Characterization of a Novel Proapoptotic Caspase-2- and Caspase-9-binding Protein*

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Caspases play important roles in regulating apoptotic signaling pathways. Here we report the cloning, by the yeast two hybrid system with dominant negative caspase-2 as “bait,” of a proapoptotic molecule named proapoptotic caspase adaptor protein (PACP), encoded by a 372-base pair open reading frame. Binding of this novel protein to caspase-2 (casp-2) was confirmed in yeast two hybrid, in vitro, and in vivo assays. The deduced amino acid sequence revealed homology to functional motifs, including ATP and cytochrome c binding sites. PACAP mRNA was widely expressed in most human tissues; in transfected cells, PACAP was diffusely expressed in the cytoplasm. Bindings studies with the PACAP recombinant protein demonstrated specific binding to casp-2 and casp-9 but not to casp-3, -4, -7, or -8 in cell extracts. Cotransfection experiments showed that PACAP binds to casp-2 and -9 in 293T cells. In addition, studies with truncated PACAP demonstrated a requirement for residues 39–72 of PACAP for specific binding to casp-2 and -9. Transient transfection of PACAP into 293T human kidney cells and rat-1 fibroblasts triggered apoptosis at 24 h, which was at least in part prevented by an inhibitor of casp-3-like enzymes. Transfection of PACAP into human B cell lines using a retroviral system also triggered apoptotic cell death. In addition, transcription of PACAP in primary human B cells was dramatically down-regulated early after cellular activation by CD40L and Staphylococcus aureus and markedly up-regulated as the cells apoptose. These findings identify a novel proapoptotic caspase adaptor protein.

Programmed cell death, or apoptosis, is essential for the development and homeostatic maintenance of many cell lineages, including the immune system, where it regulates the antigenic specificity of the system (1–3). Apoptotic signaling pathways are dependent on the activation, by proteolytic cleavage after key aspartic acid residues, of a family of cysteine proteases, termed caspases, which mediate cleavage and functional destruction of various essential intracellular proteins. Cleavage-dependent activation of caspases is mediated by other caspases or autocatalytically with the assistance of adaptor proteins, such as Apaf-1,† of nod/caspase recruitment domain 4 (CARD4), RAIDD/CRADD, DEFCAP, and NAC, which facilitate the formation of multimolecular complexes, oligomerization of included caspases, and autocatalytic activation (4–7). Fourteen structurally related mammalian caspases have been identified to date and subgrouped into three subfamilies based on phylogenetic considerations, sequence homology, predicted structure, and substrate specificity (4, 8). Various death stimuli, such as TNF α or Fas ligand, growth factor deprivation, DNA damage, certain drugs, etc., activate caspase-dependent intracellular signaling pathways (9). The “initiating” or “upstream” caspases in these pathways contain a large amino-terminal prodomain, termed a CARD, which contains motifs for protein-protein interactions, whereas effector or downstream caspases contain short prodomains (9). Activation of effector caspases leads to cleavage and functional destruction of various essential intracellular proteins and thus to apoptotic cell death (4, 8).

Several apoptotic stimuli induce the release of cytochrome c (cyt c) from mitochondria into the cytosol, where it interacts with the WD40 motif of the adaptor protein Apaf-1. Apaf-1 and cyt c complexes apparently not only facilitate the hydrolysis of dATP/ATP, but they also oligomerize via CED4 domains into larger complexes, which bind and facilitate the activation of procaspase-9 (10–12). Such apoptosome complexes also contain casp-3 and -7, indicating that casp-9-mediated activation of these effector caspases occurs in the apoptosome (13, 14). Other studies in cell-free lysates have shown that casp-9 is activated in cell free lysates in a Apaf-1-dependent manner after addition of cyt c and that Apaf-1-independent sequential activation of casp-2, -3, -6, -7, -8, and 10, but not of casp-1, -4, and -5, follows (15).

In the immune system, apoptosis triggered by ligation of T or B cell antigen receptors may trigger cellular proliferation and differentiation or, alternatively, negative signals leading to apoptosis, depending on many factors, including cellular maturation state, tissue location, and co-ligation of other cell surface receptors (2, 16). In our previous studies of apoptosis triggered by ligation of the BCR on Epstein-Barr virus-negative mature and immature human B cell lines, we found that

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1 The abbreviations used are: Apaf-1, activating proapoptotic factor 1; bp, base pair(s); CARD, caspase recruitment domain; casp, caspase; cyt c, cytochrome c; DD, death domain; DN, dominant negative; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; GST, glutathione S-transferase; IL, interleukin; PACAP, proapoptotic caspase adaptor protein; PAGE, polyacrylamide gel electrophoresis; FCR, polynucleotide chain reaction; RT, reverse transcription; SAC, S. aureus Cowan; RAIDD, RIP associated ICH-1 homologous protein with a death domain; DEFCAP, death effector filament forming CED-4-like apoptosis protein; TNF, tumor necrosis factor; BCR, B cell receptor.

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cell death proceeded via a previously unreported intracellular
signaling pathway (17). Casp-2 was activated early after B cell
receptor ligation and was required for apoptosis, whereas
casp-3, an effector caspase involved in most apoptotic path-
ways, was activated subsequently. Casp-9 was activated much
later and likely functioned to amplify the apoptotic signal.
Casp-8 and -1, which are activated by ligation of the CD95 and
TNF-R1 cell death receptors, were not involved (17). Although
casp-2 has been found to become activated upstream of casp-3 in
various primary cultures and cell lines in response to differ-
et apoptotic stimuli, including etoposide, γ-irradiation, or
growth factor withdrawal, and to induce apoptosis upon over-
expression in various cell types (18–25), its involvement early
in apoptosis triggered by ligation of a membrane receptor is
unprecedented.

Casp-2 is unique among the caspases because it exhibits
sequence similarity and shares structural features of two of the
caspase subfamilies with a CARD domain characteristic of
initiator caspases and a substrate specificity more typical of
downstream effector caspases; for these reasons, it is grouped
in a separate subfamily. Two mechanisms for casp-2 activation
have been reported. First, the CARD of casp-2 binds to the
CARD of a 22-kDa death adapter protein termed RAIDD or
CRADD (26, 27). Because RAIDD/CRADD also contains a
death domain (DD) capable of binding the DD of a protein
termed RIP, a component of the TNF-R1 and CD95 signaling
complex, it is postulated that RAIDD/CRADD couples casp-2 to
the death signaling complex, leading to casp-2 activation (26,
27). Second, casp-2 has been shown to be activated by the
cytotoxic lymphocyte granule proteins granzyme B and per-
forin (20). Furthermore, casp-2 is essential for apoptosis of B
lymphoblasts triggered by granzyme B and oocytes triggered by
doxorubicin (28) and for apoptosis of neurons stimulated by
amyloid β-peptide (25).

The present studies were initiated in order to determine the
mechanism of activation of casp-2 in apoptosis of B cells trig-
gerated by ligation of the antigen receptor. Because caspases
interact with other proteins that regulate their activation
and function, as noted above, we sought to identify a casp-2-inter-
acting protein by screening a B cell library using the yeast two
hybrid system with dominant negative casp-2 as “bait.” The
cloning and functional studies of the novel protein termed
proapoptotic caspase adaptor protein (PACAP) are presented
here.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—Human 293T embryonic kidney cells and
Rat-1 fibroblasts were grown in Dulbecco’s modified minimal essential
medium supplemented with 10% heat-inactivated fetal calf serum and
2 mM glutamine, penicillin, and streptomycin. The human Epstein-Barr
virus-negative B lymphoma lines B104 and ST486 (17) and Jurkat T
cells were grown in RPMI medium containing 10% fetal calf serum, 2
mM glutamine, penicillin, and streptomycin. FITC-labeled antibody to
CD20; allophycocyanin-labeled antibodies to CD25, CD69, and CD86;
biotin-labeled antibody to CD30; phycoerythrin-labeled antibody to
CD80; and anti-casp-2, -4, -7, and -8 were purchased from Pharmingen
(Cedarlane, Gaithersburg, MD) and reconstituted in PBS. Monoclonal
anti-casp-9 was purchased from MBL (Nagoya, Japan). Mouse monoclonal
antibody to Myc was obtained from Invitrogen (San Diego, CA), phycoerythrin-labeled streptavidin
was purchased from Pharmingen, and DevD was purchased from Calbio-
chem (San Diego, CA).

Cloning of PACAP—A cDNA fragment encoding human dominant
negative (DN) caspase-2 (17) was amplified by PCR and inserted into
the Smal site in pBD-GAL4 Cam (Stratagene, La Jolla, CA) to generate
pBD-GAL4 Cam/DN casp-2. After verification of the sequence, Saccha-
romyces cerevisiae CG1945 cells (CLONTECH, Palo Alto, CA) were
sequentially transformed with pBD-GAL4 Cam/DN casp-2 and a hu-
man B lymphocyte cDNA library (MATCHMAKER, CLONTECH; >106
cfu/ml) in pACT using a lithium acetate transformation protocol. Selec-
tion was done by growth on SD medium lacking histidine, uracil, and
tryptophan (CLONTECH). Twenty clones exhibiting activation of the
lacZ reporter gene were identified among 3 × 1010 transformants by the
β-galactosidase assay, but only one showed a reproducible interaction with
pBD-GAL4 Cam/DN. Plasmids were isolated from positive yeast colonies by
glass bead phenol-chloroform extraction protocol (CLONTECH). Escherichia
coli DH5α cells containing the pACT vector were transformed with cells
containing the pACT vector were selected in ampicillin resistant
plates. The pACT plasmids were isolated from E. coli and restriction-mapped
(XhoI), and the sequence of the insert was determined by DNA
sequencing.

The sequence upstream of the predicted ATG start site was deter-
mined. Five rapid amplification of cDNA ends (RACE) from kit (Life
Technologies, Inc., Gaithersburg, MD) with total RNA isolated with TRIZol (Life
Technologies, Inc.) from human brain and peripheral
blood leukocytes, both of which were found to express high levels of
PACAP message. Three nested primers reflecting sequences ~300 bp
downstream from the predicted start site (5′-AGACGTTTACCTGGTCCACTCTCTC-3′,
5′-CTCCGGTCCAGGACATCCGTGAT-3′, and 5′-AGTTCGGCTCTGGCTTGGCCGAT-3′) were assayed by PCR one
at a time with abridged anchor primer provided in the kit. Identical
sequences were contained in the 300–500-bp fragments amplified by
the three combinations of primers.

Assays for Binding of PACAP to DN Casp-2 and GST Recombinant
Proteins—Yeast two hybrid binding assays were employed to confirm
binding of PACAP to DN casp-2. In these experiments, CG1945 cells were sequentially transformed with purified pBD-GAL4 Cam/DN casp-2 and pACT containing the novel clone or other control
proteins, including pCL1 (coding for the wild type GAL4 protein) and
pVA3–1 (a fusion of murine p53 and GAL4DNA-BD). Interactions
between the two proteins were confirmed by activation of both reporter
genes (lacZ and LEU2).

Radialized 35S) DN casp-2 was generated using the TNT reticu-
locyte lysate system (Promega, Madison, WI). Unincorporated isotope
was removed by passage through a G25 Sephadex column (Amersham
Pharmacia Biotech, Piscataway, NJ). SDS-PAGE followed by autoradi-
ography revealed the presence of a strongly labeled 47 kDa band,
consistent with the presence of DN casp-2.

For the preparation of GST fusion protein full-length and truncated
PACAPs were subcloned into the BamHI and EcoRI sites of the pGEX-4-T bacterial expression vector (Amersham
Pharmacia Biotech), in-frame with the amino- or carboxyl-terminal GST tag. After expression in E. coli DH5α or BL21 (Life Technologies, Inc.), bacterial cells from 400-ml overnight cultures grown at 37 °C were resuspended in an
equal volume of fresh LB medium. After 30 min of additional growth at 95 °C, the harvested bacterial cells were sonicated. The mixture was added for 90 min.

Fractions containing 1 mM isopropyl-1-thio-β-D-galactopyranos-
side was added for 90 min. The induced bacteria were cultured by centrifugation, resuspended in 30 ml of cold phosphate-buffered saline, and
sonicated. The bacterial cells were further incubated with Triton
X-100 (1%) and b-lysozyme at 0.2 mg/ml at room temperature for 1 h. The
GST fusion products were purified and immobilized on glutathione-
Sepharose beads (Amersham Pharmacia Biotech).

Radialized DN casp-2 (1 × 108 cpm) was incubated with recombi-
nant PACAP-bearing glutathione-Sepharose beads bound to PACAP recombinant protein in a volume of 200 μl in the presence of EGTA (1 mM), Nonidet P-40 (0.2%), leupeptin (1 μM), aprotinin (1 μM), andphenylmethylsulfonyl fluoride (0.1 mM). After overnight incubation at 4 °C with constant rotation, the beads were washed extensively, boiled in SDS sample buffer, and run on 4–12% gradient SDS gels followed by autoradiography.

Distribution of PACAP in Human Tissues—The human Rapid-Scan-
Gene expression panel (OriGene Technologies, Rockville, MD), a semi-
quantitative PCR method, was used to evaluate the expression of
PACAP in different human tissues. The high quality first strand cDNAs
were provided in the kit were normalized using β-actin as the internal
control according to the manufacturer’s protocol and evaluated
undiluted and at a 100-fold dilution. The PCR settings were established
according to the Tm of the primers and the hot start Taq polymerase
optimal temperature requirements. The upstream and downstream
primers were 5′-CGAGGACTCATGAGGTTGCATCCTAGGCATTG-3′ and
5′-CGGGAATTCGCCAGGACATCCGTGAT-3′, respectively. The
PCR settings were 5 min at 94 °C, 45 cycles of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C;
ending with 1 min at 72 °C. The products were analyzed on 12% agarose gels followed with ethidium bromide.

GST Pull-down Assays—NH2-terminal GST-tagged PACAP expres-
sion constructs were generated in a pGEX-4-T using BamHI/EcoRI
restiction sites. PACAP mutants generated were 1–123 (full-length), 123–125 (mature peptide), 132–135, and 172–123.
Jurkat T cell lyastes (1 × 10⁶ cells) were generated with 1× lysis buffer (200 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10% Triton X-100, 25 mM sodium pyrophosphate, 10 mM glycerophosphate, 1 mM NaVO₄, 1.9 μg/ml aprotinin, 20 μg/ml, 50 mM Tris, pH 7.4, 1% Nonidet P-40, 1 mM Phenylmethylsulfon fluoride) for 1 h on ice. GST-control and GST-recombinant PACAP and mutant PACAP fusion proteins (10 μl) were incubated with the cell lysates for 3 h at 4°C with continuous rotation. Samples were pelleted and washed with saline, and proteins were resolved on SDS-PAGE gels and detected by Western blotting procedure.

**Fig. 1. Nucleotide sequence and deduced amino acid sequence of PACAP cDNA.** Start and stop codons are shown in boldface, and the polyadenylation signal is underlined.

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**Transfection and Immunoprecipitation Studies—Human 293T cells and Rat-1 fibroblasts were transiently transfected with PACAP using the calcium phosphate and LipofectAMINE (Life Technologies, Inc.) methods, respectively, according to the manufacturer's instructions. For these studies, PACAP was subcloned into engineered expression vectors (10 μl) were incubated with the cell lysates for 3 h at 4°C with continuous rotation. Samples were pelleted and washed with saline, and proteins were resolved on SDS-PAGE gels and detected by Western blotting procedure.**

**Transfection Studies in B Cell Lines by Retroviral Infection—**

**Results**

Cloning of PACAP cDNA—Screening of a human B cell cDNA library (3 × 10⁶ transformants) by yeast two hybrid system with human DN caspase-2 (C303A) as bait yielded 20 yeast transformants. Positive clones were distributed into flat-bottomed 96-well microtiter plates (100 μl/well). Activation was initiated by the addition of either 5 μg/ml CD40 ligand-α fusion protein (obtained from Dr. V. Brinkmann, Novartis Pharma, Basel, Switzerland) together with 10 ng/ml IL-4 (Sigma), or Staphylococcus aureus Cowan (CalBiochem, San Diego, CA) in Yssel's medium with 10% fetal bovine serum. Successful activation was monitored by assessing CD20, CD25, and CD69 expression on days 0, 1, 2, 3, 6 h, 1 day, 2 days, and 5 days. Total RNA was extracted with TRIzol (Life Technologies, Inc.), and PACAP mRNA expression was evaluated by RT-PCR carried out on 1 μg of RNA per sample using 5'-CGGGAATCCGCA-GATCCATAGGAGGCTGTCGTCGCTGG-3' and 5'-CGGGATCCCGA-GGAAGCCCTTTCCTTTTTT-3' as the upstream and downstream primers, respectively. These primers amplify a 600-bp sequence. **Expression of PACAP mRNA in Primary Human B Cells—**

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**Western Blotting Studies—**

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Jurkat T cell lyastes (1 × 10⁶ cells) were generated with 1× lysis buffer (200 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 10% Triton X-100, 25 mM sodium pyrophosphate, 10 mM glycerophosphate, 1 mM NaVO₄, 1.9 μg/ml aprotinin, 20 μg/ml, 50 mM Tris, pH 7.4, 1% Nonidet P-40, 1 mM Phenylmethylsulfon fluoride) for 1 h on ice. GST-control and GST-recombinant PACAP and mutant PACAP fusion proteins (10 μl) were incubated with the cell lysates for 3 h at 4°C with continuous rotation. Samples were pelleted and washed with saline, and proteins were resolved on SDS-PAGE gels and detected by Western blotting procedure. **Transfection and Immunoprecipitation Studies—Human 293T cells and Rat-1 fibroblasts were transiently transfected with PACAP using the calcium phosphate and LipofectAMINE (Life Technologies, Inc.) methods, respectively, according to the manufacturer's instructions. For these studies, PACAP was subcloned into engineered expression vectors (10 μl) were incubated with the cell lysates for 3 h at 4°C with continuous rotation. Samples were pelleted and washed with saline, and proteins were resolved on SDS-PAGE gels and detected by Western blotting procedure.**

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transfection studies in mammalian systems, as described below, suggests that residues 1–22 represent a signal peptide, which is absent from the mature protein. For these reasons, Δ23–123 PACAP is considered to represent the mature protein.

Various protein prediction programs suggest that PACAP is a globular protein with lumen and membrane binding characteristics, which would be consistent with localization in the endoplasmic reticulum or the Golgi, where casp-2 is found (34). The Prosite and Pôle Bio-Informatique Lyonnais data bases reveal several motifs in the novel protein, including two possible myristylation sites (GAAGAE, residues 83–88; and GLRSSR, residues 106–111), a consensus sequence for tyrosine kinase phosphorylation (KGRSQTSY, residues 71–78), three potential PKC phosphorylation sites (SGK, residues 69–71; SSR, residues 109–111; and SHR, residues 118–120), and one possible glycosaminoglycan attachment site (SGKG, residues 69–72) (36). In addition to these motifs, which exactly match the reported consensus sequences for these sites, PACAP also exhibits an amino-terminal sequence with 63% homology to the WD40 (Trp-Asp) motif (residues 3–17), four sequences with 57–65% homology to the ATP/GTP binding site motif A (P loop) (residues 12–29, 12–30, and 12–35) and a sequence with 69% homology to a cytochrome c binding site (residues 50–55) as well as a leucine-rich sequence (residues 1–16). PACAP also exhibited motif homologies to several enzymatic active sites, including a glycoprotease family signature (75% homology, residues 28–48), a eukaryotic cysteine protease active sites (68% homology, residues 84–94), and a serine protease active site (55% homology, residues 109–120). PACAP does not contain an identifiable CARD, DD, or death effector domain.

Data base searches using the Blast server at the National Center for Biotechnology Information identified a partially homologous cDNA sequence to PACAP, which was cloned from a CD34 + stem cell library (GenBank™ accession number AF151024). This cDNA sequence, which has not been published in the literature, is completely homologous to the 3′ nucleotide sequence including the poly(A)-tail of PACAP (about 320 bp), but the sequence upstream sequence is completely different from the cDNA sequence reported here. In addition, the deduced amino acid sequence of AF151024 does not match that of the present clone due to a missing nucleotide, which produces a frameshift. In searching the GenBank™ expressed sequence tag database, nine expressed sequence tag clones derived from eight different proliferative and nonproliferative human tissue cDNA libraries (AJ698118, AI920943, AA363205–5, AJ670058, AI004279, AW001552, AI203960, AI203981, and AI762046) showed 98–100% homology to the untranslated 3′ 300–550 bp of PACAP downstream of the stop codon, as assessed by the National Center for Biotechnology Information Blast search.

In Vitro Demonstration of PACAP Binding to DN Casp-2—An independent assay system was used to demonstrate binding of full-length PACAP to DN casp-2. For these studies, PACAP was translated in-frame with a carboxyl-terminal GST tag, purified, and immobilized on glutathione-Sepharose beads. As shown in Fig. 2B, the beads bound 35S-labeled recombinant DN casp-2 generated by the reticulocyte lysate system, confirming the ability of PACAP to bind casp-2.

Expression of PACAP mRNA in Human Tissues—A semi-quantitative PCR method was used to evaluate PACAP mRNA expression in different human tissues. The high quality first strand cDNAs from 23 human tissues were subjected to RT-PCR analyses using PACAP specific primers. As indicated in Fig. 3, PACAP mRNA was detectable in all of the tissues except placenta and fetal brain. Expression was highest in the brain, whereas heart, spleen, skin, ovary, adrenal gland, pancreas, bone marrow, small intestine, muscle, stomach, testis, and

![Fig. 2. PACAP-casp-2 binding assays.](image_url)
Peripheral blood lymphocytes exhibited intermediate levels of expression. Very low, but detectable, expression was found in kidney, liver, colon, lung, uterus, prostate, salivary gland, and thyroid.

**PACAP Binds to Casp-2 and -9 but not to Casp-3, -4, -7, or -8 in Cell Lysates**—Because we cloned PACAP using casp-2 as bait, we wanted to determine whether PACAP would bind to other caspases. Mature PACAP (23–123) was used in these studies because the full-length PACAP (1–123) protein was cleaved near the amino-terminal GST tag; therefore, the size of PACAP (1–123) recombinant protein was only slightly larger than GST and unable to bind caspases, as described below. In these experiments, glutathione-Sepharose beads bearing amino-terminal GST-tagged mature PACAP were incubated with Jurkat cell lysates for 2 h at 4 °C (Fig. 4A). Various caspases were evaluated in the eluates in Western blotting studies. As shown in Fig. 4A, casp-2 and -9 were readily detected in eluates of cell lysates incubated with GST-PACAP beads (23–123), whereas casp-3, -4, -7, and -8 were not. Controls, represented by the pre- and post-lysates, confirmed the ability of the antibodies to detect full-length casp-3, -4, -7, and -8. Thus, mature PACAP (23–123) binds casp-2 and -9 but not casp-3, -4, -7, or -8 in cell lysates. Eluates of the glutathione-Sepharose beads bearing the GST-mock and GST-PACAP (23–123) constructs were also stained with Coomassie Blue. The GST-mock and GST-PACAP (23–123) proteins exhibited apparent molecular masses of 26 and 34 kDa, respectively, in 8–12% gradient SDS-PAGE gels (Fig. 4B). The observed 34-kDa size of the GST-PACAP (23–123) construct was somewhat smaller than expected for a 26-kDa GST and 13.6-kDa PACAP fusion proteins, probably because of an additional cleavage site in the amino-terminal region of the protein.

**In Vivo Binding of PACAP to Casp-2 and -9**—In order to determine whether PACAP binds to casp-2 and -9 in intact cells, 293T cells were cotransfected with carboxy-terminal Myc-tagged PACAP and casp-2 and -9. Eighteen hours later, the cells were lysed. Myc-tagged PACAP was immunoprecipitated with anti-Myc antibodies, and binding of casp-2 and -9 to PACAP was assessed by SDS-PAGE analysis followed by Western blotting studies with antibody to casp-2 and -9. As shown in Fig. 4C, PACAP bound to both caspases in the transfected cells. Identical results were obtained in three independent experiments. The experiments were performed in the reverse direction (immunoprecipitation with antibody to casp-2 and -9 and Western blotting with anti-Myc antibody) with identical results (not shown).

The Amino-terminal Portion of PACAP Is Required for Binding to Casp-2 and -9—To evaluate binding sites in PACAP, we constructed several amino-terminal deletion constructs of the novel protein (Fig. 4D). Full-length PACAP cDNA (1–123), mature PACAP (23–123), and deletion constructs 25–123, 39–123, and 72–123 were fused with an amino-terminal GST tag in the pGEX4T1 plasmid (Fig. 4D). The recombinant proteins were obtained using two different bacterial strains, DH5α and BL21, with the same results. Proteins were evaluated in stained SDS-PAGE gradient gels. The GST-mock construct exhibited an apparent molecular mass of 26 kDa, as expected (Fig. 4E). However, the full-length GST-PACAP (1–123) construct failed to show the predicted fusion protein band, but rather exhibited only the 26-kDa GST band plus a slightly larger band (Fig. 4E, lane 1). The GST-PACAP 23–123, 32–123, and 39–123 constructs showed major bands at ~34, 33, and 32 kDa (Fig. 4E, lanes 2–4). Two additional smaller bands with approximate molecular masses of 27 and 28 kDa were also evident. These data indicate that the amino-terminal portion of the truncated PACAP constructs fused to GST is further cleaved in the bacteria at two sites. Cleavage was not observed with the GST-Δ72–123 PACAP construct, which exhibited an apparent molecular mass of 36 kDa (Fig. 4E, lane 5). The data are consistent with a cleavage site of PACAP in the bacteria between residues 39 and 72. Despite their small size, the GST-PACAP (23–123, 32–123, and 39–123) mutants bound to casp-2 as well as casp-9 (Fig. 4, F and G; lanes 2–4). The GST-Δ72–123 PACAP, however, failed to bind either of these caspases (Fig. 4, F and G; lane 5). These data localize the binding site for casp-2 and -9 to the region of the PACAP protein encompassed by residues 39–72.

**PACAP Induces Apoptosis upon Transient Transfection into Human Embryonic 293T Kidney Cells and Rat Fibroblasts**—Transient transfection studies into human 293T cells and Rat-1 fibroblasts were carried out in order to assess the function of PACAP. Mature PACAP (23–123) was cloned into the carboxy-terminal Myc-tagged pcDNA3.1 vector and transfected into the cells using the calcium phosphate method. The transfection
efficiency in human 293T cells assessed a day after transfection was 60 ± 8% (Fig. 5A). However, 69 ± 7% of the transfected cells died during the incubation period (Fig. 5E). The transfection efficiency was lower (<10%) in Rat-1 fibroblasts. As observed with the 293T cells, a large proportion (55 ± 5%) of the transfected cells died during the incubation time. Microscopic evaluation of transfected 293T cells showed that many of the cells were shrunken and had pyknotic nuclei, indicative of apoptotic cell death (Fig. 5B). Comparable findings were observed with transfected Rat-1 fibroblasts (not shown). These data indicate that overexpression of PACAP in human 293T cells and in rat fibroblasts leads to apoptotic cell death.

Immunohistochemical studies demonstrated that Myc-tagged PACAP was expressed in the cytosol (Fig. 5B, C, arrows). Immunofluorescence studies showed that the protein exhibited a fine granular distribution in the cytosol surrounding the nucleus (Fig. 5C). Very similar findings were obtained with transfected Rat-1 fibroblasts (not shown). Apoptotic cell death was, at least in part, inhibited by pretreatment of the PACAP-transfected cells with 300 nM DEVD, because cell death was reduced from 69 ± 7 to 41 ± 9% (Fig. 5E). In addition, the status of PACAP in transfected 293T cells was evaluated in Western blotting studies with antibody to the Myc tag. Under reducing conditions, carboxyl-terminal Myc-tagged PACAP exhibited an apparent molecular mass of about 14 kDa (Fig. 5D), consistent with the predicted size of a PACAP (Δ23–123) Myc fusion protein.

Transfection of PACAP into Human B Cell Lines Using a Retroviral Vector—ST486 and B104 human B cells were stably infected with a PACAP retroviral construct for further studies of the function of PACAP. Previous efforts to obtain stable B cell transfectants using transient transfection methodologies have not been successful (17). In the present studies, therefore, we used a GFP carboxyl-terminal-expressing, amphotropic retroviral vector termed PINCO, which has been reported to yield high transfection efficiencies in human hematopoietic cells (30). Three days after infection of the Epstein-Barr virus-negative ST-486 human B cell line with the vector or with the vector containing PACAP, only ~1% of the cells expressed GFP (Fig. 6, A and B), in contrast to higher levels in the published report (30). The GFP and GFP-PACAP-positive cells were then sorted and evaluated for apoptosis. Although the control vector infected B cells grew rapidly, high levels of cell death were observed in the PACAP-PINCO infected cells (Fig. 6C). Comparable results were obtained with the B104 cell line. As a control, PACAP expression was detected by RT-PCR to evaluate the presence of the gene in the cell lines (not shown). These data indicate that PACAP overexpression in human B cell lines, as in 293T embryonic kidney cells and Rat-1 fibroblasts, triggers cell apoptosis.

Expression of PACAP mRNA during Activation of Primary Human B Cells—We evaluated PACAP expression by RT-PCR during the activation of human primary B cells with SAC, which binds to the antigen receptor, as well as during activation by CD40 ligand plus IL-4 (CD40L/IL-4), which mimics T helper cell-mediated B cell activation (37, 38). Primary B cell activation by these agents is followed by proliferation and ultimately by cell death. PACAP mRNA was constitutively expressed in the primary B cells (Fig. 7, A and B). Expression abruptly decreased 3 h after activation by SAC and by CD40L/
The WD40 motif is unlikely to be functionally significant, found in several apoptotic signaling proteins, including Apaf-1.

In triplicate; error bars represent Myc expression in 293T cells transiently transfected with Myc-tagged PACAP. Extracts of the transfected 293T cells were subjected to SDS-PAGE analyses under nonreducing (a) and reducing (b) conditions. In both a and b, lane 1 represents 293T control-transfected cells, and lane 2 represents Myc-PACAP-transfected cells. E, apoptosis of PACAP-transfected 293T cells. Cells were transfected with the vector (pcDNA3.1) or the mature PACAP (Δ23–123) alone or pretreated with 300 nM DEVD. Experiments were run three times each in triplicate; error bars show the S.D.

Fig. 5. Transient transfection studies in human embryonic 293T kidney cells. A, light microscope evaluation (× 40) of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining of 293T cells transiently transfected with pCDNA3.1-LacZ to evaluate transfection efficiency. B, light microscope image (40×) of 293T cells transiently transfected with Myc-tagged PACAP. The brown cytosolic staining (horseradish peroxidase) shows Myc expression in the cells, which were also counterstained with hematoxylin. Arrows denote cells with pyknotic nuclei. C, fluorescent microscopic evaluation (40×) of 293T cells transiently transfected with Myc-tagged PACAP. The green cytosolic staining (FITC) shows the localization of Myc in the cells. The counterstain (TOPRO3) stains nuclei red. Arrows show expression of Myc-tagged PACAP. D, Western blotting analyses of Myc expression in 293T cells transiently transfected with Myc-tagged PACAP. Extracts of the transfected 293T cells were subjected to SDS-PAGE analyses under nonreducing (a) and reducing (b) conditions. In both a and b, lane 1 represents 293T control-transfected cells, and lane 2 represents Myc-PACAP-transfected cells. E, apoptosis of PACAP-transfected 293T cells. Cells were transfected with the vector (pcDNA3.1) or the mature PACAP (Δ23–123) alone or pretreated with 300 nM DEVD. Experiments were run three times each in triplicate; error bars show the S.D.

IL-4 and then returned to resting levels 12 h (SAC) or 24 h (CD40L/IL-4) after addition of the activators (Fig. 7, A and B). By 24 h (SAC) or 48 h (CD40/IL-4) after activation, PACAP mRNA levels in the activated cells considerably exceeded constitutively expressed levels. Marked activation, documented by the expression of the CD69 and CD25 activation markers, was observed 24 h after addition of the ligands (Fig. 6, C and D). Marked cell death was evident in the cultures by 3 days (SAC) and 5 days (CD40/IL-4) after stimulation (not shown). These findings indicate that primary human B cells constitutively express PACAP mRNA. They further show that PACAP mRNA expression decreases with B cell activation triggered by either ligand combination and increases as the cells begin to die.

Discussion

Because multiple homophilic and heterophilic interactions with other proteins regulate caspase actions, we used the yeast two hybrid system with DN casc-2 as bait with the goal of identifying a possible casp-2-interacting protein. These experiments identified a novel protein, termed PACAP, with a calculated molecular mass of 13.6 kDa. This is consistent with an observed molecular mass of 14 kDa for the protein on reduced SDS-PAGE gels.

PACAP triggered apoptosis upon transient transfection of human kidney cells and Rat-1 fibroblasts and early in stable transfection of human B cell lines using a retroviral vector. These findings clearly identify PACAP as a member of an apoptotic pathway. PACAP does not contain a CARD, a motif that mediates interactions between some of the caspases and certain other apoptotic signaling proteins. PACAP does, however, contain an amino-terminal WD40 repeat, a P loop nucleotide binding motif, and a cysteine binding site, motifs that are found in several apoptotic signaling proteins, including Apaf-1. The WD40 motif is unlikely to be functionally significant, because it is contained in the first 22 amino acids of the protein that appear to function as a signal peptide. PACAP also contains consensus sequences for phosphorylation by tyrosine and serine kinases, as well as potential myristylation sites. Finally, PACAP exhibits motif homologies to the active sites of a number of families of enzymes, including glycoproteases, cysteine proteases, serine proteases, and thiol proteases. The presence of multiple consensus sites characteristic of intracellular signaling pathways suggests the possibility not only that PACAP is a component of such a signaling pathway but also that it represents an adaptor protein that, in analogy to Apaf-1, interacts with cytochrome c and dATP and facilitates the activation of one or more caspases. However, because PACAP lacks a CARD, it clearly cannot bind via homophilic interactions to the CARDs of casp-2 or -9 or to another protein with this domain. The absence of a CARD plus the complete lack of sequence similarity differentiates PACAP from RAIDD/CRADD, a bipartite anchoring molecule that binds to casp-2 via its amino-terminal CARD, and to RIP, a component of the tumor necrosis factor-R1 signaling complex, via its carboxyl-terminal DD (26, 27). The absence of a CARD, lack of sequence homology, and different molecular masses also differentiate PACAP from two other recently reported casp-2-binding proteins. These include two forms of a Ced-4/Apaf-1 family member termed DEFCAP-L (1473 residues), and DEFCAP-S (1429 residues), to which casp-2 binds via homophilic CARD interactions (5). The second casp-2-binding protein is caspase-25-binding protein, a 191-residue protein that binds to the carboxyl-terminal portion of the short form of casp-2 via an unknown type of interaction and blocks its apoptotic actions (39). The absence of sequence similarity and the different molecular masses distinguish PACAP from the casp-9 binding adaptor protein, Apaf-1.

Analyses of PACAP mRNA expression revealed its presence...
in many adult tissues with different levels of expression. For example, PACAP is most highly expressed in the adult brain as is casp-9, whereas casp-2 expression is barely detectable in this organ (23). On the other hand, casp-2 mRNA is most highly expressed in the developing brain and placenta, both of which lack detectable PACAP expression (23). The message for these proteins is also expressed in other tissues, but at differing relative levels (5, 26, 27, 35, 39).

Amino-terminal deletion mutants of PACAP were generated in two bacterial strains (DH5α and BL21) in-frame with an amino-terminal GST tag in order to begin to identify binding sites for casp-2 and -9 in the molecule. The full-length PACAP peptide was generated as cleaved fragments due to a cleavage site located between residues 22 and 23. The inability of the GST amino-terminal PACAP peptide construct to bind either casp-2 or -9 indicates that the WD40 repeat contained in this portion of the protein is not important for caspase binding. The D23–123, D32–123, and D39–123 PACAP constructs were all cleaved in the bacteria, yielding GST constructs fused with short amino-terminal PACAP peptides. Those short peptides bound casp-2 and -9. The PACAP Δ72–123 peptide, which remained largely intact in the bacteria, was not able to bind casp-2 or -9. Thus, the casp-2 and -9 binding site in PACAP is located between residues 39 and 72. Studies are under way to further localize the casp-2 and -9 binding site in PACAP within this 33-residue sequence. Cotransfection studies with casp-2, casp-9, and PACAP in 293T cells indicate that PACAP (23–123) binds both caspases in intact cells. These in vivo studies support a physiological role for these interactions.

Potential proapoptotic actions of the amino-terminal truncated forms of PACAP were not evaluated in the present study. However, the clear reduction in the amounts of detectable casp-3, -4, and -7 in Jurkat T cells lysates after exposure to the GST-PACAP Δ23–123 construct, which maximally contains residues 23–72 of PACAP, strongly suggests that this portion of the protein indirectly activates these downstream effector caspases in the Jurkat T cell lysates. The ability of DEVD, an inhibitor of casp-3-like enzymes, to partially block apoptosis in PACAP-transfected 293 T cells is consistent with a role for downstream caspases in the PACAP apoptotic pathway.

The constitutive expression of an apoptotic gene in many tissues in the absence of an apoptotic stimulus is surprising. It is possible that PACAP is constitutively expressed in an inactive state and that activation (cleavage at position 22) is required in order for PACAP to bind to and regulate the acti-

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**FIG. 6.** Transfection of PACAP in human B cell lines using a retroviral system. A, cell sorting analysis of GFP expression (M1 region) in viable, sorted ST486 cells (R1 area) 3 days after infection with the GFP-expressing PINCO control retrovirus. B, cell sorting analysis of GFP expression (M1 region) in viable, sorted ST486 cells (R1 area) 3 days after infection with the GFP-expressing PACAP-PINCO retrovirus. C, evaluation of apoptosis in control and PACAP-transfected B104 and ST486 B cells. B cells were stained with propidium iodide, and apoptotic nuclei were counted in a masked fashion.

**FIG. 7.** PACAP mRNA expression after activation of primary human B cells. A, semiquantitative analysis of PACAP mRNA expression as a function of time after incubation with CD40 ligand and IL-4. B, semiquantitative analysis of PACAP mRNA expression as a function of time after incubation with SAC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was evaluated as a loading control. C and D, flow cytometric analysis of CD69 and CD25 expression 1 day after stimulation with CD40 ligand and IL-4. The dotted lines depict CD69 and CD25 expression, and the solid lines represent the control. Controls were B cells not activated with CD40L/IL-4. Experiments were run three times, each time in triplicate.
vation of casp-2 and -9. These could occur via binding of cyt c and dATP, in analogy to the activation of Apaf-1 (10), or via other processes, including membrane localization, proteolytic cleavage, and phosphorylation. We are evaluating these possibilities.

The rapid down-regulation of PACAP mRNA levels in primary B cells after stimulation with CD40L/IL-4 is also of interest. These findings suggest that PACAP expression must be up-regulated during death. This and other aspects of the functions of PACAP in resting, activated, and cycling cells are under investigation.

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REFERENCES
Characterization of a Novel Proapoptotic Caspase-2- and Caspase-9-binding Protein
Emanuela Bonfoco, Erguang Li, Frank Kolbinger and Neil R. Cooper

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