Comparative Proteomics Study Reveals That Bacterial CpG Motifs Induce Tumor Cell Autophagy in Vitro and in Vivo*§

Samuel Bertin‡§$, Michel Samson‡§¶, Catherine Pons‡§¶, Jean-Marie Guigonis‡¶ replenished, Patrick Baqué‡§**, Nicole Brossette‡§, Sophie Pagnotta‡¶¶, Jean-Ehrland Ricci§ §§, and Valérie Pierrefite-Carle‡§¶¶

Unmethylated CpG dinucleotides, present in bacterial DNA, are recognized in vertebrates via the Toll-like receptor 9 (TLR9) and are known to act as an anticancer agent by stimulating immune cells to induce a proinflammatory response. Although the effects of CpG-oligodeoxynucleotides (CpG-ODNs) in immune cells have been widely studied, little is known regarding their molecular effects in TLR9-positive tumor cells. To better understand the role of these bacterial motifs in cancer cells, we analyzed proteome modifications induced in TLR9-positive tumor cells in vitro and in vivo after CpG-ODN treatment in a rat colon carcinoma model. Proteomics analysis of tumor cells by two-dimensional gel electrophoresis followed by mass spectrometry identified several proteins modulated by bacterial CpG motifs. Among them, several are related to autophagy including potential autophagic substrates. In addition, we observed an increased glyceraldehyde-3-phosphate dehydrogenase expression, which has been shown to be sufficient to trigger an autophagic process. Autophagy is a self-digestion pathway whereby cytoplasmic material is sequestered by a structure termed the autophagosome for subsequent degradation and recycling. As bacteria are known to trigger autophagy, we assessed whether bacterial CpG motifs might induce autophagy in TLR9-positive tumor cells. We showed that CpG-ODN can induce autophagy in rodent and human tumor cell lines and was TLR9-dependent. In addition, an increase in the number of autophagosomes can also be observed in vivo after CpG motif intratumoral injection. Our findings bring new insights on the effect of bacterial CpG motifs in tumor cells and may be relevant for cancer treatment and more generally for gene therapy approaches in TLR9-positive tissues. Molecular & Cellular Proteomics 7:2311–2322, 2008.

The CpG dinucleotides are 4-fold more frequent in bacterial than in mammalian DNA, and they are generally methylated in mammals and unmethylated in bacteria (1). These differences lead the immune system to recognize unmethylated CpG sequences as a “danger signal” indicating a potential pathogen infection (2). In immune cells, recognition of these pathogen-associated molecular patterns (PAMPs)1 is mediated by the Toll-like receptor 9 (TLR9) (3) and followed by downstream signaling events leading to the activation of target genes (4). As a consequence, bacterial CpG motifs trigger pleiotropic effects in immune cells such as proliferation, activation, and cytokine/chemokine secretion (1). These proinflammatory properties led to the use of oligodeoxynucleotides (ODNs) bearing CpG motifs (CpG-ODNs) in preclinical and clinical studies to induce immune-mediated antitumoral effects in various kinds of cancers (5, 6). Although previously considered to be restricted to immune cells, expression of TLR9 by non-immune cells is now largely documented. It has recently been demonstrated that primary epithelial cells constituting a barrier between the organism and the external milieu express different TLRs (7–10). In addition, various kinds of tumor cells, including colon cancer cells, also express TLRs (11–13), and TLR9 agonists have been proposed to be involved in growth and survival of human myeloma cells (14, 15) as well as in invasion of human astrocytoma, glioblastoma, and breast cancer cells in vitro (16). Inversely CpG-ODNs have been shown to have antiproliferative and proapoptotic effects in vitro in human lung (17), breast (18), prostate (19), and colon (20) cancer cells as well as in murine glioma cells (21). Hence

1 The abbreviations used are: PAMP, pathogen-associated molecular pattern; ANXA1, annexin A1; CMA, chaperone-mediated autophagy; ODN, oligodeoxynucleotide; 2-D, two-dimensional; GFP, green fluorescent protein; GRP78, 78-kDa glucose-regulated protein; HEK, human embryonic kidney; LC3, microtubule-associated protein 1 light chain 3 protein; Met-P, SssI-methylated plasmid; P, native plasmid; PGAM1, phosphoglycerate mutase 1; PROb, DHD/K12/PROb; RH, rehydration buffer; TLR, Toll-like receptor; NCBI nr, National Center for Biotechnology non-redundant.
CpG-induced Autophagy in Cancer Cells

CpG effects in non-immune cells, and particularly in tumor cells, are still controversial, and this is, however, a crucial point as various cancer gene therapy clinical trials involve plasmid DNA or CpG-ODNs. In the present work, we analyzed proteome modifications induced in TLR9-positive colon cancer cells in vitro and in vivo 24 h after CpG-ODN treatment. As some of the modulated proteins were potentially related to autophagy, a process in which cellular organelles and bulk cytoplasm are targeted for degradation in lysosomes (22), we next analyzed the ability of bacterial CpG motifs to induce tumor cell autophagy. Our results show that, in addition to their known immunostimulatory properties, bacterial CpG motifs could exert a direct effect on TLR9-positive tumor cells in vitro and in vivo through the induction of autophagy.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotides and Plasmids**—The following sequences of the phosphorothioate-modified ODNs (Eurogentec, Seraing, Belgium) were used: for experiments in rodent cells (or human embryonal kidney (HEK) 293 cells expressing the murine TLR9): CpG-ODN 1826, 5′-TCCATGACGGTCTGACTTT-3′; and control ODN, 5′-GCCACGTCATGATGGTTTGG-3′; for experiments in human cells: CpG-ODN 2006, 5′-TGGCTCCTTGGCTTTTTGGCTTT-3′; and control ODN, 5′-TGCTCCTTGGCTTTTTGGCTTT-3′. The pCD-geo/g plasmid contains the cytosine deaminase gene under the control of the cyto-

**Plasmid Methylation**—Plasmid DNA was prepared using the Qia-

**Cell Culture**—DHD/K12/PROb (PROb) rat colon cancer cells (26) and human breast cancer MCF-7 cells were maintained in Dulbecco’s Modified Eagle’s medium in the presence or in the absence of fetal bovine serum and 10% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 10 μg/ml blasticidin (InvivoGen). For proteome analysis, PROb cells were plated into 150-mm tissue culture dishes in the presence of CpG-ODN or control ODN (10 μg/ml) or rapamycin (100 nm, Merck Chemicals) or DMSO as a control for 24 h. For electron microscopy analysis, PROb cells were plated in 6-well plates in the presence of CpG-ODN or control ODN at a final concentration of 10 μg/ml for 40 h. Medium was removed, and the cells were then cultured in complete medium in the presence or in the absence of lysosomal protease inhibitors E64d (10 μg/ml; Sigma) and pepstatin A (10 μg/ml; Merck) for the last 8 h of incubation.

**TLR9 RT-PCR Analysis**—For tissue RNA isolation, PROb tumors and healthy colon were reduced into powder mechanically in liquid nitrogen. Total RNA from tissues and cell lines was prepared by TRIzol extraction according to the manufacturer’s instructions (Invitrogen). Genomic DNA was removed by DNA-free DNase (Ambion, Courtaboeuf, France), and cDNA was synthesized using 1 μg of total RNA in a total reaction volume of 20 μl using SuperScript III reverse transcriptase and random hexamer primers (Invitrogen) according to the manufacturer’s instructions. cDNAs (100 ng) were amplified with Taq Platinum (Invitrogen). The human and rat TLR9 sequences were obtained from GenBankTM (accession numbers AF245704 andAY191271, respectively), and the following primers were used: hTLR9-forward, 5′-GGCACGTCATGATGGTTTGG-3′; and hTLR9-reverse, 5′-GCCACGTCATGATGGTTTGG-3′. For tissue RNA isolation, PROb tumors were reduced into powder mechanically in liquid nitrogen and stored at −80 °C until use. For PROb cell proteome analysis, the cells were washed twice in sucrose buffer (250 mm sucrose, 10 mm Hepes, pH 7.4) and scraped in RH buffer. The resulting cell lysates were then incubated in the presence of spermidine at a final concentration of 10 mM for 30 min at 15 °C. After centrifugation (12,000×g, 30 min, 15 °C), the supernatants containing soluble proteins were deep frozen in liquid nitrogen and stored at −80 °C until use. For PROb cell proteome analysis, the cells were washed twice in sucrose buffer (250 mm sucrose, 10 mm Hepes, pH 7.4) and scraped in RH buffer. The resulting cell lysates were then incubated in the presence of spermidine at a final concentration of 10 μM for 30 min at 15 °C. After centrifugation (12,000×g, 30 min, 15 °C), the supernatants were collected for protein concentration determination with the Bradford assay (Bio-Rad) using bovine serum albumin in RH buffer as standard curve.

**Two-dimensional (2-D) Gel Electrophoresis and Protein Staining**—2-D gel electrophoresis (pH range 3–10) and protein staining were performed as described previously (27). Gels were scanned using a
GS-800 calibrated densitometer (Bio-Rad). Protein patterns were compared using PDQuest imaging system (version 8.0, Bio-Rad). Expression levels of the proteins were quantified by analyzing the intensity of each spot. After normalization (based on total quantity of valid spots of the gels), statistical differences in expression between control ODN- and CpG-ODN-treated cells or tumors were assessed through the Student’s t test. Spots with a p value < 0.05 were considered to be statistically significant and were further processed for identification by mass spectrometry.

**In-gel Tryptic Digestion and Protein Identification by Mass Spectrometry**—Spots of interest were manually excised from the gels and destained with 50% acetonitrile in 25 mM ammonium bicarbonate. Gel pieces were crushed in Eppendorf tubes, dehydrated with acetonitrile for 5 min, and vacuum-dried. Gel pieces were first fully rehydrated with 15–50 μl of 25 mM ammonium bicarbonate, 10% acetonitrile supplemented with trypsin (5 ng/μl; Promega, Madison, WI) and then overlaid with an equal volume of buffer without trypsin. After a 20–24-h incubation at 37 °C, the incubation volume was adjusted to 100 μl with ultrapure water and acidified with 25 μl of formic acid at 25% (5% final concentration). Tryptic peptides were extracted from the gel by adding 125 μl of acetonitrile. After a 15-min incubation at room temperature, the gel pieces were spun down (8000 × g, 5 min, room temperature), the supernatant was collected, and the pellet was overlaid with 50 μl of acetonitrile and vortexed to complete peptide extrusion. The extracted material was pooled with the supernatant and vacuum-dried. Lysates were solubilized in 10 μl of 5% formic acid, 20% methanol and stored at −20 °C until used. All samples were analyzed by micro-LC/ESI/MS/MS on an LTQ/Orbitrap mass spectrometer (Thermo Fisher, Waltham, MA) coupled with pumps and autosampler under standard conditions: capillary temperature, 275 °C; and source voltage, 4500 V. Helium was used as the collision gas. Experiments were done in parallel mode (survey at 60,000 resolution and five data-dependent ion trap MS/MS scans (Top 5)). The MS/MS parameters were as follows: isolation width, 3; collision energy, 35%; micro-HPLC Surveyor system (Thermo Fisher); 60-min gradient; and BioBasic C18 (Thermo Scientific) column (100 × 0.32 mm). Such high mass accuracy on the precursor ion allowed the elimination of virtually any false positive peptide identifications, suggesting that peptides that do not match the specificity of the protease used in the digestion should not automatically be considered as false positives. Acquired MS/MS spectra were interpreted using Mascot version 2.2.0 (Matrix Science, London, UK) in-house software. Search parameters were set as follows: enzyme specificity, trypsin; one missed cleavage permitted; fixed modification, carbamidomethylation of cysteine; variable modification, methionine oxidation; mass tolerance for precursor ions, 5 ppm; mass tolerance for fragment ions, 0.3 Da; significance threshold, p < 0.05; and expect value threshold, 0.001 (to select ions with the highest individual score). Both b and y ion series were used to search against UniProt KB/Swiss-Prot/TrEMBL (database version 51.6; 257,964 sequence entries) or NCBI nr (February 16, 2007; 4,626,804 sequence entries) databases. A species restriction to mammals was applied because the only expected contaminations could be from human (mainly keratins) or bovine (fetal calf serum proteins in cell culture medium) origins (UniProt KB/Swiss-Prot/TrEMBL, 49,887 sequence entries; NCBI nr, 551,056 sequence entries). In case of peptides matching to multiple members of a protein family, the presented protein was selected based on both the highest score and the highest number of matching peptides.

**Fluorescence Confocal Microscopy**—Forty-eight hours after transfection, cells were fixed with PBS containing 1% formaldehyde for 20 min at room temperature, and coverslips were mounted in Mowiol mounting medium. Slides were examined by confocal microscopy using the Zeiss 510 Meta laser scanning microscope. Cells were scored without prior knowledge of the experimental conditions.

**RESULTS**

**CpG-ODN-induced Effects on TLR9-positive Colon Cancer Cells in Vitro and in Vivo**—To investigate the molecular events underlying the various effects of CpG-ODN reported in tumor cells (14–21), we analyzed proteome modifications induced in the PROb TLR9-positive (supplemental Fig. S1A) rat colon carcinoma cell line after CpG-ODN treatment. As a similar uptake of cyanine-3-labeled CpG-ODN can be observed in PROb cells after transfection or addition within the culture medium (supplemental Fig. S2A), the cells were incubated for 24 h with CpG-ODN or control ODN, and soluble proteins were then separated by 2-D gel electrophoresis. For each condition, triplicate experiments were independently performed. For the analysis of in vivo effects, experimental liver metastases from colon carcinoma were generated by injection of PROb cells under the liver capsule of syngenic rats. Twenty-four hours after CpG-ODN or control ODN injection, tumors were removed, and soluble proteins were separated by 2-D gel electrophoresis. Four independent tumors were analyzed in each conditions. Fig. 1 shows representative 2-D gel sections of tumor cells or tumors treated with CpG-ODN or control ODN. After gel-to-gel matching and normalization, protein spots displaying differential expression patterns among the CpG-ODN and control ODN-treated samples were digested and identified by LC/ESI/MS/MS. The proteins modulated in tumor cells upon CpG-ODN action in vitro and in vivo are presented in Tables I and II, respectively.

**Biological Pathways Affected by CpG-ODN Treatment**—We observed a majority of down-regulated proteins both in vitro and in vivo. In vitro, one overexpressed and six underexpressed proteins were reproducibly detected from CpG-ODN-treated cells compared with control ODN-treated cells. The identified proteins were grouped in the following different functional classes: 1) metabolism, 2) chaperoning and stress-related, and 3) miscellaneous. In vivo, one overexpressed and 10 underexpressed proteins were detected from CpG-ODN-injected tumors compared with control ODN-injected tumors. In addition to the functional classes found in vitro, three
proteins related to translation and protein synthesis were found to be modulated. With the exception of GRP78 (28), none of the in vitro or in vivo modulated proteins have been reported previously to be regulated by bacterial CpG motifs.

We first focused our attention on the two proteins that were found to be down-regulated by CpG-ODN both in vitro and in vivo (annexin A1 (ANXA1) (Fig. 2A) and phosphoglycerate mutase 1 (PGAM1) (Fig. 2B)). ANXA1, which is usually up-regulated in colon cancer (29), is an endogenous anti-inflammatory protein involved in many cellular functions, such as membrane aggregation, inflammation, phagocytosis, proliferation, and apoptosis (30). PGAM1 is a glycolytic enzyme interconverting 2- and 3-phosphoglycerates. Both ANXA1 and PGAM1 are known to be degraded by chaperone-medi-
all down-regulated proteins, pyruvate carboxylase (Fig. 2A) and dihydropyrimidinase-related protein 3 (CMA) (31, 32). Moreover, two other proteins, phosphoglycerate mutase-1 (PGM1) and tyrosyl-tRNA synthetase (TARS), were recently identified as being degraded during autophagy (33, 34). Finally, an increased expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and citrate synthase (CS) were described to be regulated in a cell survival mechanism, excessive autophagy can cause cell death (37). As the different pro-survival or pro-apoptotic effects of CpG-ODN in tumor cells could be explained by an upstream autophagy induction, these data prompted us to assess whether bacterial CpG motifs might induce autophagy in TLR9-positive tumor cells.

**Assessment of CpG-ODN-induced Autophagy in TLR9-positive Cancer Cells in Vitro**—To test the hypothesis that CpG-mediated autophagy induction in tumor cells, we transfected PROb cells with control or CpG-ODN and the pLC3-GFP plasmid. This plasmid encodes for the microtubule-associated protein 1 light chain 3 protein (LC3), which is a human homologue of the yeast ATG8, an essential autophagy protein. Autophagosome formation can be scored by immunofluorescence microscopy as a transition of LC3 from its diffuse cytosolic appearance to a membrane-associated, punctate intracellular distribution (24). Fig. 3A displays representative photographs of PROb cells transfected with the pLC3-GFP plasmid and control or CpG-ODN. As shown in Fig. 3B, the presence of CpG-ODN induced a significant increase in the number of autophagic cells compared with transfection in the presence of control ODN and with pLC3-GFP transfection alone. This increase was similar to the induction observed in the presence of the autophagy inducer rapamycin (38) and can be reverted in the presence of the autophagy inhibitor 3-methyladenine (39). A similar increase in the number of autophagic cells was observed when CpG-ODN was added in the culture medium (supplemental Fig. S2B). As an autophagosome ultimately fuse with lysosomes to generate single membrane-enclosed structures termed autolysosomes where material degradation occurs (24), we then mon

---

**Table I**

*Modulated proteins 24 h after CpG-ODN treatment in vitro*

All the presented proteins were statistically modulated with a *p* value<0.05 according to the Student’s *t* test.

| Spot no. | Name of protein | Accession no. | No. of peptides matched | Sequence coverage | Score | Theoretical Molecular weight | Protein function |
|----------|----------------|---------------|-------------------------|-------------------|-------|-----------------------------|-----------------|
| 8004     | Glyceraldehyde-3-phosphate dehydrogenase | P04797 | 13 | 55 | 816 | 36,090 | 8.14 | Metabolism |
| 4602     | Dihydropyrimidinase-related protein 3 | Q62952 | 6 | 15 | 294 | 62,327 | 6.04 | Metabolism |
| 7001     | Phosphoglycerate mutase-1 | P25113 | 12 | 61 | 722 | 28,928 | 6.67 | Metabolism |
| 7102     | Annexin A1 | P07150 | 19 | 62 | 1,244 | 39,147 | 6.97 | Metabolism |
| 2006     | Calponin-3 | P37397 | 8 | 31 | 437 | 36,583 | 6.57 | Structure |
| 0006     | Tropomyosin α-1 chain | Q63608 | 19 | 727 | 29,859 | 6.57 | Chaperone |
| 2004     | Prohibitin | P67779 | 11 | 51 | 672 | 29,859 | 6.57 | Chaperone |

*a* Numbers refer to the relative levels of each protein from CpG-ODN-injected cells compared with those from control ODN-treated cells.

**Table II**

*Modulated proteins 24 h after CpG-ODN treatment in vivo*

All the presented proteins were statistically modulated with a *p* value<0.05 according to the Student’s *t* test.

| Spot no. | Name of protein | Accession no. | No. of peptides matched | Sequence coverage | Score | Theoretical Molecular weight | Protein function |
|----------|----------------|---------------|-------------------------|-------------------|-------|-----------------------------|-----------------|
| 2203     | elf4A1 | AAH63812b | 20 | 73 | 1,255 | 46,426 | 5.32 | Translation |
| 6803     | Elongation factor 2 | P05197 | 9 | 19 | 557 | 96,192 | 6.41 | Translation |
| 6506     | Tyrosyl-tRNA synthetase | Q4KM49 | 8 | 21 | 437 | 59,420 | 6.57 | Translation |
| 9201     | Citrate synthase | Q8VHF5 | 8 | 21 | 449 | 52,176 | 6.53 | Metabolism |
| 5803     | Pyruvate carboxylase | P52873 | 18 | 25 | 1,181 | 130,436 | 6.34 | Metabolism |
| 7001     | Phosphoglycerate mutase-1 | P25113 | 14 | 76 | 804 | 28,928 | 6.67 | Metabolism |
| 7101     | Annexin A1 | P07150 | 16 | 50 | 1,020 | 39,147 | 6.97 | Miscellaneous |
| 9006     | Mn-superoxide dismutase | P67779 | 16 | 50 | 1,020 | 39,147 | 6.97 | Miscellaneous |
| 7502     | Chaperonin subunit 6a (ς) | 76253725b | 13 | 35 | 771 | 58,437 | 6.63 | Chaperone |
| 1601     | GRP78 | P06761 | 24 | 44 | 1,568 | 72,473 | 5.07 | Chaperone |
| 6605     | LaminA | P48679 | 28 | 48 | 1,738 | 72,473 | 5.07 | Structure |

*a* Numbers refer to the relative levels of each protein from CpG-ODN-injected tumors compared with those from control ODN-injected tumors.

**Numbers refer to the relative levels of each protein from CpG-ODN-injected tumors compared with those from control ODN-injected tumors.

**Swiss-Prot/TrEMBL database.**
monitored the number of autophagic vesicles (autophagosomes and autolysosomes) by transmission electron microscopy after incubation with control or CpG-ODN. Fig. 3C displays representative micrographs of PROb cells cultured in the presence of control or CpG-ODN. We observed a significant increase in the number of autophagosomes and autolysosomes in the presence of CpG-ODN compared with control ODN (Fig. 3D). To analyze whether this accumulation of autophagosomes was due to an increased formation or a decreased degradation, this experiment was also performed in the presence of lysosomal protease inhibitors (E64d and pepstatin A) (Fig. 3E). The accumulation of autolysosomes in the presence of inhibitors was further increased in the presence of CpG-ODN, indicating an induction, rather than a blockade, of an autophagic process by the CpG-ODN (Fig. 3F).

Finally to determine whether CpG-induced autophagy was a more general process in TLR9-positive (supplemental Fig. S1B) cancer cell lines, we transfected the MCF-7 human breast tumor cell line and the PC-3 human prostate carcinoma cells in vitro with control or CpG-ODN and the pLC3-GFP plasmid. Fig. 4, A and B, displays representative photographs of MCF-7 and PC-3 cells transfected with the pLC3-GFP plasmid and control or CpG-ODN. As shown in Fig. 4, C and D, the presence of CpG-ODN induced a significant increase in the number of autophagic cells compared with control conditions in both cell lines.

Requirement of TLR9 for CpG-induced Autophagy—To determine whether TLR9 was required for CpG-induced autophagy, we then used HEK 293 cells stably expressing (293 TLR9) or not (293 Mock) the murine TLR9. Both kinds of cells were transfected with the pLC3-GFP plasmid and CpG-ODN.
or control ODN. Representative photographs of autophagic or control 293 TLR9 cells are presented Fig. 5A. In the 293 TLR9 cell line, we observed a 6-fold induction of autophagy after CpG-ODN treatment compared with control conditions (Fig. 5B). Although rapamycin was able to induce autophagy in 293 Mock cells indicating that the autophagic pathway is func-
no difference was observed in these cells treated with CpG-ODN compared with control ODN, suggesting that the effect is TLR9-dependent (Fig. 5).

Assessment of CpG-ODN-induced Autophagy in TLR9-positive Tumor Cells in Vivo—To assess the relevance of our in vitro findings, we next examined whether CpG motifs could also induce autophagy in vivo in an experimental liver metastases model from colon carcinoma. We compared the number of autophagosomes in PROb tumors after intratumoral injection of CpG-ODN or control ODN using transmission electron microscopy (Fig. 6A). The median number of autophagosomes per field increased significantly in CpG-ODN-compared with control ODN-injected tumors (Fig. 6B). As bacterial plasmids, which present unmethylated CpG motifs, are widely used in preclinical and clinical cancer gene therapy, the same experiment was performed in tumors injected with a P, bearing active CpG sequences, or a Met-P in which the effect of CpG sequences has been abolished by in vitro methylation (25). Similarly the median number of autophagosomes per field increased significantly in P-compared with Met-P-injected tumors (Fig. 6, C and D). Thus, these data demonstrate for the first time that the presence of bacterial CpG motifs is associated with an increase of autophagy within the tumors in vivo.

Fig. 4. Autophagy induction by CpG-ODN in the human MCF-7 and PC-3 cancer cell lines. A and B, 48 h after transfection, cells were treated as described in Fig. 1A. Representative photographs of CpG-ODN- and control ODN-transfected cells in MCF-7 and PC-3 cell lines, respectively, are presented. Scale bars, 10 μm. C and D, the number of autophagic cells expressed as the median with 95% confidence intervals (700 GFP-positive cells counted in each condition) obtained with MCF-7 and PC-3 cells, respectively. Cells were scored without prior knowledge of the experimental conditions. Data are representative of three independent experiments. NS, not significant; 3-MA, 3-methyladenine.
DISCUSSION

Bacterial CpG motifs are PAMPs that are recognized by immune cells and trigger a proinflammatory immune response. Although the effects of CpG-ODN in immune cells have been widely studied, little is known regarding their molecular effects in tumor cells. In the present study, we demonstrated for the first time that bacterial CpG motifs can modulate tumor cell proteome in vitro and in vivo. Among the different proteins identified, several are related to autophagy. These proteins include potential autophagic substrates (ANXA1, PGAM1, pyruvate carboxylase, and citrate synthase) and glyceraldehyde-3-phosphate dehydrogenase for which

FIG. 5. TLR9 requirement for autophagy induction by CpG-ODN. A, 48 h after transfection, cells were treated as described in Fig. 1A. Representative photographs of CpG-ODN- and control ODN-transfected cells in the 293 TLR9 cell line are shown. Scale bars, 10 μm. The number of autophagic cells expressed as the median with 95% confidence intervals (125 GFP-positive cells counted in each condition) obtained with 293 TLR9 cells (B) and 293 Mock (C) is shown. Cells were scored without prior knowledge of the experimental conditions. Data are representative of three independent experiments. NS, not significant; 3-MA, 3-methyladenine.
an increased expression was shown to be sufficient to trigger an autophagic process (35, 36). These observations were further supported by the in vivo down-regulation of eukaryotic elongation factor 2, a key player involved in protein translation, as translation decreases toward an adaptative protein synthesis during autophagy (40). Interestingly eukaryotic elongation factor 2 was also found to be down-regulated in mouse small intestine after starvation, a process likely involving autophagy (41).

Autophagy is a process in which cellular organelles and bulk cytoplasm are targeted for degradation in lysosomes (22). Macroautophagy and CMA are two different types of autophagy that differ in the procedures used for delivery of substrates to lysosomes and the nature of these substrates. In macroautophagy (autophagy), cytoplasmic material is sequestered by a double membrane structure termed the autophagosome for subsequent degradation and recycling (22). Pyruvate carboxylase and citrate synthase were found to be degraded during autophagy (33, 34). In CMA, cytosolic proteins are delivered one by one into lysosomes (42), and ANXA1 and PGAM1 were described as substrates of CMA (31, 32). However, both autophagy and CMA are maximally activated by cellular stress, and a sequential switch from macroautophagy to CMA has been observed during starvation (43).

As macroautophagy is activated in response to extracellular pathogens such as bacteria (44), it was reasonable to think that bacterial CpG motifs, which are known as a danger signal, could trigger an autophagic process. In this study, we showed that CpG-ODN can induce autophagy in tumor cell lines from colon, breast, and prostate cancers and that TLR9 was required for this process. In addition, an increase in the number of autophagosomes can also be observed in vivo after intratumoral injection of CpG-ODN or plasmidic CpG motifs in a rat model of liver metastasis. Depending on the conditions, autophagy is able to induce cell survival or cell death (37). Thus, our data could reconcile the apparently conflicting results showing prosurvival (14–16) or proapoptotic (17–21) effects of CpG-ODN in tumor cells. Finally autophagy is also considered as an innate immunity mechanism because of its role in the removal of intracellular pathogens (44). Recent works have demonstrated that different PAMPs are able to induce autophagy via TLR3, -4, and -7 in macrophages (45, 46). Bacterial CpG motifs are defined as
a danger signal activating immune cells. In the present study, we demonstrated that these motifs can also be sensed as a danger signal in non-immune cells and trigger an autophagic process in TLR9-positive tumor cells, extending the link between TLRs and autophagy.

Acknowledgments—We thank N. Mizushima (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) for providing the pLC3 plasmid and F. Martin (Unité INSERM 517, Dijon, France) and J. Pouyssegur (UMR CNRS-UNSA 6543, Centre Antoine Lacassagne, Nice, France) for providing the PRoB and PC-3 cell lines, respectively. We also thank M. P. Nawrot (Unité INSERM 638) and P. Grattety for expert technical assistance (IFR50, Faculté de Médecine, Nice, France).

* This work was supported by the INSERM, Fondation de l’Avenir, France Cancer, and Association pour la Recherche sur le Cancer. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

REFERENCES

1. Krieg, A. M. (2002) CpG motifs in bacterial DNA and their immune effects. *Annu. Rev. Immunol.* 20, 709–760
2. Scheule, R. K. (2000) The role of CpG motifs in immunostimulation and gene therapy. *Adv. Drug Deliv. Rev.* 44, 119–134
3. Hemmi, H., Takeuchi, O., Kawai, T., Kaisho, T., Sato, S., Sanjo, H., Matsumoto, M., Hoshino, K., Wagner, H., Takeda, K., and Akira, S. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* 408, 740–745
4. Takeshita, F., Gursel, I., Ishii, K. J., Suzuki, K., Gursel, M., and Klinman, D. M. (2004) Signal transduction pathways mediated by the interaction of CpG DNA with Toll-like receptor 9. *Semin. Immunol.* 16, 17–22
5. Krieg, A. M. (2006) Therapeutic potential of Toll-like receptor 9 activation. *Nat. Rev. Drug Discov.* 5, 471–484
6. Schmidt, C. (2006) Immune system’s Toll-like receptors have good opportunity for cancer treatment. *J. Natl. Cancer Inst.* 98, 574–575
7. Platz, J., Beisswenger, C., Dalpke, A., Koczulla, R., PinKenburg, O., Vogelmeier, C., and Bals, R. (2004) Microbial DNA induces a host defense reaction of human respiratory epithelial cells. *J. Immunol.* 173, 1219–1223
8. Li, J., Ma, Z., Tang, Z. L., Stevens, T., Pitt, B., and Li, S. (2004) CpG DNA-mediated immune response in pulmonary endothelial cells. *Am. J. Physiol.* 287, L552–L558
9. Pedersen, G., Andresen, L., Matthiessen, M. W., Rask-Madsen, J., and Brynskov, J. (2005) Expression of Toll-like receptor 9 and response to bacterial CpG oligodeoxynucleotides in human intestinal epithelium. *Clin. Exp. Immunol.* 141, 289–306
10. Anderson, J. M., Al-Khairy, D., and Ingalls, R. R. (2006) Innate immunity at the mucosal surface: role of toll-like receptor 3 and toll-like receptor 9 in cervical epithelial cell responses to microbial pathogens. *Biol. Reprod.* 74, 824–831
11. Huang, B., Zhao, J., Li, H., He, K. L., Chen, Y., Chen, S. H., Mayer, L., Unkeless, J. C., and Xiong, H. (2005) Toll-like receptors on tumor cells facilitate evasion of immune surveillance. *Cancer Res.* 65, 5009–5014
12. Droemann, D., Albrecht, D., Gerdes, J., Ulmer, A. J., Branscheid, D., Vollmer, E., Dalhoff, K., Zabel, P., and Goldmann, T. (2005) Human lung cancer cells express functionally active Toll-like receptor 9. *Respir. Res.* 6, 1
13. Akhtar, M., Watson, J. L., Nazli, A., and McKay, D. M. (2003) Bacterial DNA evokes epithelial IL-8 production by a MAPK-dependent, NF-κB-indepen dent pathway. *FASEB J.* 17, 1319–1321
14. Jego, G., Bataille, R., Geoffroy-Luseau, A., Descamps, G., and Pellat-Deceunynck, C. (2006) Pathogen-associated molecular patterns are growth and survival factors for human myeloma cells through Toll-like receptors. *Leukemia* 20, 1130–1137
15. Bohnhorst, J., Rasmussen, T., Moen, S. H., Fiattum, M., Knudsen, L., Bost, M., Espesvik, T., and Sundan, A. (2006) Toll-like receptors mediate proliferation and survival of multiple myeloma cells. *Leukemia* 20, 1138–1144
16. Merrell, M. A., Ilvesaro, J. M., Lehtonen, N., Sorsa, T., Gehrs, B., Rosenthal, E., Chen, D., Shackley, B., Harris, K. W., and Selander, K. S. (2006) Toll-like receptor 9 agonists promote cellular invasion by increasing matrix metalloproteinase activity. *Mol. Cancer Res.* 4, 437–447
17. Wang, H., Rayburn, E. R., Wang, W., Kandimalla, E. R., Agrawal, S., and Zhang, R. (2006) Chemotherapy and chemosensitization of non-small cell lung cancer with a novel immunomodulatory oligonucleotide targeting Toll-like receptor 9. *Mol. Cancer Ther.* 5, 1585–1592
18. Wang, H., Rayburn, E. R., Wang, W., Kandimalla, E. R., Agrawal, S., and Zhang, R. (2006) Immunomodulatory oligonucleotides as novel therapy for breast cancer: pharmacokinetics, in vitro and in vivo antitumor activity, and potentiation of antibody therapy. *Mol. Cancer Ther.* 5, 2106–2114
19. Rayburn, E. R., Wang, W., Zhang, Z., Li, M., Zhang, R., and Wang, H. (2006) Experimental therapy of prostate cancer with an immunomodulatory oligonucleotide: effects on tumor growth, apoptosis, proliferation, and potentiation of chemotherapy. *Prostate* 66, 1653–1663
20. Rayburn, E. R., Wang, W., Zhang, R., and Wang, H. (2007) Experimental therapy for colon cancer: anti-cancer effects of TLR9 agonism, combination with other therapeutic modalities, and dependence upon p53. *Int. J. Oncol.* 30, 1511–1519
21. Andoulasis, A. E., Sonabend, A. M., Han, Y., and Lesnica, M. S. (2006) Stimulation of TLR9 with CpG ODN enhances apoptosis of glioma and prolongs the survival of mice with experimental brain tumors. *Glia* 54, 526–535
22. Dunn, W. A., Jr. (1996) Studies on the mechanisms of autophagy: formation of the autophagic vacuole. *J. Cell Biol.* 132, 1923–1933
23. Pierrefitte-Carle, V., Bague, P., Gavelli, A., Mala, M., Chazal, M., Guguen, J., Bourgeon, A., Milano, G., Staccini, P., and Rossi, B. (1999) Cytosine deaminase/5-fluorocytosine-based vaccination against liver tumors: evidence of distant bystander effect. *J. Natl. Cancer Inst.* 91, 2014–2019
24. Mizushima, N. (2004) Methods for monitoring autophagy. *Int. J. Biochem. Cell Biol.* 36, 2491–2502
25. Bertin, S., Anjuere, F., Gavelli, A., Baque, P., Sollihi, B. K., Brossette, N., Loubat, P., and Pierrefitte-Carle, V. (2008) Plasmidic CpG sequences induce tumor microenvironment modifications in a rat liver metastasis model. *Int. J. Mol. Med.* 21, 309–315
26. Martin, F., Caignard, A., Jeannin, J. F., Leclerc, A., and Martin, M. (1983) Selection by trypsin of two sublines of rat colon cancer cells forming progressive or regressive tumors. *Int. J. Cancer* 32, 623–627
27. Gayet, C., Pons, C., Guigonas, J. M., Pizzlo, J., Elies, L., Kennel, P., Rouqué, D., Bars, R., Rossi, B., and Samson, M. (2006) Protein profiling of rat ventral prostate following chronic finasteride administration: identification and localization of a novel putative androgen-regulated protein. *Mol. Cell. Proteomics* 5, 2031–2043
28. Kuo, C. C., Kuo, C. W., Liang, C. M., and Liang, S. M. (2005) A transcriptional and proteomic analysis of the effect of CpG-ODN on human THP-1 monocytic leukemia cells. *Proteomics* 5, 894–906
29. Duncan, R., Carpenter, B., Main, L. C., Telfer, C., and Murray, G. I. (2008) Characterisation and protein expression profiling of annexins in colorectal cancer. *Br. J. Cancer* 98, 426–433
30. Lim, L. H., and Pervaiz, S. (2007) Annexin 1: the new face of an old lysosomal protein. *FASEB J.* 21, 968–975
31. Cuervo, A. M., Gomes, A. V., Barnes, J. A., and Dice, J. F. (2000) Selective degradation of annexins by chaperone-mediated autophagy. *J. Biol. Chem.* 275, 33329–33335
32. Cuervo, A. M., Dice, J. F., and Knecht, E. (1997) A population of rat liver annexins by chaperone-mediated autophagy. *J. Biol. Chem.* 272, 32
33. Chandler, C. S., and Ballard, F. J. (1983) Inhibition of pyruvate carboxylase degradation and total protein breakdown by lysosomotropic agents in

**Molecular & Cellular Proteomics 7.12 2321**
CpG-induced Autophagy in Cancer Cells

3T3-L1 cells. Biochem. J. 210, 845–853
34. Watts, J. A., Kline, J. A., Thornton, L. R., Grattan, R. M., and Brar, S. S. (2004) Metabolic dysfunction and depletion of mitochondria in hearts of septic rats. J. Mol. Cell. Cardiol. 36, 141–150
35. Colell, A., Ricci, J. E., Tait, S., Milasta, S., Maurer, U., Bouchier-Hayes, L., Fitzgerald, P., Guio-Carrion, A., Waterhouse, N. J., Li, C. W., Mari, S., Barbuy, P., Newmeyer, D. D., Beere, H. M., and Green, D. R. (2007) GAPDH and autophagy preserve survival after apoptotic cytochrome c release in the absence of caspase activation. Cell 129, 983–997
36. Song, S., and Finkel, T. (2007) GAPDH and the search for alternative energy. Nat. Cell Biol. 9, 869–870
37. Debnath, J., Baehrecke, E. H., and Kroemer, G. (2005) Does autophagy contribute to cell death? Autophagy 1, 66–74
38. Noda, T., and Ohsumi, Y. (1998) Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J. Biol. Chem. 273, 3963–3966
39. Seglen, P. O., and Gordon, P. B. (1982) 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc. Natl. Acad. Sci. U. S. A. 79, 1889–1892
40. Lum, J. J., DeBerardinis, R. J., and Thompson, C. B. (2005) Autophagy in metazoans: cell survival in the land of plenty. Nat. Rev. Mol. Cell Biol. 6, 439–448
41. Lenaerts, K., Sokolović, M., Bouwman, F. G., Lamers, W. H., Mariman, E. C., and Renes, J. (2006) Starvation induces phase-specific changes in the proteome of mouse small intestine. J. Proteome Res. 5, 2113–2122
42. Massey, A., Kiffin, R., and Cuervo, A. M. (2004) Pathophysiology of chaperone-mediated autophagy. Int. J. Biochem. Cell Biol. 36, 2420–2434
43. Massey, A. C., Kaushik, S., and Cuervo, A. M. (2006) Lysosomal chat maintains the balance. Autophagy 2, 325–327
44. Deretic, V. (2006) Autophagy as an immune defense mechanism. Curr. Opin. Immunol. 18, 375–382
45. Xu, Y., Jagannath, C., Liu, X. D., Sharafkhaneh, A., Kolodziejska, K. E., and Eissa, N. T. (2007) Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. Immunity 27, 135–144
46. Delgado, M. A., Elmaoued, R. A., Davis, A. S., Kyel, G., and Deretic, V. (2008) Toll-like receptors control autophagy. EMBO J. 27, 1110–1121