Characterization and Biological Significance of Immunosuppressive Peptide D2702.75–84(E → V) Binding Protein

ISOLATION OF HEME OXYGENASE-1*

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This is the first report on peptidic inhibitors of heme oxygenase. Such peptides were originally developed from the immunomodulatory peptide 2702.75–84 which corresponds to amino acid residues 75 to 84 of the α1-helix of HLA-B2702 (2702.75–84) and has been shown to be immunosuppressive in vitro and in vivo. In vitro, 2702.75–84 inhibited cytotoxic T- and natural killer cell-mediated target cell lysis, and in vivo peptide therapy resulted in prolongation of heart and skin allograft survival in mice. The peptide was also shown to bind to heat shock protein 70. However, D-enantiomers of 2702.75–84 and derivatives thereof, while still being immunosuppressive, did not bind to heat shock protein 70. This study was designed to identify proteins binding to peptide D2702.75–84(E → V) (rvnrlarly) consisting of D-amino acids. Compared with 2702.75–84 (RENRLIRLY), glutamic acid residue 76 (E) was replaced with valine (V). Affinity chromatography using immobilized D2702.75–84(E → V) and mouse and human cell extracts, resulted in the isolation of heme oxygenase-1 (HO-1). Peptide D2702.75–84 inhibited HO activity in vitro in a dose-dependent manner. Similar to what has been observed with other inhibitors of HO, administration of peptide into mice resulted in up-regulation of HO-1 mRNA and protein, as well as enzyme activity in liver, spleen, and kidney. Other peptides derived from 2702.75–84 with similar immunomodulatory activity displayed similar effects. In contrast, inactive derivatives of 2702.75–84 had no effect on HO activity. Therefore, the immunosuppressive effects of the described immunomodulatory peptides are similar to those of cobalt-protoporphyrazin, a known up-regulator of HO-1. Our results suggest that HO-1 modulation may be a novel mechanism of immunomodulation.

Peptides derived from various regions of the HLA class I heavy chain have been shown to exert immunomodulatory effects by influencing T cell responses (1–4). Clayberger and co-workers (5, 6) studied the less polymorphic region of the HLA class I α1-helix (residues 75–84). Peptides corresponding to this region of the HLA-B7 (07.75–84) or HLA-B2702 (2702.75–84) molecule blocked the differentiation of human CTL precursors in vitro in a non-allele-restricted manner (5).

The 2702.75–84 peptide inhibited not only differentiation, but also T and NK cell-mediated lysis of target cells in a non-allele-restricted manner. Woo et al. (7) subsequently demonstrated a more powerful effect when the peptides were used in a dimeric form (2702.75–84(E → V)). These peptides have also been studied in vivo in transplantation models. In rats, 07.75–84 induced permanent acceptance of LEW heart allografts in ACI recipients when combined with subtherapeutic doses of cyclosporine A (6). In congenic LEW.1W donors and LEW.1A recipients, 07.75–84 induced long-term graft survival without additional cyclosporine A treatment (8). In a mouse heart transplant model, where C57BL/6 mice were used as donors and CBA mice as recipients, treatment with both 2702.75–84 and 2702.75–84(E → V) prolonged graft survival significantly, with B2702.75–84(E → V) showing a more powerful effect (7, 9, 10). Administration of 2702.75–84 has been shown to prolong the survival of skin allografts significantly when the tail skin of C57BL/6 mice was grafted on CBA mice (9).

Despite the peptide’s effectiveness, its mechanism of action is not fully understood. For reasons discussed elsewhere (7, 9, 10) a direct interaction with T cell receptors or NK cell inhibitory receptors can be excluded. The recent observation that both L- and D-enantiomers of peptide 2702.75–84 prolong heart allograft survival in vivo indicated that the peptides’ immunomodulatory activity is probably not based on indirect presentation by major histocompatibility class molecules (10). Similarly, the recent hypothesis that peptide 2702.75–84 modulates immune responses by binding to HSP/HSC70 can be excluded because the D-isomer of 2702.75–84 did not bind such proteins (11, 12).

Based on these observations, we looked for additional proteins that may interact with these immunomodulatory peptides. For these studies, we used a 2702.75–84-derived peptide (D2702.75–84(E → V)) with two modifications: (i) synthesis with D-amino acids and (ii) substitution of glutamic acid residue at position 76 in 2702.75–84 with valine. Compared with peptide 2702.75–84, this peptide displayed enhanced immunomodulatory activity in vitro and in vivo (12). Affinity purification using this peptide resulted in the isolation of heme oxygenase-1 (HO-1, HSP32). Effects of peptide binding to this enzyme were analyzed in vitro and in vivo.

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§ The abbreviations used are: NK, natural killer; HO, heme oxygenase; Fmoc, N-(9-fluorenyl)metoxycaronyl; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; CoPP, cobalt-protoporphyrin.
Peptide-mediated Up-regulation of HO-1

Amino acid sequencing of the ~30-kDa band was performed by Edman chemistry using an automated ABI 470 A system sequencer with an on-line ABI 128 HPLC and analyzed on ABI 610 system software at the Protein structure laboratory, University of California, Davis, CA. The sequence was compared with known sequences in the University of Wisconsin GCG data base.

Affinity Chromatography

SulfoLink gel was washed several times with 10 mM phosphate buffer containing 150 mM NaCl, 0.1% CHAPS, and protease inhibitors (wash buffer) and incubated with cell lysate for 60 min at 4 °C with continuous rocking. The gel was packed in a column and unbound cell lysate was recovered. The column was washed with 20 column volumes of wash buffer, followed by wash buffer containing 500 mM NaCl. The latter fraction was termed high salt eluate. Bound proteins were eluted with 100 mM glycine-HCl, pH 3.0, containing 0.1% CHAPS and protease inhibitors in 1-ml fractions that were immediately neutralized to pH 7.0 using 1 M Tris-HCl, pH 8.2. Protein concentration was estimated by the BCA assay (Pierce). Fractions containing protein were pooled, concentrated in a microconcentrator, and analyzed by SDS-polyacrylamide gel electrophoresis and western blotting. All fractions were stored at −80 °C.

Gel Electrophoresis and Western Blotting

Approximately 1 μg of pH 3.0 eluate, 5 μg of the high salt eluate, and 20 μg of cell lysate were boiled in SDS sample buffer containing 2-mercaptoethanol and separated on a 12% SDS-polyacrylamide gel. Proteins were visualized by silver staining (13).

Western transfer was carried out using the blot module from Novex (San Diego, CA) on nitrocellulose (Hybond-N, Amersham Corp.) in transfer buffer (12 mM Tris, 96 mM glycine, pH 8.3) at 30 V for 45 min. Efficiency of transfer was monitored by including prestained molecular weight markers (Amersham Corp.) on the gel. Nonspecific binding sites determined in the presence of purified preparations of biliverdin reductase at 4 °C. The reaction was initiated by the addition of hemin (final concentration 0.25 mM). The reaction mixture was incubated at 37 °C in the dark for 60 min. At the end of the incubation period, any insoluble material was removed by centrifugation, and supernatants were analyzed for biliverdin concentration by a modified procedure of Hillman and Beyer (Sigma Diagnostics, kit no. 552). Controls included spent samples in the absence of the NADPH-generating system and all components of the reaction mixture in the absence of spleen homogenates. Biliverdin reductase was purified from rat liver by the method described by Kutty and Malcom (14).

To determine HO activity in purified preparations of HO-1, the procedure described previously was used and the activity was determined in the presence of purified preparations of biliverdin reductase and NADPH-cytochrome P450 reductase (15).

Biliverdin Reductase—Biliverdin reductase activity was determined by measuring the rate of bilirubin formation (14). To a reaction mixture consisting of 0.1 ml Tris-HCl (pH 8.7), 0.1 mM NADPH, 5 μM (approximately 200 ng of purified enzyme protein), 50 μM biliverdin, and peptides at a final concentration of 100 μM were added. The control reaction mixture did not contain peptides. The reaction was carried out in the dark at 37 °C for 30 min, and the bilirubin formed was measured as described above.

Lactate Dehydrogenase and Horseradish Peroxidase—Lactate dehydrogenase enzyme activity was determined using whole cells (E6-1) as the source as described previously (16). Horseradish peroxidase enzyme activity was measured by addition of o-phenylenediamine. Following incubation at room temperature, the reaction was stopped by the addition of HCl, and the optical density was measured at 490 nm.
Total RNA was prepared from tissues by the method of Chirgwin et al. (20). Poly(A⁺) RNA was isolated by oligonucleotide(dT) cellulose chromatography, fractionated on a 12% agarose gel, and transferred to Nytran (Schleicher & Schuell). Prehybridization, hybridization to the appropriate 32P-labeled cDNA, and post-hybridization treatment of the blots were performed as described elsewhere (21). The signals on autoradiograms were quantitated using an LKB Ultrascan densitometer.

**In Vivo Effects of Peptide Treatment**

Normal CBA mice were treated with a single injection (intraperitoneal) of peptide D2702.75–84(E → V) at 80 mg/kg dissolved in PBS + 10% Me₂SO. Control animals were treated with an equal volume of PBS + 10% Me₂SO. At 6, 24, and 48 h post-treatment, animals were killed, and liver, spleen, kidney, heart, and blood (serum) were collected and rapidly frozen. Tissue samples were used for northern blotting to detect HO-1 mRNA and western blotting to detect HO-1 protein levels. Microsomes were prepared from spleen samples and used for HO activity measurements and western blotting.

**RESULTS**

**Isolation of Heme Oxygenase-1**—Recently, peptide 2702.75–84 (RENRLIALRY) and 2702.75–84(E → V) (RVNL- RIALRY) were shown to bind to HSC70 and HSP70 (11, 12). In contrast, β-antennomers of these peptides did not bind to HSC70, although these peptides inhibited cytotoxicity and prolonged murine heart allograft survival to a similar extent as the β-antennomers. These findings prompted us to explore additional peptide binding proteins that may mediate the peptide’s immunosuppressive effects. Whole cell lysates from Jurkat cells (Fig. 1A), a human T cell lymphoma, and YAC-1 cells (Fig. 1B), a mouse lymphoma, were incubated with an affinity matrix consisting of peptides coupled to agarose via an N-terminal cysteine residue. Proteins eluted from the affinity gel at low pH were analyzed by SDS-gel electrophoresis. Using peptide D2702.75–84(E → V) (rvnrlialry) the pH 3.0 eluate contained a major band of approximately 30 kDa (Fig. 1, lane 4). This protein was purified from both human Jurkat and murine YAC-1 cells. Control runs using SulfoLink-agarose gel containing cysteine or peptide rvntialry (negative control) did not result in the purification of the 30-kDa protein (data not shown).

Proteins in cell lysates and various fractions eluted from the affinity matrix were analyzed with various antibodies following transfer to nitrocellulose. Incubation of western blots with anti-HSC/HSP70 antibody revealed the presence of these proteins in the whole cell lysate and the high salt wash, but not in the low pH eluate of E6-1 (Fig. 2A, lane 4) or YAC-1 cell lysate (Fig. 2B, lane 4). Probing the western blots with anti-HSP25 and anti-HSP27 did not reveal the presence of these proteins in any of the fractions (data not shown). In contrast, analysis of the blots with anti-HSP32 resulted in a positive signal in the protein fraction eluted at pH 3.0. This result was obtained with proteins purified from both E6-1 (Fig. 3A) and YAC-1 (Fig. 3B) cell extracts. The positive band on the western blot corresponded to the single band observed in the SDS-polyacrylamide gel. No detectable signal was seen with whole cell lysate or the high salt fractions of either cell extract.

In an effort to confirm its identity and compare it to other published sequences, the single ~30-kDa band obtained in the pH 3 fractions that bound to anti-HSP32 antibody as well as peptide D2702.75–84(E → V), was further analyzed for partial amino acid sequence. Complete homology was observed with human and mouse HSP32 and 97% homology with rat HSP32 (Table I), thus confirming that the eluted protein was HSP32 or HO-1.

**Inhibition of Heme Oxygenase Activity by D2702.75–84(E → V)**—Heme oxygenase catalyzes the degradation of heme into biliverdin which is subsequently reduced to bilirubin by biliverdin reductase. When the activity of purified recombinant HO-1 was assessed in the presence of peptide D2702.75–84(E → V), a significant dose dependent reduction of enzyme activity was observed (Fig. 4): half-maximal inhibition of HO-1 activity occurred at about 20 μM. The inhibition of bilirubin formation was not due to the inhibition of biliverdin reductase by the peptide because addition of peptide to purified biliverdin reductase had no effect (data not shown). The specificity of the effect was also evaluated using lactate dehydrogenase and horse radish peroxidase. Addition of D2702.75–84(E → V) to these enzymes had no effect on their activity (data not shown).

The specificity of peptide-mediated inhibition of HO was studied using various different peptides. Due to the availability of limited amounts of purified recombinant HO-1, the effect of peptides on mouse and rat HO activity was assessed using spleen cell extracts (Table II). HO-1 is by far the most prevalent form of the HO system in the spleen. All of the tested peptides had been evaluated in vitro and in vivo for immunomodulatory
activity (5–10, 12). In particular, peptides D2702.75–84(E V) bound to HSP32, indicating that peptide-mediated immunomodulation of heart allograft survival is independent of binding to this heat shock protein (12). Here we demonstrated that D2702.75–84(E V) bound to HSP32 (HO-1) and inhibited enzyme activity in vitro in a dose-dependent manner. All known inhibitors of HO induce up-regulation of HO-1 transcription in cells or animals (23, 24). Similarly, in vivo administration of D2702.75–84(E V) into mice resulted in rapid up-regulation of HO-1 mRNA and HO-1 protein levels with a corresponding increase in HO activity in liver, spleen, and kidneys.

**DISCUSSION**

A single amino acid substitution in peptide D2702.75–84, replacement of glutamic acid residue 75 with valine, and peptide synthesis using D-amino acids, resulted in a peptide (D2702.75–84 (E V)) with increased immunomodulatory activity as analyzed by inhibition of cytotoxicity and prolongation of heart allograft survival. This peptide did not bind to HSC70, indicating that peptide-mediated immunomodulation is independent of binding to this heat shock protein (12).

**FIG. 4. Effect of peptide on in vitro HO activity.** The activity of purified HO-1 was measured in the presence of increasing amounts of peptide D2702.75–84(E V). Enzyme activity is expressed as nanomoles of bilirubin formed/mg of protein/min.

**TABLE I**

| Sample          | N-terminal amino acid sequence                  |
|-----------------|------------------------------------------------|
| 30-kDa band     | MERPQPDSM-QDL                                  |
| Mouse HSP32     | MERPQPDSMPQDL                                  |
| Human HSP32     | MERPQPDSMPQDL                                  |
| Rat HSP32       | MERPQLDQMSQDL                                  |

**FIG. 3. Identification of HSP32 in protein fractions separated by affinity chromatography using E6-1 (A) and YAC-1 (B) cell homogenates.** Proteins separated by SDS-gel electrophoresis were analyzed by western blotting using anti-HSP32 antibody. Lane 1, pH 3.0 fraction; lane 2, recombinant control HSP32 protein (1 μg).

**Note:**

2 G. Grassy, B. Calas, A. Yasri, R. Lahana, M. Kaczorek, J. Woo, S. Iyer, and R. Buelow, submitted for publication.

3 J. Woo, S. Iyer, M.-C. Cornejo, N. Mori, L. Gao, and R. Buelow, submitted for publication.
Peptide-mediated Up-regulation of HO-1

Inhibition of mouse and rat HO by peptides

Peptides were synthesized by Fmoc chemistry and shown to be >90% pure by analytical reverse phase HPLC. Use of “D” as prefix indicates peptide synthesis using D-isomers of all amino acids. RDP1258 is a rationally designed peptide. nL indicates a norleucine amino acid residue.

| Peptide | Amino acid sequence | Mouse HO IC₅₀ | Rat HO IC₅₀ |
|---------|---------------------|---------------|-------------|
| 2702.75–84 | RENRLIALRY | 1 mM | >1 mM |
| 2705.75–84 | REDLRTLLRY | >1 mM | >1 mM |
| 07.75–84 | RESLRNLRGY | >1 mM | 200 μM |
| 2702.75–84(E → V) | RVNLRLIALRY | 20 μM | 20 μM |
| D2702.75–84(E → V) | rvlplainrly | 20 μM | >1 mM |
| D2702.75–84(E → V, R79P) | rvlplainrly | >1 mM | 20 μM |
| RDP1258 | RnLnLnLnLnLnlnG-CONH₂ | 20 μM | 20 μM |

FIG. 5. Northern blot analysis of HO-1 mRNA levels in kidney. Poly(A)⁺ RNA was isolated from mice that had been treated with D2702.75–84(E → V) 6, 24, or 48 h before tissue collection. Each lane contained 6 μg of RNA. The blot was first hybridized with a probe for HO-1 (upper panels) and subsequently with a probe for actin (lower panels). Lanes 1 and 2, controls at 6 h; lanes 3 and 4, treated 6 h; lanes 5 and 6, controls at 24 h; lanes 7 and 8, treated 24 h; lanes 9 and 10, controls at 48 h; lanes 11 and 12, treated 48 h.

FIG. 6. Effect of D2702.75–84(E → V) administration on HO-1 protein expression in spleen homogenates from saline (lanes 1 and 3) or D2702.75–84(E → V) (lanes 2 and 4)-treated CBA-J mice. Spleen was collected at 24 and 48 h post-injection, and HO-1 protein expression was analyzed by western blotting using anti-HSP32 antibody.

In an effort to study the specificity of peptide-mediated inhibition, various modifications of the peptide sequence were analyzed for in vitro enzyme inhibitory activity. Immunomodulatory activity correlated with HO-inhibitory activity; peptides without any immunomodulatory activity did not inhibit mouse or rat HO in vitro. In contrast, all tested peptides that prolonged allograft survival in mice or rats inhibited HO activity in vitro. Peptide 2702.75–84 was shown to prolong allograft survival in mice but not in rats (6–10, 12). This peptide inhibited mouse HO but not rat HO. In contrast, peptide 07.75–84, which prolonged allograft survival in rats but not in mice, was also a more effective inhibitor of rat HO compared with mouse HO. Based on various physicochemical and structural characteristics of peptides that demonstrated in vitro immunosuppressive activity, an “in silico screening” method was used to design a set of peptides. One of these rationally designed peptides, RDP1258, was shown to prolong allograft survival in both mice and rats and was able to inhibit both rat and mouse HO activity. Thus, our results demonstrate a strong correlation between in vitro peptide mediated inhibition of HO activity and immunomodulation.

HO-1 catalyzes the metabolic degradation of heme into biliverdin, which is subsequently reduced to the potent anti-oxidant bilirubin by biliverdin reductase (25, 26). HO-1, also known as HSP32, is inducible by various forms of stress (heat, radiation, starvation, hypoxia, hyperoxia, ischemia, GSH-depletion) (26) and has been shown to be up-regulated during an inflammatory response and acute allograft rejection (27, 28). Up-regulation of HO activity has been shown to protect cells from oxidative injury (29). Apart from stress, compounds such as heme, metalloporphyrins, and heavy metals have been shown to induce expression of HO-1 in vitro (24, 26, 30, 31). Several of the investigated metalloporphyrins are known inhibitors of HO. The mechanism of feedback regulation of heme oxygenase transcription are unknown. However, one may speculate that a decrease in cellular heme oxygenase results in accumulation of heme, which subsequently induces up-regulation of HO expression.

Immunomodulatory effects similar to those mediated by D2702.75–84(E → V) were also observed following cobalt-protoporphyrin (CoPP)-induced up-regulation of HO-1. Both D2702.75–84(E → V) and CoPP inhibited HO activity in vitro. In contrast, injection of D2702.75–84 (E → V) or CoPP into mice resulted in up-regulation of HO-1 gene transcription and HO activity. HO-1 up-regulation following administration of both compounds was associated with inhibition of lymphocyte proliferation and prolongation of heart allograft survival. In summary, these observations suggest that the immunomodulatory activity of CoPP and 2702.75–84-derived peptides is due to their capability to up-regulate HO in vivo. Formal proof of this hypothesis, however, has to await the availability of genetically engineered animals.

Although the mechanism of immunosuppression by HO overexpression remains unclear, several possible explanations can be envisioned. Degradation of heme by HO results in the production of biliverdin, bilirubin, and carbon monoxide. Biliverdin, the intermediate compound of heme degradation, was shown to inhibit human complement in vitro (33). In this context, it is interesting to note that complement deposition has been observed in unsensitized rat allograft recipients (34). Based on this observation, one may speculate that increased production of biliverdin following overexpression of HO may protect a transplanted organ from complement mediated cell injury and prolong graft survival.

Bilirubin, one of the endproducts of heme degradation, has been shown to inhibit responses of human lymphocytes including phytosmaggutinin-induced proliferation, interleukin-2 production, and antibody-dependent and -independent cell-mediated cytotoxicity (35–38). Even though peptide therapy had no effect on serum bilirubin levels, one cannot exclude the
possibility that overexpression of HO-1 resulted in substantially higher intracellular bilirubin levels. Additional studies evaluating the intracellular concentration of bilirubin in spleen cells of peptide-treated mice by flow cytometry will be necessary to clarify this issue (39).

The gaseous product of heme degradation, CO, like NO, has been shown to stimulate the production of cGMP via activation of guanylate cyclase (40, 41). The secondary messenger, cGMP, has been implicated in cell growth arrest and the release of tumor necrosis factor-α by activated macrophages (42). In addition, cGMP is involved in the regulation of various protein kinases, phosphodiesterases and ion channels (22, 43–45). Thus, one may speculate that the increased cGMP levels may modulate several immune effector functions. Besides stimulation of guanylate cyclase, CO may also modulate immune responses via inhibition of inducible NO synthase. Regulation of HO-activity and NO production are intimately linked and an increase in NO production of CO causes decreased NO production and vice versa (32). Whether peptide treatment affects NO responses via inhibition of inducible NO synthase. Regulation of HO-activity and NO production are intimately linked and an increased production of CO causes decreased NO production and vice versa (32). Whether peptide treatment affects NO production is yet to be investigated.

It is interesting to discover peptides that inhibit HO-1. The mechanism of inhibition could involve factors such as the interaction of peptide with substrate and/or reductase binding sites, thus preventing binding/activation of the substrate; alternatively, the peptide could interact with HO-1 protein, changing its tertiary structure. The possibility that these peptides may bind to the substrate heme, causing inhibition of heme degradation cannot be dismissed. The latter possibility, however, is unlikely since availability of substrate during the in vitro enzyme assay is not limited and heme is concentrated in much higher proportion than the peptide. Whichever may be the case, the interaction of the peptide with HO-1 must share similarity with HO-2, in that the peptide D2702.75–84 (E→V) inhibits not only HO-1 activity but also that of HO-2, which was recently observed.4

In conclusion, our data demonstrated binding of peptide D2702.75–84 (E→V) to HO-1. The peptides and derivatives thereof inhibit HO activity in vitro. Similar to effects observed following administration of CoPP,4 injection of D2702.75–84 (E→V) resulted in up-regulation of HO activity in liver, spleen, and kidneys. The mechanism of immunomodulation by HO-1 is not resolved. Our current knowledge of the biological effects of heme degradation products (biliverdin, bilirubin, and carbon monoxide) suggest that elevated HO activity may affect multiple pathways of immune responses. Identification of compounds that specifically modulate HO activity without causing toxic side effects could lead to the development of novel immunomodulators.

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