

**Advances in Brief****Potential Role of Bcr-Abl in the Activation of JAK1 Kinase<sup>1</sup>**

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**Abstract**

To study the oncogenic role of the p210<sup>bcr-abl</sup> fusion protein in chronic myelogenous leukemia cells, we generated a mouse cell line that was stably transfected with and overexpressed the human p210<sup>bcr-abl</sup> fusion protein. We then looked for phosphorylation activation of the Janus-activated kinase (JAK) family of tyrosine-specific protein kinases by the p210<sup>bcr-abl</sup> fusion protein. We found that JAK1, which has been shown by others to be associated with the IFN- $\alpha$  and - $\gamma$  plasma membrane receptors, was phosphorylated to a much greater degree in cells containing the p210<sup>bcr-abl</sup> fusion protein than was the case in the original, untransfected cell line. In contrast, no phosphorylation of the JAK2 kinase, which is associated with the IFN- $\gamma$  but not IFN- $\alpha$  receptor, was observed either with or without p210<sup>bcr-abl</sup> protein. A substrate of JAK1, STAT1 (signal transducers and activators of transcription 1), was found to be phosphorylated in cells containing overexpressed p210<sup>bcr-abl</sup> fusion protein. These results indicate that the presence of the p210<sup>bcr-abl</sup> protein kinase within a cell is associated with phosphorylation of the JAK1 kinase and its substrate STAT1.

**Introduction**

The Philadelphia chromosome translocation t(9;22) has been found in the leukemia cells of more than 95% of CML<sup>4</sup>

patients (1, 2). As a result of this translocation, most of the *c-abl* gene, including the 3' region coding for the catalytic domain of part of the *c-abl* gene located on chromosome 9, is juxtaposed to the 5' region of the *bcr* gene on chromosome 22. The chimeric fusion gene formed by this translocation produces an 8.5-kb mRNA transcript encoding a  $M_r$  210,000 hybrid protein (p210<sup>bcr-abl</sup>; Ref. 3). The resultant fusion protein exhibits a remarkably elevated cytoplasmic tyrosine-specific protein kinase activity (4, 5), upon which its transforming properties depends. The p210<sup>bcr-abl</sup> expression can also cause anchorage-independent growth in rat embryo fibroblast cell lines when cotransfected with the c-Myc protein (6). Furthermore, the p210<sup>bcr-abl</sup> protein may be associated with a transformed phenotype in some but not all lineages of NIH 3T3 cell lines, suggesting that a particular cell-regulatory context (mutations in addition to the p210<sup>bcr-abl</sup> kinase) is required for transformation by this protein (7).

The JAK family of kinases belongs to a family of nonreceptor protein tyrosine kinases (8, 9). Several members of the JAK family kinases have already been identified, including JAK1 (10), JAK2 (11), JAK3 (12), and Tyk2 (13). JAK1 was found to be involved in both IFN- $\alpha/\beta$  and - $\gamma$  signal transduction pathways (14), whereas JAK2 was associated with the IFN- $\gamma$  signaling pathway (15) but not the IFN- $\alpha/\beta$  signaling pathway. Activation of the plasma membrane receptors associated with the JAK family of kinases triggers autophosphorylation of these receptors and thereby the activation of their transkinase activities.

We decided to test for a possible interaction between the p210<sup>bcr-abl</sup> fusion protein and the JAK family of kinases for the following reasons: (a) the p210<sup>bcr-abl</sup> fusion protein exhibits a remarkably elevated tyrosine-specific protein kinase activity (4, 5); (b) about 25% of CML patients achieve a complete remission after IFN- $\alpha$  treatment, and 30% of these patients do not respond at all (16). The JAK1 and Tyk2 kinases are associated with the cytoplasmic domains of the IFN- $\alpha$  receptors on plasma membrane; and (c) a mutation in the JAK family of kinases can cause leukemia-like abnormalities in *Drosophila* (17).

In this study, we first established a mouse cell line that overexpressed human p210<sup>bcr-abl</sup> fusion protein. Then we examined the phosphorylation status of JAK1 and JAK2. We found that JAK1 was highly phosphorylated when cells acquired the p210<sup>bcr-abl</sup> cDNA, even in the absence of stimulation of their associated receptors by extracellular growth-regulatory proteins such as IFN- $\alpha$ . Binding of IFN- $\alpha$  to the JAK-associated receptors increased the extent of phosphorylation above that seen with the presence of p210<sup>bcr-abl</sup> alone. Interestingly, JAK2 was not phosphorylated in cells transfected with *bcr-abl* cDNA. STAT1 is one of the immediate downstream substrates of JAK1 (14, 18, 19). STAT1 was found to be phosphorylated at tyrosine residue 701 after JAK1 was activated by the binding of IFN- $\alpha$ , IFN- $\gamma$ , or epidermal growth factor (18, 19) to their membrane receptor extracellular domains. STAT1 was found to be phosphorylated in 32D-*bcr/abl* cells containing activated JAK1. These data suggest that p210<sup>bcr-abl</sup> fusion protein plays an im-

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<sup>4</sup> The abbreviations used are: CML, chronic myelogenous leukemia; JAK, Janus-activated kinase; STAT1, signal transducers and activators of transcription 1; IL, interleukin; U, unit; FBS, fetal bovine serum; RT-PCR, reverse transcription PCR; SH, Src homology.

portant role in regulating the activation of JAK1. Because the JAK1 kinase is associated with the activation of a large number of growth-regulatory membrane-associated plasma membrane receptors, the activation of JAK1 phosphorylation by p210<sup>bcr-abl</sup> may be an important part of the function of the p210<sup>bcr-abl</sup> fusion protein in CML. This work may also provide important clues to the molecular origins of the sensitivity or resistance of these cells to IFN- $\alpha$ .

## Materials and Methods

**Cells.** A clone of 32D cells is an IL-3-dependent cell line isolated originally from long-term bone marrow cultures (20, 21). These cells were maintained in Iscove's modified Dulbecco's medium supplemented with 10% FBS and 10% WEHI-3 conditioned medium as a source of IL-3. Murine IFN- $\gamma$  (Life Technologies, Inc.) and murine IFN- $\alpha/\beta$  in TNE buffer (catalog No. 20051; Lee Biomolecular Research, Inc., San Diego, CA) were used at 1000 and 220 units/ml, respectively.

K562 cells (American Type Culture Collection, CCL 243) were grown in RPMI supplemented with 10% fetal bovine serum and 2 mM L-glutamine in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Induction of cells by IFN- $\gamma$  (Genentech, Inc., San Francisco, CA) was performed at 1000 U/ml.

**Construction of 32D-bcr/abl Cell Line.** A human bcr-abl cDNA subcloned in a pcDNA3 expression vector (Invitrogen), which also contained a *Neo* gene, was introduced into 32D cells by electroporation. Following electroporation, the cells were allowed to recover in complete DMEM with 10% WEHI-3 medium (positive for IL-3) for 2 days at 37°C. Total RNA and protein extract were prepared from the 32D cells transfected with pcDNA3 vector alone. The transfected cells were then plated into 0.9% methylcellulose with 30% FBS (StemCell Technology, Inc.) and selected with 0.75 mg/ml G418. No IL-3 was present in the growth medium at this point and thereafter. After 14 days, no colonies were found in control plates in which the pcDNA vector (Neo positive and bcr-abl cDNA negative) alone was transfected into the cells. Individual colonies that were growing in the plates inoculated with cells transfected with the pcDNA-p210 (Neo positive and bcr-abl cDNA positive) vector were picked and grown into complete DMEM. Total RNA and protein extract were prepared from the cells transfected with the pcDNA-p210.

**Immunoprecipitation and Immunoblotting.** 32D cells were washed free of IL-3, cultured overnight in Iscove's modified Dulbecco's medium with 10% FBS, and left unstimulated as a negative control or stimulated with interferon. Cells were harvested, washed once in PBS, and lysed in a buffer containing 1% NP40; 0.25% sodium deoxycholate; 50 mM Tris-HCl (pH 7.6); 150 mM NaCl; 1 mM EGTA; 1 mM L-phenylmethylsulfonyl fluoride; 1 mM sodium orthovanadate; 1 mM sodium fluoride; and 1  $\mu$ g/ml aprotinin, leupeptin, and pepstatin. After clarification by spinning at 15,000 rpm for 2 min in a refrigerated microcentrifuge, lysates were subjected to immunoprecipitation overnight with antiserum against JAK1 or JAK2 (Upstate Biotechnology, Inc., Lake Placid, NY) at 4°C. Immunoprecipitates were washed in lysis buffer, eluted with 1 $\times$  Laemmli's SDS sample buffer, and heated at 100°C for 5 min. Samples were separated by SDS containing 10% PAGE and electrotransferred

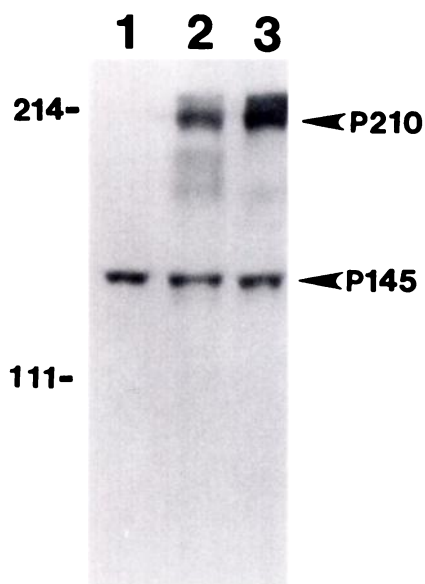
to Immobilon P membranes (Millipore, Bedford, MA). The membranes were probed with the antiphosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, Inc.), the anti-JAK1 monoclonal antibodies, or the anti-JAK2 monoclonal antibody (Transduction Laboratories, Lexington, KY). The signals on the membrane were detected by using the enhanced chemiluminescence Western blotting detection system (Amersham Corp.). For reprobing, the membranes were treated with stripping buffer composed of 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.8) at 70°C for 30 min and subsequently probed with a different antibody. The p210<sup>bcr-abl</sup> protein was detected by an anti-abl monoclonal antibody (8E9, Pharmingen).

## Results

**Establishment of a Cell Line Overexpressing Human p210<sup>bcr-abl</sup> Fusion Protein.** To study the oncogenic role of the p210<sup>bcr-abl</sup> fusion protein in CML cells, we transfected the human bcr-abl cDNA into the 32D myeloid cell line, which contains a population of immortalized but IL-3-dependent, myeloid hematopoietic progenitor cells. Two stable transfectants were tested and shown to be positive for the p210<sup>bcr-abl</sup> fusion protein. The morphology and the growth rate of these 32D cells did not change immediately following introduction of the bcr-abl cDNA (data not shown). However, these p210<sup>bcr-abl</sup>-positive 32D cells no longer required the IL-3 component of the growth media for *in vitro* survival of these cells.

Total RNA was prepared from the 32D cells and 32D cells transfected with the human bcr-abl cDNA. RT-PCR assays were performed to test for the presence of bcr-abl mRNA with primers specific for the bcr-abl mRNA. A single band in an agarose gel separation of the RT-PCR products was detected in 32D-bcr/abl cells (data not shown) after staining with ethidium bromide. No such RT-PCR product was found in parental 32D cells or in 32D cells transfected with a vector that did not contain the bcr-abl cDNA. Protein extracts were also prepared from parental 32D cells and 32D-bcr/abl cells to test for the presence of the p210<sup>bcr-abl</sup> protein. A single protein band at  $M_r$  210,000 was detected in this immunoblot of proteins from 32D-bcr/abl (Fig. 1) using an antibody against an epitope in the Abl domain of p210<sup>bcr-abl</sup> (Fig. 1, Lane 2). No protein was observed at the mobility of  $M_r$  210,000 in extracts from parental 32D cells. As a control for the amount of protein added in each sample, we used the intensity of the endogenous Abl protein, which was detectable in both the 32D and the 32D-bcr/abl protein extracts by the antibody to the Abl protein. This result suggests that the transfected bcr-abl cDNA not only produces the bcr-abl mRNA but also produces the p210<sup>bcr-abl</sup> fusion protein in 32D-bcr/abl cells.

**Phosphorylation of JAK-family Kinases in 32D-bcr/abl Cells.** The protein extracts from 32D and 32D-bcr/abl cells were prepared and tested for the presence and phosphorylation status of the JAK1 (Fig. 2) or JAK2 (Fig. 3) kinase. The JAK1 or JAK2 proteins were immunoprecipitated from protein extracts and then separated with 10% SDS-PAGE. After transferring the separated proteins onto nitrocellulose membrane, an antibody against phosphotyrosine was used to test for the phosphorylation status of the JAK1 or JAK2 protein kinase. As



**Fig. 1** Detection of p210<sup>bcr-abl</sup> protein in 32D-bcr/abl cells. Proteins from 32D (Lane 1) and 32D-bcr/abl (Lane 2) were separated on a 7.5% SDS-PAGE and electrotransferred to the Immobilon P membrane (Millipore). The membrane was then blotted with anti-Abl monoclonal antibody to detect the presence of the p210<sup>bcr-abl</sup> fusion protein (P210) and the endogenous Abl protein (P145). Proteins from K562 cells were used as a positive control for both p210<sup>bcr-abl</sup> and Abl proteins (Lane 3). The signals on the membrane were detected by using the enhanced chemiluminescence Western blotting detection system (Amersham Corp.).

shown in Fig. 2A, JAK1 from unstimulated 32D-bcr/abl cells (Fig. 2A, Lane 5) was phosphorylated to a greater degree than in the unstimulated 32D cell line (Fig. 2A, Lane 1). The level of phosphorylation on JAK1 was further intensified when IFN- $\alpha$  (Fig. 2A, Lanes 6 and 7) or IFN- $\gamma$  (Fig. 2A, Lane 8) was added to cultures of the 32D-bcr/abl cells to induce the level of phosphorylation. The phosphorylated JAK1 was undetectable in the parental 32D cells (Fig. 2A, Lane 1) and in IFN- $\alpha$ -treated 32D cells (Fig. 2A, Lanes 2 and 3). When 32D cells were treated

**Fig. 2** JAK1 protein detected in proteins isolated from 32D and 32D-bcr/abl cells. The 32D cells were grown in IL-3-free medium overnight before any treatments by IFNs. Cells were stimulated with 220 U/ml murine IFN- $\alpha/\beta$  (Lee Biomolecular Research, Inc.) for 15 min (Lanes 2 and 6), IFN- $\alpha/\beta$  for 120 min (Lanes 3 and 7), or IFN- $\gamma$  overnight (Lanes 4 and 8), or left unstimulated as a negative control (Lanes 1 and 5). Cell lysates containing 500  $\mu$ g of protein were subjected to immunoprecipitation overnight with antiserum against JAK1 from 32D cells (Lanes 1–4) and 32D-bcr/abl cells (Lanes 5–8). The immunoprecipitates were separated by a 10% SDS-PAGE and then blotted with the antiphosphotyrosine antibody (A) or the anti-JAK1 antibody (B).

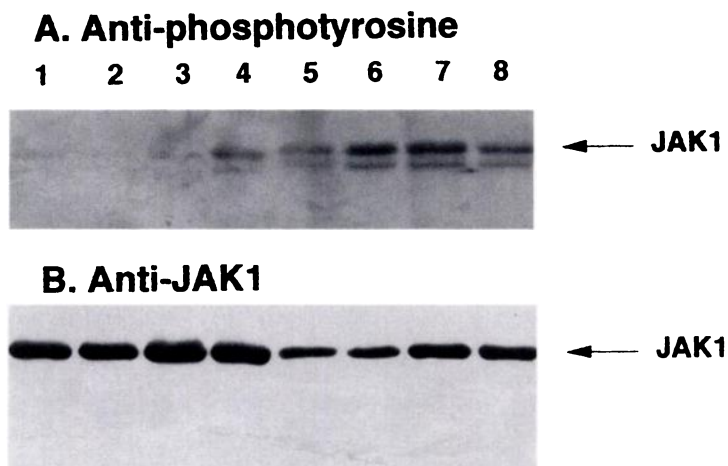
with IFN- $\gamma$ , the phosphorylation of JAK1 increased to a detectable range (Fig. 2A, Lane 4). There was no increase in the level of JAK1 protein in 32D-bcr/abl cells (Fig. 2B, Lanes 5–8) as compared to 32D cells (Fig. 2B, Lanes 1–4) as detected by the anti-JAK1 antibody (Fig. 2B).

JAK2 behaved differently from JAK1 in the 32D and 32D-bcr/abl cells. No phosphorylated JAK2 was detectable before IFN stimulation in either 32D-bcr/abl cells (Fig. 3A, Lane 2) or parental 32D cells (Fig. 3A, Lane 1). The presence of the p210<sup>bcr-abl</sup> protein was not associated with any increases in the phosphorylation of the JAK2 either in 32D-bcr/abl cells (Fig. 3A, Lane 2) or in untreated K562 cells (Fig. 3A, Lane 3). However, the JAK2 was phosphorylated in K562 cells following IFN- $\gamma$  stimulation (Fig. 3A, Lane 4). The level of JAK2 protein did not change in these cells, as was detected by anti-JAK2 antibody. In contrast to the case of JAK1, there was no increase in the level of phosphorylation of JAK2 following the stimulation of 32D and 32D-bcr/abl cells with IFN- $\alpha$  or IFN- $\gamma$  (data not shown).

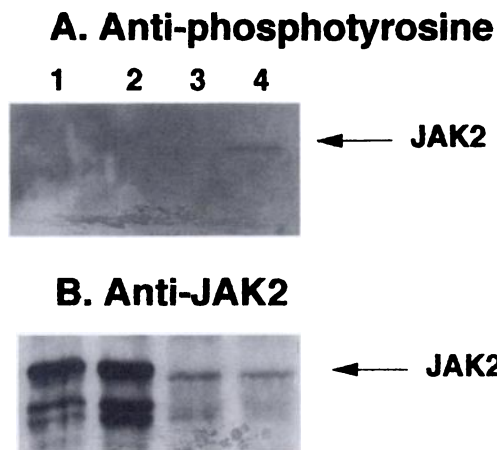
**Phosphorylation of STAT1 in 32D-bcr/abl Cells.** Because the phosphorylation of the JAK1 was increased in unstimulated p210<sup>bcr-abl</sup>-positive cells, it was of interest to test the phosphorylation status of the STAT1. The STAT1 protein was found to be phosphorylated after immunoprecipitating with an anti-STAT1 antibody from 32D-bcr/abl and then blotting with the antiphosphotyrosine antibody (Fig. 4A, Lane 6). This phosphorylation was in excess of that found when protein from 32D was immunoprecipitated with anti-STAT1 antibody (Fig. 4A, Lanes 4 and 5). No change in the level of the STAT1 protein from the 32D-bcr/abl cells (Fig. 4B, Lane 6) was detected when the STAT1 protein was immunoprecipitated and blotted with the anti-STAT1 antibody, as compared to 32D cells (Fig. 4B, Lanes 4 and 5). No specific protein or phosphorylated protein was detected when the immunoprecipitation was carried out with preimmune antibody (Fig. 4, A and B, Lanes 1–3).

## Discussion

In this study, we have shown that the autophosphorylation of the JAK1 kinase and STAT1 is increased in cells transfected with bcr-abl cDNA. Interestingly, transfection of bcr-abl cDNA





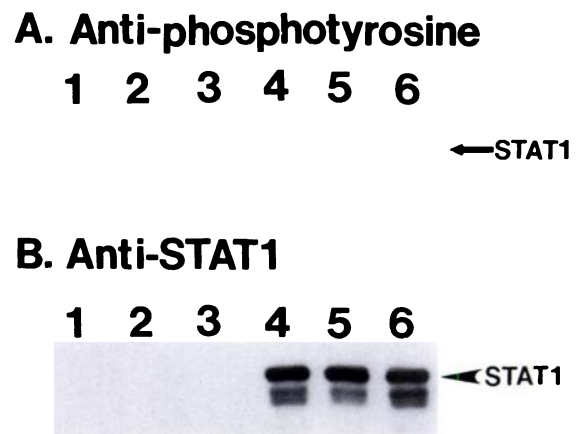


**Fig. 3** Detection of JAK2 in 32D and 32D-bcr/abl cells. Protein extracts from 32D cells (Lane 1), 32D-bcr/abl cells (Lane 2), and K562 cells (Lanes 3 and 4) were prepared and immunoprecipitated by anti-JAK2-agarose (Upstate Biotechnology, Inc.). The immunoprecipitates were separated on gel and transferred to membrane as described in the legend to Fig. 2. The membrane was blotted in panel A with the antiphosphotyrosine antibody or in panel B with the anti-JAK2 antibody (Transduction Laboratories). K562 cells were either stimulated with 1000 U/ml IFN- $\gamma$  (Genentech, Inc.) for 15 min (Lane 4) or unstimulated as control (Lane 3).

did not create any changes in the phosphorylation of the JAK2 kinase in the 32D cells.

The p210<sup>bcr-abl</sup> oncoprotein contains many functional domains, including an oligomerization domain, a SH2 domain, a SH3 domain, a kinase domain, and an actin-binding region. A number of proteins have been reported to interact with the p210<sup>bcr-abl</sup> oncoprotein, including the Grb2 and Crk1 adaptor proteins, the Bap-1 protein, and paxillin (22–24). The Grb2 adaptor protein binds to the Bcr protein domain. Both the Grb2 and Crk1 proteins also bind the Son of Sevenless (SOS) adaptor protein, which brings the p210<sup>bcr-abl</sup> into proximity with the membrane-bound Ras and thereby leads to activation of the Ras pathway. Tyrosine phosphorylation of the Bcr protein promotes the association of p210<sup>bcr-abl</sup> with the Grb2 adaptor protein. The paxillin, a focal adhesion cytoskeletal protein, becomes phosphorylated in p210<sup>bcr-abl</sup>-positive cells. This may link growth factor receptors and the p210<sup>bcr-abl</sup> fusion protein to the cytoskeleton (24).

The proto-oncogenic protein Vav, which interacts with both the Ras and JAK1 pathways, has been shown to be phosphorylated in p210<sup>bcr-abl</sup>-positive cells (25, 26). The data presented in this study show that the JAK1 kinase can be added to the list of proteins that are altered in the presence of the p210<sup>bcr-abl</sup> kinase. As shown in Fig. 2, JAK1 was highly phosphorylated in the presence of the p210<sup>bcr-abl</sup>. STAT1 was phosphorylated in the presence of activated JAK1 in 32D-bcr/abl cells (Fig. 4). Activation of STAT1 leads to a cascade of events that eventually results in the transcriptional activation of genes that are targets of the IFN interferon as well as of the Ras pathways (27–29). Interestingly, the *v-Abl* oncogene was found to activate both the JAK1 and JAK3 (30). Derived from the normal cellular homologue proto-oncogene *c-Abl*, *v-Abl* en-



**Fig. 4** Phosphorylation of STAT1 in 32D-bcr/abl cells. Protein extracts from 32D cells (Lanes 1, 2, 4, and 5) or 32D-bcr/abl cells (Lanes 3 and 6) were immunoprecipitated with preimmune serum (Lanes 1–3) or with anti-STAT1 antibody (Lanes 4–6) and then blotted in panel A with antiphosphotyrosine antibody RH-20 (Transduction Laboratories) or in panel B with anti-STAT1 antibody (Transduction Laboratories).

codes a  $M_r$  160,000 chimeric protein in which the *N*-terminal sequence of the *c-Abl* proto-oncogene was replaced by the gag moiety from the Moloney murine leukemia virus (31). The loss of the SH3 domain, which is a negative regulator of kinase activity in *v-Abl*, may dramatically enhance the tyrosine phosphorylation of associated proteins and localize the *v-Abl* to the cytoplasmic side of the plasma membrane. As compared with its counterpart p210<sup>bcr-abl</sup>, the *v-Abl* also exhibited transformation activity in a wider range of cellular systems, indicating that mechanisms involved in *v-Abl*- and p210<sup>bcr-abl</sup>-mediated transformation may be different. Nonetheless, the activation of the JAK1 by both *v-Abl* and p210<sup>bcr-abl</sup> support the hypothesis that the JAK family of kinases may be a common target of the *Abl* oncoprotein. An increased understanding of the detailed interactions of p210<sup>bcr-abl</sup> with its substrate may lead eventually to the identification of peptide elements of p210<sup>bcr-abl</sup> that exert a dominant effect on the growth phenotype of the CML cells and its response to therapy.

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