ISSN 0025-7680

International Symposium
NEW DIRECTIONS IN CANCER MANAGEMENT
Academia Nacional de Medicina
Buenos Aires, 7-8 August 2000

MEDICINA (Buenos Aires) 2000; 60 (Supl. II): 55-60

DEVELOPMENT OF MONOCLONAL OLIGOBODIES AND CHEMICALLY SYNTHESIZED OLIGOBODIES

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Abstract Oligonucleotide aptamers obtained using the SELEX procedure can recognize different molecules with high affinity. However, for proteins, this recognition is limited to native conformations and the specificity has not been clearly demonstrated by methods such as Western blotting, immunohistochemistry or immunoprecipitations. Using a library of oligonucleotides and a selection strategy based on high specificity instead of high affinity, we have reported previously the preparation of polyclonal oligobodies, reagents that recognize the protein PP2A in a very specific way[4]. Here we report a method to obtain monoclonal oligobodies. The oligobody developed specifically recognized both native and denatured states of the protein CPD1 used as a model system. We further demonstrate the specificity of the monoclonal oligobody using Western blots, immunohistochemistry, and immunoprecipitation, procedures previously limited only to antibody-based detection. In addition, a confocal microscopy is shown that was obtained using an oligobody made by chemical synthesis using an oligonucleotide synthesizer, being this the first "synthetic antibody" reported.

Key words: monoclonal oligobodies, synthetic oligobodies

Resúmen ***Desarrollo de oligoanticuerpos monoclonales y de oligoanticuerpos sintetizados químicamente.*** Aptámeros basados en oligonucleotidos y obtenidos mediante el método SELEX pueden reconocer diferentes moléculas con alta afinidad. Sin embargo, para proteínas, este reconocimiento está limitado a conformaciones nativas y la especificidad no ha sido claramente demostrada con métodos como *Western blotting,* immunohistoquímica o immunoprecipitaciones. Utilizando una biblioteca de oligonucleótidos y una estrategia de selección basada en una alta especificidad en lugar de alta afinidad, hemos publicado previamente la preparación de oligoanticuerpos policlonales, reactivos que en ese caso reconocían específicamente a la proteína PP2A de una manera muy específica[4]. Ahora describimos un método para preparar oligoanticuerpos monoclonales. El oligoanticuerpo desarrollado reconoce específicamente a la proteína nativa CPD1, usada como modelo, tanto en forma nativa como desnaturalizada. Demostramos además la especificidad de los oligoanticuerpos monoclonales utilizando *Western blots,* immunohistoquímica e immunoprecipitaciones, procedimientos previamente circunscriptos solamente a su uso con anticuerpos naturales o monoclonales. Finalmente, se muestra una imagen confocal obtenida utilizando un oligoanticuerpo sintetizado totalmente en un sintetizador de oligonucleótidos, constituyéndose así este oligoanticuerpo en el primer "anticuerpo sintético" descripto.

Libraries of oligonucleotides (aptamers) have been shown to be a very promising source of synthetic reagents capable of recognizing other molecules, with high affinity and specificity. In 1990, Tuerk and Gold[1] used a combinatorial library of oligonucleotides to produce a reagent that was capable of recognizing the bacteriophage T4 DNA polymerase. The method was called the "systematic evolution of ligands by exponential enrichment", or SELEX. In the same year, Ellington and Szostak[2] described the use of aptamers (oligonucleotides of random sequence) to form complexes with organic molecules of low molecular weight. A decade after these pioneer studies, aptamers have been developed against different proteins, displaying surprisingly high affinity (~nM). However, their ability to recognize proteins specifically, without cross-reaction with other proteins, has not yet been clearly demonstrated. Even more importantly, recognition by aptamers and the SELEX procedure has so far been applicable only to proteins in their native forms[3].

Recently, using a library of oligonucleotides we have developed oligonucleotide reagents against the protein phosphatase PP2A that behave exactly as the rabbit polyclonal antibodies raised against the same protein[4]. We have suggested the name "polyclonal oligobodies" for these reagents, in deference to their oligonucleotide origins, and their behavior as antibodies on western blots and in immunohistochemistry assays. Now, using the protein CPD1 (GenBank: U89345) as a model system, we show that it is also possible to develop "monoclonal

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oligobodies" against a target protein, even when that protein has never been purified. The only requirements are the sequence of a partial cDNA coding region, taken from a database (DNA, RNA, EST, etc.), the corresponding synthetic peptide (the "temporary target"), a synthetic oligonucleotide library, and a very crude protein extract prepared from a tissue expressing the protein of interest (the "final target"). The oligobody specificity is demonstrated by utilizing methods until now amenable only to detection with antibodies, such as western blotting, immunohistochemistry, and immunoprecipitation. Importantly, our monoclonal oligobody recognizes CPD1 in both its native and denatured states as demonstrated for the first time by a specific immunoprecipitation.

Methods

Rabbit Polyclonal Antibodies: A synthetic peptide (CPNLTYLNLSGNKIK) was custom-made at Alpha Diagnostic International Inc. (San Antonio, TX). The fragment was selected from the predicted antigenic regions of CPD1 (GenBank: U89345), using the program Peptide Structure from the Genetic Computer Group (GCG) package (Madison, WI). Polyclonal antibodies were prepared in rabbits, as described by others [5], except that the peptide was coupled to bovine serum albumin (BSA). The polyclonal antibodies were stored at -20ºC until use (with glycerol 50%, v/v). For immunohistochemistry, the primary antibody was affinity purified using the peptide bound to nitrocellulose, as previously described[5], and incubated overnight (ON) at 4ºC. A goat anti-rabbit antibody coupled to peroxidase (Promega Corporation, Madison, WI) was used as secondary antibody (incubated for one hour at room temperature (RT), at dilution 1:1 000). A goat anti-rabbit, affinity-purified antibody, coupled to alkaline phosphatase (Sigma Co., St. Louis, MO) was used as secondary antibody on western blots (1 h at RT, dilution 1:10 000)[6].

Oligonucleotide library: A library of 100-mer oligonucleotides, each with an internal 56 base pair random sequence, was custom-synthesized at DNAgency (Malvern, PA), with the following sequence: 5'-CTGCAGCCGCGGGGATCCT$(N)_{56}$ TCTAGTCGAATTCAAGCTT AGTGGC-3'. The library (concentration 0.8 µM) was amplified by PCR using the primers 5'-CTGCAGCCGCGGGGATCCT-3' and 5'-GCCACTAA GCTTGAATTCGACTAGA-3'. The library used in binding assays, at a final concentration of 17 pmoles/ml, contained approximately 1.0×10^{14} different molecules.

Binding to the temporary target, and preparation of polyclonal oligobodies: To prepare the polyclonal oligobodies, a previously-described procedure was followed [4], with few modifications. Binding to the temporary target was achieved essentially as reported elsewhere, except that after amplification by PCR, double-stranded DNA was denatured by heating at 95ºC for 10 minutes, followed by rapid cooling using cold phosphate-buffered saline (PBS), at 0-4ºC. The dilution with cold PBS must be adequately tested (we normally used 1/10 - 1/100 dilutions), to minimize reassociation, which necessarily competitively inhibits the binding of the oligonucleotide to the target. The selection process with the temporary target (synthetic peptide) involves a cycle of binding, washing, elution, PCR amplification, double-strand separation, and binding, which is repeated once or twice. After the last binding step, ^{32}P-radiolabel was incorporated during PCR amplification[4]. However, it should be noted that if the average melting temperature (T_m) of the oligonucleotides selected from the library at this step is very high, this simple procedure of heating the samples to separate strands might be ineffective (strands might re-associate very rapidly, or not dissociate at all). In such cases, strand separation using a biotinylated reverse primer, as described previously [4], is recommended.

Target switching: After detectable binding to the temporary target is achieved, the target is switched to the "final target" (CPD1, in this instance), presented in a mixture of proteins (on a western blot in the case of CPD1, discussed here), as previously described[4]. This is done to ensure that selection is based on specificity rather than affinity. Several cycles of selection and binding are performed, until no further improvement in specificity is observed (typically two or three times). A second round of selection, performed under different conditions (using various acrylamide concentrations, two-dimensional electrophoresis, etc.), might be required for other proteins at this point. In this way, polyclonal oligobodies against CPD1 were obtained.

Monoclonal oligobodies: The polyclonal oligobodies were PCR-amplified, ligated into the pGEM–T Easy vector (Promega Corporation, Madison, WI), and transfected into DH5a competent cells (Gibco BRL, Gaithersburg, MD). To isolate monoclonal oligobodies, several colonies were subcultured, alkaline lysis was performed, and the plasmids purified. The inserts were PCR-amplified (using the same primers) in the presence of ^{32}P-labeled nucleotides. The strands were separated by heating, and the ability of PCR products to bind to CPD1 on mini-western blots was tested. Several clones were screened, and the clone that produced the maximum signal was sequenced and used for final western blotting, immunohistochemistry, and immunoprecipitation. The sequence of the oligobody corresponding to this clone (after PCR amplification) is illustrated in Figure 1 (capital letters correspond to primers). After sequencing, any oligobody can be easily reproduced using an oligonucleotide synthesizer.

Western blotting and immunoprecipitation: Western blotting followed those procedures in which antibodies are normally used for detection [6]. Briefly, freshly isolated cerebella were homogenized in PBS containing a cocktail of proteinase inhibitors (final concentration: EDTA 10 mM, phenanthroline 10 mM, E-64 10 µM, leupeptin 100 µM, aprotinin 10 mg/ml, pepstatin A 10 µM) (Sigma Co., St. Louis, MO). One hundred micrograms of protein was separated electrophoretically on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel, transferred to nitrocellulose membrane, blocked with 5% BSA in PBS, and detected using either rabbit polyclonal antibodies (incubated ON at 4ºC, and visualized with secondary antibody as indicated previously), or biotinylated monoclonal oligobodies (incubated for one hour at RT, in 5% BSA in PBS, and visualized with streptavidin-alkaline phosphatase (AP)). For immunoprecipitation, we followed a previously-published procedure[7], with few modifications. Briefly, small fragments of mouse cerebellum were incubated in the presence of ^{35}S-methionine, the protein extracted, and immunoprecipitations performed using a monoclonal oligobody labeled with biotin (produced by PCR amplification in the presence of a biotinylated upstream primer), and streptavidin-paramagnetic beads (Promega Corporation, Madison, WI).

Immunohistochemistry: Mouse (C57BL/6J) cerebella were dissected at postnatal day seven, fixed in alcohol/acid (95% ethanol, 5% acetic acid) for four hours at –20ºC, dehydrated, and embedded in paraffin. Tissue slices (5 µm) were mounted on silanized (Silane, Sigma Co., St. Louis, MO) glass cover slip, deparaffinized, rehydrated, and blocked using 5% BSA in PBS for one hour. The slices were exposed to affinity-purified rabbit polyclonal antibodies overnight at 4ºC, rinsed twice with PBS, incubated with secondary antibody (goat anti-rabbit peroxidase, 1:1000) for one hour, and developed with 3,3'-diaminobenzidine (DAB) (Gibco BRL, Gaithersburg, MD). When

immunohistochemistry was performed using the monoclonal oligobody, a biotinylated monoclonal oligobody was made by adding a 5'-biotinylated primer to the PCR reaction (5'-biotin-CTGCAGCCGCGGGGATCCT-3'). After PCR amplification, the cDNA strands were separated by heating (95°C, 5 min), and diluted ten-fold with 5% BSA in PBS. After incubation of the slides with 5% BSA in PBS to block nonspecific sites, oligobodies were incubated with the tissue for one hour at RT, and then treated with streptavidin-peroxidase (Promega Corporation, Madison, WI). Control samples were prepared by preincubating oligobodies with an excess of blocking peptide (overnight at 4°C), followed by development as described above. The confocal image of Figure 4 was obtained using a LSM510 Zeiss confocal microscope. The PC12 cells were cultured in RPMI1640 (Gibco BRL, Gaithersburg, MD) medium plus L-glutamine 2 mM, $NaHCO_3$ 1.5 gr/L, glucose 4.5 gr/L, Hepes 10 mM and Na-piruvate 1 mM, horse serum 10% (Gibco BRL, Gaithersburg, MD) and fetal bovine serum (Gibco BRL, Gaithersburg, MD) 5%. The cells were cultured for five days in the presence of nerve growth factor (NGF) (Sigma Co., St. Louis, MO) 100 ng/ml added daily to obtain differentiation. The cells were fixed in paraformaldehyde 4% during 15 minutes, washed with PBS and permiabilized with 0.3 % Triton X100 in PBS for another 15'. For labeling with oligobody-Cy3, the same procedure above indicated for immunohistochemistry with monoclonal oligobodies was followed, except that streptavidin-Cy3 was used instead of streptavidin-peroxidase.

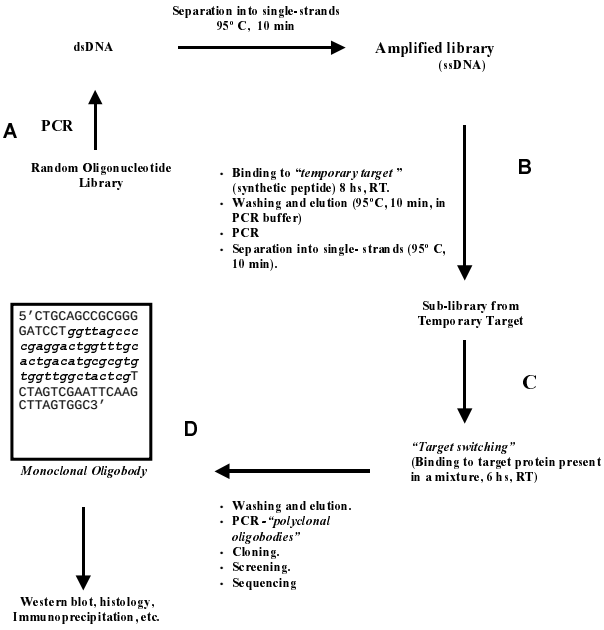

Fig. 1.- Scheme of the method to isolate monoclonal oligobodies. A. *Library.* The initial single-stranded library is PCR-amplified, and strands are separated. B. *Temporary target.* One to three cycles of selection are performed with a temporary target (synthetic peptide) to isolate a library subset. C. *Target switching.* The temporary target is replaced by the final target protein, present in a very crude preparation. This step is repeated until no further improvement in specificity is observed (typically three times), to obtain "polyclonal oligobodies". D. *Monoclonal oligobody.* The polyclonal oligobodies are PCR-amplified, ligated into the vector, transfected, and cloned. Colony-PCR was performed to select satisfactory clones (by testing the PCR products using mini-western blots, for example). Selection produces monoclonal oligobodies, each of a unique sequence.

Results

The strategy used to isolate monoclonal oligobodies is illustrated in Figure 1. Firstly, a library of 100-mer oligonucleotides, each of which has a central 56 base pair random sequence, was amplified[8] to eliminate truncated forms arising from the low efficiency inherent in the synthesis of long oligonucleotides. Secondly, synthetic peptides[9] were used as "temporary targets". Using this approach, there is no requirement for purified protein, and the sequence of a fragment of cDNA or RNA encompassing part of the coding region of the protein of interest provides sufficient information. The protein CPD1 (GenBank: U89345) was used as a model system for this work. CPD1 constitutes a good model as it has never been purified, which is also true of many sequences in various databases, and for which a function is yet to be identified. In the initial selection for optimally binding oligonucleotides from the library, a synthetic peptide corresponding to a fragment of CPD1 was used as temporary target. The peptide corresponds to a region predicted by the GCG Peptide Structure program to be antigenic, and exposed on the protein surface. The peptide was selected in this way because of the requirement to generate antibodies in rabbits as controls. Strictly speaking, the selection of peptide fragments possessing high antigenic indices is not necessary for the production of oligobodies, as their generation is independent of the immune system, and therefore does not require antigenicity. However, an elevated antigenic index may be desirable when oligobodies are required that can recognize both native and denatured proteins, as the regions of high antigenic index will probably be exposed on the protein surface in both cases (assuming that prediction of the antigenic index is accurate). Thirdly, only a small number of selection cycles (binding, elution, amplification, and binding) were applied to the temporary target, just sufficient to achieve some detectable binding (typically two or three cycles). Then the temporary target (synthetic peptide) was replaced with the final target (CPD1), present in a mixture of proteins (cerebellar extract). This step is called "target switching", and is crucial in the isolation of specific oligobodies. It should be emphasized that the final target protein should be present in a very crude extract of a tissue expressing that protein, in order to select on the basis of specificity, rather than on affinity. Two or three cycles of selection were performed with the final target present as such a mixture of proteins (separated electrophoretically on polyacrylamide gel and blotted onto membrane). This selection step was repeated before cloning, until no further improvement in specificity was observed (three times for

CPD1). In this way, specific polyclonal oligobodies against the final target were obtained. After PCR amplification, the polyclonal oligobodies were ligated into vector and transfected into bacteria. After cloning, monoclonal oligobodies were selected using mini-western blots.

The sequence corresponding to a selected clone containing the oligobody against CPD1 is shown in Figure 1. The insert corresponding to this oligobody was amplified by PCR, using a biotinylated primer. Proteins were separated electrophoretically and transferred to nylon membrane by western blotting. As shown in Figure 2, the monoclonal oligobody behaves on the western blots exactly as does its polyclonal counterpart, which was raised in rabbits using the same synthetic peptide. The only difference observed is the presence of bands corresponding to the preimmune serum, when polyclonal antibodies were used. Analysis of northern blots indicates that CPD1 transcript is expressed in the cerebellum in two isoforms ($CPD1_h$ and $CPD1_l$, of 3.4 and 1.8 kilobase pairs, respectively – results not shown). Western blots of cerebellar proteins, reacted with rabbit polyclonal antibodies or with monoclonal oligobodies raised against the synthetic peptide of CDP1, also detected two isoforms, differentially expressed during development from postnatal day five to postnatal day 17 (P5 to P17), with the 34 kDa isoform more abundant. These results were obtained by labeling the oligobodies with biotin after clon-

Fig. 2.- Western blotting and immunoprecipitation with monoclonal oligobody. Western blots (WB) of freshly isolated cerebellum samples from mice at postnatal days five to 17 (P5, P7, P11 and P17) were prepared, and CPD1 detected as described in methods. A. WB screened with a rabbit polyclonal primary antibody. B. WB treated with preimmune rabbit serum C. WB screened with the biotinylated monoclonal oligobody, and visualized with streptavidin-AP. D. Comparison of WB at P7, and immunoprecipitation of ^{35}S-CPD1: 1. WB at postnatal day seven (P7), treated with preimmune serum. 2. WB screened with rabbit polyclonal antibody. 3. WB screened with anti-CDP1 monoclonal oligobody. 4. Immunoprecipitation using anti-CDP1 monoclonal oligobody, and proteins labeled with ^{35}S. Letters “h” and “l” stand for high and low molecular weight isoforms, respectively. The arrows indicate a band corresponding to the preimmune serum.

Fig. 3.- Immunohistochemistry of CPD1: Tissue slices of mouse cerebella at postnatal day seven. A. Tissue slice treated with affinity-purified rabbit polyclonal antibodies, as indicated in methods. B. Slice treated with biotinylated monoclonal oligobody. C. Control samples prepared by preincubating the oligobody with an excess of blocking peptide (overnight, at 4ºC), followed by detection procedures as described for B.

ing, indicating that a biotin label could be added without affecting the ability of the oligobody to recognize the target. Similar results were obtained labeling the oligonucleotides with ^{32}P-dCTP during PCR amplification (results not shown). Interestingly, the oligobodies labeled with biotin were also useful in the immunoprecipitation of CPD1 (Figure 2D), implying that this oligobody can recognize both the native and the denatured forms of CPD1, as predicted by the GCG computer program.

Immunohistochemical analysis of CPD1 in slices from cerebellum was also performed. As shown in Figure 3, the results obtained by using affinity-purified polyclonal antibodies made in rabbits (Figure 3A) were indistinguishable from those obtained with the monoclonal oligobody (Figure 3B). Negative controls, prepared by pre-incubation of the monoclonal oligobody with the synthetic peptide corresponding to CPD1, showed no staining (Figure 3C). Staining for CPD1 was observed in both granule cells and in Purkinje cells. Finally, the sequence from Figure 1 was used to construct the first synthetic oligobody, by using an oligonucleotide synthesizer. This synthetic oligobody was labeled during synthesis with biotin and was used to confirm the nuclear localization of CPD1. The synthetic oligobody was incubated with differentiated PC12 cells followed by incubation with streptavidin-CY3. The corresponding confocal image (Figure 4) shows a strong signal in the nucleus, although some cytoplasmic localization can also be observed. This figure constitute the first “immunohistochemistry” made with a “synthetic antibody” constructed in an oligonucleotide synthesizer.

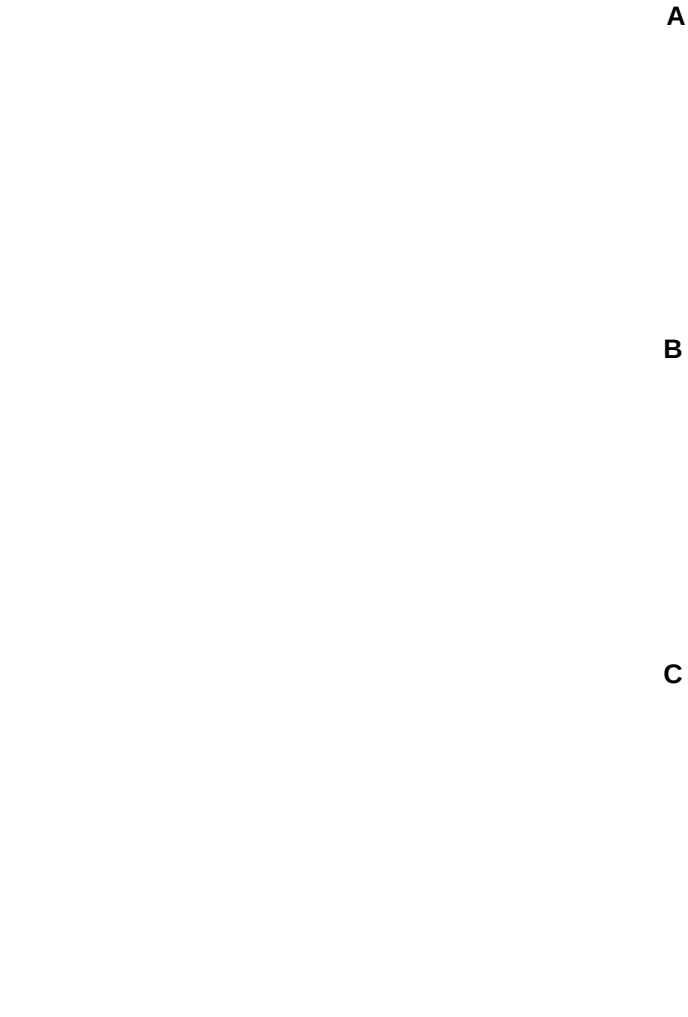

Fig. 4.- Confocal microscopy of CPD1 using a synthetic Cy3-oligobody: The figure shows the results obtained by using a 100% synthetic oligobody made in a Oligo1000M synthesizer. Biotin label was added at the end of the synthesis and streptavidin-Cy3 was used to develop the CPD1 localization, which is mainly nuclear. A: Cy3 channel; B: visible, transmitted light; C: A + B.

Discussion

After the original ideas of Tuerk and Gold[1] and Ellington and Szostak[2], several reagents have been developed that bind different molecules with high affinity, including proteins, nucleic acids, dyes, and other small molecules[10, 11]. However, the specificity of aptamers for target proteins has not been clearly demonstrated. Few assays have been performed to show aptamer specificity for protein, and these have generally been done utilizing only one or two pure proteins, and not with a heterogeneous mixture of proteins such as that found in crude extract, by which aptamer specificity could be demonstrated unequivocally. Another important limitation of aptamers has been their ability to recognize only proteins that are fully accessible in a stable, soluble and native conformation, as remarked by Weiss *et al*[9].

The main impediment to the development of reagents that can behave like antibodies has been the search for high affinity compounds, rather than for highly specific ones. The procedures typically used to isolate aptamers involve many cycles of binding, elution, amplification, and binding. This is done with the goal of obtaining high affinity aptamers. The outcome of such a selection procedure is a few molecular species of very high affinity indeed. However, the most important feature of any library (and also of nature) is its diversity, and this is irretrievably lost during so many cycles of selection, as species of high affinity are not abundant. Consequently, after so many cycles it is too late to attempt selection based on specificity. Therefore, to obtain oligobodies we used the opposite strategy, using selection procedures that isolate oligonucleotides with high specificity for CPD1, and doing so very early in the process, in order to maintain maximum diversity. Considerations of affinity are deferred until specificity is achieved. We also initially used a fragmented, temporary target, for presentation as the antigen to the

library, and subsequently switched targets. Once high specificity was achieved, further selection was undertaken to obtain an isolate with adequate affinity for our detection purposes, producing in this way a "monoclonal oligobody". In other words, we followed a procedure that resembles the mechanism used by nature in the production of antibodies, first selecting for high specificity, and then for high affinity.

It is important to note here that the selection performed after switching from the temporary to the final target probably should be done with the final target present in its native conformation, if the intention is to use the oligobodies in tests that maintain the native conformation of the protein (i.e., gel shifts, column chromatography, immunoprecipitation, etc.). In the same way, the target protein probably should be in its denatured form for procedures that produce denatured forms of the protein (western blotting, immunoprecipitations after SDS extraction, etc.). This could be critical because oligobodies, like antibodies, may be sensitive to conformational changes in the target protein (aptamers derived from native proteins by SELEX fail to recognize denatured proteins). However, sometimes the oligobodies selected using western blots might be able to recognize both the native and the denatured protein, as occurs here with CPD1 oligobodies (immunoprecipitaton), probably because the synthetic peptide used corresponds to a region predicted to be exposed on the molecular surface. It is also important to note the ability of the oligobody to immunoprecipitate CPD1 in a very specific way. This ability has been reserved so far only to natural antibodies.

The sequence obtained after cloning of the monoclonal oligobody, shown in Figure 1, was used to make a "synthetic oligobody" in an oligonucleotide synthesizer. The oligobody was labeled with biotin during synthesis and streptavidin-Cy3 was used for detection of the confocal image shown in Figure 4. This result confirm the nuclear localization of CPD1, but most importantly, constitute the first "Immunohistochemistry" made with a reagent 100% synthetic. This oligobody was able also to "immunoprecipitate" CPD1 or to recognize CPD1 in Western blots exactly as the monoclonal oligobody (results not shown).

Oligobodies may prove very useful reagents in many different fields, as they are relatively easy to prepare, the cost of production is low, and the procedure is rapid. Most importantly, their production requires no immune response or animals, no pure proteins, and no costly culture and purification procedures. Only a partial coding sequence taken from a database is required. Finally, it is interesting to note that the size of oligobodies (aptamers) is very small compared to antibodies (about 1/10 after subtracting the primers). Therefore, if their *in vivo* stability and clearance prove to be adequate, as suggested[12], they might be excellent vehicles for the delivery of drugs into tumors. In such cases, since the time required to develop oligobodies is short, it might even be possible to develop anti-tumor oligobodies customized to the requirements of each patient, and for the particular stage of their disease.

Acknowledgements: we thank Professor Dr. Marcelo Dankert (Instituto de Investigaciones Bioquímicas – Fundación Campomar) for his continuing interest in and support of our work.

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