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Research Article

Hsp90 binds CpG oligonucleotides directly: implications for Hsp90 as a missing link in CpG signaling and recognition

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Abstract. CpG motifs originating from bacterial DNA (CpG DNA) can act as danger signals for the mammalian immune system. These CpG DNA motifs like many other pathogen-associated molecular patterns are believed to be recognized by a member of the toll-like receptor family, TLR-9. Here we show results suggesting that heat shock protein 90 (hsp90) is also implicated in the recognition of CpG DNA. Hsp90 was characterized as a binder to oligodeoxynucleotides (ODNs) containing CpG motifs (CpG ODNs) after several purification steps from crude protein extracts of peripheral blood mononuclear cells. This finding was further supported by direct binding of CpG ODNs to commercially available human hsp90. Additionally, immunohistochemistry studies showed redistribution of hsp90 upon CpG ODN uptake. Thus, we propose that hsp90 can act as a ligand transfer molecule and/or play a central role in the signaling cascade induced by CpG DNA.

Key words. CpG DNA; TLR-9; CpG signaling; pattern recognition; innate immunity.

Utilization of DNA as a delivery vehicle, for the antigen of interest, is a rapidly developing vaccination strategy. During the development of this strategy, bacterial DNA was found to have direct effects on immune cells and on the outcome of the immune response, adding a further chapter to a story which is now over 100 years old [1]: the view on DNA has changed in an unexpected way – once considered immunologically inert, it is now recognized as being an immune effector. Certain immunostimulatory DNA sequences have thus been identified in the bacterial DNA backbone. One of these, the unmethylated CpG dinucleotide in a particular sequence context, is known as the CpG motif [2]. In mammalian genomes, these CpG motifs are under-represented in frequency and often methylated. Thus, unmethylated CpG motifs fulfill criteria for a pathogen-associated molecular pattern (PAMP) that can be recognized selectively by the mammalian immune system as a danger signal based on pattern discrimination between self and nonself.

Most research on CpG DNA has highlighted its effects on immune cells, but much remains to be investigated on the signaling pathways involved. Recent studies on knockout mice have shown that signaling by CpG DNA is mediated through a member of the toll-like receptor (TLR) family, TLR-9 [3]. However, no direct binding between TLR-9 and CpG DNA has been demonstrated. TLRs are present in mammals, invertebrates and plants, and represent conserved gene-encoded pattern recognition receptors (PRRs) that function to detect a potential invader [reviewed in ref. 4].

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In general, PRRs in vertebrates like the TLRs have evolved to recognize highly conserved PAMPs, i.e. CpG DNA, peptidoglycan (PG), lipopolysaccarides (LPS) and viral double-stranded RNA (dsRNA) and thus to initiate the immune alarm [5]. Despite their conservation and ancient origin, these receptors have adopted connections to highly advanced functions of adaptive immunity such as mediation of the Th1 response pathway by CpG DNA.

The present study was performed to identify protein(s) which directly bind oligo deoxynucleotides (ODNs) containing CpG motifs (CpG ODNs), and which might be involved in the signaling of CpG DNA. CpG ODN-binding protein(s) were purified from crude protein extracts of peripheral blood mononuclear cells (PBMCs), through monitoring of the CpG ODN-binding activity with a electromobility shift assay (EMSA). Sequence analysis of CpG material in a purified protein fraction indicated involvement of the cytoplasmic or membrane forms of heat shock protein 90 (hsp90). Binding was reproduced using commercially available hsp90. Furthermore, immunohistochemistry showed redistribution of hsp90 upon CpG ODN treatment.

Based on our results and those of other studies that link heat shock proteins to other TLRs [6–8], we suggest that hsp90 functions as a ligand transfer molecule in TLR-9 signaling.

Materials and methods

Cells

Buffy coats from healthy blood donors were obtained from Karolinska Hospital, Stockholm, Sweden, and PBMCs were isolated by Ficoll-Hypaque gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden).

Cell lines

All cell lines were of human origin: 293 (an embryonic kidney cell line), U937 (a monocytic cell line), K562 (a multipotent hematopoietic cell line), Raji (a B cell line) and Jurkat (a T cell line). Cells were maintained in RPMI 1640 culture medium (GIBCO) supplemented with 5% fetal calf serum (FCS) (GIBCO) and penicillin-streptomycin. For microscopy, LCL cells (lymphoblastoid B cell line 94C1) were utilized and grown in Iscove's modified Dulbecco's cell medium (GIBCO) containing 10% FCS and penicillin-streptomycin.

Oligodeoxynucleotides

All ODNs (phosphodiester) were purchased from Amersham Pharmacia Biotech and are listed in table 1.

Immunofluorescence microscopy

LCL cells (5×10^5) were incubated with different concentrations (0.2, 1, 2, 4 nmol/ml) of FITC-labeled ODNs for 5, 20 and 45 min at 37 °C. Cells were then cytospun onto glass slides, fixed with methanol/acetone (1 : 1) at –20 °C and rehydrated in PBS. Cells were then incubated with primary antibodies in a moist chamber using a rabbit polyclonal anti-hsp90α and anti-hsp90β (Sigma) or a rabbit polyclonal anti-hsp70 (Santa Cruz) or a mouse monoclonal anti-vimentin (DAKO). TRITC-conjugated pig anti-rabbit (DAKO) or Texas red-conjugated horse anti-mouse antibody (Vector) were used as secondary antibodies. All antibodies were diluted in blocking buffer (2% BSA/0.2% Tween-20/10% glycerol/0.05% NaN_3 in PBS) and DNA was stained with Hoechst 33258. The glass slides were mounted with 70% glycerol containing 2.5% DABCO anti-fading agent (Sigma). The following controls were used: (i) untreated cells (without ODN) stained for hsp90 or (ii) ODN-treated cells stained only with the secondary antibodies. Glass slides were viewed for intracellular distribution of FITC-labeled ODNs and hsp90, hsp70 or vimentin staining. Images were collected using a Leitz DM RB microscope, equipped with a Leica PL Fluotar 100 × oil immersion objective. Composite filter cubes were used for the FITC, Texas red/rhodamine and Hoechst 33258 fluorescence. Pictures were captured with a Hamamatsu dual-mode cooled CCD camera

Table 1. Oligodeoxynucleotides (ODNs) used in EMSA for binding activity.

Description	Name used in text	Sequence 5′ to 3′
Starting sequence [13]	1 AACGTT	ACCGATAACGTTGCCGGTGACG
CpG inversion of 1 AACGTT	2 AAGCTT	ACCGATAAGCTTGCCGGTGACG
Two base pair-disrupted CpG motif of 1 AACGTT	3 ACCATT	ACCGATACCATTGCCG GTGACG
Methylated CpG motif of 1 AACGTT	4 AAOGTT	ACCGATAAOGTTGCCGGTGACG
Alternative CpG motif	5 GACGTT	ACCGATGACGTTGCCGGTGACG
CpG inversion of 5 GACGTT	6 GAGCTT	ACCGATGAGCTTGCCGGTGACG
A human CpG motif [15]	7 TCGTCGTT	TCGTCGTTCC CCCCCCCCCC
CpG inversion of 7 TCGTCGTT [15]	8 TGCTGCTT	TGCTGCTTC CCCCCCCCCCC
Methylated CpG motif of 7 TCGTCGTT [15]	9 TOGTOGTT	TOGTOGTTCCCCC CCCCCCC
A random 19-mer	10 Random	GTACATGAC CTTTACGGGA

O indicates a methylated cytosine.

(C4880), and recorded and analyzed on a Pentium PC computer equipped with an AFG VISIONplus-AT frame grabber board using Hipic5.1.0 (Hamamatsu) and Image-Pro Plus (Media Cybernetics). Digital images were assembled using Adobe Photoshop software.

Preparation of protein extracts from PBMCs and cell lines

Protein extracts from PBMCs or the different human cell lines were prepared as previously described [9]. Approximately 500×10^6 cells from PBMCs or 60×10^6 cells from spinner cultures were used. The supernatants from the nuclear (N) fraction and cytoplasmic/membrane (C/M) fraction were stored at – 70 °C prior to use. Protein concentration was measured using a BioRad protein assay according to the manufacturer's instruction.

Electrophoretic mobility shift assay

To detect DNA-binding activity for the different ODNs, protein extracts were analyzed by EMSA using the ODNs listed in table 1 as probes. ODNs (1.5 pmol) were end-labeled with T4-polynucleotide kinase (GIBCO) and (γ^{32}P)-ATP (Amersham Pharmacia Biotech) ($\approx 1.0 \times 10^6$ cpm/pmol when measured). Binding reactions were performed with approximately 5 µg C/M or N fractions of the different protein extracts, chromatographic fractions, or 3 µg commercially available hsp90 (Sigma). Electrophoresis of the different samples was carried out in 6% nondenaturating polyacrylamide gels (Invitrogen) at 125 V in 0.5 × Tris-borate-EDTA (TBE). Results were visualized by exposure to X-ray film, or by phosphorimager (Molecular Dynamics).

Reverse-phase chromatography

The C/M protein extract (1 ml) was applied onto an OASIS (Waters) cartridge activated with methanol and equilibrated in water. Before a stepwise elution with increasing methanol concentrations, the cartridge was washed with 2 ml water. All fractions, including the flow through and washings, were collected and tested for ODN binding by EMSA using 1 AACGTT.

High-performance liquid chromatography

An ÄKTA purifier system (Amersham Pharmacia Biotech) was utilized for high-performance liquid chromatography (HPLC) and the column effluent was monitored at 214 and 280 nm. All chromatography was performed in a modified binding buffer, pH 7.0 (20 mM Hepes pH 7.9, 100 mM NaCl, 1 mM EDTA). For cation exchange chromatography, a Mini S PE 4.6/50 in the modified binding buffer was used. The column was first washed with 2 column volumes (CVs) of the buffer and then eluted with 10 CVs of 1 M NaCl in the buffer. The flow rate was 0.5 ml/min and 0.5-ml fractions were collected. Size exclusion chromatography was performed on a Superdex 200 HR 10/30 column in the modified binding buffer supplemented with 0.5 mM dithiothireitol (DTT) and 0.04 mM phenylmethyl sulfonyl fluoride (PMSF) and elution was in 1.5 CVs at a flow rate of 0.5-ml/min with collection of 0.5-ml fractions. The anion exchange chromatography was with a Mini Q PE 4.6/50 column in binding buffer (pH 8.0), containing 0.5 mM DTT and 0.04 mM PMSF. At this step, the column was also washed with 2 CVs of the buffer and 0.25-ml fractions were collected. Subsequent elution was with 10 CVs in the same buffer with 1 M NaCl and fractionated in 0.5 ml.

Gel electrophoresis

Proteins were separated according to size in 10% Tris/glycine gels (Invitrogen). Detection was accomplished with silver staining for the analytical runs and with Coomassie staining for the preparative procedure according to the manufacturer's instructions (Invitrogen).

In situ digestion of proteins from SDS/PAGE

Digestion of proteins from one-dimensional (1 D) gels was accomplished as described by Shevchenko et al. [10].

Nano-HPLC microelectrospray ionization mass spectrometry and database search

Mass spectra were acquired using the combination of nanoflow HPLC and microelectrospray ionization mass spectrometry on an LCQ ion trap mass spectrometer (ThermoFinnigan) as described previously [11, 12].

Results

Electromobility shift assay

To detect components that might be involved in direct binding of CpG DNA and that mediate signaling in innate immunity, we used an EMSA with nuclear and cytoplasmic/membrane extracts prepared from PBMCs. A shifted band was detected in EMSA using a ^{32}P-end-labeled ODN containing a CpG motif, 1 AACGTT [13] (table 1, fig. 1 A). This experiment was reproduced for all blood donors used (data not shown). The study was further extended to include EMSA on C/M and N protein extracts (data not shown) prepared from different human cell lines, all of which showed the same shifted band (fig. 1 B). In summary, these results indicated that protein(s) detected in the EMSA were not specific for a particular cell type. In the further studies, we focused on the C/M fraction.

Sequence specificity of CpG ODN-binding protein(s)

To assess specificity of the CpG ODN-binding protein(s), we performed the EMSA on C/M extracts from PBMCs

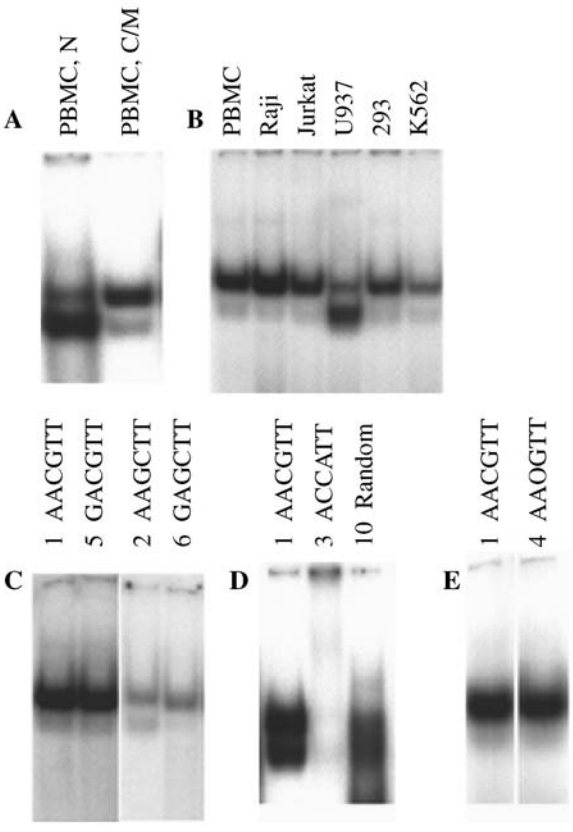

Figure 1. EMSA using protein extracts from PBMCs and several cell lines. Protein extracts from PBMCs were incubated with 1.5 pmol of ^{32}P-end-labeled ODNs in a total volume of 10 µl for 40 min on ice and then subjected to EMSA. (*A*) Nuclear (N) and cytoplasmic/membrane (C/M) fractions from PBMCs using 1 AACGTT. (*B*) C/M fractions (5 µg) from PBMCs and the human cell lines Raji, Jurkat, U937, 293 and K562 using 1 AACGTT. In all these extracts, a shifted band was detected. For sequence dependency, the C/M fraction of PBMCs was used with ODNs with the CpG motifs 1 AACGTT and 5 GACGTT and their GC-inverted controls 2 AAGCTT and 6 GAGCTT (*C*), the ODN with CpG motif 1 AACGTT, the ODN with disrupted CpG motif 3 ACCATT, and a random 19-mer, 10 Random (*D*) and ODN 1 AACGTT with a CpG motif and ODN 4 AAOGTT with a methylated cytosine (*E*). Protein binding was decreased when the CpG was absent, while methylation did not affect the binding. (*C*) Two parts from the same gel and the same exposure time.

using different ODNs. This set of experiments demonstrated that the DNA-binding protein(s) have similar binding capacity to an ODN containing another CpG motif known to stimulate immune cells [2], 5 GACGTT (fig. 1C), while non-CpG motifs containing ODNs (2 AAGCTT, 6 GAGCTT, 3 ACCATT, 10 Random) resulted in no binding or weaker binding than that observed for 1 AACGTT (fig. 1C, D). However, methylation of the cytosine in the CpG motif (4 AAOGTT) did not eliminate binding (fig. 1E). We conclude that protein(s) in the C/M extract interact with CpG ODNs in a manner that is independent of the cytosine methylation but affected by flanking residues.

Isolation and characterization of hsp90

Starting material for the isolation procedure was C/M protein extracts prepared from 5×10^8 human blood cells, enriched for PBMCs. To characterize the protein that binds to the CpG ODN (1 AACGTT), we employed a separation scheme that involved reverse phase with OASIS cartridges followed by cationic exchange, gel filtration and anionic exchange chromatography. EMSA was employed to analyze fractions from each chromatography step. After all these separation steps, we detected binding with the CpG ODN (1 AACGTT) in a fraction that contained two dominating protein bands (fig. 2).

For step one, C/M extract was loaded onto a reverse-phase OASIS cartridge, in water. Elution was accomplished stepwise with increasing concentrations of methanol. Protein binding was detected in the flow-through fraction. This was lyophilized and the sample was resuspended in binding buffer (pH 7), loaded onto a cationic exchange column and eluted with a 1 M NaCl gradient. Twenty-four fractions were collected. CpG ODN-binding protein(s) eluted in fractions 2–3 and in fraction 10 (fig. 3A). The sample responsible for CpG binding in fraction 10 could not be recovered after the next separation step. Material in fractions 2–3 was pooled and fractionated by size exclusion chromatography, utilizing a Superdex 200 column. Seventy fractions were collected (fig. 2B) and CpG binding protein was detected in fractions 20–21. Material

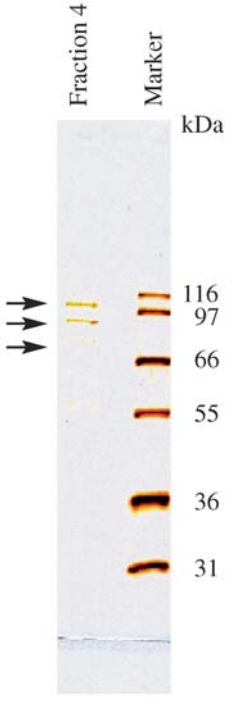

Figure 2. SDS/PAGE on the active fraction from the final purification step. Fraction 4 (10 µl of 250 µl) from the anion exchange chromatography was analyzed by SDS/PAGE. By silver staining, two dominant protein bands of 100 and 80 kDa were visualized.

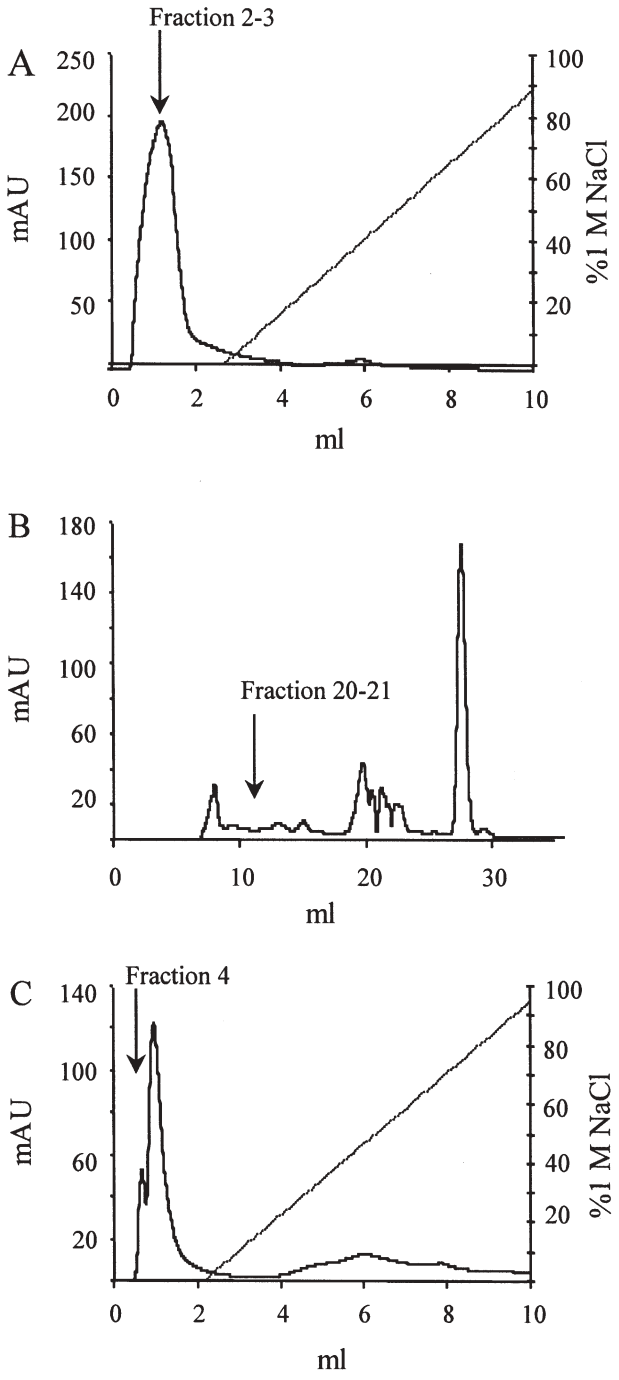

Figure 3. Purification steps of a CpG ODN-binding protein from C/M protein extracts. EMSA was used for detection of CpG ODN-binding protein in chromatographic fractions. Fractions with CpG-binding activity are indicated by vertical arrows. (*A*) Cation exchange chromatography. (*B*) Size exclusion chromatography. (*C*) Anion exchange chromatography.

from these two fractions was pooled, concentrated in binding buffer at pH 8, and subjected to anionic exchange chromatography on a Mini Q column using a gradient of 1 M NaCl. Twenty-seven fractions were collected and binding activity was detected in fraction 4 (fig. 2C). A 10-µl aliquot of this fraction was loaded on SDS/PAGE. Two dominan bands at 100 and 80 kDa, respectively, were detected (fig. 2). For sequence analysis, all the material in fraction 4 was run on SDS/PAGE, stained with Coomassie blue, and three bands were detected. These were cut from the gel, proteins in each band were digested with trypsin, and the resulting tryptic peptides were then analyzed by nano-HPLC microelectrospray ionization mass spectrometry on an ion trap mass spectrometer. Multiple peptides from heat shock protein hsp90α and hsp90β were found in all three bands analyzed. In the band containing the 80-kDa protein, 21 and 13 peptides from hsp90α and hsp90β were detected, respectively. These represent 40% of the hsp90α sequence and 26% of the hsp90β sequence.

Direct binding of CpG ODN to hsp90

To confirm the above result, we performed CpG-binding experiments on commercially available human hsp90α and hsp90β proteins. We found that hsp90 protein bound to the ODNs with the same specificity as the original extract (fig. 4A, B). During the course of this work, ODN 1 AACGTT was defined as a good stimulant for murine cells [13]. In addition, an ODN containing this CpG motif has previously been shown to stimulate human lymphocytes [14]. When we tested defined ODNs that were optimal for stimulation of human B cells [15] table 1), binding to hsp90 was readily detected (data not shown).

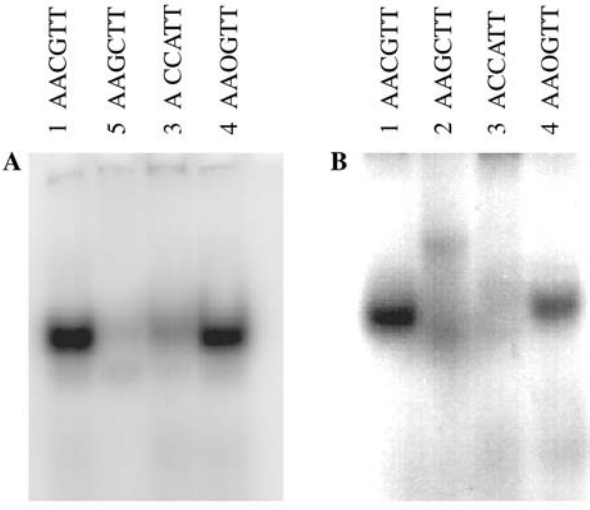

Figure 4. The protein hsp90 binds CpG ODNs. (*A*) A set of ODNs were utilized for binding specificity of C/M protein extracts (5 µg) from PBMCs. (*B*) The same set of ODNs were used for binding of commercially available hsp90 (3 µg).

Visualization of redistribution of hsp90 after CpG ODN treatment

Since EMSA analysis demonstrated direct binding of hsp90 and CpG DNA, we next attempted to visualize whether a cellular distribution of hsp90 could be linked to the intracellular localization of FITC-labeled CpG ODN. For this purpose, a B cell line (LCL 94C1) was treated with 1 AACGTT and 4 AAOGTT and then stained for hsp90. Interestingly, microscopic examination showed a significantly higher cellular uptake for the CpG ODN (18%) than for the methylated ODN (5%). After ODN uptake by healthy cells (fig. 5A), the number of apoptotic cells increased. In nonapoptotic cells containing CpG ODN, a redistribution of hsp90 from the cytoplasm into the nuclear compartments was observed after 20 and 45 min (fig. 5B, C). This redistribution was not observed with hsp70 (fig. 5D) or with vimentin (data not shown). This demonstrated that the response was specific to hsp90 and not shared by other heat shock proteins. Of interest, furthermore, is that a CpG nonresponder, the epithelial cell line 293, did not show this redistribution of hsp90. Thus, the binding of hsp90 to the CpG ODN seems to be connected to a cellular redistribution of hsp90.

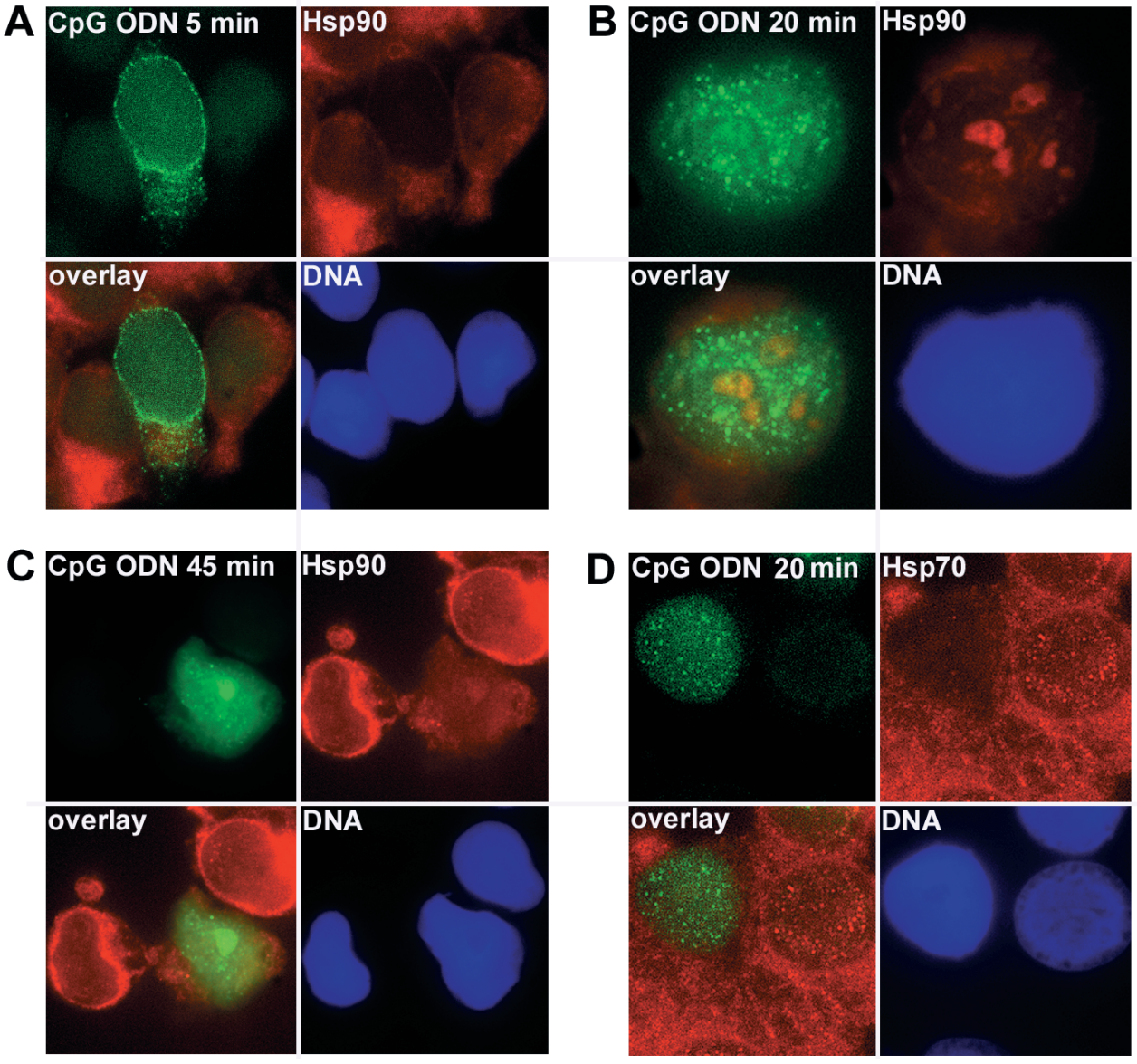

Figure 5. ODN 1 AACGTT uptake and redistribution of hsp90. Immunohistochemisry on LCL 94C1 cells incubated at different time points with FITC-labeled ODN 1 AACGTT and anti-hsp90 at different incubation times of 5 min (*A*), 20 min (*B*), 45 min (*C*) and FITC-labeled ODN 1 AACGTT and anti-hsp70 after 20 min (*D*). In all cases, 2 nmol/ml of ODN was used. The ODN was taken up, detected in cytoplasm (*A*), and transported to the nucleus as was hsp90 during this process (*B*, *C*). The ODN and hsp90 were colocalized in nuclear bodies (*B*). No redistribution of the hsp70 protein was detected (*D*).

Discussion

Recent research on PAMPs connected to immunity has highlighted the importance of innate responses [16]. The patterns in question involve PG, LPS, bacterial flagellin, lipoteichoic acid, dsRNA and bacterial DNA. These materials are specific to bacteria and viruses and could signal the presence of a potential infectious agent. DNA is special since it has the same chemical composition and double-helical structure whether it originates from viruses, bacteria or vertebrates. Discrimination between self and foreign DNA depends on certain sequence motifs and modifications such as methylation [1]. Recognition of these patterns can initiate signal transduction within cells and communication between cells and thus marks the set point for the immune response with secretion of cytokines that activate the Th1 pathway and upregulation of costimulatory molecules needed for the adaptive response.

Functional analyses in gene-deficient mice have identified TLR-9 as the only receptor-like molecule known so far to be necessary for responses mediated by immunostimulatory CpG DNA sequences [3]. The main model for CpG DNA recognition and signaling suggests that TLR-9 activation and signal transduction from the endosome to the cytoplasm activates myeloid differentiation marker (MyD88), IL-1 receptor-associated kinase (IRAK) and tumor necrosis receptor-associated factor 6 (TRAF6) [17]. These cytoplasmic mediators induce further downstream transduction through activation of MAP kinases and nuclear translocation of the transcription factor NF-κB, which initiates transcription of many immune response genes [18, 19]. Of importance here is that direct binding between PAMPs and TLRs has not been shown experimentally.

Here, we demonstrated that hsp90 selectively binds ODNs containing immunostimulatory CpG motifs. This protein was isolated from extracts of human blood cells enriched for PBMCs by a series of chromatographic steps. CpG ODN-binding activity was detected in chromatographic fractions by EMSA. After the final purification step, protein in the active fraction was separated by SDS/PAGE, digested in situ, and sequenced by nano-HPLC mass spectrometry. Hsp90 was identified as the major protein in the fraction with the CpG ODN-binding activity. Commercially available hsp90 was also shown to bind ODNs containing CpG motifs and comigrated with isolated hsp90 in the EMSA. Hsp90 was also shown to bind ODNs containing either a methylated or nonmethylated cytosine in CpG motifs. Interestingly, cellular uptake of CpG ODNs was shown by immunohistochemistry to promote redistribution of hsp90 to specific nuclear bodies together with the CpG ODN. Location of hsp70 in the cell was not affected by CpG ODN uptake. Moreover, a CpG-nonresponding cell line did not show hsp90 redistribution.

Heat shock proteins were originally defined as chaperones that promoted folding. Later, they were also associated with delivery of peptide antigens to antigen-presenting cells and activation of cytotoxic T lymphocytes specific for the antigen [20]. Hsp60 can also stimulate the production of tumor necrosis-factor-α via the membrane receptors CD14 and TLR-4 [21]. Thus, heat shock proteins facilitate immune responses through stimulation and antigen presentation. Accordingly, heat shock proteins have been suggested to be one of the first endogenous mammalian adjuvants [20].

In the present work, we identified hsp90 as a binder of immunostimulatory ODNs. This result suggests an interactive link between hsp90 and TLR-9. In fact, TLR-9 has also been shown to colocalize with CpG ODNs during stimulation [22]. Of relevance here is that several recent reports demonstrate a link between heat shock proteins and other TLR receptors. Heat shock proteins hsp60, hsp70 and gp96 can activate intracellular innate signaling through TLR-2 and TLR-4 [6–8].

Recent work has shown hsp70 and hsp90 to be intermediate components of the LPS signal pathway [23, 24]. The suggested model for this involved CD14 binding to LPS and then delivery of LPS to a membrane-associated signal-transducing multimeric complex containing hsp70 and hsp90 [23]. TLR-4 is also known to be important for LPS signaling but the study from Triantafilou et al. [24] did not characterize TLR-4 as a part of the multimeric signal complex. Indeed, there are similarities in LPS and CpG signaling with involvement of TLR-4 and TLR-9, respectively, and activation of similar intracellular pathways.

Taken together, these results are especially interesting in connection with the TLR receptors, as one main obstacle in understanding their signal mediations has been the lack of evidence for direct binding of PAMPs to the corresponding TLR. These receptors could be parts of multimeric complexes where the ligands are only briefly associated with the TLR in a selection process. From partially purified fractions with CpG ODN-binding activity, we obtained evidence for several proteins by in gel digestion with trypsin and subsequent peptide mapping. One peptide pattern indicated the presence of TLR-9 (data not shown), suggesting an interaction between TLR-9 and hsp90. In support of our data, a recent paper shows that the specific inhibitor of hsp90, geldanamycin, inhibits CpG cellular stimulation [25]. Geldanamycin did not affect CpG ODN binding in our EMSA. This could be due to binding at a different site, or due to higher affinity binding of the ODN to the activation site under the assay conditions.

Beside TLRs and their adaptors, DNA protein kinases (DNA-PKs) are clearly necessary in the intracellular chain of events in CpG signaling [26]. Such a link to TLR-9 has not been established, although parallel activation seems likely. Interestingly, DNA-PK causes nuclear retention of the interferon regulatory factor-3 known to mediate transcription of immune response genes [27]. A similar con-

nection to hsp90 is conceivable, as we demonstrated that hsp90 is quickly translocated to the nucleus upon CpG stimulation. In addition, hsp90 has been found involved in the assembly of transcriptional complexes [28] and in this way might activate gene transcription that is dependent on CpG DNA stimulation.

Of interest is to consider the two main models for immunity, i. e. the self-nonself model [29] and the danger model [30]. In the first model, recognition of foreign structures by receptors like TLRs is necessary to mediate costimulation needed for responses, while the danger model emphasizes recognition of endogenous molecules that are released due to injury. Indeed, heat shock proteins were included as signal mediators in the danger model. In this study, we demonstrated how a foreign pattern like CpG ODNs is bound to endogeneous hsp90, and TLR-9 is known to be necessary for this signaling. Hence, the sequence order could be binding of CpG DNA to a complex of hsp90 and TLR-9 where the hsp90 is sequence specific but TLR-9 discriminates based on methylation.

In summary, a double function for the heat shock proteins may be suggested. One function is to present PAMPs including CpG ODNs to the TLRs in analogy with peptide delivery in the antigen specific response. The other function is to mediate intracellular signaling with recruitment of hsp90 to the nucleus.

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