QM, a Putative Tumor Suppressor, Regulates Proto-oncogene c-Yes*

Received for publication, February 25, 2002, and in revised form, July 1, 2002
Published, JBC Papers in Press, July 22, 2002, DOI 10.1074/jbc.M201859200

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The QM gene encodes a 24.5 kDa ribosomal protein L10 known to be highly homologous to a Jun-binding protein (Jif-1), which inhibits the formation of Jun-Jun dimers. Here we have carried out screening with the c-Yes protein and found that a QM homologous protein showed interactions with c-Yes and other Src family members. We have found that two different regions of QM protein were associated with the SH3 domain of c-Yes. The QM protein does not contain canonical SH3 binding motifs or previously reported amino acid fragments showing interaction with SH3 domains. Several c-Yes kinase activity assays indicated that the QM protein reduced c-Yes kinase activity by 70% and that this suppression is related not only to the two SH3 binding regions but also to the C-terminal region of QM. Moreover, our autophosphorylation assays clarified that this regulation resulted from the inhibition of c-Yes autophosphorylation. Immunofluorescence studies showed that the QM proteins and c-Yes are able to interact in various tumor cell lines in vivo. The increases of the QM protein and mRNA levels were detected when the QM was transfected. These results suggest that the QM protein might be a regulator for various signal transduction pathways involving SH3 domain-containing membrane proteins.

Src family kinases are involved in many intracellular functions, including cell stability, division, proliferation, migration, and differentiation. c-Yes is one of the Src family kinases and is expressed ubiquitously in most tissues and presumably is involved in many signaling pathways (1). c-Yes has not been studied extensively compared with other members of Src families because of the high similarity to Src. However, various studies demonstrated that yes and src genes have different characteristics in expression, activity, and function (2). Several proteins have been reported to influence Src family kinase activity (3, 4, 5), and in some cases, binding proteins have different effects on each type of kinase activity (6). Src family kinases contain SH3 and SH2 domains that are involved in many protein-protein interactions. SH2 domains usually mediate protein-protein interactions by tyrosine-phosphorylated proteins or ligands (7, 8). SH3 domains are composed of about 60 amino acids and are found in many signaling proteins (9). They are highly homologous and have diverse mediating roles in protein interactions. SH3 domains are one of the well-characterized protein binding domains. Many studies revealed that they recognize the proline-rich consensus sequence containing the PXXP motif in the context of the left-handed helical polyproline-2 (PPII) helix (10–12). Recently, several other SH3-recognizing amino acid sequences, such as RXKXXXY and WXXQF, have been reported (13, 14), and they play a different role in relation to each Src kinase.

The QM/Wilms' tumor suppressor gene was first identified from the Wilms' tumor cell line (15), and its genomic location was reported within Xq28 (16). QM is also highly homologous to the Jun-binding protein (Jif-1) as a putative tumor suppressor (17), but it has been observed that QM and Jun have a rare interrelationship in vivo (18). Most QM proteins are localized in the cytoplasm, and subcellular fractionation assays have shown that QM is peripherally localized to the endoplasmic reticulum (19). In contrast, another study contended that QM was associated with presenilin 1 (PS1) and translocates from cytoplasm to nucleus resulting in suppression of the complex formation of the c-Jun homodimer (20). In another report, it has been demonstrated that yeast QM homologous genes, such as GR5 and QSR1, participate in translational control of gene expression in yeast (21). These are especially required for cell growth and differentiation throughout mRNA translation, because they take part in the recombination of 60 and 40 S ribosomal protein subunits. Several studies showed that QM proteins are expressed in many different species, and sequence analysis has proven that they are highly conserved throughout (22). Embryos and bone development studies indicate that QM proteins are highly expressed in undifferentiated cells, while differentiated and adult tissues tend not to maintain QM proteins (23–25).

Despite the importance of QM proteins in cell stability, differentiation, and development, it has not been revealed that QM is involved in intra- or extracellular signaling pathways. In our study, we showed that QM influenced the membrane protein, c-Yes, directly or indirectly. From these results, it was possible to deduce that the QM protein takes part in signal transduction through association to c-Yes. Moreover, since the QM protein also shows interactions with other Src family members, it can be suggested that the QM protein might be an important regulator or mediator of many other SH3-domain-containing proteins and other cytoplasmic molecules, even in translation and transcription processes.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—Matchmaker LexA two-hybrid system and human HeLa cell library were purchased from CLONTECH, c-yes-encoding SH3-SH2-PTK, kindly provided by Dr. Marius Sudol, was amplified by the PCR method and was cloned into pLexA as a bait protein. Saccharomyces cerevisiae EGY48 (p5op-lacZ), host strain, was...
transformed simultaneously with pLux-c-Yes and with the human HeLa DNA library. About 10^6 yeast colonies were transformed; the double transformants were grown on appropriate S.D. minimal medium, and a β-galactosidase assay was performed. Putative positive selected clones were analyzed by restriction enzyme digestion and sequenced.

**DNA Construction**—All the fragments were amplified by PCR and subcloned into appropriate expression vectors. For yeast expression, an SH3-SH2-PTK (c-Yes) fragment was generated with BamHI/XhoI and cloned in pBR322 containing pGEX vectors (pGEX-Fyn-SH3, pGEX-Hck-SH3, pGEX-Lyn-SH3, pGEX-Src-SH3) were kindly provided by Dr. Thomas E. Conti. 

The BOSC 23 cell line was kindly provided by Dr. S. Ryu and Dr. S. Cho (KRIBB) and was maintained in DMEM—GALACT-5C-15% FCS-100 units/ml penicillin, 100 μg/ml streptomycin, 1% nonidet P-40, 1% sodium deoxycholate, 0.15 mM NaCl, 10 mM sodium phosphate (pH 7.0), 100 μM sodium vanadate, 50 mM NaF, 50 mM leupeptin, 1% aprotinin, 2 mM EDTA, and 1 mM diithiothreitol) was added. The plate was incubated for 20 min at 4 °C with agitation. The cells were scraped and centrifuged at 12,000 rpm for 30 min. The cell lysates (1 ml) were added to the prepared 50% slurry GST-agarose beads. Following incubations in the cold room at 4 °C by gentle rocking, the samples were centrifuged at 10,000 rpm for 3 min and then washed in dialysis buffer. The 5× SDS loading buffer was added to the beads, boiled for 5 min, and subjected to 12% SDS-PAGE. The binding of the two proteins was detected by Western blot analysis.

**Commmunoprecipitation**—Cells were washed in PBS and lysates were prepared as described above. Precleared 200–500 μg of lysates with 500 μl of GST-agarose beads were incubated overnight with 10 μl of anti-c-Yes-agarose affinity beads. After washing three times with 1× PBS, the protein complexes were eluted with 0.1 M glycine/250 mM NaCl, pH 2.5, and concentrated and analyzed by SDS-PAGE. The binding of the two proteins was detected by Western blot analysis.

**Protein Interactions under Native Conditions**—For the detection of endogenous protein-protein interactions, 2× 10^6 HeLa or AGS cells were transfected for 2 days, and the lysates were prepared according to the method described above. The collected cell lysates were incubated with 10 μl of anti-c-Yes or anti-QM (C-17) rabbit polyclonal antibody (Santa Cruz Biotechnology) for 3 h. Gel pull-down assays of each GST-SH3 (Fyn, Hck, Lyn, Src, and Yes) were also performed with native HeLa cell lysates and overexpressed GST-SH3s from E. coli. We incubated 30 μl of GST-SH3s bound to GST-agarose and 1 mg of the HeLa cell lysate for 4 h at 4 °C. They were washed four times with lysis buffer. Native QM protein was immunoprecipitated with 5 μg of the anti-QM antibody from the 0.5 mg of precleared cell lysate. After 1 h of incubation, 0.5 mg of GST-SH3 containing E. coli cell lysates, and 40 μl of prewashed protein A/G-agarose were added and communoprecipitated for 3 h.

**Immunofluorescence**—1× 10^6 cells were plated on coverslips. Fixation was followed by a slightly modified methanol fixation method. The fixed cells were permeabilized for 3 min at 4 °C in PBS supplemented with 0.1% Triton X-100. After washing three times with PBS, the coverslips were soaked in blocking buffer (0.5 g/100 ml of bovine serum albumin in PBS) for 15 min at room temperature. The cells were then incubated with the anti-c-Yes mouse and the anti-QM rabbit polyclonal antibody (each 1:300 dilution in blocking buffer) for 1 h. Following three 5-min washes, the coverslips were incubated for 5 min with rhodamine-conjugated goat anti-rabbit (1:100, Santa Cruz Biotechnology) and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody (1:100, Sigma). After the third 5-min wash with PBS, the coverslips were mounted, and visualization was performed using an Axiovert 25 fluorescent microscope (Carl Zeiss).

**In Vitro/in Vitro Kinase Assays**—c-Yes derivative-transformed BOSC 23 cells were cultured with 0.1% DMSO in DMEM supplemented with 10% heat-inactivated FBS, 10 mM Tris-HCl, pH 7.5, 12 mM β-glycerophosphate, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 1% Triton X-100, 500 units/ml penicillin, 100 units/ml streptomycin, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin, and the extracts were incubated with anti-FLAG M2-agarose (Sigma) for 2 h at 4 °C. The protein-bound agarose was washed three times with kinase wash buffer (150 mM NaCl, 5 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 20 mM Tris-HCl, pH 7.5) and detected by Western blot analysis. Kinase assays were carried out in 30 μl of the kinase reaction buffer (20 mM Tris-HCl, pH 7.5, 20 mM MgCl2, 20 mM cold ATP, and 0.3 μC of γ-32P]ATP (Amerham Biosciences)), and 0.4 mg/ml of poly-3EY-14:4 (Sigma) was used as a substrate. Cotransfected cell lysates were used in in vitro kinase assays without any exogenous protein addition. For the in vitro kinase assays, 5 μg of purified GST fusion proteins were added to pulled-down c-Yes derivatives. The kinase assays were based on those previously reported (26). The reactions were performed for 30 min at 30 °C and were terminated by adding 6× SDS loading buffer. The samples were boiled and subjected to 12% SDS-PAGE. The gel was exposed to an x-ray film (Agfa) at −70 °C overnight. For measurement of the specific activity of radiolabeled peptides, the bands were excised and counted in a liquid scintillation counter (Wallac 1409).

**Autophosphorylation and QM Protein Phosphorylation Study**—Autophosphorylation assay was initiated by addition of the kinase reaction buffer without poly-3EY-4:1 for 30 min at 30 °C. Autophosphorylation inhibition studies were carried out with several cotransfected cell lysates incubated anti-FLAG M2-agarose or with addition of purified...
GST-fused QM subcloned protein derivatives. The reaction was halted by boiling in SDS loading buffer, and the samples were resolved by SDS-PAGE. After the gel was autoradiographed for 1 or 2 days at 70 °C, 32P-labeled proteins were excised and quantified on the liquid scintillation counter.

For the QM protein phosphorylation study, c-Yes-bound beads were incubated with 10–40 μg of purified GST-QM or thrombin-treated QM for 1 h at 30 °C in 40 μl of the kinase reaction buffer and terminated by adding the SDS loading buffer. For concentration, samples were dried and subjected to 12% SDS-PAGE.

Protein Expression and Purification—All the QM derivatives were inserted into pGEX-4T at EcoRI/XhoI sites. Bacterial cell line BL21-CodonPlus (DE3)-RIL (Stratagene) was transformed, and a single colony was inoculated in the LB culture medium with ampicillin and cultured overnight at 37 °C. The culture was diluted 10-fold and incubated at 30 °C. Following induction with 0.5 mM isopropyl-1-thio-D-galactopyranoside for 4 to 6 h, the collected cells were resuspended with PBS containing 0.5% N-lauroyl sarcosine, 1 mM dithiothreitol, 1 mM EDTA, and appropriate protease inhibitors (27). After incubation of the resuspended cells with lysozyme for 20 min on ice, they were briefly sonicated and centrifuged at 30,000 rpm (Beckman). Following the centrifugation, the supernatant was removed and subjected to prewashed GST-agarose beads (Peptron, Korea) and incubated for 20 min at 4 °C with agitation. These protein-complexed beads were packed into a column for washing. The column was washed with 30-fold bead volume of 0.1% N-lauroyl sarcosine PBS. Proteins were eluted with 5 mM reduced glutathione (Sigma) and collected for concentration. The concentration was performed by a Vivaspin 15 ml concentrator (MW 10,000, Vivaspin) and dialyzed for 24–36 h with the buffer changed every 12 h in 2 liters of PBS containing 0.05% N-lauroyl sarcosine. The dialyzed proteins were centrifuged for 20 min at 30,000 rpm to remove insoluble materials. The protein was diluted to 1–2 mg/ml with the dialysis buffer. The concentration was quantified by Bradford assay.

RT-PCR—AGS, HeLa, and HepG2 cell lines were transfected with pEBG-QM. After 40 h of incubation, total RNA was prepared with the TRIZOL reagent (Invitrogen), and reverse transcription was carried out with 2 μg of total RNA for 1 h at 42 °C in a total volume of 40 μl. After the reaction was terminated at 95 °C for 10 min, PCR was performed with the pair of primers, whose action was designed to amplify the PTK coding region (5′-A-TGT-CCC-ACG-GTC-GAC-CCA-CAA-ACA-C-3′/5′-ACT-GCT-ACA-GAA-CCA-CAG-TAC-CAG-C-3′). To denature the samples, 30-μl reaction mixtures were incubated at 95 °C for 5 min, followed by the next three cycles 25 times. The reaction mixtures were denatured at 94 °C for 30 s, annealed at 50 °C for 40 s, and extended at 72 °C for 1 min. After final elongation was conducted at 72 °C for 5 min, PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. Equal quantification of mRNA was normalized by PCR with β-actin primers (Promega) and analyzed densitometrically.

RESULTS

QM Sequence Is Analyzed and QM Protein Interacts with the SH3 Domain of c-Yes—c-Yes was screened by a yeast two-hybrid system with the human HeLa cell library, and several
intense blue colonies were selected from more than 200 blue colonies, the putative positives. The cDNA inserts of these colonies were sequenced, and homologies were analyzed using GenBank\textsuperscript{TM}. One of the proteins was 99\% homologous to QM/Wilms' tumor suppressor (15) with the absence of cysteine at the 23rd position in both mouse and human QM and an amino acid change from serine to asparagine at the 202nd position in both mouse and human QM/GenBank\textsuperscript{TM} access number AAL88713). Our motif analysis revealed that there were two sites for amidation (1–4: 4MGRI; 35–38: LGRI), myristylation (24–29: GVPDAK, 113–118: GMGraf), and PKC phosphorylation (136–138: SIR; 167–169: SKK) (by ScanProsite, ExPASy). To confirm the protein-protein interaction and to investigate the binding domain within c-Yes, SH3-SH2-PTK, SH2-PTK, and PTK were constructed (Fig. 1). Immunoprecipitation and GST pull-down assay showed that QM protein binds to c-Yes in an SH3 domain-mediated manner (Fig. 2, A and B). QM did not bind to the SH2 or PTK domains (Fig. 2, A, lanes 3 and 4; B, lanes 2 and 3).

Two Regions of QM Protein Bind to c-Yes—To identify the c-Yes binding region of QM, N- and C-terminal deletion mutants were constructed and assayed (Fig. 1). From the computer analysis (Compute pl/Mw, ExPASy) of the QM protein, it was predicted that QM is highly basic (pl is greater than 11) and that there are four α-helices. N- and C-terminal-deleted subclones were designed to have at least one α-helix (pEBG-QM2, pEBG-QM3, pEBG-QM4, pEBG-QM3c, pEBG-QM2c, and pEBG-QM1c). Unexpectedly, all the QM fragments were associated with c-Yes except for the C-terminal region (Fig. 3, A, B, D, and E). This implies there is more than one binding site in the QM protein. To verify the more specific regions, several other QM subclones were constructed. N terminus (QM1: 1–49) and a second α-helix-containing region (QM23: 97–141) were found to be associated with c-Yes (Fig. 3, B and C). The N-terminal region (QM1: 1–49) contains some SH3 binding candidate peptides, but QM23 shows no previously reported SH3 binding amino acid sequences or motifs. The SH3 domain is one of the well established protein binding domains, and several consensus amino acid sequences have been reported that normally contain proline-rich residues, such as PXXP (9). The QM protein sequence analysis showed that there are 8 proline residues, but there are no conserved SH3 binding sequences within the two SH3 binding regions of the QM protein. PYP (aa 16–18) of the QM protein is the only proline-rich site, although from several crystallographic studies it has been proved that a PYP amino acid fragment is not suited for SH3 hydrophobic pockets. There should be two amino acids between prolines for matching the proline-rich binding groove of the SH3 domain (29, 30). The most feasible SH3 binding peptide consensus of QM protein is the RPACYR (aa 4–10) fragment. Even though it has been reported that the PXXY motif is bound to the SH3 domain of Epas8 (31), the aspartic acid of the motif is a critical amino acid to bind to the SH3 domain. For the replacement of the aspartic acid with cysteine, the RPACYR peptide might not perform as a binding motif of SH3 like PXXYD. One other study gave the more noteworthy result that the VPMRLR peptide of YAP (Yes-associated protein) interacts with the SH3 domain of p53BP-2 (p53-binding protein-2) (32). The SPOT test has revealed that the critical amino acids for binding to the SH3 domain are the valine, proline, and the italic arginine, and they are conserved in the RPACYR peptide except for the valine. This study also showed binding specificity of the SH3 domain with the VPMRLR peptide. The SH3 domain of p53BP-2 has two unique amino acids, Tyr\textsuperscript{576} and Leu\textsuperscript{580} and they are required for the interaction between the VPMRLR peptide and the SH3 domain in p53BP-2. However neither the Tyr nor the Leu is conserved in the SH3 domain of c-Yes. From these results, the QM protein might not bind to the SH3 domain through the PXXP motif or other previously reported consensus sequences. The intensity of protein-protein associations did not show any significant difference between the two binding regions of QM according to Western blotting.

QM Protein Also Interacts with Other Src Family Members—QM protein and c-Yes bound under native conditions (Fig. 4, A and B). Not only did QM protein interact with c-Yes, but also with other Src family members, such as Lyn, Lck, and Src. We tried several times to detect associations between QM and GST-fused SH3 domains of Src family members, and all the SH3 domains bound to QM proteins although binding intensities were different (Fig. 4, C and D). The SH3 domain of Src showed similar binding intensities compared with the SH3 of Yes, and the SH3 domains of Fyn gave stronger signals than that of Yes. The SH3 domains of Lyn and Hck showed weaker binding intensities than other Src family members. We could not always detect a signal between the SH3 of Lyn and QM protein.

Co-localization of c-Yes and QM Protein in Several Tumor Cell Lines—Because the binding assays showed that QM protein and c-Yes were co-localized, we tried to prove the correlation between them in vivo. Immunofluorescence data indicate that QM proteins are highly expressed in various tumor cell lines, and most of them are localized within the cytoplasm (Fig. 5, column 1). Our data show the localization of QM and c-Yes proteins overlap in the cytoplasm (Fig. 5, column 3). Several other cell lines (HT29, KatoIII, SNU5, SNU16, and SW480) showed the same results (data not shown).

QM Protein Suppresses c-Yes Kinase Activity—Because SH3 is known to be an important domain in Src family kinase regulation, we wanted to ascertain how QM protein affects the
kinase activity of c-Yes. As shown in Fig. 6A, SH3 domain-containing c-Yes was significantly suppressed by QM, whereas SH3-deleted SH2-PTK and PTK were not affected by QM in their kinase activity. For the more specific effects of QM on c-Yes, GST-fused QM was purified, and in vitro kinase assays were performed. There was an 80% reduction of relative c-Yes kinase activity when 5 μg of GST-QM was incubated with 15 μl of FLAG-c-Yes-bound anti-FLAG-agarose (Fig. 6B).

The C Terminus of QM Protein Is Also Important for Suppressing c-Yes Kinase Activity—To identify which QM domains play a key role in c-Yes kinase suppression, subcloned QM derivatives were cotransfected with c-Yes, and kinase assays were conducted. The C-terminal region (144–213) of the QM protein showed no suppression activity of c-Yes (Fig. 6C, lane 3). In vitro assays were conducted with all the other subclones to confirm this result. Every subcloned protein was obtained from E. coli and purified. Unexpectedly, the two binding regions of the QM protein did not fully contribute to the suppression of c-Yes activity when assayed independently (Fig. 7, columns 3 and 8). More particularly, QM1 and QM23, which include SH3 binding regions, reduced the kinase activity of c-Yes by less than 30% individually, and when working in concert, they suppressed it up to 50%. The C terminus of QM did not function effectively in preventing the kinase activity of c-Yes by itself (Fig. 7, column 10). However, QM2c and QM14, which contain the C terminus of QM, inhibited activity by more than 60 and 40%, respectively. The full-length QM protein restricted c-Yes activity by 80%. Our results presented here support the contention that the C terminus of QM is an important region for the suppression of c-Yes activity. However it functions more effectively when the C terminus of QM is accompanied by the N terminus (QM1: 1–49) or the middle (QM23: 97–141) section of the QM protein than when the C terminus of QM works alone, and both binding domains are required for full suppression of kinase activity (Fig. 7, columns 3–6 and 10–12). The reason why the C terminus of QM properly affects c-Yes with the two SH3 binding regions of QM is that the C terminus of QM does not directly bind to c-Yes alone. Only on one occasion could we detect a weak interaction between the C terminus of QM and c-Yes in binding assays (Fig. 6B).

Fig. 3. The SH3 domain of c-Yes is associated with two regions of QM protein. Subcloned QM in pEBG and pFLAG-c-Yes were cotransfected into the BOSC 23 cell line and detected with anti-c-Yes antibody. A, C-terminal deletion studies showed that the N terminus of the QM protein is associated with the c-Yes. B, data showed that the C terminus (144–213) and the acidic domain (52–90) of the QM protein did not interact with c-Yes. C–E, pFLAG-c-Yes and QM-subcloned pEBG vectors were cotransfected, and immunoprecipitation was performed with anti-c-Yes and detected with anti-GST antibody (Amersham Biosciences) by Western blot. C, lane 1, N terminus of QM bound to c-Yes; lane 2, acidic domain was not detected; lanes 3 and 4, glycine/alanine loop bound to c-Yes. D, N-terminal deletion assay of QM. Lanes 1 and 2, glycine/alanine loop bound to c-Yes; lane 3, weak interaction was detected between QM C terminus and c-Yes. E, C-terminal deletion assay of QM. Lane 1, GST was detected (pEBG-transfected cell lysate); lane 2, GST did not associate with c-Yes; lanes 3–6, N terminus of QM bound to c-Yes. F and G, pEBG-QM derivatives and pFLAG-c-Yes-cotransfected cell lysates were probed by anti-GST antibody (F, Anygen, Korea) or by anti-c-Yes antibody (G).
**Effect of Ribosomal Protein QM on c-Yes**

**DISCUSSION**

In this study, we have screened the HeLa cell cDNA library with c-Yes protein and found several c-Yes-interacting proteins. One of them was QM, known as a key protein involved in the cytoplasm. Co-immunofluorescence was performed, and red-stained cells show that most of the QM protein exists in cytoplasm, and c-Yes proteins were detected by green fluorescence. Yellow indicates co-localizations of QM and c-Yes protein in the cytoplasm.

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Effect of Ribosomal Protein QM on c-Yes

Fig. 6. QM protein suppresses c-Yes kinase activity. A, pEBG-QM or pEBG were cotransfected with each c-yes subcloned pFLAG vectors, and kinase assays were performed with purified c-Yes kinase by anti-FLAG antibody agarose from BOSC 23 cell lysates. c-Yes kinase activity was reduced by QM. Black bars (■) represent the kinase activity in which c-yes and its derivatives were cotransfected with pEBG for controls. Other bars represent the kinase activity in which the pEBG-QM and c-yes subclones were cotransfected. The following indicate cotransfected derivatives: Column 1, pFLAG-c-Yes and pEBG-QM; Column 2, pFLAG-SH2-PTK and pEBG-QM; Column 3, pFLAG-PTK and pEBG-QM. B, c-Yes kinase assay with dose depending on QM protein. Each lane shows the following amount of purified GST or GST-QM added to 15 μl of c-Yes bound anti-FLAG-agarose. Lane 1, 5 μg of purified GST; lanes 2-9, 0-7 μg of GST-QM. C, pFLAG-c-Yes was cotransfected with each N terminus-deleted QM subclone, and c-Yes kinase activity was assayed. More than five individual experiment sets were performed. Control shows kinase activity without QM and its derivatives. Results depict the average of more than three valid data points, p < 0.01.

Fig. 7. The kinase activity assays of c-Yes, SH2-PTK, and PTK with QM protein derivatives. c-Yes, SH2-PTK, and PTK were purified from cotransfected BOSC 23 cell lysates. The first, second, and third line of each column represents the autophosphorylation of PTK, SH2-PTK, and c-Yes, respectively, and 5 μg of the following purified proteins were added for the kinase inhibition assay. Column 1, GST; column 2, control; column 3, GST-QM1; column 4, GST-QM2; column 5, GST-QM3; column 6, GST-QM4; column 7, GST-QM12; column 8, GST-QM33; column 9, GST-QM14; column 10, GST-QM3c; column 11, GST-QM2c; column 12, GST-QM; [], PTK; ■, SH2-PTK; E3, c-Yes). More than three individual experiment sets were performed. Results represent the average of more than three valid data points, p < 0.01; **, p < 0.05 (analysis of variance).

Binding of 40 and 60 S ribosomal subunits (34). From our binding assays, it has been revealed that two regions of the QM protein are bound to the SH3 domain of c-Yes (Fig. 2, A and B). According to sequence analysis of the QM protein, the RPARC-YR (aa 4–10) peptide is the most plausible consensus for the interaction between the QM and c-yes proteins. Even though the reported environments of the RPARC-YR peptide and the SH3 domain of c-Yes did not exactly satisfy each of the binding conditions of the PXXDY peptides or the VPMRLR peptide, we can assume that the concerted conditions of both peptides can be applied to the RPARC-YR peptide. It means that the specificity of the RPARC-YR peptide might be lower than the PXXDY motif or the VPMRLR peptide. Even though QM also associated with other Src family members, their binding intensities varied. Sequence similarities among the SH3 domains can imply the binding strengths between the QM protein and SH3 domains. The SH3 of Src, Yes, and Fyn shows more similar sequence homologues than those of Lyn or Hck, and our data showed that the SH3 domains of Lyn and Hck produced weaker binding strengths than those of the others. However, the conserved binding regions of SH3 are the same except for the case of Lyn. This means that other factors also contribute to binding strength.

Recently, several studies have tried to elucidate the effect of substrate binding in kinase activities. One of the most interesting proteins is the Nef protein of HIV-1, because it has been revealed that Nef induced kinase activity in Hck when Nef is associated with the SH3 domain of Hck (35). QM also binds to the SH3 domain of c-Yes but the QM protein showed inhibition of c-Yes activity in contrast to Nef and Hck. The binding of the QM protein to the SH3 domain is important in the inhibition of c-Yes kinase activity, although even more interesting is the fact that the binding region has to be accompanied by the C terminus (144–213) of QM for the complete suppression of c-Yes activity. Although the C terminus of QM was predicted to have a random coiling status, the C terminus was required to block
the kinase activity. Therefore, we suggest that because of the C-terminal flexibility, when QM binds to the SH3 domain of c-Yes, the C terminus of QM might be closely located to the PTK domain and stabilize the c-Yes protein in the inactive conformation.

Our data suggested that kinase activity and autophosphorylation assays were compatible and that these results clarify understanding of how QM protein regulates c-Yes kinase. In Hck, the Nef protein binds to the SH3 domain and helps induce conformational changes in Hck to easily coordinate ATP (35, 36). This means that conformational changes leading to stability for autophosphorylation induce the activation of Hck when other proteins are associated with the SH3 domain. It can be easily assumed that ligand binding to the SH3 or SH2 domain induces disruption of the inactive state. On the contrary, to stabilize a kinase in the inactive form, QM binding to the SH3 domain has to reduce c-Yes conformational change or disturb active site opening. Such a mechanism was also proven by our in vivo and in vitro studies. The C terminus of QM protein brought about inhibition of autophosphorylation with support of the SH3 binding domains (QM1 and QM23) of the QM protein (Fig. 8D). We also performed time-dependent assays. Every 10 min, QM proteins were added during autophosphorylation assays, confirming that the QM addition kept c-Yes from further autophosphorylation (data not shown). From these results, we can suggest that the blocking of autophosphorylation by the QM protein results in suppression of the kinase activity of c-Yes and this might be caused by the conformational change of c-Yes preventing ATP introduction with the support of the C terminus and SH3 binding domains of the QM protein.

The data showing the suppression and autophosphorylation inhibition of c-Yes by QM derivatives also indirectly illuminate the QM protein and SH3 domain binding mechanism whereby QM1 and QM23 would not bind to the same site of SH3 of c-Yes. If QM1 and QM23 were associated with the same site of the SH3 domain of c-Yes, they should compete with each other. This would result in similar levels of suppression of kinase activity and autophosphorylation between the incubation of QM1 or QM23 with c-Yes and the incubation of QM3 or QM4 with c-Yes, even though QM3 and QM4 contain two SH3 binding regions of the QM protein. However, our data contradicted the above prediction, and QM3 and QM4 induced 1.5-fold more suppressed c-Yes activity and autophosphorylation than did QM1 or QM23 (Fig. 7, columns 3, 5, 6, and 8 and Fig. 8C). This means that QM1 and QM23 influence c-Yes in their own way, and the binding sites on SH3 might be different as well. Therefore, we suggest two steps in the mechanism of QM and c-Yes interaction: (i) QM holds the c-Yes protein with two different SH3 binding domains, and this initially stabilizes c-Yes in an inactive form. (ii) Finally, the C-terminal region of QM, close to the PTK domain, helps to keep c-Yes in an inactive conformation and completes the blocking of ATP access. To clarify the

\[ \text{FIG. 8. QM protein inhibits c-Yes autophosphorylation.} \]
\[ \text{QM protein inhibits c-Yes autophosphorylation. A, lane 1, GST-QM1; lane 2, GST-QM2; lane 3, GST-QM3; lane 4, GST-QM4; lane 5, GST-QM; lane 6, control. B, SH2-PTK and PTK autophosphorylations were not inhibited as much as c-Yes. C, in vitro autophosphorylation inhibition. The first, second, and third line of each column represents the autophosphorylation of c-Yes, SH2-PTK, and PTK, respectively. Each column represents incubations with the following proteins: column 1, GST; column 2, control; column 3, GST-QM1; column 4, GST-QM2; column 5, GST-QM3; column 6, GST-QM4; column 7, GST-QM; column 8, GST-QM23. Control shows kinase autophosphorylation without QM protein. D, in vivo autophosphorylation inhibition. First, second, and third line of each column represent c-Yes, SH2-PTK, and PTK, respectively. Each line of the column shows that pFLAG-c-Yes, pFLAG-SH2-PTK, or pFLAG-PTK and the following vectors were cotransfected: column 1, pEBG; column 2, control; column 3, pEBG-QM; column 4, pEBG-QM1c; column 5, pEBG-QM2c; column 6, pEBG-QM3c. More than three individual experiment sets were performed. Results represent the average of more than three valid data points, } \]
\[ p < 0.01; **, p < 0.05 \text{ (analysis of variance).} \]
suppression mechanism, a study of conformational changes is required. For the identification of binding sites between QM and SH3, more specific studies have to be carried out, such as point mutations and/or SPOT tests using synthesized peptides of QM and c-Yes.

From previously reported experiments, QM has become known as a candidate for playing a key role in cell signaling and development (37, 38). Since the QM protein exists in the cytoplasm (Fig. 5) and c-Yes is one of the membrane proteins (39, 40), QM protein presumably comes into contact with and influences c-Yes in vivo. In the case of QM overexpression, not only c-Yes protein but c-yes mRNA levels increased around 20–40% compared with the control (Fig. 10, A and B). This result demonstrates that the QM protein also has an impact on c-Yes within cells, and we would like to suggest two explanations for this phenomenon. One is that highly expressed QM influences c-Yes activity by QM protein for the maintenance of signal transduction. The other explanation is that c-Yes is required more to minimize the effect of suppressed ribosomal subunit aggregation (19). The other explanation is that c-Yes is required more to minimize the effect of suppressed ribosomal subunit aggregation (19). The other explanation is that c-Yes is required more to minimize the effect of suppressed ribosomal subunit aggregation (19). The other explanation is that c-Yes is required more to minimize the effect of suppressed ribosomal subunit aggregation (19). The other explanation is that c-Yes is required more to minimize the effect of suppressed ribosomal subunit aggregation (19). The other explanation is that c-Yes is required more to minimize the effect of suppressed ribosomal subunit aggregation (19).

It is clear that the QM protein also has an impact on c-Yes within cells, and we would like to suggest two explanations for this phenomenon. One is that highly expressed QM influences c-Yes activity by QM protein for the maintenance of signal transduction. The other explanation is that c-Yes is required more to minimize the effect of suppressed ribosomal subunit aggregation (19). The other explanation is that c-Yes is required more to minimize the effect of suppressed ribosomal subunit aggregation (19). The other explanation is that c-Yes is required more to minimize the effect of suppressed ribosomal subunit aggregation (19). The other explanation is that c-Yes is required more to minimize the effect of suppressed ribosomal subunit aggregation (19).

Even though further studies are needed to clarify the precise effects and exact mechanism of the interaction between Src family members and the QM protein, our study has presented evidence that the QM protein and c-Yes have a close interrelationship in vitro and in vivo.

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