THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 52, Issue of December 26, pp. 52188–52194, 2003
Printed in U.S.A.
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Physical and Functional Interaction between Hck Tyrosine Kinase and Guanine Nucleotide Exchange Factor C3G Results in Apoptosis, Which Is Independent of C3G Catalytic Domain*

Received for publication, September 26, 2003
Published, JBC Papers in Press, October 9, 2003, DOI 10.1074/jbc.M310656200

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The hematopoietic cell kinase Hck is a Src family tyrosine kinase expressed in cells of myelomonocytic lineage, B lymphocytes, and embryonic stem cells. To study its role in signaling pathways we used the Hck-SH3 domain in protein interaction cloning and identified C3G, the guanine nucleotide exchange factor for Rap1 and R-Ras, as a protein that associated with Hck. This interaction was direct and was mediated partly through the proline-rich region of C3G. C3G could be co-immunoprecipitated with Hck from Cos-1 cells transfected with Hck and C3G. C3G was phosphorylated on tyrosine 504 in cells when coexpressed with Hck but not with a catalytically inactive mutant of Hck. Phosphorylation of endogenous C3G at Tyr-504 was increased by treatment of human myelomonocytic THP-1 cells with mercuric chloride, which is known to activate Hck tyrosine kinase specifically. Coexpression of Hck with C3G induced a high level of apoptosis in many cell lines by 30–42 h of transfection. Induction of apoptosis was not dependent on Tyr-504 phosphorylation or the catalytic domain of C3G but required the catalytic activity of Hck. Using dominant negative constructs of caspases we found that caspase-1, -8, and -9 are involved in this apoptotic pathway. These results suggest that C3G and Hck interact physically and functionally *in vivo* to activate kinase-dependent and caspase-mediated apoptosis, which is independent of catalytic domain of C3G.

The Src family tyrosine kinases play an important role in linking signals received by transmembrane receptors and a variety of intracellular pathways, thereby regulating diverse cellular responses such as proliferation, differentiation, and cell death (1, 2). This is achieved through their non-catalytic sequences, which enable multiple interactions with cellular proteins, and through the kinase domain, which phosphorylates substrates to alter their activity, change their subcellular location, and effect their intermolecular interactions. The hematopoietic cell kinase (Hck)[1] is a Src family member that is expressed in cells of myelomonocytic lineage, B lymphocytes, and embryonic stem cells with higher levels in differentiated cells, suggesting a role for this enzyme in signaling pathways of mature hematopoietic cells (3–5). Hck is activated by agents that induce macrophage differentiation and in response to cytokines such as interleukin-3, granulocyte-macrophage colony stimulating factor, and leukemia inhibitory factor. It is also involved in cytokine production in macrophages in response to lipopolysaccharide and viral infection (6–9).

Structurally, Hck is similar to other members of the Src family in that it has a catalytic domain at the C terminus that is preceded by a 100-amino acid SH2 domain and a 50-amino acid SH3 domain. The SH2 and SH3 domains are protein interaction modules that mediate either intramolecular or intermolecular associations. SH2 domains bind to phosphorylated tyrosine residues in a specific amino acid context, whereas SH3 domain interacts with polyproline tracts in polypeptides with optimal affinity for P*XX*P. Intramolecular interaction of the SH2 domain with the C-terminal phosphotyrosine and SH3 domain with proline sequences present between the catalytic domain and SH2 domain keep the Hck molecule in a closed inactive configuration (10). Dephosphorylation of C-terminal tyrosine or binding of ligands to SH2 or SH3 domains results in its activation. The Hck gene encodes two polypeptides (p61 and p59 in human cells and p59 and p56 in murine cells) that are derived from alternate translation initiation sites (11).

C3G is an ubiquitously expressed 140-kDa protein with a C-terminal catalytic region that has guanine nucleotide exchange factor activity toward Rap-1, Rap-2, and R-Ras (12–16). The central region has four proline-rich motifs that have been shown to interact with the SH3 domain of the adaptor molecule Crk (12, 13). The N-terminal one-third of the molecule has been less well defined. Various isoforms of C3G arising by alternate splicing of the primary transcript exist that show tissue and cell type-specific expression (17, 18). Through its ability to activate Rap1, C3G has been shown to counteract signaling through the Ras/mitogen-activated protein kinase pathway and has also been shown to transmit signals through the stress kinase c-Jun NH_2-terminal kinase pathway (19). C3G knockout mice are embryonic-lethal. Fibroblasts deficient of C3G function show impaired cell adhesion, delayed cell spreading, and accelerated cell migration, suggesting the requirement of C3G-dependent activation of G-proteins for adhesion and for early embryonic development (20, 21). Integrin-mediated cell adhesion leads to transient increase in tyrosine phosphorylation of C3G, but the identity of the kinase or site of phosphorylation are not known (22). Upon coexpression of CrkI, C3G is activated by phosphorylation of Tyr-504, but the kinase mediating this phosphorylation has not been identified (23). Recently

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[1] The abbreviations used are: Hck, hematopoietic cell kinase; SH, Src homology; GST, glutathione *S*-transferase; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; GFP, green fluorescent protein.

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growth hormone-induced activation and phosphorylation of C3G has been shown to require c-Src and Jak2 kinases (24). C3G shows transformation suppression activity that does not require its catalytic activity but requires non-catalytic sequences (25).

In the present study we have identified C3G as a protein that interacts with Hck physically and functionally *in vivo*. We have analyzed the structural requirements and some of the functional consequences of the interaction of Hck with C3G. This interaction resulted in Hck catalytic activity-dependent activation of an apoptotic pathway mediated by caspases.

EXPERIMENTAL PROCEDURES

Antibodies—C3G antibody was obtained from Santa Cruz Biotechnology Inc. It was a rabbit polyclonal antibody that recognized the C-terminal 19 amino acids of human C3G. Phosphotyrosine antibody was a mouse monoclonal antibody, PY20, from Santa Cruz. Hck polyclonal antibody from Santa Cruz recognized an epitope in the unique domain of Hck. A mouse monoclonal 3E9, which recognizes SH3 domain of Hck, was made in our laboratory (26). P-C3G (Tyr-504) antibodies were SC-12926, a goat polyclonal, and SC-12926R, a rabbit polyclonal, from Santa Cruz, which recognize human C3G phosphorylated on tyrosine 504.

Expression Vectors—Mutants of human C3G, ΔN-C3G lacking the N-terminal 579 amino acids, ΔC-C3G lacking the catalytic domain of C3G, and the Y504F mutant of C3G in which tyrosine 504 is mutated to phenylalanine were kindly provided by Dr. M. Matsuda, Department of Pathology, Research Institute, International Medical Centre of Japan, Tokyo, and have been described by Ichiba *et al.* (23). Full-length human C3G cloned in pcDNA3-FLAG was kindly provided by Dr. S. Tanaka (27). Rat Hck cDNAs cloned in pCI plasmid (Promega), which drives expression under the control of cytomegalovirus promoter, have been described (28). The $p59^{hck}$ construct expressed both p59 and p56 isoforms. A catalytically inactive mutant of p59 Hck (mHck) construct was made by mutating lysine 267 to arginine in $p59^{hck}$ construct using a PCR-based site-directed mutagenesis approach. This vector expressed catalytically inactive Hck proteins. Clone C-9 of rat C3G has been described previously (17). Clone C-9 and its fragments were cloned in the EcoRI site or BamHI site of pGEX vectors to produce GST fusion proteins. Human procaspase-1 cDNA (α-form) cloned in $pCB6^{+}$ expression vector and mutant procaspase-1 prepared by replacing Cys-285 (TGC) with Ala (GCC) have been described (29). The nucleotide sequence of the mutant Hck cDNA and other constructs was confirmed by automated sequencing. Plasmid expressing caspase-9s, a dominant negative inhibitor of caspase-9, was a gift from Dr. D. W. Seol (30). The green fluorescence protein (GFP) expression plasmid pEGFP-N1 was from Clontech.

Cell Culture, Transfections, and Immunofluorescence Staining—Cos-1, HeLa, MCF-7, and J774 cells were grown as monolayers in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics in a humidified 37 °C incubator with 5% CO_2. THP-1 human myelomonocytic cell line was grown in RPMI with 10% heat-inactivated fetal calf serum. Transient transfections were done using Qiagen column-purified plasmids and LipofectAMINE reagent (Invitrogen) according to the manufacturer's protocol and as described previously (31). For immunofluorescence staining, cells grown on coverslips were transfected with the required plasmids, fixed after 30–42 h, and stained with the required antibodies as described earlier (31). Dual labeling for Hck and C3G was performed by incubating the cells serially with C3G antibody, anti-rabbit Cy3, mouse monoclonal anti-Hck, and anti-mouse fluorescein isothiocyanate.

Treatment of Cells with Mercuric Chloride—THP-1 cells were grown in RPMI medium with 10% heat-inactivated fetal calf serum. These cells were differentiated to a macrophage lineage by the addition of 20 ng ml^{-1} 12-*O*-tetradecanoylphorbol-13-acetate for 48 h. Differentiated cells were subjected to $HgCl_2$ treatment as described by Robbins *et al.* (32). Briefly, the cells were washed with phosphate-buffered saline and treated with 0.5 mM $HgCl_2$ for 15 min at room temperature before preparation of whole cell lysates in SDS sample buffer for Western blotting.

Apoptosis Assays—Quantitative analysis of apoptotic cells was carried out essentially as described previously (28, 33). Cells grown on coverslips were transfected with the required plasmids and processed for immunofluorescence staining using appropriate antibodies. Cells were mounted in 90% glycerol containing 1 mg/ml *para*-phenylenediamine (antifade) and 0.5 μg/ml DAPI (4′6-diamidino-2-phenylindole) to stain the DNA. Cells showing immunofluorescence staining were counted, and those cells that showed loss of refraction, condensed chromatin, apoptotic bodies, or cell shrinkage were scored as apoptotic. At least 200 expressing cells were counted in each coverslip. The data represent the mean ± S.D. from at least three independent experiments. Cells not expressing the transfected protein were also counted in each coverslip. Generally 1–3% of non-expressing cells showed apoptosis. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay was carried out as described previously (28). Apoptosis assays in J774 cells were performed by cotransfecting expression plasmid for EGFP with Hck and C3G plasmids and observing the phenotype of GFP-expressing cells.

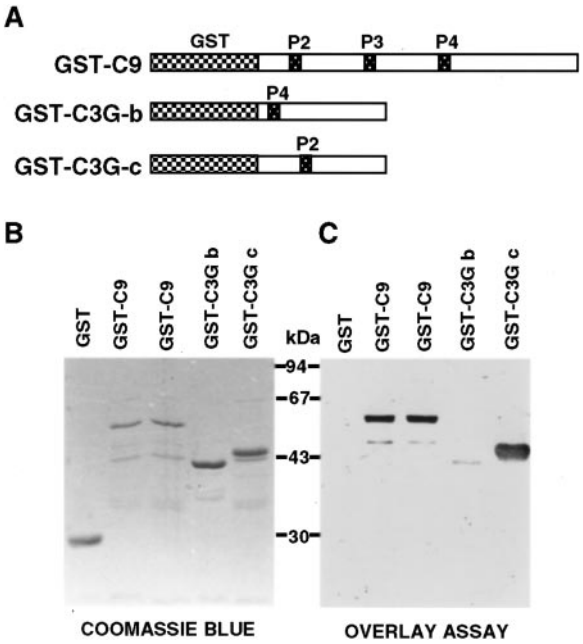

FIG. 1. **Interaction of SH3 domain of Hck with proline-rich region of C3G.** *Panel A*, schematic presentation of GST-C3G fusion proteins. P2, P3, and P4 represent proline-rich regions. *Panel B*, Coomassie Blue-stained SDS-PAGE gel showing various GST fusion proteins used for overlay assay. *C*, overlay assay showing interaction of GST-C3G fusion proteins with SH3 domain of Hck. GST (*lane 1*) was used as a control. For the overlay assay, proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membrane was incubated with purified GST-SH3 fusion protein and then with a monoclonal antibody that recognizes SH3 domain of Hck.

Overlay Assay and Western Blotting—GST and GST fusion proteins were expressed in *Escherichia coli* by pGEX vectors and purified using the glutathione-agarose affinity method as described (34). Purified GST fusion proteins were resolved in SDS-PAGE and then transferred onto nitrocellulose membrane. The blot was blocked overnight at 4 °C with 2% bovine serum albumin containing 1 mM dithiothreitol made in TBSTG (10 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 0.05% Tween 20, 0.2% gelatin). The blot was then incubated for 1.5 h with purified GST-SH3 fusion proteins (30–50 μg/ml) of Hck. After this the blot was washed in TBSTG for 15 min (3 washes each for 5 min). The blot was then processed with primary antibody against SH3 domain of Hck followed by secondary antibody as described for Western blotting (26, 31). Detection of primary antibodies was either by ECL or by color development using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate for alkaline phosphatase-conjugated secondary antibodies.

Immunoprecipitation and in Vitro Phosphorylation—Whole cell lysates were prepared by extracting with 2× IP buffer (40 mM Tris, pH 7.2, 2% Triton X-100, 1% sodium deoxycholate, 300 mM sodium chloride, 2 mM phenylmethylsulfonyl fluoride, 2 μg/ml protease inhibitors, soybean trypsin inhibitor, leupeptin, and aprotinin) for 15 min on ice. The cell debris was removed by spinning at 10,000 rpm for 15 min at 4 °C. The supernatant was diluted 1:1 with water and incubated with the antibody for 1 h. After this, protein A-agarose beads were added, and the incubation was carried out for one more hour. The beads with the antibody attached to them were pelleted by spinning at 4000 rpm for 2 min and washed with 1× IP buffer three times and either boiled with 1× sample buffer for Western blot or processed for kinase assay.

Immunoprecipitated proteins attached to the protein A-agarose

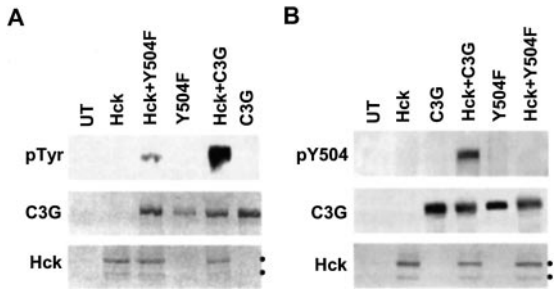

FIG. 2. **Phosphorylation of C3G at Tyr-504 by coexpression of Hck.** Cos-1 cells were transfected with various expression vectors as indicated, and after 24 h of transfection whole cell lysates were used for Western blotting with either antiphosphotyrosine antibody (*panel A*) or antibody against Tyr-504-phosphorylated C3G (*panel B*). The same blots were reprobed with C3G and Hck antibodies to show their expression levels. Anti-Tyr(P) (*pTyr*) and Tyr(P)-504 (*pY504*) were detected by ECL, whereas Hck and C3G were detected by color development using alkaline phosphatase-conjugated secondary antibodies. The *dots* indicate the p59 and p56 isoforms of Hck. *UT*, untransfected cells.

beads were phosphorylated in kinase buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 2 μg/ml protease inhibitors soybean trypsin inhibitor, leupeptin, aprotinin), 1 mM phenylmethylsulfonyl fluoride, and 2 μM[γ-^{32}P] ATP in a total volume of 50 μl for 1 h on ice. The reaction was stopped by adding 25 μl of 3× sample buffer and boiling for 5 min. The samples were then resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and exposed for autoradiography.

RESULTS

Interaction of SH3 Domain of Hck with C3G—In our screen for proteins that interact with the SH3 domain of Hck tyrosine kinase, we obtained a clone (C9) from a rat spleen cDNA library using protein interaction cloning strategy (17). This clone showed an open reading frame of 210 amino acids and the presence of 3 proline-rich motifs that corresponded with the proline-rich sequences 2, 3, and 4 (P2, P3, P4) of the guanine nucleotide exchange factor, C3G (12, 13). Earlier, these sequences were shown to mediate interaction with Crk (13). To analyze the interaction between Hck and C3G, different segments of clone C9 containing the proline-rich region P2 (GST-C3G-c), P4 (GST-C3G-b), and P2, P3, and P4 (GST-C9) were expressed as GST fusion proteins (Fig. 1, *A* and *B*). These fusion proteins, which were expressed to a comparable extent (Fig. 1*B*), were transferred to a membrane and incubated with purified GST-SH3 (Hck) fusion protein, and bound fusion proteins were detected using a monoclonal antibody against the Hck-SH3 domain. Hck-SH3 domain interacted with C3G sequences containing either P2 or P4 and also all three proline-rich regions, whereas no interaction was found with GST alone (Fig. 1*C*), suggesting that the Hck-SH3 domain is able to interact directly with the C3G proline-rich regions. The interaction with P2 was stronger than with P4, suggesting that interactions were specific and not because of overexpression.

Phosphorylation of C3G at Tyr-504 by Expression of Hck—Earlier it was shown that human C3G, which shows 87.3% amino acid identity to rat C3G, can complement C3G function in knockout mice (20). Human C3G is phosphorylated on tyrosine 504 upon coexpression of Crk1, and this modification enhances its exchange factor activity toward Rap1 (23). Because Hck-SH3 interacted with proline-rich regions of C3G, we explored the possibility of human C3G being a substrate of Hck *in vivo*. Cos-1 cells were transfected with various expression constructs, and whole cell lysates were blotted with antiphosphotyrosine antibody. It was seen that a 140-kDa protein that migrates similarly to C3G was phosphorylated on tyrosine when C3G was cotransfected with Hck (Fig. 2*A*). The 140-kDa protein detected by antiphosphotyrosine antibody is likely to be

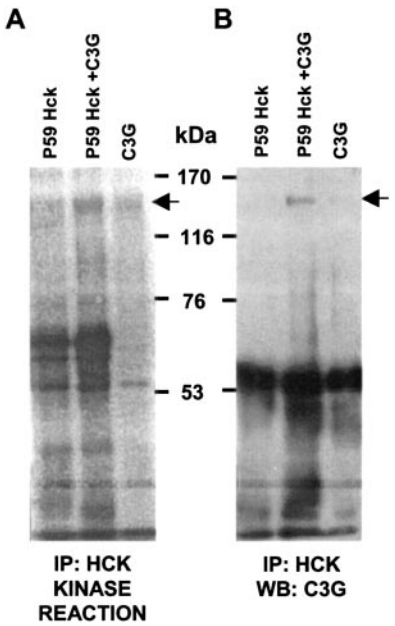

FIG. 3. **Interaction of C3G with Hck.** Cos-1 cells were transfected with indicated constructs, and the cell extracts were subjected to immunoprecipitation (*IP*) with Hck polyclonal antibody. *Panel A* shows *in vitro* phosphorylation of Hck immunoprecipitate, and *panel B* shows a Western blot (*WB*) with C3G antibody.

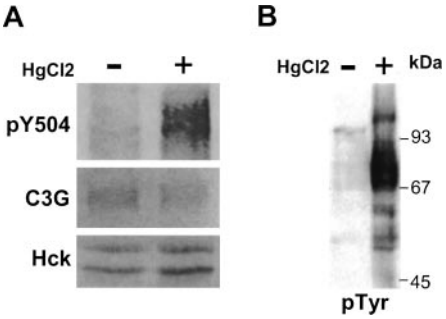

FIG. 4. **Increase in Tyr-504 phosphorylation of endogenous C3G upon activation of Hck.** Whole cell lysates were prepared from differentiated THP-1 cells with or without $HgCl_2$ treatment and subjected to Western blotting with antibodies for pTyr-504 (*pY504*)-C3G (*panel A*). The same blot was reprobed with C3G and Hck antibodies to detect their levels. *Panel B* is a Western blot showing phosphotyrosine content of cellular proteins upon $HgCl_2$ treatment.

C3G itself because very little phosphotyrosine was seen on the Y504F mutant of C3G when coexpressed with Hck (Fig. 2*A*). A lower level of phosphorylation of the Y504F mutant of C3G was not due to a lower level of expression because C3G and Hck levels were comparable, as determined by reprobing the blot with anti C3G and anti-Hck antibodies (Fig. 2*A*).

These results suggested that C3G was phosphorylated on tyrosine when coexpressed with Hck, and the major site of Hck-mediated phosphorylation on C3G was tyrosine 504. The phosphorylation of C3G at Tyr-504 was confirmed by Western blotting using two different antibodies specific to Tyr-504-phosphorylated C3G. As shown in Fig. 2*B* coexpression of Hck with C3G, but not with Y504F mutant of C3G, showed phosphorylation. This antibody (raised in goat, SC-12926) did not detect phosphorylation at other residues in C3G as seen by the total absence of a signal on Y504F C3G (Fig. 2*B*).

In vivo C3G could be a direct substrate of Hck or could be the substrate of another tyrosine kinase activated by Hck. We examined the ability of Hck to phosphorylate C3G *in vitro*. Cos-1 cells were transfected with either Hck, C3G, or Hck and

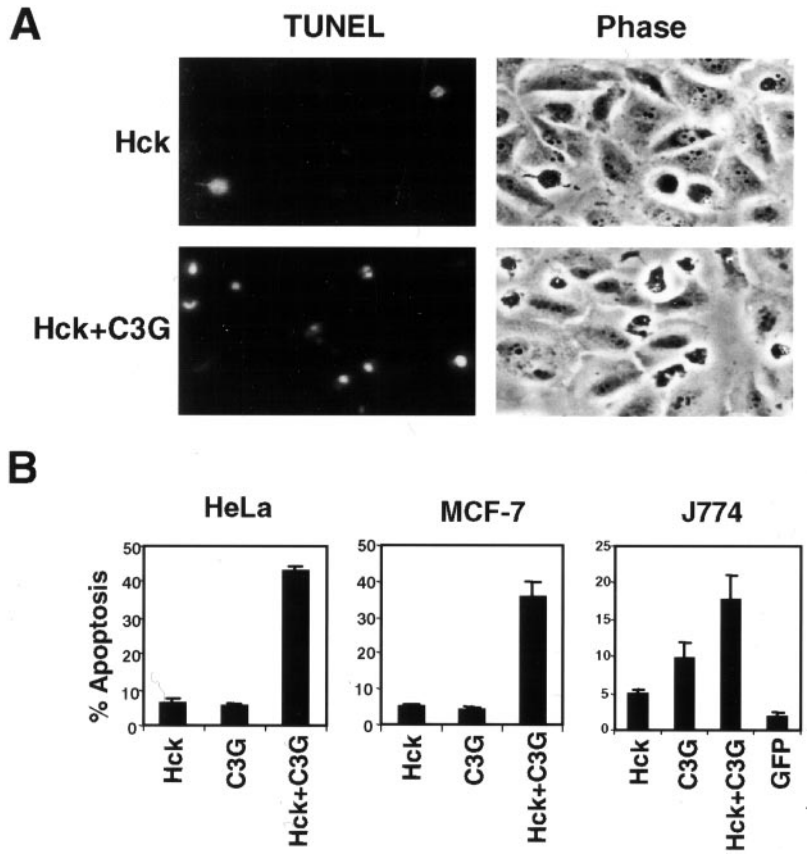

FIG. 5. **Induction of apoptosis by coexpression of Hck and C3G.** HeLa cells transfected with Hck or Hck and C3G were subjected to TUNEL staining after 42 h of transfection (*panel A*). HeLa and MCF-7 cells were transfected with the indicated constructs, and the extent of apoptosis was determined in expressing and non-expressing cells after staining with Hck and C3G antibodies after 42 h of transfection (*panel B*). J774 cells were transfected with Hck and C3G expression plasmids along with GFP. Cells were fixed after 30 h, and the extent of apoptosis was determined in GFP-expressing and non-expressing cells after staining with DAPI (4′6-diamidino-2-phenylindole). The percentage of apoptotic cells was calculated as the number of expressing cells with apoptotic morphology divided by the total number of expressing cells. A minimum of 200 expressing cells were counted from microscopic fields. Data represent mean ± S.D. of at least three independent experiments.

C3G, and lysates were immunoprecipitated using Hck antibody. The immunoprecipitates were subjected to *in vitro* kinase reactions (Fig. 3*A*) or blotted with anti C3G antibody (Fig. 3*B*). C3G was present in Hck immunoprecipitates, suggesting their interaction *in vivo* (Fig. 3*B*) and that it was phosphorylated in the Hck immunoprecipitate to some extent (Fig. 3*A*).

Phosphorylation of Endogenous C3G upon Activation of Hck—Treatment of myelomonocytic cells with mercuric chloride has been shown to specifically activate Hck, and this approach has been used to identify Hck substrates (32, 35). Lyn, the closest homolog of Hck among the Src family kinases is also expressed in these cells but is not activated by $HgCl_2$ (32). To find out whether endogenous C3G is phosphorylated upon activation of endogenous Hck we used the myelomonocytic cell line THP-1, which shows good expression of C3G protein. THP-1 cells were differentiated with 12-*O*-tetradecanoylphorbol-13-acetate for better expression of Hck and then subjected to $HgCl_2$ treatment to activate Hck. It was observed that there was a dramatic increase in Tyr-504 phosphorylation of C3G upon $HgCl_2$ treatment, as determined by Western blotting using phospho-C3G (Tyr-504)-specific antibody (Fig. 4*A*). The levels of C3G and Hck were unchanged under these conditions (Fig. 4*A*), but as expected, there was a large increase in the tyrosine phosphorylation of cellular proteins (Fig. 4*B*). Direct interaction between Hck and C3G in THP-1 cells could not be demonstrated by coimmunoprecipitation experiments even after treatment of cells with $HgCl_2$ (data not shown), indicating, therefore, that this interaction may be weak or transient.

Activation of Apoptosis by Coexpression of Hck and C3G—We explored the physiological consequence of Hck-C3G interaction by studying the effects of Hck and C3G coexpression on cell survival. HeLa cells were transiently transfected with Hck alone or with C3G and labeled using the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling) reagent after 42 h to detect apoptosis. It was observed that a larger number of cells that were cotransfected with Hck and C3G showed TUNEL positivity, suggesting that coexpression of these two molecules triggers a pathway leading to apoptosis (Fig. 5*A*).

To quantitate the extent of apoptosis, HeLa cells grown on coverslips were transfected with Hck and C3G and stained for Hck and C3G using specific antibodies. The stained cells were examined by microscopy, and it was observed that a large number of cells (about 45%) that expressed Hck and C3G showed apoptosis, as determined by morphological criteria such as shrinkage of cells, chromatin condensation, and fragmentation. Those cells that expressed only C3G or Hck did not show high level of apoptosis (Fig. 5*B*). Untransfected cells that did not express C3G or Hck showed only basal levels of apoptosis (3–4%). Expression of C3G with green fluorescence protein showed apoptosis only in about 6% of cells. Similar results were obtained in MCF-7 cells and the mouse macrophage cell line J774 (Fig. 5*B*). Apoptosis was assessed 30 h after transfection in J774 cells because a large number of apoptotic cells showed detachment from the coverslips at later points of time. The higher level of apoptosis induced by C3G alone in J774 cells may be due to the fact that these cells express Hck constitutively. These results suggest that coexpression of Hck and C3G leads to high levels of apoptosis.

Apoptosis induced by a variety of inducers is generally mediated by caspases (36). C3G- and Hck-mediated apoptosis was inhibited by cotransfection with a catalytic mutant of procaspase-1 in HeLa cells (Fig. 6). This mutant procaspase-1 inhibited apoptosis induced by expression of caspase-1 but not by caspase-3 or caspase-4 (29). Coexpression of catalytically inactive mutant of caspase-8 as well as a dominant negative isoform of caspase-9 (caspase-9s) also inhibited apoptosis induced by Hck and C3G. These results suggest that caspase-1, -8, and -9 are required for induction of apoptosis by C3G and Hck. Expression of C3G and Hck induced apoptosis in 36% of

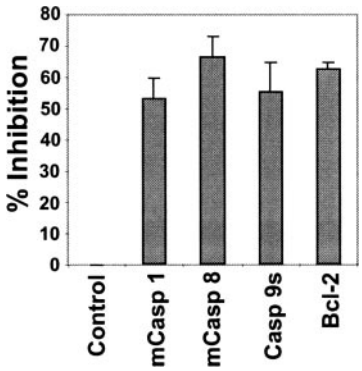

FIG. 6. **Inhibition of C3G and Hck induced apoptosis by expression of dominant negative forms of caspases.** HeLa cells were transiently transfected with p59 Hck and C3G along with either control plasmid, mutant caspase-1 (*mCasp-1*), mutant caspase-8 (*mCasp-8*), caspase-9s, or Bcl2. Extent of inhibition of apoptosis by the various constructs is depicted relative to the control plasmid taken as 0% inhibition.

MCF-7 cells (Fig. 4*B*). MCF-7 cells lack functional caspase-3 protein due to a deletion in the gene (37), indicating thereby that caspase-3 function is not an absolute requirement for induction of apoptosis by C3G and Hck. Coexpression of anti-apoptotic protein Bcl2 inhibited apoptosis induced by C3G and Hck (Fig. 6).

Catalytic Domain of C3G Is Not Required for Apoptosis Induced by Coexpression of Hck and C3G—We explored the possibility of an apoptotic pathway being induced as a consequence of Tyr-504 phosphorylation of C3G. For this purpose we compared the extent of apoptosis in cells expressing both Hck and Y504F mutant of C3G with those expressing Hck and wild type C3G. As seen in Fig. 7*B* there was no significant reduction in the number of apoptotic cells when Y504F C3G was expressed along with Hck, indicating that Hck-mediated Tyr-504 phosphorylation of C3G is not a requirement for triggering the apoptotic cascade. To identify whether the catalytic activity of C3G was required for Hck- and C3G-induced apoptosis, we quantitated the extent of apoptosis induced by two deletion mutants of C3G, one lacking the C-terminal catalytic domain (ΔC-C3G) and the other lacking the N-terminal 579 amino acids (ΔN-C3G) and possessing only one proline-rich sequence and the catalytic domain (Fig. 7*A*). Neither of these constructs induced apoptosis when expressed alone. As shown in Fig. 7*B*, we observed that apoptosis was very low when Hck was coexpressed with ΔN-C3G, but a large number (67%) of cells that coexpressed Hck and ΔC-C3G showed apoptosis. These results suggested that the catalytic sequences of C3G were not required, but the N-terminal half of the C3G, which has the proline sequences, was required for induction of apoptosis by coexpression with Hck.

Kinase Activity of Hck Is Required for Hck- and C3G-induced Apoptosis—Because Hck- and C3G-induced apoptosis was not dependent on Tyr-504 phosphorylation of C3G, we investigated the requirement of Hck catalytic activity for inducing apoptosis. A catalytically inactive mutant of p59 Hck (mHck), which showed similar expression and subcellular localization as wild type Hck but showed no antiphosphotyrosine staining (Fig. 8*A*), was used along with C3G in cotransfection experiments. Cells that coexpressed mHck and C3G showed a low apoptotic index (Fig. 8*B*), indicating the requirement of Hck catalytic activity for triggering apoptosis upon coexpression with C3G. C3G was not phosphorylated when coexpressed with mHck, showing the requirement of Hck catalytic activity for phosphorylation of C3G (Fig. 8*C*).

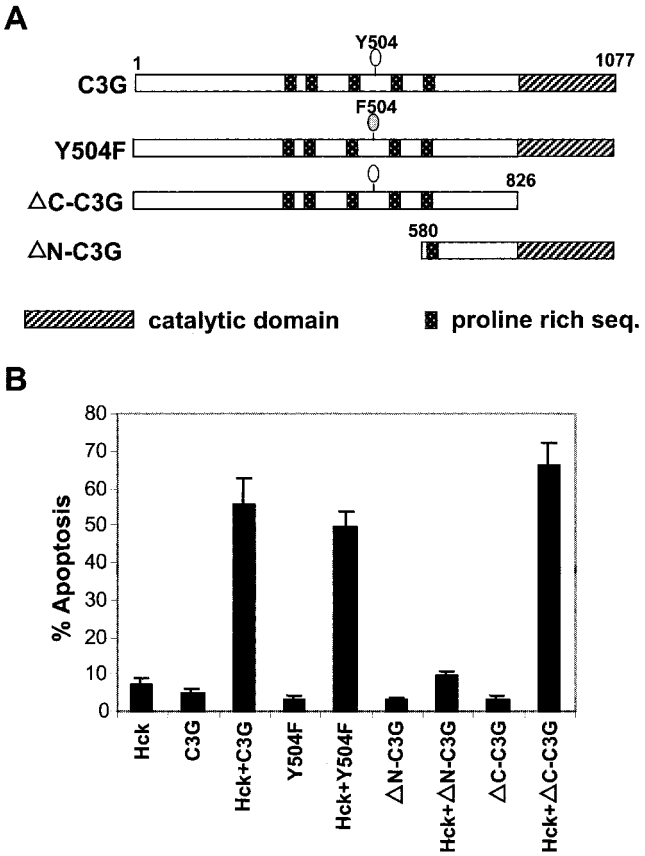

FIG. 7. **Induction of apoptosis by Hck and C3G expression is independent of C3G phosphorylation and its catalytic activity.** C3G, Y504F, ΔN-C3G, and ΔC-C3G (shown in *Panel A*, *line diagram*) were either transfected alone or with Hck in Cos-1 cells and stained for Hck, C3G, and DNA after 42 h of transfection. The percentage of apoptotic cells were scored among expressing and non-expressing cells using morphological criteria (*panel B*). Data represent the ± S.D. of at least three independent experiments after background subtraction.

DISCUSSION

Our results presented here show that Hck tyrosine kinase physically interacts with guanine nucleotide exchange factor C3G. This interaction may be mediated at least partly by the SH3 domain of Hck and proline-rich regions of C3G located in the central domain. The interaction between Hck and C3G appears to be very specific because it results in the phosphorylation of C3G predominantly at Tyr-504, although there are many tyrosines in C3G. Phosphorylation at this site has previously been shown to increase catalytic activity of C3G (23). Our results are consistent with the suggestion that Hck directly phosphorylates C3G *in vivo*. However, the possibility that Hck activates another tyrosine kinase, which then phosphorylates C3G, cannot be ruled out because *in vitro* phosphorylation of C3G by Hck was inefficient. A possible explanation for inefficient *in vitro* phosphorylation of C3G by Hck is that *in vivo* another factor (such as a protein) may be required for efficient phosphorylation of C3G by Hck. Bcr-Abl, Ras-GTPase-activating protein (GAP), Cbl, Wasp, ELMO, ADAM15, and STAT3 have been identified as substrates of Hck that interact through SH3 domain (35, 38–43). Thus, the interaction through SH3 domain may be a general feature of substrate recognition by Hck. The sequence surrounding the Tyr-504 of C3G does not have any acidic amino acids (IPSVPYAPFAA) toward the N terminus as required for recognition specificity of Src family kinases (44). However, to our knowledge the sequence requirement for phosphorylation by Hck tyrosine kinase has not yet been determined.

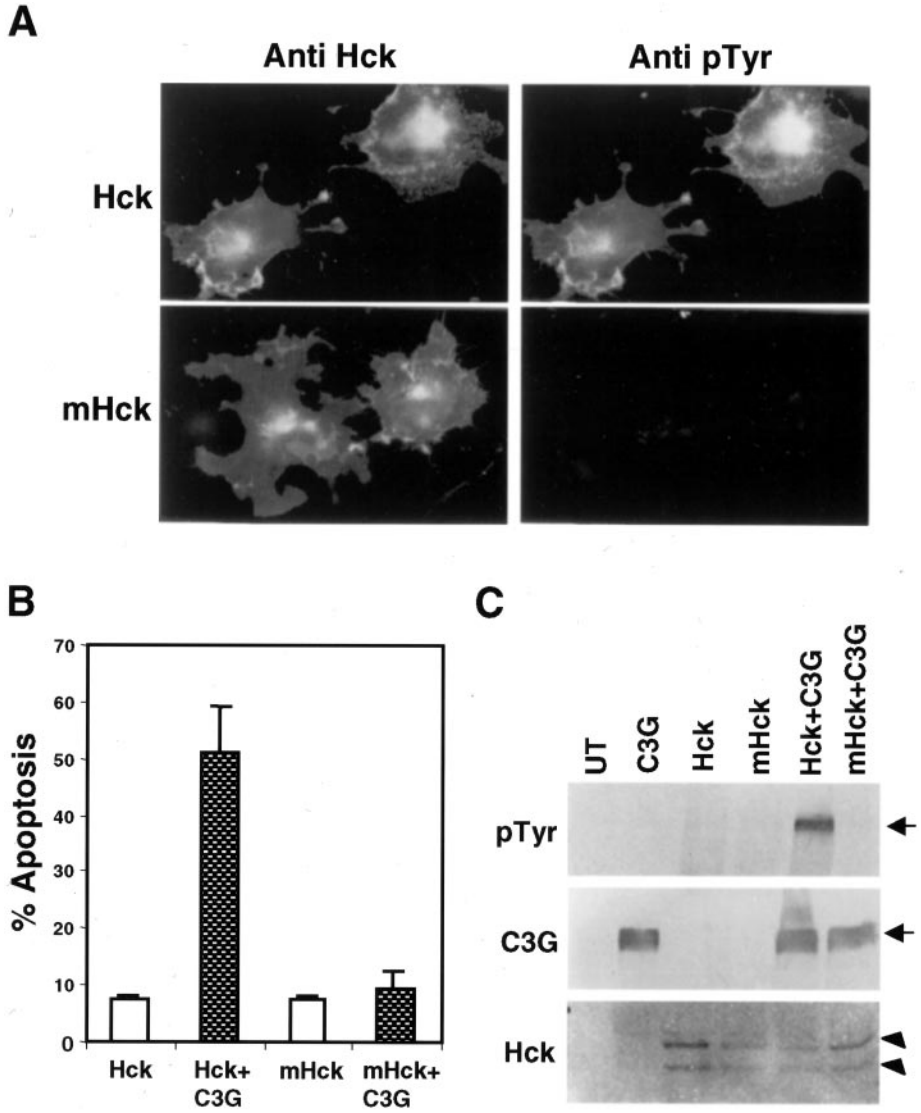

FIG. 8. **Apoptosis induced by Hck and C3G expression requires catalytic activity of Hck.** *Panel A*, Cos-1 cells transfected with Hck or mHck were dual-stained with phosphotyrosine (*pTyr*) and Hck antibodies. *Panel B*, apoptosis was scored in Cos-1 cells transfected with either Hck and C3G or mHck and C3G after 42 h of transfection. In mHck the catalytic lysine was replaced by arginine. *Panel C*, catalytically inactive mutant of Hck does not induce tyrosine phosphorylation of C3G. Cos-1 cell lysates expressing the indicated proteins were used for immunoblotting with phosphotyrosine, C3G, and Hck antibodies. *UT*, untransfected cells.

Recently Src and Jak2 have been implicated in the phosphorylation of C3G in response to growth hormone stimulation of NIH 3T3 cells leading to Rap1 activation (24). By using dominant negative mutants of c-Src and Jak2, it was shown that the activity of both the kinases was required for C3G phosphorylation in response to growth hormone. It was suggested that the endogenous C3G gets phosphorylated at Tyr-504 because exogenously expressed Y504F mutant of C3G was not phosphorylated. Using phosphospecific antibodies, we have for the first time unequivocally demonstrated the phosphorylation of endogenous C3G at Tyr-504, which increased in response to activation of endogenous Hck.

One of the consequences of interaction of C3G and Hck was the induction of apoptosis, which was inhibited by antiapoptotic protein Bcl2. Catalytic activity of Hck was required for activation of this apoptotic pathway, which requires caspase-8, -1, and -9. Neither the catalytic activity of C3G nor Tyr-504 phosphorylation of C3G were required for induction of apoptosis by expression of Hck and C3G. Then what is the role of C3G in the induction of apoptosis? Activation of Hck by mutation of negative regulatory tyrosine phosphorylation site or by the SH3 ligand Nef results in fibroblast cell transformation (45–47). This is due to large increases in kinase activity of Hck. Expression of Y504F C3G or C3G showed only marginal increases in phosphorylation of certain endogenous polypeptides by Hck, as determined by phosphotyrosine immunoblotting of the whole cell lysates (data not shown). C3G is a large molecule, and it could conceivably provide a binding site for a potential substrate of Hck. It is likely that the interaction between Hck and C3G may result in transient phosphorylation of certain specific proteins by Hck, which may lead to activation of an apoptotic pathway.

Tyrosine kinase activity of growth factor receptors is required for survival of cells. Lyn, Syk, and Lck tyrosine kinases are required for inhibition of apoptosis by cytokines (48, 49). There are only a few examples of tyrosine kinases, such as c-Abl, Lyn, and AATYK, which have been implicated in the induction of apoptosis (50–52). Depending upon the physiological context, Lyn is involved in the induction or inhibition of apoptosis (48, 51). Our results show the potential of Hck to induce kinase activity-dependent apoptosis in the presence of C3G. Further work is required to understand the physiological significance of this novel property of Hck of inducing apoptosis.

We have shown physical and functional interaction between Hck tyrosine kinase and guanine nucleotide exchange factor C3G in which C3G acts upstream as well as downstream of Hck-mediated signaling. On one hand activation of Hck results in phosphorylation of C3G at Tyr-504, a phosphorylation known to increase C3G catalytic activity. On the other hand, through its non-catalytic sequences, C3G alters the signaling downstream of Hck in such a way that it leads to induction of apoptosis, which requires the catalytic activity of Hck.

Acknowledgments—We thank Dr. M. Matsuda of the Department of Pathology, Research Institute, International Medical Centre of Japan, Tokyo for generously providing various human C3G mutants and Dr. S. Tanaka for providing normal C3G expression plasmid.

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