced into *Bam*HI site of pGEX-3X giving construct pGST-SH3-b.

A fragment of 419 bp containing Abl SH3 domain (nucleotides 2734 to 3152 of the p210 Bcr-Abl fusion gene) was cloned using primers p210F2-*Bam*HI (CCC**GGATCC**TTTCTGAATGTCATCGTCC) and p210R2-*Eco*RI (CCC**GAATTC**AGAAGCTGCCATTGATCC) and introduced into *Bam*HI-*Eco*RI sites of pGEX-4T-1 generating pGST-SH3Abl.

Bacterial expression of GST fusion proteins

Constructs pGST-CAT, pGST-C3G12 [1], pGST-SH3-b, pGST-p87, pGST-P2 and pGST-SH3Abl were expressed in BL21 pLys strain of *Escherichia coli* and the corresponding GST-fusion proteins purified using the glutathione-agarose affinity method as described [18]. pGST-RalGDS-RBD (GST fused to the Ras binding domain of RalGDS, amino acid residues 701-851), was obtained from Dr. P. Crespo (University of Cantabria/Instituto de Investigaciones Biomédicas, Santander, Cantabria, Spain).

Rap1 activity assay

Boff 210 cells were growth to 1.5 x 10^5 cells/ml and serum starved for 20-24 h followed by incubation with EGF (epidermal growth factor) or IFN-α (interferon-α).

After treatment, cells were washed twice with cold PBS and lysed in 500 μl of Mg^{2+} lysis/wash buffer (MLB) containing 25 mM HEPES, pH 7.5; 150 mM NaCl, 1% igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 1 mM Na_3VO_4, 25 mM NaF, 1mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin. Fresh cell lysates were diluted to roughly 1 μg/μl total cell protein with MLB and the lysates were precleared with glutathione agarose beads. After that, 1 mg of cell lysate was incubated with 10-15 μg pGST-RalGDS-RBD glutathione agarose conjugated per assay and the mixture was gently rocked at 4°C for 30 min. Agarose beads were collected by pulsing and the supernatant was drained off. The beads were washed 3 times with MLB, resuspended in appropriate amount of Laemmli sample buffer and boiled for 5 min. Supernatants were collected and loaded onto 12% SDS-PAGE. Rap1-GTP was detected with 1μg/ml anti-Rap1 antibodies.

Pull down experiments

Pull downs from cell extracts were carried out by incubating 1 mg of protein extract in lysis buffer with 0.1 to 2 μg of GST-fusion proteins, bound to glutathione-sepharose beads, for 2 hours at 4°C. Complexes were subjected to 3 washes in lysis buffer and boiled in loading buffer before SDS- PAGE.

In vitro mutagenesis

Plasmid pGST-p87, containing whole p87C3G clone, was subjected to *in vitro* mutagenesis by PCR using Pfu TURBO DNA polymerase (Stratagene) and the following pairs of oligos: CSH3b2-MutF (GCAGACAGATACG**G**CA**G**CTGCTCTC**G**CCGAGAAGAAGCG) and CSH3b2-MutR (CGCTTCTTCTCGG**C**GAGAGCAG**C**TG**C**CGTATCTGTCTGC); CSH3b3-

MutF (GACCCAGAAAAACCA**G**CT**G**CTCTA**G**CAGAGAAGAAAAA) and CSH3b3-MutR (TTTTTCTTCTCTG**C**TAGAG**C**AG**C**TGGTTTTTCTGGGTC); CSH3b4-MutF (GAGCTGGCCCCG**G**CG**G**CCGCCCTA**G**CCCCCAAGCAGCGG) and CSH3b4-MutR (CCGCTGCTTGGGGG**C**TAGGGCGG**C**CG**C**CGGGGCCAGCTC), where the mutated nucleotides are in bold (Proline to Alanine substitutions).

The PCR reactions were carried on at 95º, 30s; 55º 1 min; 68º, 11 min for 12 cycles and the mutations verified by automatic sequencing.

Results

Detection of a novel C3G isoform in K562 and Boff 210 cells

Type I and type II interferons, often used as a treatment for CML, activate CrkL-Rap1 signaling pathway in hematopoietic cells, presumably through the activation of C3G [19, 20]. To further study the involvement of C3G in this pathway, our first goal was to determine the expression of C3G in two models of CML, human K562 cells and mouse Boff 210 cells, as well as in primary CML patient samples. Both, K562 cells, growing in the presence (C) or absence (st) of 10% fetal bovine serum, and Boff 210 cells, untreated or treated with doxycycline (20 μg/ml, 48 h) and/or IL3 (10% of a conditioned media from WEHI cells), expressed good amounts of p210 Bcr-Abl (Figure 1A). Immunoblotting of total cell lysates from both K562 (Figure 1B) and Boff 210 (Figure 1C) cells with anti-C3G (c-19) antibodies detected a putative C3G isoform of approximately 87 kDa which is much more abundant than full length, 140 kDa, C3G form. Similarly, p87C3G is highly overexpressed in samples from CML patients at time of diagnosis as compared to healthy individuals (Figure 1D). This CML-associated p87C3G expression returns to basal levels after treatment with STI571 (Figure 1D). The authenticity of this band was confirmed by specific blockage with the corresponding blocking peptide, which completely competed both p140C3G and p87C3G bands (Figure 1B,C right panel, Figure 1D middle panel). Furthermore, the p87C3G band is not recognized by an anti-C3G antibody against the 300 first amino acids of the protein (C3G H-300), which indicates that p87C3G conserves the C-terminus of full length C3G but it is truncated at its N-terminus (Figure 1E).

Cloning of the 5´end of p87C3G by RACE: estimation of p87C3G start point

The above results with anti-C3G (H-300) and anti-C3G (c-19) antibodies suggest that p87C3G lacks, at least, the first 300 amino acids of the entire C3G protein, while it contains an intact C-terminus. To determine the start point of this isoform, we performed 5´RACE analysis using total RNA from Boff 210 cells and two sets of three adjacent oligonucleotides, mC3G-C1, 2, 3 and mC3G-N1, 2, 3 oligos (Figure 2A).

We sequenced a total of 23 clones from de N-terminal amplification and 10 clones from the C-terminal amplification, which size ranged between 252-494 nucleotides and 677-1162 nucleotides, respectively. The longer, most 5´, fragment from the N-terminal amplification started at nucleotide 971 of the mouse sequence and the longer fragment from the C-terminal amplification started at 2037. Thus, we could not obtain a single clone containing the complete p87C3G cDNA, probably due to limitations of the technique. There is an ATG site located at position 961, just ten nucleotides upstream the beginning of the longer N-terminal RACE clone, which corresponds to amino acid 321. This Met residue is very likely the start position of p87C3G as discussed below (see Discussion Section). In human C3G, the equivalent ATG starts at nucleotide 916 and corresponds to amino acid 306.

Additionally, Northern analysis on Boff 210 total RNA revealed two transcripts of 7.5 and 4.4 kb (Figure 3). The 7.5 kb transcript presumably encodes full-length 140kDa C3G protein, as it is ubiquitously detected [1, 21]. The shorter, 4.4 kb transcript has been previously described in adult human heart and skeletal muscle where, in concordance, a 80-90 kDa transcript was also detected [1]. The presence of two transcripts in Boff 210 is in agreement with the detection of the two C3G isoforms (p140 and p87) in these cells.

All these results suggest that p87C3G isoform is not originated from full length C3G by proteolytic digestion but it is translated from a spliced C3G mRNA.

Interaction of p87C3G with CrkL and Bcr-Abl

Immunoblotting of CrkL immunoprecipitates with anti-C3G (c-19) antibody revealed that p87C3G interacts with CrkL in K562 and Boff 210 cells (Figure 4A). Under our experimental conditions, we were unable to detect this interaction with full length p140C3G. The interaction between p87C3G and CrkL increased in cells treated with EGF, a known activator of the CrkL-Rap1 pathway [22] and when the expression of Bcr-Abl decreases (Boff 210 cells treated with doxycycline, Figure 1A, lower panel) (Figure 4A). In agreement with these results, Rap1 activation by EGF or IFN-α increased dramatically in Boff 210 cells previously treated with doxycycline (Figure 4 B). All these results are in concordance with a previous report suggesting competition between Bcr-Abl and C3G in their binding to CrkL [6].

Next, we studied whether p87C3G was also able to interact with Bcr-Abl in K562 and Boff 210 cells. We immunoprecipitated Bcr-Abl with anti-Abl antibodies and detected the presence of p87C3G in the Abl immunoprecipitates using anti-C3G antibodies (Figure 4C). Same results were obtained with anti-Bcr antibodies (data not shown). As expected, interaction between p87C3G and Bcr-Abl in Boff 210 cells decreases in the presence of doxycycline (Figure 4C, right panel). Interestingly, we could also observe this interaction in mononuclear cells lysate from a primary CML sample at the time of diagnosis but not in a healthy individual or a CML patient in complete remission after treatment with STI571 (Figure 4D), further confirming its relevance *in vivo*.

p87C3G interacts with Bcr-Abl through its SH3-binding domain

To characterize the p87C3G region involved in the interaction with Bcr-Abl, we performed pull down experiments of K562 lysates with several constructs of C3G fused to GST (Figure 2B). Figure 5A shows that only pGST-C3G12 and pGST-p87, containing, respectively, most of p87C3G and putative full length p87C3G, were able to pull down Bcr-Abl under these conditions, whereas constructs containing only the catalytic domain (GST-CAT) or the SH3-binding domain complete or partial (GST-SH3-b and GST-P2), failed to do so. As expected, constructs containing the SH3-binding domain complete or partial (but not the catalytic domain) were also able to interact with CrkL.

Bcr-Abl protein contains a SH3 domain that could potentially interact with the SH3-binding domain of C3G. To test this hypothesis we cloned the SH3 domain of Bcr-Abl fused to GST and performed pull down assays in K562 lysates with the resulting construct pGST-SH3Abl. Figure 5B shows that the SH3 domain of Bcr-Abl interacts with both p87C3G and CrkL. This interaction is specific of the SH3 domain of Bcr-Abl, since GST alone did not interact with p87C3G or with CrkL.

Contribution of the P2 region of the C3G SH3-binding domain is essential for its interaction with Bcr-Abl

To further confirm the specificity of the interaction between p87C3G and Bcr-Abl, we performed *in vitro* mutagenesis of the C3G SH3-binding domain, using pGST-p87 construct. We designed three different mutants of the C3G SH3-binding region, each one mutated in a different polyproline region (P2, P3 and P4, Figure 2B) with all the Prolines substituted by Glycines. Pull down experiments with these mutants (mP2, mP3 and mP4) showed that only mutations in the P2 region of the C3G SH3-binding

domain significantly decrease the interaction between p87C3G and Bcr-Abl (Figure 5C), suggesting that this region is the most important in the interaction between p87C3G and Bcr-Abl. Mutations in this polyproline stretch did not significantly affect to the interaction with CrkL (Figure 5C).

p87C3G is phosphorylated through a Bcr-Abl kinase-dependent mechanism

C3G is phosphorylated on Tyr-504 upon integrin-mediated cell adhesion [6] or coexpression with CrkI [13]. Recently the Src family tyrosine kinase Hck, which is specifically activated by Bcr-Abl in hematopoietic cells [14], was described to phosphorylate C3G in this cell type, where both proteins play a role in apoptosis [16]. Related to this, both CrkL and p87C3G are constitutively tyrosine phosphorylated in Boff 210 and K562 cells (Figure 6A). In Boff 210 cells, p87C3G phosphorylation increased in the presence of doxycycline suggesting a negative dependence on Bcr-Abl in this process. Additionally, IFN-α, an antagonist of Bcr-Abl function slightly increased p87C3G tyrosine phosphorylation in K562 cells.

To further investigate the role of Bcr-Abl on C3G phosphorylation in CML cells we performed *in vitro* kinase assays on K562 cell lysates immunoprecipitated with anti-Abl, anti-C3G (c-19) or anti-Hck antibodies. The reaction products were detected by autoradiography (Figures 6B,C) or with anti-p-Tyr antibodies (Figure 6D). Both p140C3G and p87C3G were found phosphorylated in the C3G immunoprecipitates, indicating that C3G interacts and is a substrate for cellular kinase activities (Figure 6B). Besides, p87C3G was also tyrosine phosphorylated in the Abl immunocomplexes, presumably through the kinase activity of Bcr-Abl, as this phosphorylation decreases in cells treated with STI571, a known inhibitor of the Bcr-Abl kinase function, (Figure 6C and Figure 6D, upper panel). In agreement with previous reports [16], p87C3G was also

found tyrosine phosphorylated in Hck immunoprecipitates being this phosphorylation also dependent on Bcr-Abl kinase (Figure 6D). The presence and phosphorylation of p87C3G and Hck in the immunoprecipitates are specific as these proteins are absent in control cell lysate incubated with beads (data not shown). Thus, the phosphorylation of p87C3G seems to occur in a complex with Bcr-Abl and Hck and is, at least in part, dependent on Bcr-Abl activity.

Discussion

In this paper, we described the detection and characterization of a novel C3G isoform, p87C3G, which may play a putative role in the pathogenesis of CML since its expression is significantly increased in untreated CML patients, as compared to normal healthy donors or patients treated with STI571, and interacts with the Bcr-Abl oncogene responsible of the malignant transformation of CML cells. p87C3G is also detected in unrelated tissues, like NIH 3T3 fibroblasts, although at very low levels compared to CML cell lines (data not shown). In addition to the ubiquitous 7.5 kb transcript, corresponding to full length C3G, a second transcript of about 4.4 kb was detected in Boff 210 RNA, in agreement with the presence of p87C3G in these cells. A shorter transcript of similar size, containing the 3´end of C3G but lacking the 5´end, was also previously described in tissues such as human heart and skeletal muscle where, in accordance, a band of 80-90 kDa was also present at the protein level [1].

Under our experimental conditions, we have been unable to detect interaction between Bcr-Abl and full length C3G, probably due to its low expression in CML cells. A recent article by Cho et al, showed p140C3G/Bcr-Abl interaction, although it has to be mention that the authors only detected this interaction in COS cells transfected with the corresponding DNAs but not in K562 cells of in MEFs from Bcr-Abl p190 transgenic mice [23]. Also results by these authors suggesting cooperation between Rap1 pathway and Bcr-Abl are in disagreement with our results where Rap1 is highly activated when Bcr-Abl expression decreases by the presence of doxycycline (Figure 4B). The antagonism between the CrkL-Rap1 and the Bcr-Abl pathways has been reported by other authors demonstrating that, in fact, the CrkL-Rap1 pathway exhibits suppressive effects on Bcr-Abl expressing cells [20, 24, 25].

Using rapid amplification of 5´cDNA ends (RACE) technique, we identified the putative start codon of p87C3G at positions 321 and 306 of mouse and human C3G proteins respectively, located after the first Proline-rich stretch. RACE allowed us to generate fragments upon 1 kb in length when using oligonucleotides from the 3´end of C3G gene (C-T oligos). The fact that all the N-T fragments obtained were smaller than 500 bp strongly suggests these fragments nearly reached the 5´ end of the corresponding mRNA. Nevertheless, it is surprising that we could not obtain larger fragments with the N-T oligonucleotides, considering that both mRNA species (full length p140C3G and p87C3G) must coexist in the cell. A plausible explanation is that p87C3G is much more abundant and/or more stable than full length C3G in these cells, thus favouring the amplification of p87C3G RNA in the RACE reaction. We consider that Met 306 of human C3G (321 of mouse protein) is the putative start of p87C3G for the following reasons: (i) Previous Met is located at position 250 (human C3G). Considering that antibody anti-C3G H-300 does not recognize p87C3G, it is very unlikely this isoform contains residues upstream aminoacid 300. In fact, this antibody perfectly recognizes the peptide encoded by a fragment containing the C3G SH3-b domain that starts, just exactly, at residue 250 (data not shown). (ii) Next Met is located at residue 551. Again, this Met can not be the starting of p87C3G since, as shown in Figure 6, this isoform is tyrosine phosphorylated and also recognized by an antibody specifically raised against the phosphorylated Tyr 504 (data not shown), the only Tyr residue susceptible of phosphorylation in C3G [13]. (iii) Finally, mutations in the P2 polyproline stretch (~aa 440-460) revealed that this segment is involved in p87C3G/Bcr-Abl interaction. Fluorescence in situ chromosomal hybridization (FISH) revealed that there is a single chromosomal loci for C3G gene at 9q34.3 [26], strongly suggesting that the p87C3G isoform arises from alternative splicing of a unique transcript. Besides, Shrivakrupa *et*

al. cloned a C3G fragment (C9) from a rat spleen cDNA library that also started just after the first Proline-rich stretch, similarly to our p87C3G clone [27], further supporting the idea of p87C3G being an alternative spliced form of C3G.

Both p87C3G and C3G12 (lacking the first 86 amino acids of p87C3G) interact with CrkL and Bcr-Abl. These interactions were specific of the mentioned fragments, as the catalytic region alone did not interact either with CrkL (as expected) or with Bcr-Abl. Small fragments containing (totally or partially) just the SH3-binding region failed to pull down Bcr-Abl, whereas they interacted with CrkL. It is possible that the interaction Bcr-Abl/p87C3G involves only the SH3-b domain of p87C3G although the rest of the p87C3G protein is necessary for the stability of the binding.

In addition, our data indicate that at least the SH3 domain of Abl is involved in the interaction between Bcr-Abl and p87C3G. A previous report established that the C3G SH3-binding domain only interacts with CrkL and, weakly, with Grb2 N-SH3 domain but not with Abl SH3 domain [28], although It should be mentioned that these experiments were performed *in vitro* and in HeLa cells. Surprisingly, the SH3 domain of Bcr-Abl also interacts with CrkL. This would constitute an additional interaction site between these two molecules, since it is known that Bcr-Abl also interacts with CrkL through the SH3-binding domain of Bcr-Abl and the N-T SH3 domain of CrkL [12, 29]. There is evidence for a potential SH3-binding domain inside the SH2 domain of CrkL that may explain this interaction [30].

In vitro mutagenesis experiments demonstrated that the second Proline-rich domain (P2) of C3G (i.e. the first polyproline stretch in p87C3G) is the most critical in the interaction with Bcr-Abl and confirm the specificity of such interaction as mutations in this domain only affect to the interaction of p87C3G with Bcr-Abl but not to the interaction with CrkL (Figure 5C).

Kinase studies performed in Figure 6 revealed that (i) p87C3G is found tyrosine phosphorylated in Boff210 and K562 cells, (ii) C3G proteins coimmunoprecipitate with a tyrosine kinase activity, (iii) only p87C3G, but not p140C3G (at least under the experimental conditions used) is phosphorylated *in vitro* in a Bcr-Abl-dependent manner, and (iv) p87C3G is also phosphorylated in Hck immunocomplexes by a Bcr-Abl kinase-dependent mechanism. Thus, the phosphorylation of p87C3G seems to occur in a complex with Bcr-Abl and Hck and is dependent, at least partially, on Bcr-Abl activity since addition of STI571 to the reaction decreases p87C3G phosphorylation. We also found Hck to be tyrosine phosphorylated in both C3G and Hck immunocomplexes, being this phosphorylation independent on Bcr-Abl catalytic activity, in agreement with a previous report [15]. Nevertheless, contrarily to those authors, we were unable to detect Hck in Abl immunocomplexes (Figure 6D, lower panel). One interesting point is that, although p87C3G phosphorylation seems to be mediated by Bcr-Abl catalytic activity, the interaction between Bcr-Abl and p87C3G is independent on Bcr-Abl kinase as it does not vary in the presence of STI571 (Figure 6D, lower panel).

On the other hand, data in Figure 6A also indicate that p87C3G is constitutively phosphorylated on tyrosine residues in K562 and Boff 210 cells and that decreasing of Bcr-Abl expression (treatment with doxycycline in Boff 210 cells and treatment with IFN-α in K562 cells) increases p87C3G phosphorylation. These observations are in agreement with previous results showing that there is competition between the Bcr-Abl oncogene and cellular proteins, such as C3G, for their binding to the adapter proteins CrkL and Crk [6, 31] and suggest that C3G is involved in p210 Bcr-Abl signaling. Nevertheless, these results are in apparent contradiction with the kinase assay results (Figures 6B-D) showing a dependence of p87C3G phosphorylation on Bcr-Abl kinase

function. One possible explanation is that several pathways phosphorylating C3G coexist in hematopoietic cells. Thus, in Figure 6B-C we are detecting *only* p87C3G directly phosphorylated by kinase activities that form complexes with C3G, presumably Bcr-Abl and Hck, while in Figure 6A we are detecting *all* phosphorylated p87C3G in the cell. It is well known that C3G phosphorylation is modulated by cell adhesion and that this property is altered in CML cells. In fact, CML cells express an adhesion-inhibitory variant of β1 integrin that is not found in normal progenitors [10]. In the presence of Dx, when Bcr-Abl expression is decreased, there is an increase in p87C3G phosphorylation (Figure 6A), probably due to an increase in CrkL available to interact with p87C3G (Figure 4A) which results in an increase in Rap1 activation (Figure 4B). In hematopoietic cells activation of Rap1 triggers an "inside out" signal that activates integrin-mediated cell adhesion [10, 32]. Reciprocally, it also possible that when the expression of Bcr-Abl decreases there is an increase in β integrins-mediated cell adhesion that can activates the CrkL-C3G-Rap1 pathway through an "outside in" signal [6, 8, 10, 32-34].

Curiously, the SH3 domain of Bcr-Abl is the one involved in regulation of adhesion to laminin and collagens of leukemic cells through the formation of $\alpha_2\beta_1$ integrin complexes [35], which is in agreement with a functional cooperation between p87C3G and Bcr-Abl by its SH3 domain.

In conclusion, we have described a novel isoform of C3G (p87C3G) abundantly expressed in CML cells that, through its interaction with the SH3 regulatory domain of Bcr-Abl, may play a role in the pathogenesis of CML. Further studies will be necessary to investigate the significance of this novel connection between Bcr-Abl and p87C3G, and whether it is related to the adhesive properties of leukemic cells.

Acknowledgments

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Ethical approval was given by our Institution Ethical Committee (Center for Cancer Research, IBMCC, CSIC-USAL). All patients provided written informed consent.

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Legends to Figures

Figure 1. **Expression of p87C3G in Bcr-Abl-expressing cells.** (A) Bcr-Abl expression in K562 and Boff 210 cell lines. Lysates were immunoprecipitated with anti-Abl antibodies and analyzed by immunoblotting with antibodies to Bcr. (B,C) C3G expression in K562 and Boff 210 cell lysates (20 µg) using anti-C3G (c-19) antibody (left panels, 5 min exposure with ECL). Both p140 and p87 bands were completely competed using the corresponding blocking peptide (right panels, 2 h exposure with ECL plus). (D) C3G expression in primary mononuclear cells from healthy individuals (H) and CML patient samples at different stages of treatment with STI571 (30 µg) using anti-C3G (c-19) antibody (upper panel, 2 min exposure with ECL). p140 and p87C3G were completely competed with blocking peptide (middle panel, 30 min exposure with ECL plus). >90%, CML patient samples containing more than 90% Ph+ cells; 50%, CML patients containing about 50% Ph+ cells; 0%, samples from CML patients in total remission after treatment with STI571 (0% Ph+ cells). (E) Antibody anti-C3G (H-300) does not recognize p87C3G in Boff 210 cell lysates. Actin or tubulin were used as control of protein loading. C, exponentially growing cells; st, serum-starved cells; IL3, interleukine 3; Dx, doxycycline; BP, blocking peptide. Blots are representative of 2 or 3 independent experiments.

Figure 2. Strategy of p87C3G cloning and constructs in pGEX vectors. (A) Schematic representation of C3G indicating the putative start of p87C3G and the position of the sets of oligonucleotides used in 5´RACE analysis. SH3-b, SH3-binding region; REM, Ras exchange motif; CDC25-H, catalytic or exchange domain. Regions recognized by C3G (c-19) and C3G (H-300) antibodies are indicated. (B) Schematic representation of C3G fragments cloned as GST fusion proteins, indicating the names of

the resulting constructs. P2, P3 and P4 correspond to the polyproline stretches 2, 3 and 4 of the SH3-b domain. Numbers correspond to amino acid positions of human C3G protein.

Figure 3. Northern analysis of Boff 210 total RNA. A 0.96 Kb PCR fragment, containing nucleotides 2272 to 3231 of human C3G was [^{32}P]α-dCTP labelled and hybridized to a Northern blot containing 20μg of total RNA from Boff 210 cells. The sizes of the two transcripts detected and the 28S rRNA position are indicated. M, molecular weight marker.

Figure 4. **p87C3G interacts with CrkL and Bcr-Abl.** (A) Lysates (1 mg) of Boff 210 cells (untreated or treated with 20 μg/ml doxycycline for 48 h) and K562 cells (untreated and treated for 2 min with 200 ng/ml EGF) were immunoprecipitated with anti-CrkL antibodies and immunoblotted using anti-C3G (c-19) antibodies. Immunoblots showing the amount of p87C3G in the lysates are presented in the lower panels. (B) Rap1-GTP from Boff 210 cells, treated with EGF or IFN-α in the presence or absence of doxycycline, was pull down with purified GST-RalGDS RBD-glutathione agarose beads and detected with anti-Rap1 antibodies (upper panel). Total Rap1 was detected from the same lysates by Western blot with same antibodies (lower panel). (C) K562 and Boff 210 cell lysates were immunoprecipitated with anti-Abl or anti-C3G (c-19) antibodies and analyzed by immunoblotting with antibodies to C3G (c-19) or CrkL. Immunoblots in lower panels show equal amounts of p87C3G in all lysates. C, exponentially growing cells; st, serum-starved cells; Dx, doxycycline. Panels are representative of 3 similar experiments. (D) Mononuclear cell lysates (300 μg) were immunoprecipitated with anti-Abl antibodies, and p87C3G detected with anti-C3G (c-

19) antibodies. H, healthy individual; >90%, CML patient at the time of diagnosis; 50%, CML patient containing about 50% Ph+ cells; 0%, CML patient with complete cytogenetic response; b, beads incubated with cell lysate from a 90% Ph+ patient.

Figure 5. **p87C3G interacts with Bcr-Abl through its SH3-b domain.** (A) Total cell lysates of K562 were pulled down with C3G12, CAT, SH3-b, p87 and P2 GST-fusion proteins bound to glutathione-sepharose beads, and analyzed by immunoblotting with antibodies to Abl (upper panel) and CrkL (middle panel). (B) K562 cell lysates were pulled down with GST alone or fused to the SH3 domain of Abl and analyzed by immunoblotting with antibodies to C3G (c-19) (upper panel) and CrkL (middle panel). (C) Pull down analysis with anti-Abl (upper panel) or anti-CrkL (middle panel) antibodies of K562 lysates incubated with wild type GST-p87 fusion protein (p87) or versions of GST-p87 mutated in the P2 (p87-mP2), P3 (p87-mP3) or P4 (p87-mP4) polyproline stretches. (A-C) Bottom panels: Coomassie stained gels of corresponding GST-fusion proteins. In all cases, equal volumes of purified protein, obtained from the same amount of bacteria, were loaded. b, beads incubated with cell lysate; L, cell lysate. All panels are representative of 3 independent experiments.

Figure 6. **p87C3G is tyrosine phosphorylated by two different mechanisms.** (A) p87C3G and CrkL are constitutively phosphorylated in Boff 210 and K562 cells. Cell lysates, treated as indicated, were immunoprecipitated with anti-p-Tyr antibodies and immunoblotted with antibodies to C3G (c-19) or CrkL. Lower panels show similar amount of p87C3G in all samples. St, starved cells; Dx, doxycycline; C, serum-growing cells. (B) K562 cells were immunoprecipitated with anti-C3G (c-19) antibodies and subjected to *in vitro* kinase reactions with [γ^{32}P]ATP. (C) K562 cells untreated or

treated with STI571 were immunoprecipitated with anti-Abl antibodies and subjected to *in vitro* kinase reactions with [γ^{32}P]ATP. ^{32}P-phosphorylated proteins were detected by autoradiography. b, beads incubated with cell lysate. (D) Upper panel, lysates of K562 cells treated or not with STI571 were immunoprecipitated with anti-Abl, anti-C3G (c-19) and anti-Hck (N-30) antibodies as indicated and subjected to *in vitro* kinase assay by incubation with cold ATP. Phosphorylated proteins were detected by Western blot with anti p-Tyr antibodies; lower panel, same blot stripped and incubated with anti-C3G (c-19) and anti-Hck polyclonal (two left lanes) or monoclonal (rest of lanes) antibodies. The blots are representative of 5 experiments.

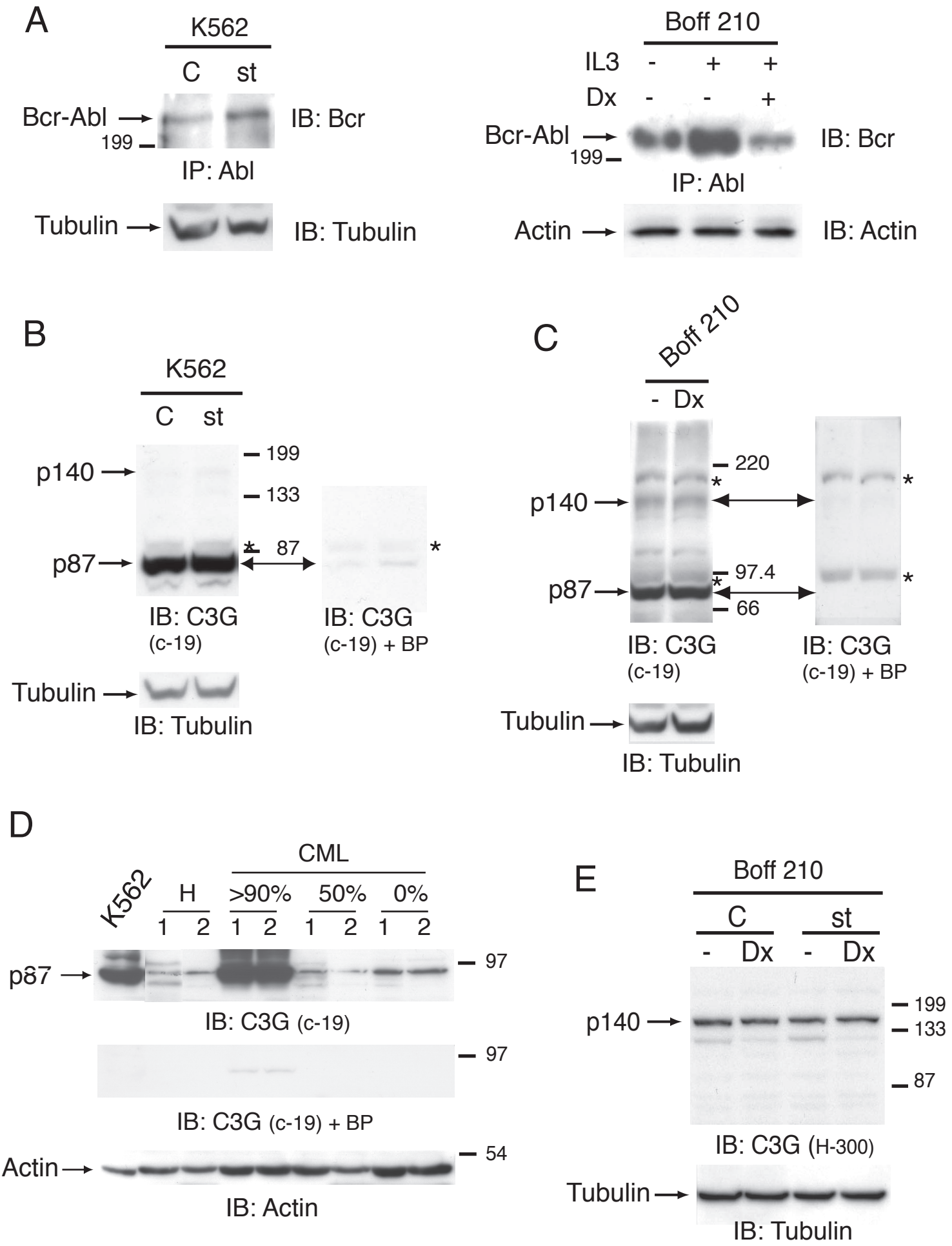
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Fig. 1

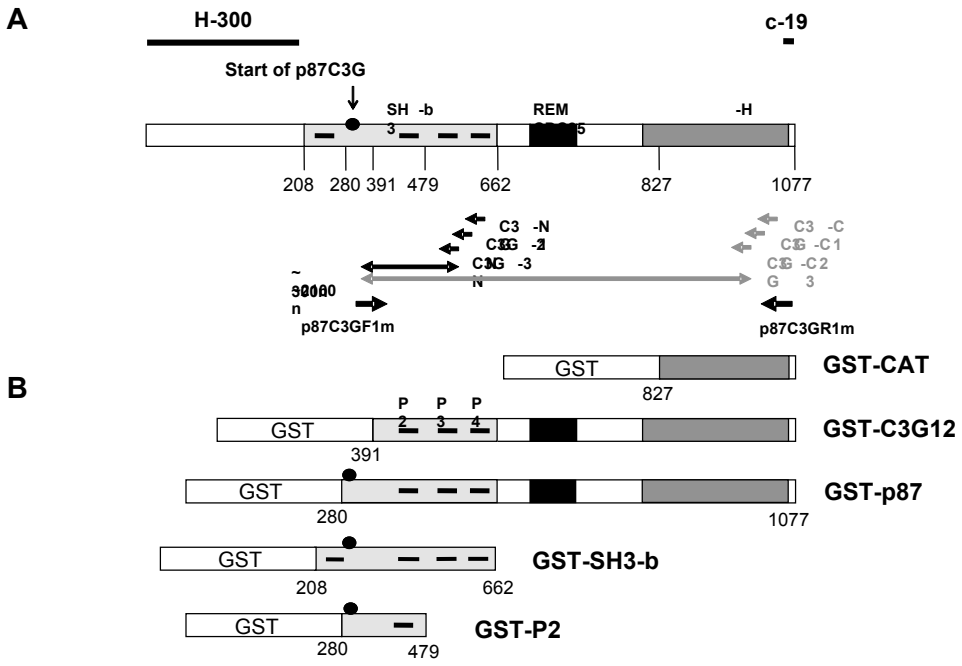
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Fig. 2

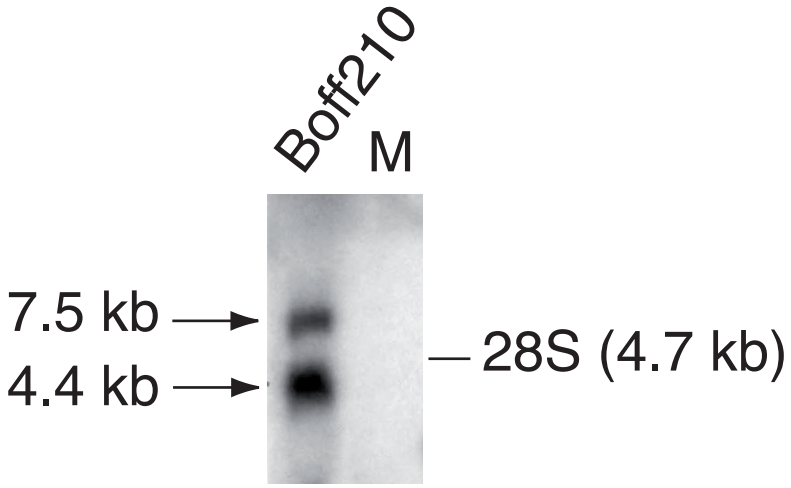

Fig. 3

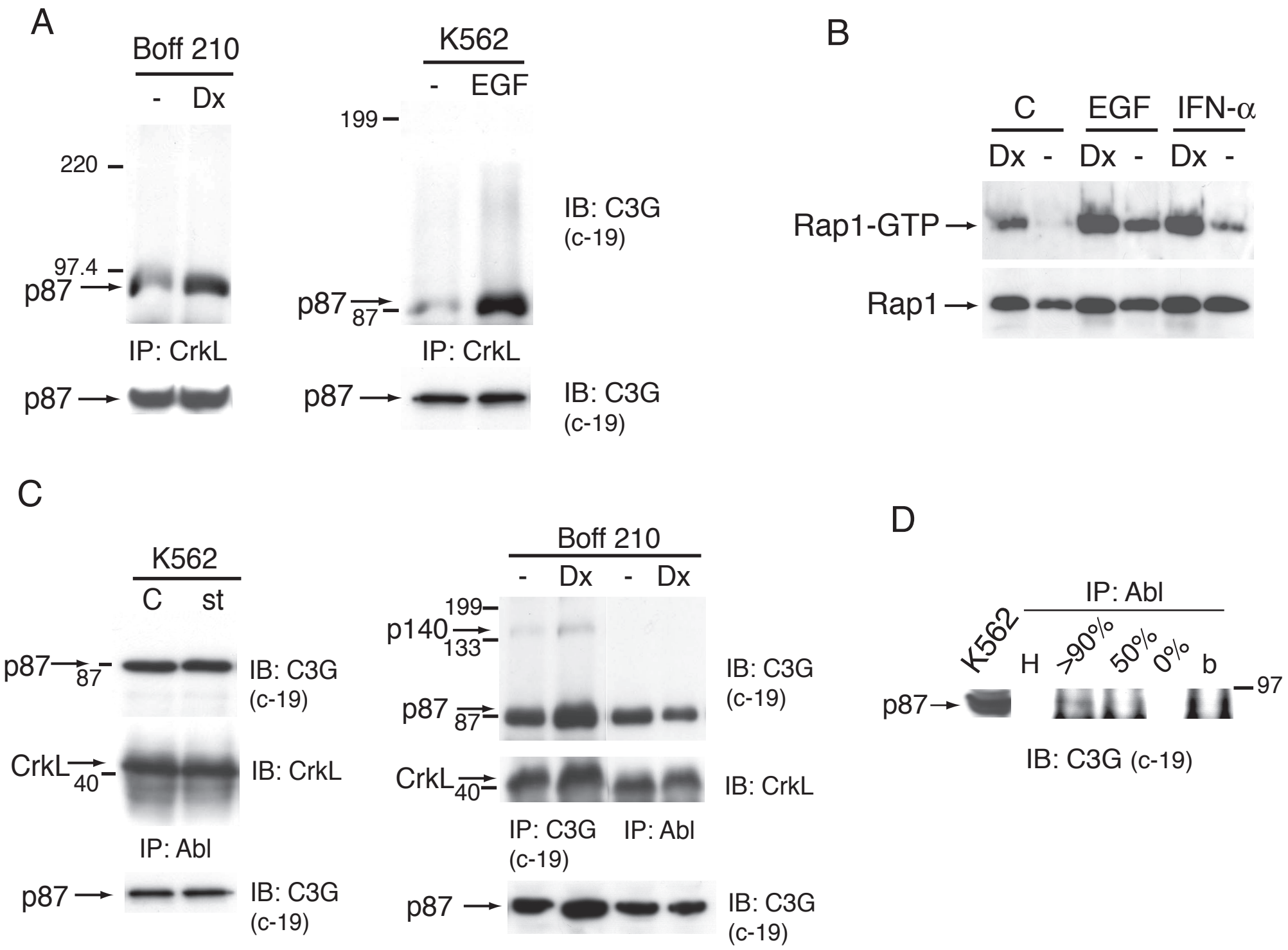
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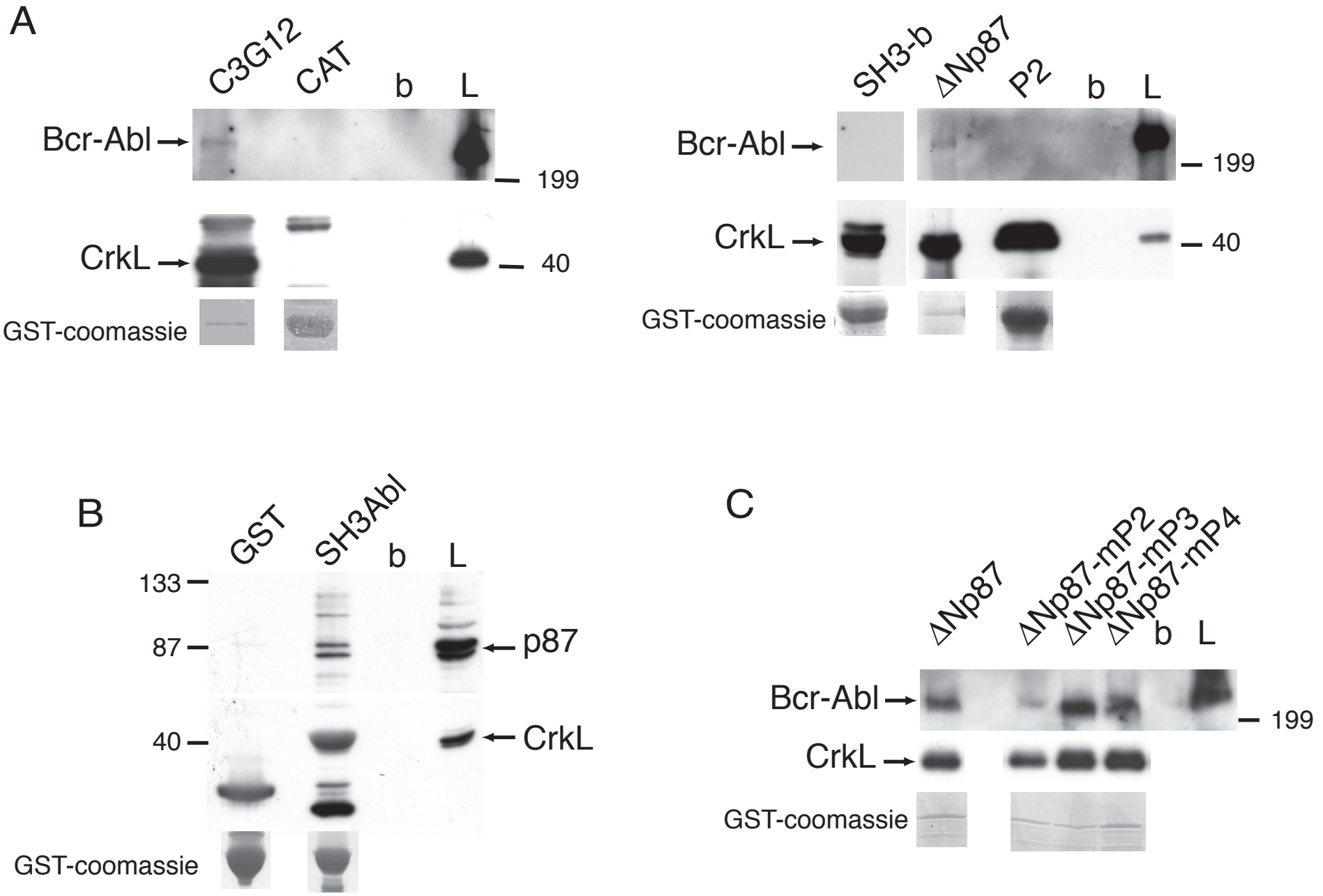
Fig. 4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CrkL
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GST-coomassie
B
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133
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Fig. 5

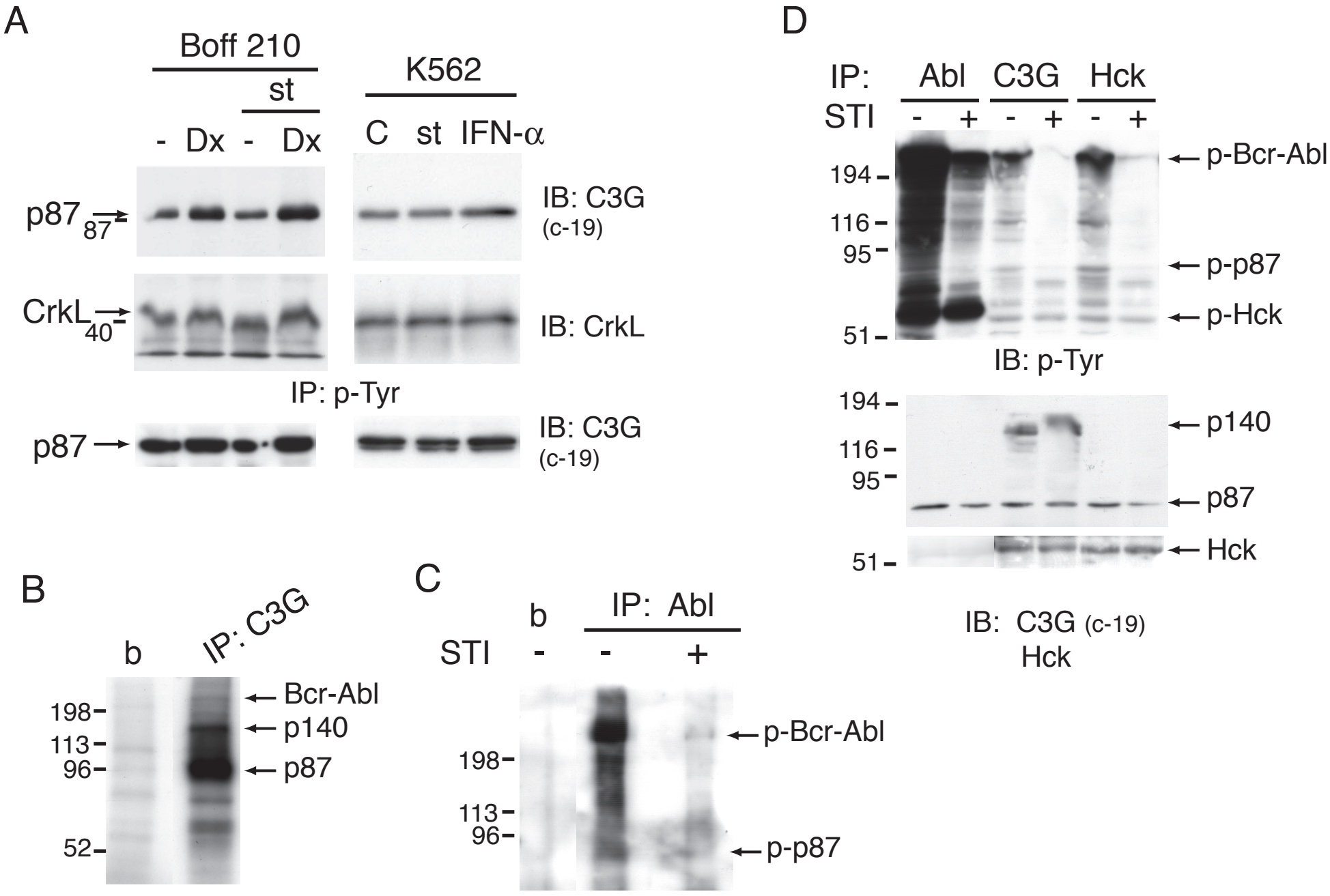
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Fig. 6