The 62-kDa protein associated with Ras GTPase-activating protein (RasGAP) was originally described as a tyrosine-phosphorylated protein in cells transformed by v-Src, v-Fps, and v-Abl as well as fibroblasts stimulated with epidermal growth factor (1). Subsequent work has demonstrated that a 62-kDa protein is tyrosine-phosphorylated and associates with Ras-GAP upon stimulation of cells with platelet-derived growth factor (2, 3), colony-stimulating factor-1 (4), insulin (5, 6), insulin-like growth factor-1 (7), vascular endothelial growth factor (8, 9), and stimulation of B cell receptors or Fcγ receptors (9, 10). Thus, p62 tyrosine phosphorylation is a common signaling event following activation of receptor and cytosolic tyrosine kinases. Isolation of a cDNA for p62 was reported, but it became clear that this protein was Sam68 and is distinct from the RasGAP-associated p62 (11–15). Recently, the RasGAP-associated p62 was cloned from cells transformed by oncogenic Abl proteins and was termed p62dok (downstream of tyrosine kinases) (16, 17).

The amino terminus of p62dok has partial sequence homology to a pleckstrin homology domain and part of a phosphotyrosine binding domain. The protein is relatively rich in serines and threonines and has a C-terminal domain that is proline-rich (16, 17). These findings suggest multiple potential protein-protein interactions with p62dok.

Activation of the Abl oncogene has been associated with a variety of human leukemias. A Bcr-Abl fusion protein of 210 kDa, in which the first exon of c-Abl has been replaced by Bcr sequences encoding 927 or 902 amino acids is present in 95% of patients with chronic myelogenous leukemia (18, 19). Another Bcr-Abl fusion protein, p185bcr-abl, contains only sequences from Bcr exon 1 (426 amino acids) fused to exons 2–11 of c-Abl and is seen in 5–10% of de novo acute lymphocytic leukemias (20, 21). The tyrosine kinase activity of these proteins has been shown to be essential for the transforming activity of the Bcr-Abl protein (22–24).

Although numerous tyrosine-phosphorylated proteins have been identified in Bcr-Abl-transformed cells, the necessity of these proteins for Bcr-Abl transforming function are unknown. p120 RasGAP and associated proteins, p190 and p62 are tyrosine-phosphorylated in Bcr-Abl cell lines, and p62 was shown to be a major tyrosine-phosphorylated protein in progenitor cells from chronic myelogenous leukemia patients in early phases of the disease (25, 26). Further, tyrosine phosphorylation of p62 has been shown to correlate with the ability of Bcr-Abl to transform fibroblasts (24, 27). These findings prompted us to analyze whether p62dok is the tyrosine-phosphorylated 62-kDa protein that has been described in Bcr-Abl-transformed cells, to determine whether this protein is the tyrosine-phosphorylated protein that correlates with fibroblast transformation, and to investigate protein-protein interactions of p62dok in Bcr-Abl-transformed cells.

MATERIALS AND METHODS

Plasmid Constructions—The p210bcr-abl SH2 domain deletion (p210ΔSH2) and kinase-defective mutants were previously described.

Interactions of p62dok with p210bcr-abl and Bcr-Abl-associated Proteins

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A 62-kDa Ras GTPase-activating protein (RasGAP)-associated protein is tyrosine-phosphorylated under a variety of circumstances including growth factor stimulation and in cells transformed by activated tyrosine kinases. A cDNA for p62dok, reported to be the RasGAP-associated 62-kDa protein, was recently cloned from Abl-transformed cells. In this study, the interactions of p62dok with Bcr-Abl and associated proteins were examined. In 32D myeloid cells and Rat-1 fibroblasts transformed by p210bcr-abl, p62dok is tyrosine-phosphorylated and co-immunoprecipitates with Bcr-Abl, RasGAP, and CrkL, a Src homology 2 (SH2) and SH3 domain-containing adaptor protein. Tyrosine-phosphorylated p62dok from cells expressing p210bcr-abl bound directly to the SH2 domains of Abl and CrkL in a gel overlay assay. Previous work has shown that an SH2 domain deletion mutant of Bcr-Abl is defective in transforming fibroblasts but not with the induction of growth factor independence. In both fibroblasts and myeloid cells expressing this mutant, p62dok is underphosphorylated as compared with cells expressing full-length p210bcr-abl but remains capable of associating with Bcr-Abl. However, in a gel overlay assay, p62dok from cells expressing the SH2 domain deletion was incapable of associating directly with SH2 domains of Abl and CrkL. Interestingly, no direct binding between Bcr-Abl and p62dok could be demonstrated in a yeast two-hybrid assay. These data suggest that indirect interactions mediate the interaction between Bcr-Abl and p62dok and that the SH2 domain of Bcr-Abl is required for hyperphosphorylation of p62dok. Further, hyperphosphorylation of p62dok correlates with the ability of Bcr-Abl to transform fibroblasts but not with the induction of growth factor independence in myeloid cells.
Interactions of p62<sub>doc</sub> in Bcr-Abl-transformed Cells

(24) and were subcloned into the retroviral vector pGDI (gift of G. Daley, Massachusetts Institute of Technology). A 185 bp cDNA was gifted by O. Witte (UCLA). The SH2 domain deletion in p185 was created by replacing the Kpr1 to Aft1 fragment of p185 with the equivalent fragment from p310ΑSH2. The GST-CrkL fusion proteins were generated as described (24-11 and K-12). p62<sub>doc</sub>, p120<sub>doc</sub>-SH3 were generated as described (32). Single clones were analyzed for Bcr-Abl expression and used for further experiments.

**Yeast Two-hybrid Assay—**Analysis of interactions using the yeast two-hybrid system were performed as described (33). A full-length p185<sub>cbl</sub>-abl was expressed as a LexA fusion protein. Full-length p62<sub>doc</sub> (gift of B. Stillman and N. Carpio) was expressed in frame fused to the acidic activation domain VP16. Interaction was identified on plates lacking histidine and analyzed for β-galactosidase production as described (33). Controls included Lex A, VP-16, and LexA-lamin.

Antiser—Antibodies recognizing Abl proteins (24-11 and K-12), p62<sub>doc</sub>, p120<sub>doc</sub>-SH3, and GST were purchased from Santa Cruz Biotechnology. The hemagglutinin (12CA5), and protein expression was induced by adding 250 μM isopropyl-1-thio-β-D-galactopyranoside to exponentially growing cells. The GST fusion proteins were isolated from sonicated bacterial lysates using glutathione-Sepharose beads (Amersham Pharmacia Biochemicals). Coomassie-stained gels of the GST fusion proteins were used to normalize for expression of the various GST fusion proteins. Between 2.5 and 5 μg of GST fusion proteins were incubated with 30 μl of glutathione-Sepharose beads in bacterial lysate buffer (150 mM NaCl, 16 mM NaH<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, 4 mM NaH<sub>2</sub>P<sub>2</sub>0<sub>4</sub>, pH 7.3, containing 10 μg/ml aprotinin, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, and 0.1% β-mercaptoethanol). The beads were washed four times in ice-cold PBS and incubated for 4 h with normal cell lysates. The beads were washed three times with PBS and boiled in SDS-sample buffer. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes for immunoblot analysis.

** Gel Overlay Assay—**GST, GST-Abl SH2, GST-Abl SH3, GST-CrkL, GST-CrkL SH2, and GST p185<sub>doc</sub> fusion proteins were used for this assay. PVDF membranes were blocked overnight at 4 °C in PBS with 0.05% Tween 20 and 5% nonfat dry milk. The blots were washed twice in PBS with 0.05% Tween 20 (PBS-T) and incubated for 2 h at room temperature with either GST only or with GST-Abl or GST-CrkL fusion protein (2 μg/ml) in binding buffer (25 mM sodium phosphate, pH 7.20, 150 mM NaCl, 0.1% Tween 20, 2.5 mM EDTA, 20 mM NaF, 1% nonfat milk, 1 mM diethiothreitol, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Bound GST protein was detected by incubation with anti-GST antibody (Santa Cruz Biotechnology) diluted 1:500 in binding buffer excluding milk, diethiothreitol, and protease inhibitors. Antibody reactions were developed using enhanced chemiluminescence. The blots were washed for 1 h in PBS-T between each of the steps.

**RESULTS**

**Tyrosine Phosphorylation of p62<sub>doc</sub> in Bcr-Abl-expressing Cells—**Bcr-Abl-expressing cells contain numerous tyrosine-phosphorylated proteins as compared with control cells, and one of the most prominently tyrosine-phosphorylated proteins is a protein with a relative molecular mass of 62 kDa. We have previously shown that tyrosine phosphorylation of this protein is significantly decreased in a Bcr-Abl mutant with a deletion of the SH2 domain (24). Decreased tyrosine phosphorylation of a 62-kDa protein was seen in 32D cells, a murine myeloid cell line, and Rat-1 fibroblasts expressing the SH2 domain deletion mutant (Fig. 1, A and B). This mutant renders 32D myeloid cells growth factor-independent but is defective in fibroblast transformation as determined by the inability to form colonies in soft agar (24, 38, 39). Thus, p62<sub>doc</sub> tyrosine phosphorylation correlates with fibroblast transformation by Bcr-Abl but not its ability to induce factor-independent growth.

Immunoprecipitation of p120<sub>RasGAP</sub> followed by phosphorylase immuno blotting demonstrated a marked decrease in the tyrosine phosphorylation of the RasGAP-associated p62<sub>doc</sub> in cells expressing the SH2 domain deletion of Bcr-Abl as compared with full-length Bcr-Abl (Fig. 1C). The RasGAP-associated p62<sub>doc</sub> protein is tyrosine-phosphorylated in the SH2-expressing cells as compared with control cells, but its phosphorylation is significantly less than in cells expressing full-length Bcr-Abl. To determine whether this 62-kDa tyrosine-phosphorylated protein was the recently cloned p62<sub>doc</sub> (<sup>7</sup.jboss) anti-p62<sub>doc</sub> immuno reactive band found to be phosphorylated (37). Bcr-Abl SH2 cell lysates were incubated with the p62<sub>doc</sub> antibody followed by Western blot analysis.
phosphorylation (Fig. 2A, lane 2). Equal amounts of p62<sub>dok</sub> were immunoprecipitated from each cell line (Fig. 2B). Of note, in Fig. 2A, there is a tyrosine-phosphorylated protein in p62<sub>dok</sub> immunoprecipitates that migrates below p62<sub>dok</sub>. This protein is not recognized by p62<sub>dok</sub> immunoblotting of p62<sub>dok</sub> immunoprecipitates (Fig. 2B), suggesting that it is distinct from p62<sub>dok</sub> and is probably a p62<sub>dok</sub>-associated tyrosine-phosphorylated protein.

To determine whether the pattern of p62<sub>dok</sub> mobility as determined by SDS-PAGE was solely due to phosphorylation, p62<sub>dok</sub> was immunoprecipitated from both the 32Dp210 and 32Dp210ΔSH2 cell lines, treated with purified calcineurin, a serine/threonine phosphatase, or with purified protein-tyrosine phosphatase-1. Phosphatase-treated immunoprecipitates were separated by SDS-PAGE and immunoblotted with p62<sub>dok</sub> antiserum. As shown in Fig. 3, calcineurin treatment of p62<sub>dok</sub> had no effect on the mobility of p62<sub>dok</sub>, whereas treatment of p62<sub>dok</sub> immunoprecipitates with protein-tyrosine phosphatase-1 eliminated the more slowly migrating forms of p62<sub>dok</sub>. These data suggest that the retarded mobility of p62<sub>dok</sub> seen in the 32Dp210 cells compared with the 32Dp210ΔSH2 cells was due to differences in tyrosine phosphorylation.

**Association of p62<sub>dok</sub> and Bcr-Abl**—We have previously demonstrated that Bcr-Abl and RasGAP co-immunoprecipitate (26). To determine whether p62<sub>dok</sub> was present in this complex, p62<sub>dok</sub> immunoprecipitations followed by Abl immunoblots or Abl immunoprecipitations followed by p62<sub>dok</sub> immunoblots were performed. In both cases, an association between p62<sub>dok</sub> and Bcr-Abl is detected (Fig. 4). This association is also seen in cells expressing the SH2 domain deletion of Bcr-Abl; however, less Bcr-Abl is present in the complex in these cells. These experiments have been performed normalizing for levels of
protein or amount of Bcr-Abl or p62\textit{dok} immunoprecipitated and have demonstrated consistently less Bcr-Abl in association with p62\textit{dok} in cells expressing the D\textsubscript{SH2} mutant.

The data presented above suggest a possible interaction between tyrosine-phosphorylated p62\textit{dok} and the SH2 domain of Abl. To assist in localizing the domains of Bcr-Abl that may interact with p62\textit{dok}, full-length Bcr-Abl or the Abl kinase domain tagged with an N-terminal hemagglutinin (12CA5) epitope was expressed in SF9 cells. Infected cells were lysed, and Bcr-Abl or the Abl kinase were purified by binding to 12CA5-Sepharose. Binding of p62\textit{dok} to the purified Bcr-Abl or Abl kinase domain was analyzed by incubating cellular lysates with these immobilized constructs followed by immunoblotting of bound protein for p62\textit{dok} (Fig. 5). Full-length p210\textit{bcr-abl}, but not the kinase domain of Abl, interacted with p62\textit{dok} from both the 32Dp210 and the 32Dp210\textit{D\textsubscript{SH2}} cell lines (Fig. 5). No p62\textit{dok} binding was seen using lysates from 32D cells or 32D cells expressing the kinase-inactive Bcr-Abl (data not shown). These data suggested that tyrosine phosphorylation of p62\textit{dok} was required for binding to Bcr-Abl and that a domain outside the kinase domain of Abl mediates the binding of p62\textit{dok} to Bcr-Abl. To further narrow the domain of Bcr-Abl that may be interacting with p62\textit{dok}, GST binding assays using the Abl SH2 or SH3 or the SH2/SH3 domains were performed. In these experiments, the SH2 domain of Abl interacted with p62\textit{dok} from both the 32Dp210 and 32Dp210\textit{D\textsubscript{SH2}} cell lines (Fig. 6).

In the experiments presented in Figs. 5 and 6, both the hyperphosphorylated p62\textit{dok} from 32Dp210 cells and the hypophosphorylated p62\textit{dok} from 32Dp210 cells and the hypophosphorylated p62\textit{dok}...
phorylated p62dok from 32Dp210ΔSH2 cells bound to Bcr-Abl (Fig. 5) or to the Abl SH2 domain (Fig. 6).

The above data are consistent with an interaction between the Abl SH2 domain and tyrosine-phosphorylated p62dok but cannot address the question of whether these interactions are direct or indirect. To address this question, a gel overlay assay was performed using GST-Abl SH2 and SH3 domains as probes. p62dok immunoprecipitates from Bcr-Abl-expressing cells were run over the columns containing GST-Abl SH2 or GST-Abl SH3 and probed using the Abl SH2 or SH3 domains. Fig. 7 shows that p62dok immunoprecipitated from 32Dp210 cells bound to the SH2 domain of Abl, but no direct interaction was observed in p62dok immunoprecipitates from cells expressing either kinase-inactive Bcr-Abl or the SH2 domain deletion of Bcr-Abl. No direct interaction was observed between the SH3 domain of Abl and p62dok in this assay (data not shown). These data demonstrate that the isolated SH2 domain of Abl can bind directly to tyrosine-phosphorylated p62dok from 32Dp210 cells.

To further analyze whether a direct interaction between p62dok and Bcr-Abl occurs in vivo, in the context of a full-length Bcr-Abl, a yeast two-hybrid analysis was performed. For these experiments, full-length p185bcr-abl was expressed as a LexA DNA binding domain fusion protein, and p62dok was expressed as a VP16 activation domain protein. These constructs were transformed into a yeast strain containing a histidine gene under the control of a LexA promoter. Interactions between the proteins of interest are scored by growth on plates lacking histidine. Kinase-active Bcr-Abl was expressed in yeast as demonstrated by anti-phosphotyrosine immunoblotting (data not shown) and its ability to interact with Grb-2 (Fig. 8), an interaction that requires tyrosine phosphorylation of Bcr-Abl.
Expression was confirmed by a positive interaction with Grb-2 (data not shown). In this system, we could not detect a direct interaction between p62<sub>dok</sub> and Bcr-Abl (Fig. 8).

Association of p62<sub>dok</sub> with Other Signaling Proteins—The above data are consistent with the model of Bcr-Abl interaction with p62<sub>dok</sub> shown in Fig. 10. In this model, an initial indirect interaction between p62<sub>dok</sub> and Bcr-Abl occurs, regardless of the presence or absence of the SH2 domain of Abl. In the presence of the SH2 domain of Abl, p62<sub>dok</sub> becomes hyperphosphorylated and is then capable of binding directly to the SH2 domain of Abl. To test this model, p62<sub>dok</sub> interactions in Bcr-Abl-expressing cells were analyzed. p62<sub>dok</sub> has multiple consensus binding sites for SH2 domain-containing signaling molecules including Abl, RasGAP, and Crk proteins. As shown in Fig. 4, p62<sub>dok</sub> and Bcr-Abl co-immunoprecipitate, and as expected, p62<sub>dok</sub> and p120<sup>RasGAP</sup> are present in an immune complex (Fig. 9A).

CrkL has been identified as a prominently tyrosine-phosphorylated protein in Bcr-Abl-expressing cells; therefore, the association of p62<sub>dok</sub> with CrkL was analyzed. p62<sub>dok</sub> and CrkL co-immunoprecipitate from lysates of the indicated cells were separated on 8% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated with bacterially expressed Abl SH2 domain, and binding was detected with a GST antibody (A). No binding was detected using GST alone or the Abl SH3 domain (data not shown). The immunoblot was stripped and reprobed with p62<sub>dok</sub> antisera (B). The migration of p62<sub>dok</sub> and the antibody heavy chain (VH) is indicated to the right of each panel.
Interactions of p62\textsuperscript{dok} in Bcr-Abl-transformed Cells

Fig. 9. Interactions of p62\textsuperscript{dok} with p120\textsuperscript{RasGAP} and CrkL. Lysates of the indicated cells were immunoprecipitated with p62\textsuperscript{dok} (A) or CrkL antisera (B) and immunoblotted with RasGAP or p62\textsuperscript{dok} antisera as indicated. C, lysates of 32Dp210 (top) or 32Dp210\DeltaSH2 (bottom) were analyzed for p62\textsuperscript{dok} binding to the indicated GST-CrkL or GST-Crk II fusion proteins. K39 is a Lys to Arg mutant in the FLVRES SH2 phosphotyrosine binding domain of CrkL, and SH3 is the amino-terminal SH3 domain of CrkL. Glutathione beads (beads) and bacterially expressed GST (GST) were used as controls. WCL was used to determine the presence and migration of p62\textsuperscript{dok}. D, p62\textsuperscript{dok} immunoprecipitates from lysates of the indicated cells were separated on 8% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were incubated with bacterially expressed CrkL SH2 domain, and binding was detected with a GST antibody (A). No binding was detected using GST alone or using the CrkL SH3 domain (data not shown). The migration of p62\textsuperscript{dok} and the antibody heavy chain (VH) is indicated to the right.

The interaction of p62\textsuperscript{dok} with CrkL was also analyzed. As shown in Fig. 9B, CrkL immunoprecipitates from Bcr-Abl-expressing cells contain p62\textsuperscript{dok} as do cells expressing the SH2 domain deletion mutant. Various domains of CrkL expressed as GST fusion proteins were analyzed for their ability to bind to p62\textsuperscript{dok}. As seen in Fig. 9C, full-length CrkL was capable of interacting with p62\textsuperscript{dok} from 32Dp210 cells, but this interaction was reduced in cells expressing p210\DeltaSH2. In a gel overlay assay, the
CrkL SH2 domain was capable of binding directly to p62 dok immunoprecipitated from Bcr-Abl-expressing cells (Fig. 9D, lane 2). However, no direct binding of CrkL was observed in p62 dok immunoprecipitates from cells expressing the SH2 domain deletion mutant (Fig. 9D, lane 3). No binding was detected using the SH3 domain of CrkL (data not shown).

**DISCUSSION**

In this paper, we have demonstrated that p62 dok is hyperphosphorylated on tyrosine in Bcr-Abl-expressing cells but minimally phosphorylated in cells expressing an SH2 domain deletion of Bcr-Abl. In addition, p62 dok is present in an immune complex with Bcr-Abl and RasGAP, suggesting that p62 dok is the RasGAP-associated p62 protein. Further, p62 dok, when hyperphosphorylated, is capable of binding directly to the SH2 domain of Abl and CrkL. However, no direct binding of p62 dok to Bcr-Abl is seen in the yeast two-hybrid system. Thus, our current model of p62 dok and Bcr-Abl interaction is that indirect interactions mediate initial binding of p62 dok to Bcr-Abl, regardless of the presence or absence of the SH2 domain of Abl. Hyperphosphorylation of p62 dok requires the presence of the SH2 domain of Abl, and once hyperphosphorylated, p62 dok can bind directly to the SH2 domain of Abl or to other Bcr-Abl-associated proteins (Fig. 10). The finding that the SH2 domain of Abl is required for hyperphosphorylation of a substrate is similar to data obtained with the C-terminal domain of RNA polymerase II (42).

The identity of the protein that mediates the initial binding of p62 dok to Bcr-Abl is presently unknown. We have previously shown that CrkL binds directly to a proline-rich region in the C terminus of Abl (29). Our data suggest that CrkL is not responsible for mediating the initial interaction of p62 dok and Bcr-Abl, since direct binding between CrkL and p62 dok can only be demonstrated when p62 dok is hyperphosphorylated. Another candidate for mediating the interaction is p120 RasGAP, since this protein has been demonstrated to be present in an immune complex with Bcr-Abl and p62 dok. Further, this complex is present in cells expressing the SH2 domain mutant of Bcr-Abl. Since the mechanism of RasGAP and Bcr-Abl interaction is not known, additional experiments will be required to test this hypothesis. Preliminary data from the yeast two-hybrid system suggest that p62 dok and Grb-2 interact directly. Since Grb-2 is known to bind to tyrosine 177 of Bcr-Abl, Grb-2 is another candidate for mediating the interaction between p62 dok and Bcr-Abl.

The data presented here support the conclusion that p62 dok is the bona fide RasGAP-associated p62. Previous experiments have demonstrated that a RasGAP-associated tyrosine-phosphorylated protein of 62 kDa is present in Bcr-Abl-transformed cells. This is supported by our finding of p62 dok tyrosine phosphorylation and association with RasGAP in Bcr-Abl-expressing cells. It has also been noted that tyrosine phosphorylation of a 62-kDa RasGAP-associated protein correlates with fibroblast transformation by Bcr-Abl (26, 27). A similar RasGAP-associated tyrosine-phosphorylated protein is seen in cells transformed by other activated tyrosine kinases such as Src and Fps, and lack of tyrosine phosphorylation of this protein has also been observed in SH2 domain mutants of these proteins (43, 44). Our data demonstrate that p62 dok is minimally tyrosine-phosphorylated in cells expressing an SH2 domain deletion of Bcr-Abl as compared with wild type Bcr-Abl.

The lack of increase in phosphorylation of p62 dok is seen in both fibroblasts and myeloid cells expressing the SH2 domain deletion mutant. The phenotype of the Bcr-Abl SH2 domain mutant is that it is defective for fibroblast transformation but remains capable of inducing factor independent growth in myeloid cells. Thus, the hyperphosphorylation of p62 dok correlates with fibroblast transformation but not induction of growth factor independence by Bcr-Abl.

In chronic myelogenous leukemia cells, Bcr-Abl is postulated to have several possible functions. These include an increased proliferation of progenitor cells, protection from programmed cell death, and an adherence defect leading to increased numbers of circulating immature cells. Since the function of p62 dok is currently unknown, it is not clear which of these possible functions are impacted by p62 dok hyperphosphorylation. However, the correlation of p62 dok hyperphosphorylation with the induction of anchorage-independent growth in fibroblasts suggests a possible role for p62 dok in mediating the adherence defect of Bcr-Abl-expressing cells. In the absence of a biochemical activity, placement of p62 dok function in a specific pathway will require additional knowledge of p62 dok interactions or generation of p62 dok null mice.

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32368

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